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Handbook of Animal Models and its Uses in Cancer Research

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With 129 Figures and 77 Tables

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Preface

The *Handbook of Animal Models and Its Uses in Cancer Research* brings forward the most cutting-edge developments in animal model systems for translational cancer research. Animal models are becoming increasingly significant in cancer research because they serve as a vital link between laboratory-based findings and human clinical trials. Translational research requires ideal animal models that replicate genetic, anatomical, physiological, and pathological features of human diseases. Over the last years, there has been an incredible gain in our understanding of the basic molecular mechanisms and translational observations that have led to the development of recent ex vivo and in vitro approaches and various research methods that could further improve our holistic and incomplete genetic insight of the disease that is associated with the advancement of cancer and its response to various therapies. In consequence, animal welfare concerns have increased worldwide and traditional ethical standards about animals, such as anti-cruelty laws, have been replaced. It is important to respect the biological and psychological nature of animals in terms of pain and distress. A painless death should be the norm when killing research animals. It is not only morally important to minimize animal distress and pain, but it is also scientifically important because uncontrolled distress and pain can greatly invert variables. In addition, ethical issues related to animal research must be seriously addressed by the research community. Moreover, this understanding assures that upgraded approaches for screening, treatment, and prevention will be advanced more efficiently for patients' benefit.

This book describes preclinical and clinical cancer research by using animal models for discerning all features of cancer biology, from the development of tumor to the underlying mechanisms in resistance or response to the treatment, and includes syngeneic models, stem cell models, orthotopic models, metastatic models, transgenic models, and gene knockout models.

The reader can find under this one volume, three major sub-sections disseminating virtually all types of existing and emerging animal models in cancer research. The book encompasses areas that provide essential information vital to developing a new drug into practice in terms of efficacy and safety with the utilization of animals in research. Animal models such as mice, rats, rabbits, etc. are crucial to investigate mechanistic information that will promote preclinical drug/therapy design, which will aim at a particular component associated with the disease pathogenesis.

The goal of this book is to compile together the recent discoveries and developments in various in vitro, in vivo, and ex vivo studies of cancer research. It will provide knowledge and a better understanding of the advancement of the molecular and cellular mechanisms associated with the progression, formation, and clinical results of various types of cancer from the evidence collected from various animal models which are utilized for cancer research. There is a fundamental need in our society for animal research and the advancement of medicine that has helped reduce suffering and improve quality of life. This book is a valuable resource for basic and translational cancer researchers, drug discovery researchers, contract research organizations, and knowledge seekers at all levels of biomedical research.

Unique Features of the Project

1. ***This book will provide an up-to-date knowledge on the latest and different animal models used in cancer research***.
2. ***The book will serve as a guide for investigation towards the valuable analysis of the problems of limited models for high throughput screening of various drugs for therapy***.
3. ***The three sub-sections in the book are designed to provide a full explanation of the ongoing research and laboratory protocols involving various animal models in cancer research***.
4. ***Underpins the molecular and pathological mechanisms associated with various cancer and translational impacts through examining animal models***.

Chennai, India — Surajit Pathak
Chennai, India — Antara Banerjee
Adana, Turkey — Atil Bisgin
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Prof. Surajit Pathak, PhD is currently working as a Professor at Chettinad Academy of Research and Education, Chennai, India (from 2015 till date). He worked in Linkoping University, Linkoping, Sweden, as a Principal Research Scientist (June 2022–Dec 2022). He has received his Ph.D. degree from India in 2007 and completed his postdoctoral training (2007–2015) from the University of Alabama, USA, University of Padova, Italy, and University of Linkoping, Sweden.

Dr. Pathak's current research focuses on **"Biomarker discoveries for early detection of colorectal cancer."** He has published more than **100 research articles** in peer-reviewed international journals of repute with 2100 citations and 27 H-index. He received various research grants including Core International and National research grants from SERB-CRG (DST, Govt of India) and DST Indo-Italy bilateral research projects and from other international funding agencies. He has various active international and national research collaborations.

Dr. Pathak is the editorial board member of various renowned international high impact journals. He was also an editor of two books entitled *Cancer Stem Cells: New Horizon Cancer Therapy* published by Springer Nature in 2020 and *Stem Cells and Aging* published by Elsevier in 2021. He is the member of

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Dr. Antara Banerjee is presently working as an Associate Professor at Chettinad Academy of Research and Education (CARE), Chennai. After completing her PhD in 2008 from India, Dr. Banerjee pursued her postdoctoral research at university of Padova, Italy. Later she worked as a Research Associate at Linkoping University, Sweden, till 2015. She has published **over 90 peer-reviewed research articles** in international and national journals with **citation index-1247 and H-index 18**. Her primary expertise and research interest are to investigate aspects the tumor specific niche as a therapeutic target for colon cancer including the epigenetic signals with the goal of identifying novel therapeutic strategies to improve treatment outcome in patients. The Banerjee's lab at Chettinad Hospital & Research Institute is now focused on understanding the role of mesenchymal stem cells niche in tissue regeneration and exploring signaling pathways in governing stem cell fate. Dr. Banerjee received various research grants including Early Career research grants from SERB-ECR, Govt of India, and from other pharmaceutical companies. She is the Editor of a book entitled *Cancer Stem Cells: New Horizon Cancer Therapy* published by Springer Nature in 2020 and *Stem Cells and Aging* published by Elsevier in 2021. She is also a member of various professional research bodies in India and abroad including the Indian Association of Cancer Research and Indian Science Congress.

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Dr. Atil Bisgin was born in 1979 and had medical education in Cukurova University, then Medical Genetics in Akdeniz University. In 2015, he established AGENTEM where more than 15,000 molecular tests per annum have been performed, including NGS-multi-gene panels, clinical exome, whole exome, and whole genome sequencing. He performed more than 400 presentations, 150 as an invited speaker, and has more than 100 published papers in indexed journals. He has ongoing international research projects and multicentric, multiphase clinical trials in oncology and rare diseases. He is a board member of human/medical genetics societies about novel technologies in diagnostics, quality assessments, and establishment of national databases for several countries. He is working in the Medical Genetics Department at Cukurova University since this was established in 2013 with international institutional cooperation. Dr. Bisgin provides training courses for newly established next-generation-sequencing laboratories and multiomics research centers, designs (inter) nationwide targeted multigene panels for clinical practice and pipelines, and also provides services for the genetic testing with clinical reporting via his Infogenom-AB initiative. He also provides lecture in medical faculty and in-charge for bioinformatics, immuno/oncogenetics, translational medicine and biotechnology masters' degree and PhD training in Institutes of Natural and Applied Sciences, Health Sciences and Addiction, and Forensic Sciences.

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Part I

Animal Models for Cancer Research

Importance of Animal Models in the Field of Cancer Research

1

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Abstract

Cancer has traditionally been the center of human interest all around the world, making it a medical research hotspot. Animal models are categorized based on the method used to induce cancer in the animal. The mouse has been the standard animal model for fundamental and preclinical cancer research, although other species, such as zebrafish, serve essential and complementary roles as cancer research models. A number of treatments, including chemical or physical mutagenesis, viral infection, transgene insertion, homologous recombination, and the recently established gene edition, have resulted in genetically altered mouse and zebrafish cancer models. As research advances, the methods for creating cancer animal models become increasingly diversified, including chemical induction, xenotransplantation, gene programming, and so forth. The introduction of genetically engineered animal models has greatly aided in the understanding of the illness. Animal models can be utilized not only to study the biochemical and physiological mechanisms of cancer incidence and progression in objects but also for cancer medication screening and gene therapy research. Animal models are useful for researching the biology and genetics of human malignancies, as well as for preclinical research into anti-cancer medicines and cancer prevention. Major strides have been achieved in the development of animal models of cancer, which have become increasingly complex via the use of new technologies and the incorporation of clinical data from patients.

Keywords

Cancer · Animal model · Mouse · Zebrafish · Anticancer medicines

Introduction

Within the animal kingdom, animals are worshiped, eaten, admired, feared, cared for, respected, relied on, and fed in a variety of ways. It is a part of the daily lives, livelihood, and sociology. Early investigations of processes in animal models provided most of the basic understanding of human biochemistry, pharmacology, endocrinology, and physiology (Coffey and Isaacs 1980). Over the course of history, researchers have experimented with the purpose of acquiring knowledge about the anatomy and function of animals and humans. Throughout human history, animals have been employed in study and research for millennia. Evidence shows that Aristotle employed animals in his studies, even in ancient Greece, largely to enhance the understanding of live animals. The development of animal models expanded after the eighteenth and

nineteenth centuries, when many scientists, researchers such as Pasteur, Lazzaro Spallanzani, John Needham, and Lavoisier conducted experiments to study the origin of animals. They published their findings in 1957 (Oparin 1957). In nearly every field of research, animal models are used, as the chapters in this book illustrate.

Animals in Research: Past and Present

In the past, animals were used in basic and applied research, but nowadays, that practice is being questioned. There has been an increase in both scientists and animals as the medical, biological, and pharmaceutical sciences expanded over the last century.

Biological and Medical Research Involving Animals During the Early Twentieth Century

Every year, between 50 billion and 100 billion animals are used in scientific research. Research using animals in the UK reached 72 million in 2003 (Miele 2016). The use of animals was doubled 30 years ago. Since ancient Greece, animals have been used in scientific research for thousands of years. Natural philosophers and physicians of that period were interested in learning more about how complex creatures like humans and animals functioned. To understand and produce better treatments and cures, they study malfunctions, disease development, and the effects of injuries. In addition to pursuing knowledge in its own right, they sought to understand how and why illnesses and injuries happen. Since humans and animals have many biological similarities, physiological processes described in human studies may also apply to animal models. In some societies, animal research continued for another 2000 years, becoming a part of systematic scientific investigation through the Roman era (c. 510 BC to 455 AD) and the rise of early Arabic medicine (between the fall of Rome and the fifteenth century).

History of medical experts agrees that physiology's early discoveries were largely based on studies of animals. In 1628, William Harvey discovered blood circulation, while in 1667, Robert Hooke discovered lung function, and in 1733, Stephen Hales discovered blood pressure. These developments further validated the findings of earlier researchers.

Animal Models Concept

A model is a person or item that resembles another, anything that accurately looks like something else (Dictionary OE 1989). A variety of biochemical and physiological processes can be observed in animal models in ways that simulate human diseases or behaviors or mimic events in other biotas (Hau et al. 2002). The same event is like that of an animal. The similarity of this animal's physical behavior to other species is the subject of inquiry, not the image of the animal utilized. In this

context, speaking to animals as "human models" would be more precise. Comparative medicine, animal laboratory research, and animal experiments are more important to mammals than to other animal species (Svendsen and Hau 1994).

The validity and relevance of the "add-on" outcomes in the usage of animal models depend on how well the animal model is chosen. A solid understanding of comparative anatomy and physiology is required to build an animal model. The evolution of the animal kingdom has led to a variety of animal models as well as human physiology studies in species completely different from humans. Pharmaceutical development and the study of molecular biological mechanisms often require the use of animal models. There has been extensive research done evaluating various animal models for the prediction of carcinogens and to determine the mechanisms of carcinogenesis (Teicher and Andrews 2004). It is time-consuming and expensive to use chronic exposure to a carcinogen in drug development, so it is not widely applicable. Although mouse models are less costly, easier to handle, and have known genetic information, they are still more appealing than large animal models (Cheon and Orsulic 2011). These models have the benefits of reproducibility, the ability to induce a variety of tumor types, and immunity. Often, however, the outcome of this model differs from that of in vitro experiments performed with human cancer cells. A method developed by the National Cancer Institute (NCI) overcomes this disadvantage by injecting human cancer cells into a mouse with an immunodeficient immune system. Eight xenograft models are being developed using different NCI cancer cell lines (brain, colon, leukemia, lung, melanoma, ovary, prostate, and renal). For the evaluation of new drugs' efficacy and toxicity, various methods for generating mouse models have been developed.

Tumor Xenograft Animal Model

Research on tumor xenograft animals is primarily concerned with bridging research in basic and clinical fields, as well as proving the usefulness of in vitro models (Cekanova and Rathore 2014). Animal models of tumor xenograft provide a more sophisticated platform for studying tumorigenesis in vivo. The platform allows us to uncover the related signaling pathways and disease mechanisms of certain oncogenes and tumor suppressors, which may play a role in tumor development (Khaled and Liu 2014). In addition to determining the drug toxicity, pharmacokinetics, and pharmacodynamics, these models can be used for evaluating preclinical drug response by determining antitumor efficacy (Kelland 2004). In addition to evaluating drug response, these models can also provide a platform for testing the usefulness and practicality of new tumor detection reagents or methods.

Several key advantages compel mice to be used for tumor xenograft models, including their small genome size, short reproductive cycle, large litter size, low maintenance costs, and ease of manipulation (Khaled and Liu 2014). For cancer research, different strains of mice with different levels of immunodeficiency are used, including athymic nude mice, SCID mice, and NOD/SCID mice (SCID mice with an additional level of immunodeficiency). Based on the lack of or defect in

nearly all types of immune cells (B cells, T cells, dendritic cells, macrophages, and natural killer cells), NOD/SCID mice have the most severe immune deficiency, followed by SCID mice lacking B cells and T cells and athymic nude mice lacking T cells (Cho et al. 2016). A variety of strains are used for research purposes depending on their level of immunodeficiency. In light of the costs and characteristics of the various mice strains, athymic nude and SCID mice are most appropriate for implanting human tumor cell lines, whereas NOD/SCID and SCID mice are more suitable for transplanting human tumors.

What Does Research Involving Animals Consist Of?

In science, the majority of animals are employed in fundamental research as well as "translational and applied research." Research expenditures for basic research, translational research, and applied research are closely connected in the medical field. A medical study uses animal models to uncover previously unknown biological processes and basic relationships. As a result, these findings can help improve human health care. Animals raised for research are not subjected to experimental treatments while alive but are put down as donors so they can be used for cell or tissue research. Many animal tests are carried out within the consumer protection framework and are required by law (so-called "regulatory purposes").

Do Researchers Use Any Species of Animal in Their Research?

Small animals such as guinea pigs, rats, rabbits, and mice are commonly utilized in studies; fish and birds are used for particular investigations. As well as rats and mice were most commonly used in medical research for their genome similarity with humans and sacrificed for their organs. From a technical standpoint, decoding the mouse genome a few years ago made it relatively simple to manipulate it; the mouse is the most important research species ever discovered because it provides insight into the genetic foundations of life processes. The slight decline in animal experiment numbers over the past 2 years is mainly due to a decrease in the number of rats and mice.

The use of fish has increased over the years as the zebrafish genome was decoded and enabled insight into the origin of life processes in vertebrates. Other species are used only to a minor extent.

The Mouse

A mouse is a small mammal of the mammal family Rodentia. For research, the most commonly used species in Europe and North America is the house mouse, *Mus musculus*. Humans are likely to have colonized other continents after discovering and settling in Eurasia and then spread mouse populations to other continents. Many

of the laboratory mice used today are descendants of mice fans that existed approximately the turn of the twentieth century. About 95% of all animals in the laboratory are mice and rats. For several reasons, including small-scale (hosting and maintenance ease), mice are typically selected for the animal model. There are a variety of traits described in this book, including being generally gentle and polite; their short reproductive cycle and short life span, anatomy, genetics, biology, and physiology of animals as well as how to create spontaneous mutations via genetic manipulation. Mice have been employed as study subjects in various fields, including biology, psychology, and engineering. They are utilized to create models of human disease in order to discover therapies or cures. There is such a wide range of diseases they modeled, including high blood pressure, cataracts, diabetes, obesity, breathing problems, seizures, deafness, muscular dystrophy, spinal cord injuries, Parkinson's disease, Alzheimer's disease, numerous types of cancer, cystic fibrosis, heart disease, and acquired immunodeficiency syndrome (AIDS). Several studies have used mice to examine behavior, sensory perception, aging, and nutrition.

The Rat

The rats were used as test subjects for the first time in the eighteenth century (1828), for the medical research. The Wistar Institute of Philadelphia, the oldest independent scientific organization in America, carried out the study employing laboratory rats in 1894. Secondly, *Rattus norvegicus* is merely one of the experimental animals most frequently employed. Animal models like rats can be used to test a variety of hypotheses. In addition to known genetic background, rats also exhibit the following characteristics: small size, short generation time, and known microbial status. Rodents that tend to be tractable typically do well in a laboratory setting. Rat handlers are rarely bitten by their animals unless they are suffering from extreme pain or stress. In a number of research disciplines, animal models have been applied, including earth study and space exploration, to discuss more basic scientific issues on nutrition, genetics, immunology, neurology, metabolic illness, and compliance. Efficacy, drug discovery, and toxicity investigations are some of their most common applications.

The Rabbit

Approximately 2% of rabbits inhabiting the Iberian Peninsula are European rabbits (*Oryctolagus cuniculus*) (Hardy et al. 1995). Based on archaeological evidence from Nice, France, rabbits and humans have coexisted for about 120,000 years (Dickenson 2013). European rabbits were used as research animals in the nineteenth century. The early studies on the rabbit focused on comparing its anatomical characteristics to those of other animals, such as the heart and circulatory characteristics of the rabbits and frogs (Champneys 1874). In numerous investigations, Louis Pasteur used rabbits that helped to establish the first rabies in the world

(Rappuoli 2014). While many so-called "fancy" races of rabbits are available in the livestock trade, studies outline the types of species utilized frequently. The New Zealand White (NZW) is the race utilized most frequently in rabbit studies. Rabbit breeds are also sometimes utilized in California and Dutch. Japan's researchers studied Watanabe heritable hyperlipidemia rabbits (WHHL) and WHHL rabbits susceptible to myocardial infarction and measured cholesterol levels as a way to detect dyslipidemia symptoms like atherosclerosis (Shiomi and Ito 2009). Rabbits are used to simulate pregnancy and to make polyclonal antibodies during immunological research by different researchers (Ema et al. 2010). Routine researchers are using rabbits to study atherosclerosis, osteoporosis, ophthalmic, and immunological conditions (Arslan et al. 2003). Because of the increased size of the blood, rabbits produce polyclonal antibodies more than rodents (Hanly et al. 1995). They can be surgically implanted with biomedical devices due to their tractable nature and large size (Ronisz et al. 2013). Rabbits have also been used for testing the teratogenicity of new medicines in pharmacological studies (Oi et al. 2011).

Zebra Fish

The zebrafish (*Danio rerio*) is a relatively small predatory teleost with a yellow and dark blue striped body. It is popular among aquarium hobbyists, and it is becoming more popular in research laboratories. There are two pairs of barbels on the body of adult fish, and the lateral line is incomplete (Laale 1977). The male mainly has yellow anal fins, while the female genital papilla is situated only in the anal fin (Creaser 1934). Zebrafish are commonly found in the slow-flowing waters of rivers, streams, and ponds in South Asia, India, Bangladesh, and Nepal (Spence et al. 2008). The water is usually low, with different sizes of clay, silt, or stone substrate (McClure et al. 2006). The main foodstuffs of zebrafish are insects and plankton, with indications suggesting they are fed along the water column and the sea surface (Engeszer et al. 2007). These study models include translucent, unfertilized eggs, fast-growth, and large genomes combining their tiny size with the capacity to spawn several times each year. At least 70% of zebrafish genes are orthologous to human genes (Howe et al. 2013). Zebrafish were initially utilized in the study of developmental biology. Research on the behavioral and genetic effects of zebrafish has increased greatly in the field of biochemistry, molecular biology, cell biology, and neuroscience.

Birds

In the Aves taxonomic order, birds are classified by physiological, anatomical, and genetic characteristics. Passeriformes is the largest order of birds that include perching and songbirds birds such as finches, canaries, and cardinals. Pigeons, doves, and pigeon-like birds belong to the order Columbiformes; African gray

buds and parrots are in the order Psittaciformes, while domestic poultry like quail and chicken are in the orders Galliformes. Researchers have used birds as models for aging, reproduction, parasitology, memory, atherosclerosis, and infectious disease, among others (Austad 1997). Genome sequencing of several bird species has now been completed (Cracraft et al. 2015). *Gallus gallusdomesticus* has been the most widely studied bird species and serves as the perfect model in the fields of immunology, infectious diseases, virology, toxicology, and embryology (Kaiser 2012). The reproductive development and retinal diseases of chickens are also studied. In addition to vaccines (human influenza), these transgenic chicken embryos are being created for developmental analysis using viral vectors, such as lentiviruses. Inbred lines that are resistant to diseases and future transgenic technologies can allow embryos to generate therapeutic proteins as bioreactors and perhaps create chickens with increased pathogenic resistance (Bacon et al. 2000). It is estimated that up to 35% of chickens are subjected to spontaneous ovarian cancer, and the test is widely used in the research of ovarian cancer in people (Bähr and Wolf 2012). Quails (*Coturnix coturnix* and *Coturnix japonica*) are also widely studied in several academic areas. They are one of the species of birds with the shortest life spans because of their smaller size, making their studies useful (Austad 1997). In captivity, the Japanese quail (*C. japonica*) has shown social behaviors such as mate selection; therefore, it has been used to assess these phenomena (Ball and Balthazart 2010). Methods for studying transgenic quail are now accessible, just as they are for chicken, and they give a helpful tool for studying gene function (Seidl et al. 2013). Amazon parrots, Psittaciformes, and budgies (*Melopsittacus undulates*) have also been the focus of studies, with veterinary diagnostics, behavior, cognition, aging, and sensory investigations (Austad 2011). Investigations have looked into the cognitive abilities and communication skills of African grey parrots (Hesse and Potter 2004). In the laboratory research, the European starling (*Sternus vulgaris* and *Sternus roseus*), the zebra finch (*Taeniopygia guttata*), and the house sparrow (*Passar domesticus*) were studied (Bateson and Feenders 2010). Generally, songbirds can learn and communicate complex songs and adapt to changing environments. Aging and neurogenesis are among the traits studied most frequently in zebra finches (Harding 2004). Zebra finches, canaries, and other tiny finches like *Lonchura stratum Domestica* are among the most favored songbird species for neurological study, because their tiny size makes it easier in groups to maintain and propagate the zebra finches in research settings (Schmidt 2010). Additionally, studies are conducted on their behavioral characteristics, including sexual dimorphisms, ability to sing all year round, and ability to learn songs with age (Fee and Scharff 2010). A variety of psychological and neuroscientific studies have been conducted on pigeons (*Columba livia*), including tests for cognitive functions, neural anatomy, and neuroendocrinology (Shanahan et al. 2013). To gain an understanding of their homing, vision, and discrimination abilities, the barn owl (*Tytoalba*), an example of a nocturnal bird species, is designed to understand auditory spatial mapping processes, neuroanatomy, learning, and vision (Peña and DeBello 2010).

Armadillo

One of the fascinating examples of tiny mammals is the nine-banded Armadillo (*Dasypus novemcinctus*), which can be found from the south of North America to the north of Argentina (Balamayooran et al. 2015). Armadillo has an enveloping carapace, and more importantly, 33–35 °C body temperature. The breeding occurs during the summer, but the implantation of embryons is delayed until the end of October, when the same quadruples are always born (Balamayooran et al. 2015). Armadillos are good models for studying leprosy due to their low body temperatures, sensitivity, and their ability to respond physiologically to infection (Balamayooran et al. 2015). The animal's persistent polyembryony has also made it a model of interest in studying the many features of twins (Blickstein and Keith 2007).

Guinea Pigs

The Hystricomorpha suborder rodents included pigs from Guinee (*Cavia porcellus*), which are associated with porcupines and chinchillas. Their native habitats of guinea pigs include the grasslands and mountains of southern America, such as the Andes Mountains. The females are smaller than the males, with shorter legs, stocky, non-boring, crepuscular, and short tails, weighing between 700 and 1200 g (Harkness et al. 2010). Guinea pigs have been employed in scientific studies since the 1600s, when they were initially used in physiological investigations (Pritt 2012). *Staphylococcus aureus* is a pathogen that causes most nosocomial infections in guinea pigs, as well as Legionnaires' disease, Tuberculosis, Chlamydia, and Syphilis in humans (Padilla-Carlin et al. 2008). The analysis of infectious diseases caused by bacteria, parasites, and viruses include leptospirosis, which is traditionally studied using guinea pigs, the leishmaniasis situation, acute respiratory syndrome (SARS), and other viral disorders, including Ebola, have escalated (Wahl-Jensen et al. 2012). Studies of cholesterol metabolism, aspects of asthma, fetal and placental development, and Alzheimer's disease have been conducted on guinea pigs as well (Bähr and Wolf 2012). Guinea pigs are comparable to humans physiologically, immunologically, and physically and are susceptible to scurvy, contrary to the intake of other rodents and primates (including humans) (Gresham and Haines 2012). Guinea pigs are maintained like other rodents but need more room than smaller rodents.

Amphibians and Reptiles

The Reptilia class is divided into four orders, each of which is categorized as follows: Chelonia, Squamata, Rhinocephalia, and Crocodilia. Anura, which consists of frogs and toads (these creatures include Xenopus, Buffo, Ranas, Hyla, and Dendrobate), are often seen in research settings. Species like *Ambystoma mexicanum* and *Ambystoma tigrinum* can also be found in Caudata. In the reptile class, the

lizards and the snake are of Squamata order; the chelonians are of the Chelonian order (including turtles, tortoises, and geese), and animals belonging to the Crocodilian order include crocodiles, caimans, and the alligators. Studies of human diseases need to be done on reptiles and amphibians more than on mammals (Pough 1991). Frogs and salamanders serve an important role in studying the physiological changes, regeneration, the development of embryos, metamorphoses, and climatic change (Burggren and Warburton 2007). Reptiles have frequently been researched because of their fundamental circulatory systems, immune responses, hormonal control, and unique reproductive behaviors; studies are being focused on the African clawed frog (*Xenopus laevis*) and the western clawed frog (*Xenopus tropical*). In recent years, *Xenopus laevis* has been the subject of extensive comparative medicine research and development (O'Rourke 2007). Observation of embryonic development is easier with larger eggs, and development, neurobiology, regeneration, endocrinology, and toxicology can be studied more efficiently (Koustubhan et al. 2008). The chytrid fungus *Batrachochytrium dendrobatidis* has been inspected using *Rana catsbiana* (bullfrog) for developmental and toxicological investigations as well as infectious disease studies (Alworth and Vazquez 2009). Molecular studies are being done on *A. mexicanum*, in particular, to uncover how amputated organs can regenerate (Rao et al. 2014). Studies have been conducted on *Ambystoma tigrarium* to study general declines in biodiversity in North America, environmental pollutants like pesticides, and the impact of *A. tigrarium* infection (Kerby et al. 2011). A Carolinaensis (green anole) has been used in traditional medicine for its reproductive biology effects (Lovern et al. 2004). In addition to *Caiman crocodylus* and *Alligator mississippiensis* (crocodiles), studies have also been conducted on *Trachemys scripta elegans* (red-eared slider) (O'Rourke and Lertpiriyapong 2015).

Hamsters

Hamsters belong to the order Rodentia (with the mouse and rat) and suborder Myomorpha. The literature documented over 24 species of hamsters; however, research is primarily conducted on golden hamsters (Harkness et al. 2010). Three to four littermates collected there in 1930 are believed to be part of the golden lineage of Syrian hamsters (Adler 1948). As the name implies, the common wild clothes are reddish-gold on the dorsal side, with a brown underside. Grasshoppers weigh 85–150 g and weigh more in females than in males; they have shorter legs and shorter tails, along with larger cheek pouches (Harkness et al. 2010). Their distinctive anatomical and physiological characteristics, particularly their sensitivity to illness and infection, make a suitable study model. Infectious illnesses, parasites, and dental problems were initially investigated in Hamsters before they were transferred to cancer research in the 1960s (Suckow et al. 2012). Diabetes, reproductive endocrinology, cardiovascular illness, and cancer continue to be studied in Hamster (Hein et al. 2013). For instance, diabetes mellitus research has been conducted using Chinese and African hamsters; Hamsters of Italy and Turkey

were utilized to examine features of hibernation, and scientists studied their behavior and pineal activities in the circadian rhythms of Turkish and Siberian hamsters (Butler et al. 2008).

Gerbils

There are numerous varieties of rodents utilized in studies, including gerbils. Even though there are over 100 species of gerbil-like rodents, the Mongolian gerbil (*Meriones unguiculatus*) is the most commonly used in the United States. Mongolian gerbils originate in the mountainous region of Mongolia and northeastern China and are long-tailed, burrowing, and the herbivorous rodent with 55–130 g of body weight (Harkness et al. 2010). Because of structural changes in the blood flow to the brain in the "Circle of Willis" anatomical area, the use of gerbils as models for stroke or cerebral ischemia has been particularly well established (Hickman et al. 2017).

Chinchillas

Chinchillas are members of the Hystricomorpha suborder of the order Rodentia. There are long-tailed chinchillas, short-tailed chinchillas, chinchillas, and *Chinchilla lanigera*. Chinchillas originated from South America's Andes Mountains (Suckow et al. 2012). It is found that females weigh more than males; the fur coats on their rear limbs are dense (Alworth and Harvey 2012). The coat's lushness in the mid-1900s caused them to almost extinct in the wild (Jiménez 1996). Chinchillas are famous for having huge heads and ears in addition to big eyes. Chinchillas are traditionally used for hearing tests because of their extraordinary detail about the otitis media and large inner ear (Morton et al. 2012) (Fig. 1 and Table 1).

Research on Animals: What Are They Used For?

The human population uses animals for many different purposes, including research. Nearly 260 million Americans kept approximately 110 million dogs and cats as pets. Each year, in the United States, more than 5 billion animals are killed for food. Moreover, animals are used for companionship, transportation, entertainment, and sport.

Animals are also utilized to better understand living things and diseases that affect people and other animals. When a novel medicine or operative method is developed, societies believe using it first among humans is not ethical because damage instead of assistance is conceivable. Instead, animals are used to test the drug or technology to ensure that it is both safe and effective.

Experiments on animals may offer valuable experimental models that would be impossible to replicate on human subjects. Animals can be fed uniformly and closely monitored diets. Members of some animal species are genetically identical, which

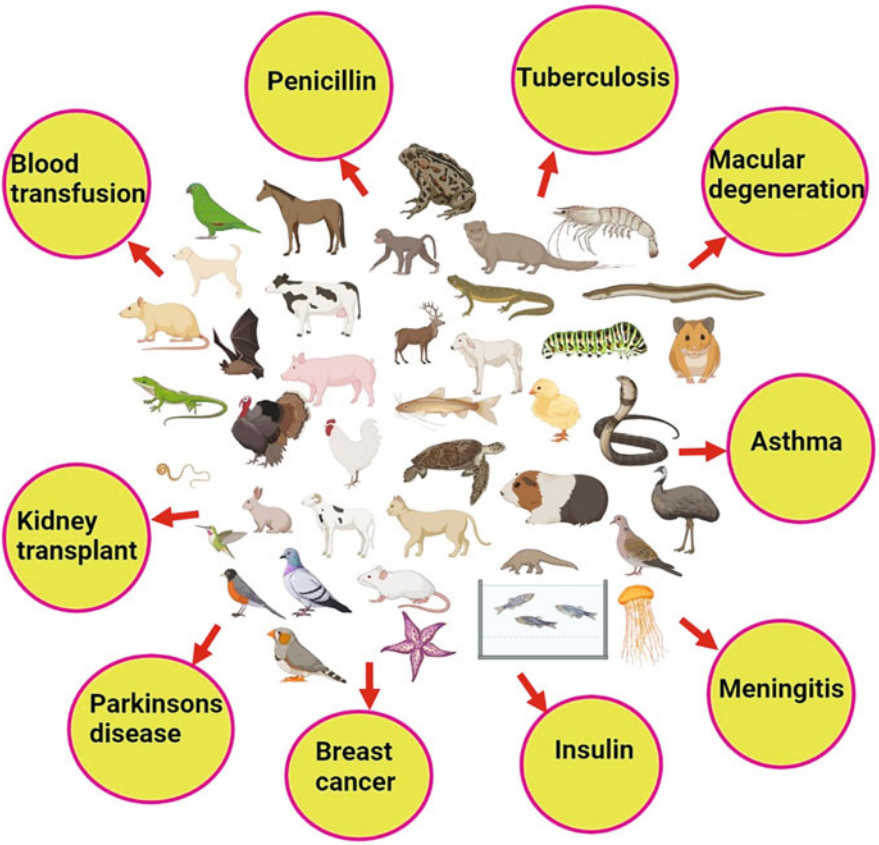

Fig. 1 Application of animals in medical therapy development

allows researchers to compare processes among similar animals. As some animals are biologically similar to humans, they can be used as models for particular diseases, such as rabbits for atherosclerosis or monkeys for polio (Special tests are still conducted on monkeys to determine the safety of the polio vaccine). Animals can also be used to generate, test, and develop new products, like as monoclonal antibodies, in the fast-developing biotechnology industry.

The researchers can study a wide range of living organisms, including microbes and humans. Since they are the simplest to cultivate or research, a number of basic biological processes in single cells, tissue crops, and plants are best explored. In addition to insects and nematodes, researchers study a wide range of animals, including monkeys, dogs, and cats. Scientists are interested in mammals as the majority of diseases that affect people also afflict other mammals because they are closest to us in terms of evolutionary distance, as an example, albeit not present in plants, insects, or germs.

Table 1 Advantage of various model organisms

Species	Experimental advantages
Yeast (Foury 1997)	Secondary screening process. Molecular methods that are extremely effective, genes are simple to clone, and the genome sequence is complete. All fundamental eukaryotic cell organelles are present, and cell cycle regulation is identical to that of mammals
Nematode	Hermaphrodites, self-fertilization, and screens for suppressors and enhancers at the second location; molecular methods that are extremely effective; genes are simple to clone. SNPs, transposons, and transposons may all be easily detected. Cosmid rescue in a hurry, RNAi is effective, and deletion collections cover the genome. Sequences of the genome, there are 959 cells and 302 neurons in total, with a fully described morphology, serial EM reconstruction, identification of all cell lineages, time-lapse imaging of the development process, and laser ablation of single recognized cells
Fruit fly (Bernards and Hariharan 2001)	Targeted gene disruption, genome sequence complete, fast generation time, RNAi effective, the use of second-site suppressors and enhancers, highly powerful molecular techniques, efficient cloning methods, transposon tagging, SNP mapping, and easily generated transgenic animals; mosaic analysis: determining where a gene functions by using targeted gene misexpression in space and time
Zebrafish (Barut and Zon 2000)	Simplest vertebrate with decent genetics: zygotic patterning mutants are almost saturated, a genome analysis is in progress (good SNPs and mapping of embryonic pathways), ease of morphological defect examination (clear embryos), possible embryological manipulation, vertebrate organ systems similar to other vertebrates (e.g., blood, gastrointestinal tract, eyes, heart), rapid vertebrate development
Chicken	Well suited for embryological manipulation, availability, outside of the mother, low-cost accessibility; transplantation of limbs, notochords, neural crests, easily infected by retroviruses in birds
Mouse (Benavides and Guenet 2001)	"Reverse" genetics: targeting genes by homologous replacements, recombination procedure, mammals with brains similar to those of humans, a large collection of mutants, it is possible to create chimeric embryos. Material availability at all phases, primary cell source for culture
Monkey	Human-like in appearance, postnatal developmental connections and physiology anatomy of learning, injury responses
Frog	A vertebrate Early embryos may have ectopic gene expression; despite the difficulty of manipulating levels, the embryo is accessible (without a shell); there are excellent experimental embryology grafting induction preparations (Keller sandwiches/animal caps, etc.); and RNA is injected into identifiable blastomeres

In comparison with other objectives, very few animals are utilized in research. Research, education, and testing use about 17 to 22 million vertebrates annually, less than 1% of what is killed and consumed by humans each year (U.S. Congress 1986). Rats and mice that have been raised for study account for around 85% of these animals. In the fiscal year 1988, approximately 52,000 cats and 142,000 dogs were used in the experiment, of which 40,000 to 50,000 were obtained exclusively for

Table 2 Animal experiments' contribution to medical advancement

Timeline	Animal experiments have made possible advances
1881	Germ disease theory
1898	Cycle of life of the malaria-parasite found
1902	Genetics and mice
1905	First human transplant operation
1913	Immunization-protected diphtheria
1914	Found out vitamin A
1915	Transfusion of blood
1921	Neurotransmission was shown
1922	Isolation of insulin
1926	Calvary anemia
1937	Heparin was utilized as an antibiotic agent
1940	Penicillin protects the mouse from infection
1945	Kidney insufficiency dialysis
1955	Developed polio vaccine
1958	Pacemaker for the heart
1961	Artificial valves for the heart
1967	Successful human cardiac transplants
1977	Vaccination to eliminate smallpox
1982	Developed leprosy treatments
1987	AZT-the first HIV medicine
1992	Vaccine for Hib meningitis
1996	Dolly, a sheep, is being cloned
2002	The mouse genome has been sequenced and analyzed
2008	First whole organ transplant tissue-engineered

research. Between 50,000 to 60,000 non-human primates are tested annually, many of them from American breeding facilities, such as apes and chimpanzees (Enforcement 1989) (Table 2).

Why Is There a Need to Use Animals?

Chemical reactions power all life processes, which are found in all species. When an animal is introduced to a substance, it can interact with it in many places on the body, and the effect on one process can lead to unpredictable results in others (Fig. 2). Experimental animals are important because their complexity cannot be replicated in cell culture or nonliving systems. For example, toxicity can be affected by the speed with which the substance enters the system; the liver and the surrounding tissues are affected by changes in liver activity. Some response is influenced by the tissue characteristics (In other words, the liver differs from the kidney). The use of beneficial chemicals, such as drugs, is primarily employed to avoid excessive exposure to the tissues and organs that will receive the benefit. Often, laboratory

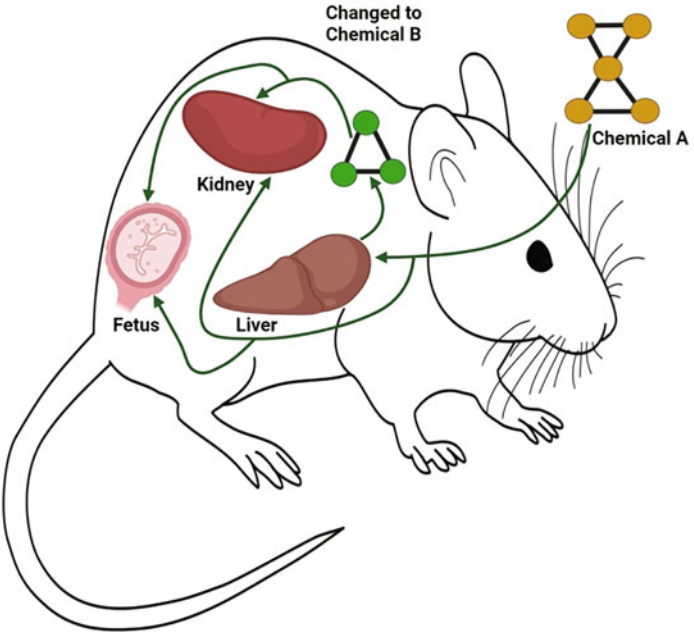

Fig. 2 Relevance of animals in human and environmental sciences in discovering important hazardous characteristics of chemical compounds

equipment cannot mimic such complex phenomena. Animal testing is, in the end, the most effective approach to discover side effects such as cancer and birth abnormalities.

What Are the Advantages of Employing Animals in the Study, and How Does Animal Research Assist Animals?

The study of animals can be beneficial both for humans and animals. For decades, scientists have used animals to advance knowledge of humans, veterinary medicine, and chemicals. A century ago, people would have been quite different if they had ceased using animals for scientific and medicinal study.

The use of animals helps to develop knowledge is a significant problem. The second is to decrease the animal's possible discomfort or misery. Exploring the circulation of blood, lung function, and the hormonal system in humans includes research on animals. Animal research and testing have been used to develop important treatments and preventive therapies, such as antibiotics, insulin, vaccines, and organ transplants over the last few decades. These studies have also provided insights into some of the more complicated diseases, such as tuberculosis, malaria, cancer, human immunodeficiency virus (HIV), heart disease, and depression. The development of novel veterinary medicines and vaccinations has

benefitted both farm animals and pets. Those in favor of animal research claim that it is necessary to continue such research on both ethical and scientific grounds (Hendriksen 2005).

Numerous animal species have benefitted from methods developed in humans to prevent and treat disease (Sechzer 1983). Animal medical practices, such as vaccines, antibiotics, anesthetics, and surgical procedures, have been adapted for human use from studies performed on animals. Animal research has resulted in pets, animals living longer, and livestock, more comfortable and healthier lives in zoos.

Many animal-specific therapies have been developed. Vaccines for rabies, feline leukemia, canine parvovirus, and distemper have prevented a large number of animals from acquiring these lethal diseases. Scientists have found therapy in animal studies for heartworm infection (a painful and eventually lethal fatal illness), cholera in pigs, tuberculosis, and brucellosis.

Animal studies also assisted the protection of a number of endangered species. Several species have been made healthier and more successful by eliminating parasites, treating and reproducing illnesses, using anesthetics, and advancing many technologies. Embryo transfer and insemination techniques enable species that are endangered or extinct to be conserved and managed. Many endangered species have been reintroduced into the wild due to research about sexual behavior in animals (Table 3).

What Are the "Animal Rights" Issues?

An idea called "animal rights" drives a major portion of the resistance to animal research. In this context, animals are, just as human beings, legally and morally inherent. This means that it is wrong to use animals as animals, such as food, clothing, entertainment, study, or any other reason (Regan 2004).

In other words, whether or not animals have them is determined by the definition of the phrase "rights." If living things had a right to survive, animals would be entitled to it. Nevertheless, the word is not used widely by most ethicists. Generally, they only provide rights to society members who are able to implement mutually recognized moral standards under certain conditions (LaFollette and Shanks 2020). Animals cannot create or join such communities. In this light, they cannot be empowered.

There are also some philosophically untenable conclusions based on the animal rights approach. In its most extreme version, it suggests that all creatures, including humans, have the same life. On the other hand, a human being's death is not comparable with a mouse's death. In perspective, reducing the number of rats in sewers or bugs in homes is not unethical. Keeping animals as animals is not regarded as moral slavery comparable.

The use of animals in research is addressed by a number of organizations in the United States. Various groups have different positions, and addressing the animal rights movement as a whole is oversimplifying. However, despite their divergence of opinions, it is crucial to distinguish between some who think that animal research

Table 3 Lab animals' contributions to science (Colby et al. 2019)

Animal model	Contribution to modern medicine
Mice, dogs, sheep, cattle, chickens, monkeys	For the development of a new treatment for disease caused by roundworm parasites and a new treatment for malaria
Rats	Cells that make up the brain's location system have been discovered (an inner GPS)
Hamsters, mice	The discovery of machinery that controls vesicle traffic, a crucial mode of transport of cells
Mice, flies	The stimulation of innate immunity has resulted in new discoveries
Frogs, mice	For discovering that adult cells can be converted into pluripotent stem cells
Mice	The dendritic cell was discovered and its involvement in adaptive immunity was discovered
Rabbits	In vitro fertilization's development
Protozoan, mouse, frog	The discovery of telomeres and the enzyme telomerase in the protection of chromosomes
Hamster, mouse, cow	Human papillomaviruses (HPVs) responsible for cervical cancer have been discovered
Monkey, chimpanzee, mouse	The human immunodeficiency virus (HIV) was discovered
Mouse, chick	Principles for using embryonic stem cells to introduce particular gene changes in mice have been discovered
Nematode roundworm	The discovery of double-stranded RNA for RNA interference gene silencing
Piglet	The bacteria *Helicobacter pylori* was discovered, and its function in gastritis and peptic ulcer disease was discovered
Mouse, *Drosophila* (fruit flies)	The organization of the olfactory system and the discovery of odorant receptors
Clam, mouse, dog, rat, chimpanzee, pig, rabbit, frog	Magnetic resonance imaging (MRI) breakthroughs

should go on. Some believe that it should be permitted, but with changes and limitations. These latter people, who constitute a strong minority in society, are encompassed under the word "movement to protect animal rights."

In media, legislatures, schools, and in many cases, their efforts have been a tremendous success; they worked tirelessly and effectively to advance their cause. In addition to self-regulation by scientists, external regulations have increased the cost and difficulty of animal research. Some animal scientists have left the region, while newer scientists have decided not to go there. Many people believe that too much animal research is carried out, not based on evidence but based on repeated arguments.

Some animal rights movement participants utilize more severe tactics that are often supported by more liberal elements (Horton 1989). Since 1980, more than 30 acts of sabotage, theft, and vandalism to research facilities have resulted in millions of dollars in losses (Franco 2013). A decade's worth of records has been destroyed. Threats and harassment were directed against researchers and their families.

Even though these acts have been forgiven and supported by leading representatives and organizations of the animal rights movement, the scientific community finds no moral justification for them. Medical research committed to enhancing human well-being has been hindered by vandalism and intimidation. Individuals sabotaging laboratories and harassing researchers violate not only the law but also damage people who today are suffering from terminal sickness by physically injuring researchers.

Animal Use in Research: Ethical Concerns

The ethical considerations of animal experimentation are always paramount over scientific concerns, aside from sufficient technical knowledge. Each researcher should be well-versed in the animal model being utilized and the biology and behavior of that species. Researchers should be aware of the importance of their work and evaluate all of the premises that provide each research with a sound scientific foundation.

The theory of 3R also emerged from the United Kingdom. A young zoologist, William Russell, who also worked as a psychologist, and Rex Burch, a microbiologist who introduced ethical aspects using laboratory techniques, produced a report that served as the foundation for the 3Rs theory (Russell and Burch 1959). Each R represents an ethical guideline regarding the use of animals in research.

Reduction is a method that allows a protocol to use a smaller number of animals. It is possible to accomplish this by planning the experiments in detail, ensuring that the results will have statistical significance.

Refinement includes using methods that avoid animal suffering, such as the use of anesthesia during a procedure and an analgesic regimen for pain relief during recovery; use of noninvasive techniques; training animals to cooperate with procedures, and providing a comfortable and secure environment for their housing.

Replacement for the use of animals is the major goal in science. This involves replacing other models, such as bacteria or other invertebrates, cell cultures, organs, or even cellular fractions, which can be used instead of animals. A technique done without the use of animals would be the perfect substitute.

The 3R principles have become ubiquitous and are used to govern animal research in many nations. The scientific community is becoming more committed to implementing Russell-in Burch's use of experimental animals; Russell and Burch (1959) advocated "reduction, replacement, and refinement."

Alternatives to Animal Research

According to a theory, many animal studies might be replaced with trials that yield equivalent information without employing animals. Tissue cultures, microbes, and computer simulations are currently unable to substitute animal research. Researchers have replaced some animal trials, and the hunt for alternatives is still ongoing. But if scientists can replace large numbers of animal experiments with experiments that do

not use animals, they will because animals are expensive and difficult to use and because scientists do not want to experiment unnecessarily on animals. The best way for researchers to obtain pertinent information is through the use of animals.

The number of animals used in experimentation is being reduced, which is a positive step forward. In the process of testing consumer products, primarily drugs, chemicals, and cosmetics, animals, primarily rats, and mice are used. Many scientists are actively examining ways to minimize animal use in testing even more, and these efforts can ultimately remove the need for animals in research.

Options include several possibilities. An alternative is characterized in the scientific community as decreasing the number of animals used, modifying experimental designs to keep the animals from suffering pain or discomfort, and excluding other animals or approaches (Russell and Burch 1959). As a result, an option might still entail using animals, but with fewer of them or in other ways.

Most researchers usually believe that non-animal experiments are helpful rather than a substitute for animal experiments. Non-animal studies can yield a lot of useful information, but they cannot entirely replace the data acquired from animal research. Only animals may be observed to have the effects of a disease, damage, therapy, or prevention on a complex organism. For instance, certain elements of the etiology, treatment, or blindness prevention cannot be examined in bacteria because of lack of eyes; Protozoa lack heart or blood vessels, so they cannot be used to study hypertension or arthritis. Tissue culture cells are also required to study bone and joints.

Using new experimental methods can often reduce the need to use animals, especially when testing for toxicity of new chemical compounds. For example, new chemical compounds are now frequently assessed and not given to animals in cell cultures when they are risky. But animals cannot be replaced by cell cultures. Even if a chemical in the cultivation of cells is harmless, the effect on a complex creature must be assessed by an animal. In the same way, computer modeling and other non-animal alternatives can complement animal experiments more and more, rather than replacing them.

Conclusion and Perspective

Animal research is currently the backbone of biomedical research; its translational value should be improved as much as possible for significant scientific breakthroughs in uncovering human diseases and improving healthcare. The selection of a proper animal model is vital to biomedical research's success. Good quality of life is imperative for every species used in biomedical research. The needs of an animal must be met both biologically and behaviorally for good science to take place. Developing new drugs, understanding disease pathways, and designing vaccines, an animal model is crucial. Infectious diseases have been studied using animal models for decades, and new therapies have been discovered because of that use. Nearly all the therapeutics currently being used depend on animal models for preliminary safety and effectiveness testing, and human testing for risky or ineffective therapies has therefore been reduced.

To translate results into real-world products, researchers should identify models that closely mimic the target species and should not be driven by low cost or easy handling. This saves money, time, and resources in the long run. To better understand or discover novel therapies, future investigators should embrace a variety of animal models – flies, zebrafish, mice, rats, pigs, birds, rabbits, etc. Laboratory animal research protocols are ethically justified by scientists' desire and responsibility to reduce the number and use of animals in their research and perhaps replace animals fully. A trusting relationship between scientists and society will be built through this process. The next generation of scientists should also be educated on how to think about both ethics and science. In terms of animal welfare, it is clear that good science must be coupled with ethics to improve biomedical research involving animals. Today, by not utilizing the animals in the study, Future men's health and welfare should not be jeopardized. The benefits of each animal model will be combined to improve understanding of all these aspects, thus paving the way for future animal models. In conjunction with different technologies and efforts, more efficient models can be developed to study vaccine development. A carefully controlled animal model study will continue to be a valuable tool for identifying new antibiotics and improving the dosing regimen of existing antibiotics. Considering the possible harm that research may do in future generations, it would be immoral and egoist to stop utilizing animals in research now. Experiments using animals must be based on ethics and integrity and certainly must justify using animals. To the best of their abilities, researchers should use animals ethically and responsibly in their academic activities, contributing to the spread of knowledge while remaining attentive to legal principles. In an unified effort, providing better conditions and treatment for animals still used in research would reduce the number of animal experiments while simultaneously improving the quality of life for everyone.

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Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Declarations No animal and human data are involved.

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Cancer Study: Cell to the Animal Models

2

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Contents

Abstract

Cancer still represents one of the most common causes of death, despite remarkable oncology progresses. Based on the new research findings, this up-to-date chapter is reviewing the literature data regarding the hallmarks of cancer cells and their study provided by the use of animal models. Interesting data have arisen in scientific literature regarding functional genomics and proteomics, which provide

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novel knowledge useful in the development of modern treatment strategies. Considering the theory of cancer stem cells (CSCs), this distinct population is responsible for tumor promotion, growth, metastasis, recurrences, chemoresistance, and drug resistance to the routine oncological and radiotherapy treatment protocols, and, as a consequence, the mechanisms involved in these processes are currently under research. An important mechanism involved in tumor progression, in association to stemness, is represented by epithelial-mesenchymal transition (EMT), used by cancer cells to improve their invasive abilities. Another well-studied molecular phenotype in cancer research is that of microsatellite instability (MSI), which is important in colorectal cancer and endometrial or gastric cancer subtyping and management. Cancer research may use cell lines, experimental models, and patient-derived tumor xenografts (PDTXs) to assess the therapeutic performance of potential anticancer agents. Currently, the treatment and diagnosis of several types of cancer can be significantly improved by using nanomaterials, considering their biological and chemical properties. Nanomedicine has a huge potential in oncology, opening promising perspectives in cancer diagnosis and management.

Keywords

Genomics · Proteomics · Cancer stem cells (CSCs) · Epithelial-mesenchymal transition (EMT) · Cell lines · Experimental models · Patient-derived tumor xenografts (PDTXs) · Nanoparticles

Introduction

Despite considerable progress in oncology, cancer still represents one of the most common causes of death over the world, with huge number of cases every year (i.e., approximately 10 million deaths in 2020) which is expected to increase by 2040 (Catara et al. 2021; Sung et al. 2021).

It is well known that an effective treatment plan includes the multidisciplinary team, with specialities such as surgery, histopathology, oncology, immunotherapy, and radiotherapy. A successful oncological treatment depends on the understanding of the effects and mechanisms of the disease on one side and the development of an adequate therapeutic method correlated with genetics and molecular pathways on the other. Current cancer research is based on functional genomics and proteomics, which help in the understanding of complex molecular signalling pathways and the development of strategies for prevention and treatment (Swanson et al. 2004).

Currently, two possible theories are recognized with respect to the occurrence of carcinogenesis: the stochastic model, on one side, which claims that each cancer cell has an equal ability to initiate and support tumor growth and the theory of cancer stem cells (CSCs), on the other, which considers that tumors have a hierarchical organization and only CSCs have a carcinogenic potential (Samonig et al. 2020).

CSCs form a population of neoplastic cells that are responsible for tumor promotion, growth, metastasis, and recurrences. Furthermore, CSCs possess

chemoresistance and drug resistance to the routine oncological and radiotherapy treatment protocols. This makes them a major concern in therapy. For example, their high amount in histological examination after neoadjuvant chemoradiotherapy, in locally advanced colorectal cancer, was associated with a lack of treatment response and poor prognosis (Zhao et al. 2016).

A well-studied molecular phenotype in cancer research is microsatellite instability (MSI). MSI has been reported in approximately 15% of sporadic colorectal cancer and endometrial or gastric cancer and at lower frequencies in other cancers, such as ovarian cancer, prostate cancer, or glioblastomas (Cortes-Ciriano et al. 2017).

It is already known that molecular genetic experiments are permanently strengthening our knowledge, by the study of functional genomic and proteomic characteristics and their associations responsible for neoplastic phenotypes.

Both in vitro and in vivo studies are widely used in order to improve the spectrum of anticancer therapies, considering the analogies with human tumors molecular pathways involved in development and progression (Ferone et al. 2019). Most in vivo studies are using mouse models providing important data for cancer biology (Hagerling et al. 2019).

Medical cancer research uses cell lines as one of their main tools. They are used to assess the therapeutic performance of potential anticancer agents due to low price and synchronous tumor development. There are two methods used to obtain transplantable cell line: from mice (allografts) and from human cancers (xenografts). These models are useful in quick testing of probable cancer and metastasis-associated genes, as well as for drug testing in preclinical stages.

Patient-derived tumor xenografts (PDTXs) are the result of the transplantation of human tumor biopsies in immunodeficient mice. The main advantage of PDTX is the conservation of genetic, molecular, and histological diversity usually found in cancer, even after transplantation in mice (Hidalgo et al. 2014).

The treatment and diagnosis of several types of cancer can be significantly improved by using nanomaterials, considering their biological and chemical properties. Moreover, nanotechnology reveals multiple opportunities in oncology, such as in vivo bioimaging, drug delivery, and detection of the molecular markers, or in diagnosis and imaging (Augustine et al. 2021). Nowadays, there are multiple types of nanoparticles which can be used in cancer nanomedicine.

From improving the effectiveness of therapies and finding new imaging techniques, nanotechnology has a huge potential in oncology. Its potential is promising in cancer diagnosis and management.

Proteomics of Cancer Stem Cells, Molecular Pathways, and Epithelial-Mesenchymal Transition (EMT)

The first acknowledgment of CSCs was done in 1997, when Dick et al. transplanted CD34 and CD38 acute myeloid leukemia stem cells in severe combined immunedeficient mice, leading to initiation of acute myeloid leukemia in hosts (Kim and Ryu 2017). Moreover, several experiments were focused on CSC populations, which

were detected in different tumor tissues, thus demonstrating their contribution in cancer initiation and progression (Barbato et al. 2019).

There is no doubt that the assessments of CSC involvement may allow a more rational understanding of the intracellular processes of cancer, such as cellular differentiation, growth, motility, proliferation, malignancy, survivability, and death (Buzdin et al. 2021). Several studies have tried to investigate the malignant properties of CSCs, in order to discover novel candidates for new targeted therapies and for controlling the progression of cancer (Barbato et al. 2019). Additionally, there is a need for a better understanding of the molecular regulation of malignant mechanisms of CSCs for each type of cancer, in order to identify those markers valuable for diagnosis, prognosis, and development of individualized targeted therapies.

In order to investigate the malignant properties of CSC, different cancer studies tried to identify useful tools in order to describe CSC-like stem cell markers. Several stem cell markers, including CD34, CD44, CD123, CD133, ABCG2, ALDH, Nanog, and c-kit, were isolated from a large variety of malignant solid tumor (breast, ovary, colon, gastric, and pancreatic cancer), according to literature (Bao et al. 2013). CD133 is one of the most studied cell markers and was identified in CSC population from solid tumors, including brain, ovary, pancreas, breast, stomach, and colon cancer, but also identified in neural and hematopoietic stem cells. Its expression was detected in 44 normal human tissue and 22 of 82 cell types (Huang et al. 2020). Currently, as a result of the accumulated data, several markers of common types of cancer are known, as following: CD44+, CD24−, and ALDH+ in breast cancer; CD44+, a2b1+, and ALDH+ in prostate cancer; CD133+, CD44+, ALDH+, and CD117+ in lung cancer; and CD133+, CD44+, CD24+, and ESA+ in pancreatic cancer (Barbato et al. 2019).

The main challenge in cancer treatment is chemoresistance and recurrence after chemotherapy, which lead to poor prognosis and survival in cancer patients. The tumor resistance is frequently associated with the presence of aggressive cancer cells that have strong mechanisms of chemoresistance, like atypical initiation and aberrant development of variable oncogenic signalling pathways (Catara et al. 2021).

There is an extensive range of factors with significant involvement in CSC radiochemotherapy resistance, including a high ability of DNA repair mechanism, increased level of ATP-binding cassette (ABC) transporters, resistance to apoptosis, and increased levels of aldehyde dehydrogenases (Barbato et al. 2019) (Fig. 1).

Another widely studied factor which determines CSC drug resistance is epithelial-mesenchymal transition (EMT), as a change of epithelial phenotype into a mesenchymal and migratory phenotype. The entire process is associated with loss of epithelial hallmarks, such as adherence junction proteins E-cadherin and β-catenin and an increase in fibroblast-like cells, along with mesenchymal markers expression, such as vimentin and fibronectin (Grassi et al. 2017).

The literature suggests different mechanisms of EMT involvement in drug resistance, such as stimulation of angiogenesis; decreased reactivity to proapoptotic signals; reduced immune response; enhanced DNA repair, in addition to a reduction in DNA damage; and an enhanced activity of the export pumps, which are responsible for drug elimination from cells (Williams et al. 2019). EMT process is

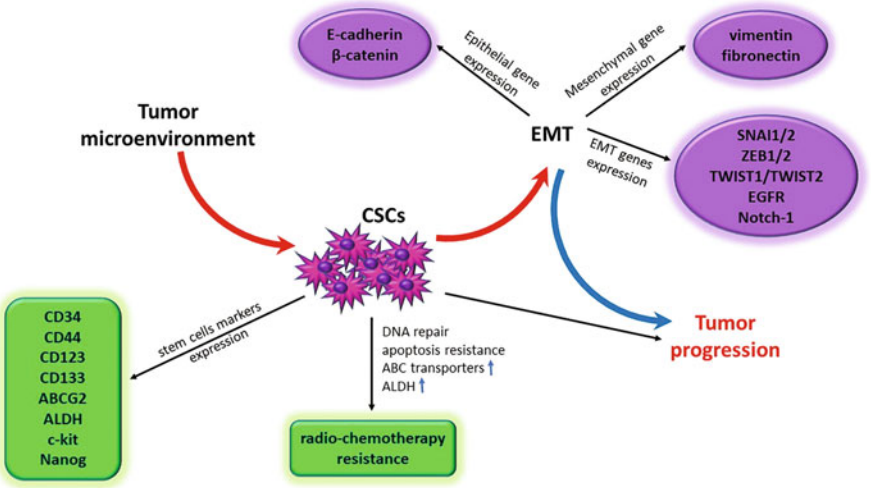

Fig. 1 Cancer stem cells' influence in tumor microenvironment and epithelial-mesenchymal transition. Cancers stem cells induce tumor growing by upregulation of epithelial and mesenchymal gene expression that induces the epithelial-to-mesenchymal transition. Supplementary, an increased level of ATP-binding cassette (ABC) transporters, resistance to apoptosis, DNA repair mechanism, and increased levels of aldehyde dehydrogenases contribute to their radio- and chemotherapy resistance in tumor microenvironment. *ABCG2* ATP-binding cassette super-family G member 2; *ABC transporters* ATP-binding cassette transporters; *ALDH* aldehyde dehydrogenases; *CD* clusters of differentiation; *c-kit* tyrosine-protein kinase Kit (CD117); *CSCs* cancer stem cells; *DNA* deoxyribonucleic acid; *E-cadherin* epithelial cadherin; *EGFR* epidermal growth factor receptor; *EMT* epithelial-to-mesenchymal transition; *Nanog* Nanog homeobox; *Notch-1* Notch homolog 1; *SNAI1/2* snail family transcriptional repressor 1/2; *TWIST1/TWIST2* twist family BHLH transcription factor 1/2; *ZEB1/2* zinc finger E-box binding homeobox 1/2; *β-catenin* beta-catenin; ↑ increased level

coordinated by the transcription regulators containing zinc finger proteins (SNAI1/2 and ZEB1/2) and helix-loop-helix proteins twist (TWIST1/TWIST2). Moreover, microRNAs (miRNAs) and growth factors also act as a trigger of EMT initiation (Vergara et al. 2016). For instance, epidermal growth factor (EGFR) is acting like an EMT promoter in variable types of gynecological cancers, including endometrial or ovarian cancers, with high levels of EGFR expression being correlated with a poor prognosis and a low rate of survival (Grassi et al. 2017).

The result of EMT process is associated with cancer cell dissemination and metastasis in distant organs and resistance to oncological treatments and targeted therapy (Grassi et al. 2017) (Fig. 1). In order to identify optimal therapies against chemoresistance, EMT resistance mechanisms have been widely investigated in several studies, showing that blood of patients with distant metastasis contains circulating tumor cells which co-manifested stem cell markers and EMT markers (Phi et al. 2018). Therefore, cancer cells acquired stem cell-like properties during the initiation of EMT or stimulation of EMT markers and EMT transcription factors (Phi

et al. 2018), in correlation with metastasization or chemoresistance, respectively. For instance, activation of EMT transcription factors, like Notch-1, induces resistant lung cancer cells to gefitinib, an EGFR inhibitor (Williams et al. 2019). Similarly, EMT leads to drug resistance in ALK-positive lung cancer, which is treated with a targeted agent, named crizotinib (Williams et al. 2019).

Meanwhile, a strengthened expression of EMT markers and increased malignant abilities, such as invasiveness and chemoresistance, could be predictor of anti-EGFR antibody response (Williams et al. 2019).

Reduced E-cadherin expression, correlated to EMT, and miR-200c have been correlated with poor prognosis, low rates of cancer-free survivability, and a lack of response to neoadjuvant radio-chemotherapy, in rectal cancer (Williams et al. 2019). In addition, previous data revealed that radiotherapy can enhance the rate of recurrences through the initiation of EMT process in patients with rectal cancer, while the level of EMT-positive circulating tumor cells is higher in non-small-cell lung cancer, after radiotherapy (Petrova et al. 2016; Williams et al. 2019).

The loss of expression of E-cadherin, a tumor suppression protein, results in cell detachment from the tumor and invasion of surrounding tissues and dissemination to distant organs (Petrova et al. 2016). In order to identify the clinical relevance of EMT proteins for development of targeted therapies, variable studies have associated the loss of E-cadherin expression with clinical profile of multiple tumor types. Thus, in squamous cell lung cancer, increased E-cadherin expression can be a predictor for disease-free survival and overall survival (Petrova et al. 2016). Similarly, highly E-cadherin loss is associated with poor prognostic for patients with oral squamous cell carcinoma (Petrova et al. 2016). Other examples are the correlation between E-cadherin and the poor differentiation grade in esophageal cancer, while E-cadherin is revealed as a predictor factor for overall survival in gastric cancer (Vergara et al. 2016).

Another marker of EMT process is vimentin, a component of the intermediate filament (IF) family of proteins, a component of normal mesenchymal cells, involved in cellular integrity maintenance. A high expression of vimentin has been found in numerous types of neoplasms, such as breast, endometrial, prostate, lung, and gastrointestinal cancer and malignant melanoma. There are multiple evidence which highlight that neoplasm's vimentin high expression has a stimulating effect of growth and invasiveness, making it an important factor of poor prognosis (Satelli and Li 2011). Therefore, the expression of vimentin is a marker of poor outcome, in epithelial cancers, being correlated with the undifferentiated histological grade, chemoresistance, and disease progression (Satelli and Li 2011). The role of vimentin in cancer prognosis has been also reported in triple-negative breast cancer, where increased levels of vimentin are correlated with younger patients and a high level of proliferation marker Ki-67 (Satelli and Li 2011). Similar data has been reported about the role of vimentin as a prognostic marker in erlotinib (EGFR tyrosine kinase inhibitor) treatment of patients with non-small-cell lung cancer and a negative predictor for overall survival in squamous histology type (Vergara et al. 2016).

More than EMT protein role in maintaining the cells' adherences and transcription elements, other potential functions may be identified due to the complexity of

these processes. Due to the clinical and pathology diversity, as well as the cancer-to-cancer biological variation, there is a certain need for multiple studies to focus on EMT markers. Since the main benefits of cancer markers are for diagnosis, screening, and monitoring of disease progression, potential EMT biomarkers can be determined by proteomics, offering the opportunity to discover new challenges in the area of EMT (Vergara et al. 2016).

Proteomics of Cancer Cells and Microsatellite Instability

New research progress in the proteomics and genomics of cancers revealed biomarkers which can be good candidates for a better understanding of carcinogenesis pathways. This could allow medical decisions to be more accurate when it comes to targeted therapies, by correlating the genetic profile with the molecular phenotype of a tumor (Alves Martins et al. 2019).

MSI appears when insertion or deletion mutations cause a large number of errors in repetitive sequences. This is a result of the damaged DNA repair mechanisms, which leads to abnormal mismatch repair activity (MMR) (Michalak et al. 2020) (Fig. 2). In this repair process, proteins, such as MSH2, MSH6, MSH1, and PMS2, are used to repair MSI (Michalak et al. 2020).

Germline and somatic mutations in these proteins (mainly MLH1 and MSH2) are the results of a deficiency in the MMR system and is usually detected in hereditary nonpolyposis colorectal cancer (HNPCC), also named Lynch syndrome (Shirazi and Sepulveda 2018). Besides, germline 3-end deletions in EPCAM gene are implicated in MSH2 methylation (Shirazi and Sepulveda 2018).

There are two different mechanisms involved in the occurrence of MSI in hereditary and sporadic colorectal cancer. A germline mutation in a mismatch repair protein as well as a more than 90% deficiency of the MLH1 and MSH2 mismatch repair genes has been detected in HNPCC (Soreide et al. 2009). MSI is the consequence of MLH1 expression loss, determined by epigenetic silencing, in sporadic colorectal cancer (Soreide et al. 2009).

Literature data revealed that MSI status, especially high levels of MSI (MSI-H), is reported as a prognostic biomarker in colorectal cancer due to its molecular alterations (Saeed et al. 2021). Thus, the detection of mismatch repair deficiency and microsatellite status is considered a rational algorithm in the management of patients diagnosed with colorectal cancer. The main approach used for the investigation of MMR insufficiency in malignancies is the immunohistochemical examination, which establishes the expression of the four major MMR proteins (MLH1, MSH2, MSH6, and PMS2) and polymerase chain reaction (PCR) to assess MSI status (Shirazi and Sepulveda 2018). It is worth mentioning the limitations of these methods. Thus, immunohistochemistry is not able to identify cases when the protein has a qualitative dysfunction, but it is still antigenically active and is prone to false-deficient MMR results due to variable patterns in MSS tumors or errors in other proteins which are part of the MMR pathway (Shirazi and Sepulveda 2018). MSI PCR method is a comparative test between errors in the length of microsatellite short

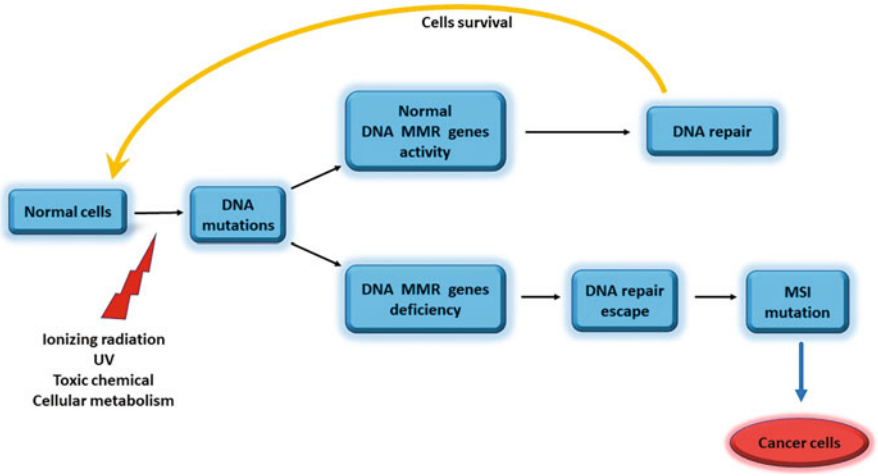

Fig. 2 Relation between microsatellite instability and proteomics tumor cells. DNA damages in normal cells induce overexpression of MMR genes that contribute to DNA repair and cell survival. MMR system deficiency is associated with DNA repair escape, MSI mutation, and tumor progression. *DNA* deoxyribonucleic acid; *MMR* mismatch repair activity; *MSI* microsatellite instability; *UV* ultraviolet light radiation

repeats in the tumor and the normal tissue, and it is capable to detect the functional errors of the proteins involved in the MMR pathway. Each sample of DNA extracted from tumor is strengthened by PCR applied to a reference panel (NCI-MSI panel), according to the recommendations of the National Cancer Institute, from 1997 (Valeri et al. 2014). NCI-MSI panel has two types of markers, including three dinucleotides (D2S123, D17S250, and D5S346) and two mononucleotides (BATS25 and BATS26), and according to it, a tumor can belong to one of the following categories: MSS (no markers of instability), MSI-L (low microsatellite instability or instability less than 30%), and MSI-H (high microsatellite instability or instability in 30% or more) (Hong et al. 2012).

MSI has been widely investigated in different types of cancers, such as colorectal, gastric, or endometrial cancer. There are three clinical features which highlight the importance of MSI phenotype in clinical settings, as following: the screening marker for Lynch syndrome, its prognostic value in colorectal cancer and also in other tumors, and its value as an indicator of response to chemotherapy (Sepulveda et al. 2017). Recent studies have demonstrated the role of MSI as an initiator of immune checkpoint blockade, and, thus, their expression is useful for the identification of the tumors that may have a good response to immunotherapy (Lee et al. 2016). In this direction, Cortes-Ciriano et al. assessed the frequency of MSI in a study on 7919 tumors, including 23 types of different cancers (Cortes-Ciriano et al. 2017). The findings of the study revealed a different MSI frequency between different tumors, up to 28.3% in endometrial adenocarcinoma and less than 0.2% in lung adenocarcinomas (Cortes-Ciriano et al. 2017). Furthermore, five types of tumors were named

MSI-prone tumors (such as esophageal, gastric, colon, rectal, and endometrial carcinoma), which are associated with a high frequency of MSI compared with other malignancies (Cortes-Ciriano et al. 2017). Similarly, Bonneville et al. analyzed the frequency of MSI in 27 different tumor types, and they found a significant variation in the prevalence of MSI events between cancers, ranging from 0.25% in glioblastoma multiforme to 31.4% in endometrial adenocarcinoma (Bonneville et al. 2017).

To date, it is certain that MSI has prognostic and predictive implications in different types of cancer, mainly in colorectal cancer. It is already known that 85% of sporadic CRC occurs due to chromosomal instability and approximately 15% of cases are the results of MSI phenotype. MSI colorectal cancer has an individual phenotype, particularly in cases with MSI-H, which are characterized by diploid DNA, low differentiation histological grade, mucinous cell type, and infiltration with tumor lymphocytes. Furthermore, there is data which shows that cases with MSI-H phenotype have a better prognosis than those with MSS phenotype owing to a lower recurrence rate and early-stage diagnosis (Hong et al. 2012). The improved prognosis of MSI-H is the result of an increased antitumor immune response with increased tumor-infiltrating T cells, which respond to specific tumor antigens. This was partly a result of the production of truncated peptides, with role of neoantigens, because of the frequent frameshift mutations (Shirazi and Sepulveda 2018). The neoantigens are recognized by cytotoxic T cells and induce an antitumor immune response and an upregulation of some immunomodulatory genes (antigen chaperone molecules, proinflammatory cytokines, and cytotoxic mediators) (Shirazi and Sepulveda 2018). Moreover, MSI was found to be a factor of nonresponse to 5-flurouracil-based chemotherapy in locally advanced stage colorectal cancer, but the mechanism is not completely understood. Thus, further studies are needed to assess the response of 5-FU chemotherapy regimen in MSI colorectal cancer (Sargent et al. 2010).

MSI phenotype exhibits different molecular characteristics that may represent a useful tool for a future targeted therapy, in gastric cancer. Usually, the patients with MSI gastric cancer are women diagnosed at an older age but with a better overall survival rate (Zhu et al. 2015). In a meta-analysis on 1976 patients diagnosed with gastric cancer, Zhu et al. investigated the MSI frequency which ranged from 11.68% to 33.82% (Zhu et al. 2015). Moreover, patients with MSI phenotype were found to have a better prognosis, with limited lymph node metastasis and decreased mortality rate (Zhu et al. 2015). The main mechanism of MSI gastric cancer is correlated with *Helicobacter pylori* chronic gastritis, associated with atrophic gastritis and intestinal metaplasia, according to literature data (Zhu et al. 2015).

In recent years, different studies have highlighted the ability of MSI to predict immunotherapy potency, emphasizing the significant association between MSI and programmed cell death-1 (PD-1) and programmed death-ligand 1 (PDL-1). Based on clinical information, it was demonstrated that MSI-H and MMR are strongly correlated with a good prognosis in colorectal cancer and other cancers which are treated with immune checkpoint inhibitors (Zhao et al. 2019). According to the US Food and Drug Administration (FDA), a tumor with MSI-H expression can be treated with immune checkpoint blockade (ICB) therapies, especially PD-L1and PD-1 (Zhao

et al. 2019). Thus, pembrolizumab, the anti-programmed cell death-1 inhibitor, is available for MSI-H metastatic cancer, while nivolumab is the preferred treatment for patients with MSI-H colorectal cancer (Zhao et al. 2019).

Numerous studies tried to investigate the impact of pembrolizumab in patients with MSI-H tumors, due to its ability to predict therapeutic response. In a clinical trial, the results of pembrolizumab administration in patients with refractory MSI-H tumors after standard chemotherapy were studied (Zhao et al. 2019). The results of this clinical trial were significant, showing a duration of treatment benefits yielding from 1.6 to 27 months and overall response rate of 39.6% (Zhao et al. 2019). Due to a stable response and long survival rates, FDA proposed pembrolizumab as a second-line medication for inoperable or metastatic dMMR (mismatch repair deficiency)/MSI-H cancers (Zhao et al. 2019).

Similarly, another study analyzed the efficacy of nivolumab administered in dMMR/MSI-H metastatic colorectal cancers, in patients treated with standard chemotherapy, such as fluoropyrimidine, oxaliplatin, or irinotecan (Lemery et al. 2017). Nivolumab showed a significant clinical benefit, with overall survival rate being 73% and progression-free survival at one year 50% (Lemery et al. 2017). Based on these results, FDA approved nivolumab for metastatic colorectal cancer with MSI-H, in patients who had an inappropriate response to the first line of chemotherapy (Lemery et al. 2017).

Multiple studies have demonstrated that nivolumab combined with ipilimumab is more potent than nivolumab alone in the treatment of patients with melanoma or small-cell lung cancer (Chalabi et al. 2020). The patients who received nivolumab and ipilimumab were free from cancer progression and had a 71% survivability rate after one year, and the overall survivability was 85% (Chalabi et al. 2020). Furthermore, the efficacy of nivolumab and ipilimumab combination was assessed in another trial, as a preoperative therapy in resectable colon cancer with dMMR or pMMR (mismatch repair proficient) (Chalabi et al. 2020). Impressive results were observed in 100% of patients with dMMR colorectal cancer, while pMMR patients had no response (Chalabi et al. 2020). It is obvious that further investigations are mandatory and necessary, since immunotherapy has an inspiring and promising benefit in MSI-H tumors (Yi et al. 2018).

Genomics and Cell Lines for Animal Models and Genetic Modification Technologies in Cancer Models

Considering the importance of genetics in the therapy management of cancers, different experimental models provide useful data which may be extended to oncologic patients' prognosis and treatment.

Nowadays, in vitro studies are widely used to obtain precious cellular and molecular information, even if there is an inability to describe the entire pathological interactions within a living entity. The main aim of animal models and in vitro cell line testing is to simplify the process of anticancer therapy development, since

in vivo tumor model is a great instrument which mimics the morphology and genetics of cancer (Ferone et al. 2019).

More than 95% of in vivo studies are using mice considering their genomic heterogeneity and physiological similarity to humans, resulting in simplification of the investigations of cancer genetics, environment or epigenetics, and molecular transformations under oncological conditions (Onaciu et al. 2020). Mice are considered an excellent experimental model due to their small size, easy care, and their fast reproduction rate due to a short gestation time. Though mice and humans have some similar characteristics, one of the fundamental differences between the two species is the tumors' origin. While mice usually have sarcomas or lymphomas of mesenchymal origin, humans develop tumors of epithelial origin like lung, breast, or colon cancer. Genetic studies show that mouse models are an important tool for the understanding of cancer biology, especially when considering the following three models: humanized, transplantable, and genetically engineered tumor models (Hagerling et al. 2019).

Chorioallantoic membrane (CAM) represents another attractive in vivo animal model in preclinical cancer research, being rapid and reproducible and with a good cost-efficient ratio. Used since the 1980s as a tool to study cancer metastasis, CAM presents an important vascular network that provides a valuable mean for the study of tumor angiogenesis and analysis of novel angiogenic factors. Hitherto, CAM has been used to assess tumor growth and progression in several types of cancers, such as pancreas, ovarian, breast, prostate, and head and neck carcinomas, as well as the antitumoral activity of cancer therapeutics, such as cisplatin, cyclophosphamide, aloin, paclitaxel, mitomycin C, vincristine, actinomycin-D, camptothecin, and melphalan (Lokman et al. 2020).

Numerous mouse models were proposed for variable types of tumors. Usually, studies have used both immunocompetent and immunodeficient mice with xenografted tumor characteristics. Generally, the tumor cells are transplanted subcutaneously or orthotopically and are generally used for tumor generation and relapse testing (Lampreht Tratar et al. 2018). Literature reports describe variable methods for transgenic mice generation, which are using the addition of DNA sequences into the genome of the animal models (Lampreht Tratar et al. 2018). Three of the most known methods are (1) spontaneous mutations, spontaneously arising in breeding colonies, (2) induced mutations by chemical factors or radiation, (3) retroviral infection, and (4) microinjection of DNA agents (Doyle et al. 2012).

The first model based on spontaneous mutations mentions Hermansky-Pudlak syndrome, which contains oculocutaneous pigment mutations and severe B and T cell dysfunction which are involved in severe combined immunodeficiency (SCID). Despite its advantages, e.g., low cost in introduction of mutation, this method is limited because it requires a large validation of the unique role of the alteration and there are difficulties in detecting if the mutation is associated or not with phenotypic changes (Onaciu et al. 2020).

The approach of using a retroviral infection has the advantage of inserting individual genes, but it is limited by the size of the gene which a vector can accommodate (Onaciu et al. 2020). The method is applied on infected embryos,

implanted into female mice, which are evaluated for the presence of genetic alterations. This technique is not widely used because the expression of the viral genes and their insertion into the mice genome are influenced by the mechanism of novel DNA methylation (Onaciu et al. 2020). Similarly, the method of DNA microinjection is based on the direct introduction of DNA agents into a fertilized embryo, followed by its transfer into a recipient female. The samples are analyzed for the presence or absence of the transgenes (Onaciu et al. 2020). The mutations induced by the last method allow the evaluation of the capacity of different environmental agents to cause neoplasia after mice exposure to different carcinogens, like radiations, viruses, or hormones (Ferone et al. 2019). The induced cancer in animal models is widely used in laboratory tests due to the ability to mimic human tumor types, which result following environmental exposure to carcinogens (Onaciu et al. 2020).

The combination of carcinogens, such as irradiations, chemicals, and viruses, gene editing, or homologous recombination can be the main strategy for cancer induction in laboratory mice (Ferone et al. 2019). A common method easily performed in laboratory is the induction of local tumors, by applying a topical carcinogen, e.g., squamous cell carcinomas induced in mice by exposure to ultraviolet light (Ferone et al. 2019). Similarly, the association between the initiator 7,12-dimethylbenz[a]anthracene (DMBA) and the promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) can generate papilloma and squamous cell carcinoma (Ferone et al. 2019). Furthermore, other laboratory methods are also used, to obtain not only skin cancers but also colon cancers (by association of azoxymethane and dextran sodium sulfate, as a proinflammatory agent), lung cancer (by urethane), and breast cancer (by association of DMBA and progesterone receptor stimulators) (Ferone et al. 2019).

As a result of recent progress in genetics and oncology, a relatively new method, genetically engineered mouse models (GEMMs), is approved for the alteration of different oncogenes and tumor suppression genes, which initiate spontaneous mice tumors (Hagerling and Werb 2016). Furthermore, spontaneous tumor growth was found to reveal a multitude of stages involved in human tumor development and the components of the tumor microenvironment (Hagerling and Werb 2016).

GEMM mostly uses tissue-specific promoters to lead to the expression of a viral oncogene, like SV40 large T antigen, or oncogenes involved in cancer initiating, such as BRAFV600E, in malignant melanoma or Kras and MYC in breast cancer (Olson et al. 2018). Moreover, these models are also utilizing the expression of recombinase enzyme, for example, APC in colon cancer or PTEN in prostate cancer, to initiate deletion of tumor suppressors (Olson et al. 2018). Studies report more examples of genetically engineered models, such as RIP-Tag2 and KrasGD12 in pancreatic cancer, MMTV-PyMT and MMTV-Neu in breast cancer, or K14-HPV16 in papilloma (Hagerling and Werb 2016).

The results of these genomic alterations can lead to the development of either autochthonous invasive cancer or precancerous lesions, such as pancreatic or prostatic intraepithelial neoplasia (Olson et al. 2018). GEMM is also appropriate for the analysis of the interplay between cancer cells and immune cells and the interactions

between innate immune cells and adaptive cells over tumor growth (Olson et al. 2018). More than that, GEMM method is a useful tool to analyze the role of neutrophils and different genes which are expressed by them in cancer development. In a recent study reported by Finisguerra et al., the deletion of the hepatocyte growth factor receptor, Met, reduces the amount of antitumor neutrophils but not the protumoral type (Finisguerra et al. 2015). Met is considered a precondition for the cytotoxic status of antitumor neutrophils, with an important role in tumor growth decrease and metastasis development (Hagerling and Werb 2016).

GEMMs are created by several methods, such as (1) mouse embryo manipulation, (2) Cre-Lox recombination, (3) hydrodynamic injection, and (4) CRISPR-Cas9 (Brown et al. 2018). The manipulation of the mouse embryo is done in two ways: either by pronuclear injection, the commonest method used to generate transgenic mice, by the injection of the needed genetic material into the fertilized egg nucleus, or by embryonic stem cell transfer (Brown et al. 2018). The embryonic stem cell transfer involves the selection of those embryonic stem cells which were modified with a DNA construct and introduced into the blastocysts (Brown et al. 2018). For example, there are three types of hepatocellular carcinoma, based on DNA sequencing and mutation analysis (Brown et al. 2018). Moreover, GEMM has been developed by disabling the cellular tumor antigen p53, which in mice is encoded by Trp53-alone or in association with other genetic modification, for liver cancer (Brown et al. 2018). In the study reported by Katz et al., the method Cre-Lox recombination induced hepatic cancer after 14 months in AlfpCre +Trp53D2–10/D2–10-modified mice with deleted p53 (Katz et al. 2012). Liu et al. detected macroscopic tumors in mice infected with hepatitis B virus, as result of p53 deletion, in association with phosphatase and tensin homolog (Liu et al. 2017). More than that, this study also utilized hydrodynamic injection with the aim to introduce plasmids into hepatocytes, by injecting them into the tail vein of mice (Liu et al. 2017). The result of the hydrodynamic injection is an increased vena cava pressure and cellular permeability, which allows the transport of plasmids into the liver cells and an enhanced level of transgenes, in the animal model (Brown et al. 2018).

One of the most studied examples of cancer that has been modelled in mice is APCMin model, which causes approximately 80% of human colon cancer (Moser et al. 1995). Moser et al. demonstrated that the application of N-ethyl-N-nitrosourea can determine a nonsense mutation in codon 380 of the Apc gene (Moser et al. 1995). The method used was that of somatic inactivation of Apc, mediated by Cre-loxP (Moser et al. 1995). In that way, the activation of Cre-recombinase determined the deletion of all genes flanked by loxP recombination sites (Burtin et al. 2020). Furthermore, adenovirus-mediated delivery of Cre-recombinase was determined by the APC loss in intestinal epithelial tissue, followed by a spontaneous occurrence of colorectal adenomas, which have similar characteristics with adenomatosis polyposis coli (Burtin et al. 2020). Thus, the result of introducing mutations correlated with a particular type of neoplasia is a mouse model with similar molecular and clinicopathological characteristics of human tumors (Walrath et al. 2010).

Cell Line-Derived Mouse Cancer Models

Cell lines are used to test in preclinical phases potential anticancer agents or to identify genes involved in carcinogenesis and metastasis, by exploiting two methods to obtain transplantable cell line: mice allografts and human xenografts. For instance, xenotransplantation research is used in the study of colorectal cancer resistance mechanisms to vemurafenib (Kersten et al. 2017). The xenotransplantation model feasibility has been evaluated in a clinical trial where patients diagnosed with colorectal cancer were treated with a combination therapy targeting BRAF V600E and EGFR (Kersten et al. 2017).

Some studies based on xenografts were able to reveal different gene expression patterns that interfere with organ-specific models of metastatic colonization (Hernandez et al. 2016). The recognition of some elements correlated with disease subtypes and outcome in cancer cell lines led to the discovery of pathway-targeted therapeutic advantages. Some particularities discovered about gene expression patterns, mutations, or variations in the number of copies of cell lines led to classifying them in histologic subtypes (Hernandez et al. 2016).

Other studies identified two systems, which describe both cell lines and tumors at the DNA copy number, mRNA expression, and mutation levels, for a variety of cancer types. One of them, the Cancer Genome Atlas (TCGA), characterizes the expression profile of more than 500 tissue specimens per tumor form and their genome (Domcke et al. 2013). The other system, Broad-Novartis Cancer Cell Line Encyclopedia (CCLE), is comprised of genomic profiles of about 1000 cell lines and is used as a model for multiple tumor types (Domcke et al. 2013).

By comparing data from TCGA and CCLE, significant differences are revealed between some of the most frequently used cell line models for ovarian cancer (Maru and Hippo 2019). For example, A2780, CAOV3, IGROV1, OVCAR-3, and SK-OV-3 cell lines are used in the study of high-grade serous carcinoma (HGSC), the commonest histological subtype of ovarian cancer (Maru and Hippo 2019). Although TP53 mutation is a distinctive feature in HGSC in approximately 95% of cases, genomic profiling of A2780 and SK-OV-3 tests revealed that TP53 was intact (Maru and Hippo 2019). Moreover, mutations such as ARID1A, BRAF, PIK3CA, or PTEN, which were usually found in other subtypes of ovarian cancer, were also observed in HGSC (Maru and Hippo 2019). These results suggest that the histopathological origin is not yet fully deciphered and there is a need for adequately characterized cell lines as models for each subtype of ovarian neoplasia (Maru and Hippo 2019).

In prostate cancer study, some of the most popular prostate cancer cell lines were DU145, LNCaP, and PC-3 (Namekawa et al. 2019). In the last decade, new prostate cancer cell lines have been developed from prostate cancer xenograft models, metastases, tumors, and even from those three initially popular prostate cancer cell lines, principally the LNCaP cell line (Namekawa et al. 2019). It should be noted that LNCaP cells – LNCaP-abl and LNCaP-LTAD sublines – are androgen-insensitive, while the original LNCaP cells are androgen-sensible (Namekawa et al. 2019). Furthermore, a few multidrug-resistant prostate cancer sublines have been produced

by combining the original cell lines with cytotoxic drugs or antiandrogenic agents (Namekawa et al. 2019).

The drug resistance field has made impressive improvements with the help of studies which used cancer cell lines. Although facing a series of limitations, cell lines are widely used in large-scale studies on drug resistance in human cancer models (Dong et al. 2021). The key limitation of cell lines is that they were removed from their tissue of origin. This means they have fewer cytokine interactions and a different drug distribution and are no longer associated with other cell types normally found in that tissue (Weinstein 2012).

Using CCLE, Barretina et al. predicted the sensibility profiles of different types of neoplasia through the association between drug activity and mutations in special genes, such as AHR, IGF1R, NRAS, or SLFN11 (Weistein 2012).

Similarly, Garnett et al. analyzed different genetic aberrations, such as gene amplifications and deletions, point mutations, or microsatellite instability, for several cancer cell line variations (Garnett et al. 2012). The results of the study showed a gene-to-treatment relationship and a significant sensibility to inhibitors of PARPs (a group of DNA repair proteins) for Ewing's sarcoma cells with the EWS-FLI1 gene translocation (Weistein 2012).

Patient-Derived Tumor Xenografts (PDTXs) in Cancer Models

PDTXs have the advantage of preservation of the human tumors diversity, even after transplantation in mice (Hidalgo et al. 2014), being helpful in the study of numerous malignancies, including breast, pancreatic, colorectal, and prostate cancer and chronic lymphocytic leukemia (Lai et al. 2017). Supplementarily, PDX models are a valuable tool for the understanding of tumor biology and for the preclinical assessment of new therapeutic drugs used in different cancers.

PDTX models are analyzed in immunodeficient mice models like the athymic nude, NOD-SCID, SCID, or recombination-activating gene 2 knockout mice (Rag 2). They are used to generate xenograft cell lines and to develop transplantable tumor xenografts. In order to perform such roles, mice models must have a severe immunodeficiency. One example is the NOD/SCID model with IL2rg mutations (NSG), which has the capacity to engraft all cancer types, as a result of their amplified immunodeficiency (Lampreht Tratar et al. 2018).

Currently, PDTXs are usually used in mouse, chicken, and zebrafish animal models. In these studies, the human tumoral cells are represented by primary patient tumor material or cell lines, which are xenotransplanted on the animal model in an orthotopic manner (human cells are implanted into a similar site in the animal model) or heterotopic manner (human cells implanted into other sites, such as intraperitoneal injection of breast cancer cells) (Berman et al. 2014).

Previous studies have showed the importance of PDX models in cancer studies, by identification of different pathways involved in tumorigenesis and metastasis and also of gene expression profile, genetic mutations, or treatment tumor response. Due to their ability to mimic human cancer, PDX models can be used to describe cellular,

molecular, and sub-clonal profiles for different cancer types (Lai et al. 2017; Ferone et al. 2019). In this direction, Ebinger et al. identified a subpopulation of relapse-inducing cells in acute lymphoblastic leukemia (ALL), by using genetic engineering and PDX models. A dormant ALL subpopulation was also discovered to exhibit drug resistance. The analysis of single-cell and bulk expression from this population showed comparable features with ALL cells isolated from adult and pediatric individuals with minimal residual disease (Ebinger et al. 2016). It must be noted that the dormant cells became drug-sensible and started to proliferate when they were removed from in vivo setting (Ebinger et al. 2016). Another study evaluated the antiangiogenic effects of the first-line therapy, at the same time with the second-line therapy, by transplanting primary tumor biopsies or metastatic tissue from patients diagnosed with renal cell carcinoma into mice, with an engraftment efficiency of about 75%, after 5 months (Suárez et al. 2016). Interestingly, the same histological subtype of tumor and characteristics of the metastases were registered (Suárez et al. 2016).

The PDX model attributes as reference for tumor cell expansion and molecular agonist or inhibitor were demonstrated in the inhibition of Musashi (Msi), which is a critical agent for pancreatic cancer recurrence in patient-derived xenografts (Fox et al. 2016). Msi inhibition was associated with a reduction in the growth of primary PDX (Fox et al. 2016). Additionally, PDX preclinical investigations had a significant contribution in determining the right antitumor therapy for aggressive pediatric neoplasia (Nicolle et al. 2016). Nicolle et al. have analyzed pediatric liver cancer, by studying 24 tumor-derived xenografts including malignant rhabdoid tumors, hepatoblastomas, hepatocellular carcinoma, and transitional liver cell tumor (Nicolle et al. 2016). The engraftment rate for metastatic tumors was about 75%, while it was 33.3% for primary tumors (Nicolle et al. 2016). Furthermore, PDX models demonstrated that in combined treatment, such as irinotecan and temozolomide, tumor regression has improved, while the use of a single drug was associated with poorer response (Nicolle et al. 2016).

Considering the mentioned findings, PDX has become a reference model in preclinical oncological research for the assessment of antitumor therapy and for a new perspective in tumor biology study. Moreover, the ability of preserving the same properties of the original tumor makes the PDX an accurate model of real-life tumors, for several types of neoplasia (Nicolle et al. 2016).

Experimental Models and Nanotechnologies

Nanomaterials exhibit special properties opening perspectives of new developments of cancer nanomedicine. Some examples of nanomaterials are VYXEOS™, which can deliver synergistic ratios of two therapeutic agents; NBTXR3/Hensify, a nanoparticle which is radio-enhanced and synergizes with the radiotherapy; and Patisiran/ONPATTRO®, the first RNAi (RNA interference) therapy approved by the FDA (Augustine et al. 2021). Other particles, such as albumin NPs, liposomes, or polymeric micelles are approved for oncologic therapies, while some

nanotechnology-enabled curative methods, like radiotherapy, chemotherapy, immunotherapy, and RNA interference therapy, are under clinical evaluation (Shi et al. 2017).

Nanotechnology has the potential to significantly improve drug-delivery methods and to reduce the side effects of cancer drugs (Cuenca et al. 2006). This is achieved by augmenting the therapeutic dosage, circulation time, and efficacy of the treatment. Clinically validated nanotechnology application leads to the improvement of the therapeutic ratio of anticancer drugs, by altering the pathophysiological modulation and by changing tissue drug distribution and pharmacokinetics (Hare et al. 2017).

Liposomes, the first class of nanoparticles (NPs) with therapeutic properties, are one of the most used NPs for cancer treatment. Liposomal doxorubicin (Caelyx), approved in 1995 by the FDA, was the first antitumor NPs used in breast cancer (Hare et al. 2017).

An albumin-bound novel 130-nm nanoparticle, Abraxane (ABI-007), has been developed to enhance intratumor concentrations of active drugs by using endogenous albumin pathways, with benefits in the treatment of advanced breast cancer or other solid malignancies (Cuenca et al. 2006).

Nanoparticles are also a valuable tool in imaging, which uses radioisotopes in combinations with fluorescent antibodies, considering their ability to be "read" by the magnetic resonance imaging (MRI) (Craig and Jensen 2017). Computed tomography by lymphotropic tracers is a technique used for diagnostic imaging in animal models. This technique has a drawback though, as the quantity of gold nanoparticle required was too large based on pigs and mice models (Craig and Jensen 2017). In mouse models, this imaging system was used to trace the lymphatic system, due to its role in metastasis distribution. Anti-CD45 antibodies marked with these gold nanoparticles had a high affinity for axillary and popliteal lymph nodes, in a relatively recent study (Craig and Jensen 2017).

Radioactive nanoliposome applications in imaging are designed to target the Thomsen-Friedenreich (TF) antigen, which is usually overexpressed in colorectal tumors (Sakuma et al. 2015). In a rat model, a nanobeacon was used to detect TF antigens, using a fluorescent microendoscope (Sakuma et al. 2015; Craig and Jensen 2017). After injection with HCT116, a human colorectal carcinoma cell line, into rats' descending colon, tumor progression was followed up with the help of this beacon, for both its evolution and regression after treatment (Sakuma et al. 2015; Craig and Jensen 2017).

In addition to their role in imaging, radioactive nanoliposomes can also have a therapeutic effect, such as the use of radioactive rhenium (188Re)-labelled nanoliposomes in a mouse model with colon peritoneal carcinomatosis (Craig and Jensen 2017). Following their administration, a better treatment outcome when compared to 5-fluorouracil chemotherapy has been registered, with regard to tumor progression and growth (Craig and Jensen 2017).

Nanotechnology has a huge potential in oncology, providing new perspectives in cancer diagnosis and targeted therapies. However, more research is needed for its optimization.

Conclusions and Perspectives

Cancer management needs a multidisciplinary team, and molecular targets revealed by genomics and proteomics may be useful for the development of new therapies.

Interesting data about CSCs, in association to EMT, provide novel markers to prevent promotion, growth, invasion, metastasis, chemoresistance, drug resistance, and recurrences.

In selected cancers, the identification of MSI is important in subtyping and, consequently, in personalized management.

In the last few years, the results obtained using animal models and patient-derived tumor xenografts have been translated into clinical trials, opening new therapeutic approaches in oncological patients. One of the limits of animal model exploitation is that human cancer stromal components might be replaced in the host animal, resulting in different interactions with tumor cells. However, current studies performed on cell lines and on animal models designed to evaluate different therapies' efficiency and the use of novel anticancer drugs lead to the development of targeted approaches.

The cancer management may benefit by the discovery of nanomaterials, with promising perspectives in medicine applications of nanotechnology.

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Animal Models for Cancer Research: The Choice of the Right Model System

3

Sinan Kandir

Contents

Abstract

The human is a complex organism, so if translational research is conducted, it should similarly mimic that complexity. Model systems comprise mathematical, computational, in silico, ex vivo, in vitro, and in vivo models in cancer research. Alternative model selections are the best practice for the reduction of experimental animal usage. The aim of animal usage in cancer research is to well-understand the physiopathology of different types of cancer, from genomics/proteomics to metabolomics levels, to screen the behaviors of the cancer cells in living organisms, and the efficiency of the treatment methods that mirror precision medical areas. Various animals can be used as model organisms. The most important point in experimental animal usage is ethics. This chapter will primarily focus on the fundamentals of the model systems with the comparisons of in silico and in vitro as alternatives to animal models. Then, the chapter will discuss the in vivo models

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with ethical issues of animal experimentation, the R principles, and the selection of the suitable animal models in cancer research.

Keywords

Animal · Cancer · Humanized · Oncology · In vivo · 3R

Introduction

The high-throughput, accurate, robust, validatable, reproducible, and transparent data are vital in order to maintain high-quality research and obtain translatable results. The primary step of a state-of-the-art study begins with planning from head to toe and a well-designed methodology, while model selection is the core step. The animal models are essentially used for biomedical research to understand physiological and physiopathological conditions, and to develop new therapeutics for centuries. The most important point in experimental animal usage is ethics. Animals are living organisms like humans, and modern humankind could occasionally neglect its position through the evolutionary axis. Thus, acquiring knowledge about legal regulations and ethical rules, in particular the 3Rs, is the basic procedure of biomedical research before taking action. Furthermore, alternative model selections are the best practice for the reduction of experimental animal usage.

Alternative Model Systems

The model systems in biomedical research could be defined as controlled experimental setups that are mimicked similarly or identically target organisms (human or animal) systematically with reproducible, inspectable, and transparent features for developing hypotheses to understand the mechanisms and discover solutions to complex biological problems.

In Silico Model Systems

Mathematical oncology is modelling and simulating cancer models by using applied mathematics with knowledge of calculus, differential equations, statistics, and mathematical theories (e.g., game theory, Heisenberg's uncertainty principle, chaos theory, fractals, quantum mechanics, etc.), to predict the cancer dynamics and behavior, personalized medicine, and effectiveness of treatments (Anderson and Maini 2018; Anderson and Quaranta 2008). Because of reducing the number of experimental animals and robust reproducibility, mathematical oncology has gained importance exponentially (Anderson and Maini 2018). The road map of mathematical oncology has not only focused on cancer dynamics, therapeutic response, and personalized medicine, but also evaluated patient-specific big data and improve early

detection strategies with the statistical science in the last decade (Rockne et al. 2019; Anderson and Quaranta 2008). These types of models are named *mechanistic models* which are integrated whole data incomes from patients or experiments to clinical outcomes (Baker et al. 2018; Gaw et al. 2019).

The primary motivation of mathematical oncology is the transference of big data to clinical predictions of the likelihood of real scenarios (Rockne and Scott 2019; Rockne et al. 2019). The mechanistic models in cancer research are integrated with the mathematical formulas into machine learning and test the prognostic hypotheses and predictions with the offer of the best treatment options. Hence, basic cancer research takes advantage of *intersection shapes richness* – an ecological concept that defines the richness of biodiversity at the intersection areas – of various disciplines, from mathematics, physics, and biology to computational science. However, mathematical models can mostly focus on a specific and small area in the face of cancer complexity. The recent advances in computer science have boosted the mathematical model systems to a more reliable form of computational biology. The computational models which originate from mathematical formulas are becoming backbones in cancer research (Gaw et al. 2019; Anderson and Quaranta 2008; Anderson and Maini 2018; Baker et al. 2018; Bekisz and Geris 2020).

In this perspective, the in silico models are established. Basically, the frameworks of in silico models are the way of the translational phase of fundamental mathematical formulas to computer programs, bioinformatics knowledge with machine learning and artificial intelligence, to –omics area, and straightforward to silicon chip technologies of microfluidic physiological systems (microphysiological systems; lab-on-a-chip, organ-on-a-chip) to clinical applications. These sophisticated, cost-saving, flexible, lab-handled in silico model systems have promise for the future owing to a great opportunity in the preclinical cancer research with virtual screening of the cancer dynamics, drug design to interactions, treatment efficiency in nano-scale, and also, quite favorable in respect of R principles (Stillman et al. 2020; Niarakis and Helikar 2021; Jean-Quartier et al. 2018). In spite of the offered advantages of in silico model systems, these are still juvenile in comparison to in vivo models, and have pitfalls and limitations such as inability to simulate whole organisms throughout cancer homeostasis and allostatic mechanisms, algorithmic challenges and complexity, and need large-scale datasets to produce accurate computational data (Fig. 1) (Sacan et al. 2012; Bray 2015).

In Vitro Model Systems

The in vitro model systems are powerful candidates of alternative models for animal experiments because of their adjustable and easy integration capabilities with in silico models. Literally, knowledge about the history of the in vitro models started with microbiological and pharmacokinetic studies in the early 1950s (Grasso 1985). The cell-based in vitro models are preferred owing to their cost-effective and time-shortened nature, well-controlled environmental circumstances, enabling them to study specific cell lines with distinctive molecular pathways, ethical flexibility,

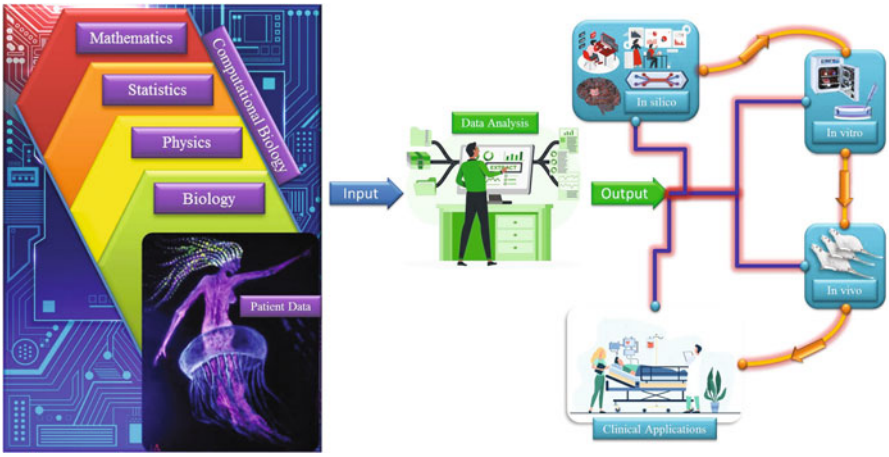

Fig. 1 Cancer research models, from basic science to clinical applications

standardization, and reproducible properties (Arantes-Rodrigues et al. 2013; Nikolic et al. 2018; Katt et al. 2016). Current scientific biotechnological innovations have paved the way for ultrafast developments in cutting-edge in vitro model technologies. Classical in vitro models have been comprised of two-dimensional (2D) monolayer cell cultures coated on a plate by a selected cell line; they are inaccurate to mimic a dynamic tumor microenvironment (complex cellular and extracellular matrix interactions). Thus, recently, more complex spheroids and organoid (organ-like) models via 3D cell cultures have been produced that enable mechanically active and reliable simultaneous molecular response (Yip and Cho 2013; Birgersdotter et al. 2005; Rodrigues et al. 2021). Furthermore, 4D semi-active organoids are available, which are more similar to the real tissue with extracellular matrix, heterogeneity, vascularization, epithelial tissue properties, regulable and dynamic microenvironment, such as ex vivo models besides 3D matrix composite cell lines (Fig. 2) (Jensen and Teng 2020; Charbe et al. 2017; Zhao et al. 2021; Langhans 2018; Wessels et al. 2022; Kuhl et al. 2016). *Tech-feed-tech*, so, contemporary 3D bioprinting technologies shed light on rapid improvements to in vitro model systems. The development of in vitro models facilitates the translational potency of basic science to clinical applications.

The 2D models are limited, due to their monolayer single-cell designed homogeneous structure, by cellular drug response and screening the basic cellular behaviors (migration, proliferation, apoptosis, etc.), whereas the 3D models are used to mimic the tumor microenvironment, metastatic behaviors; moreover, the 4D models are more realistic and complex than other in vitro models and comprise high-throughput imaging analysis by adding in silico data, with their heterogeneous microenvironment properties, progressive, metastatic, individual, and collective behaviors of the cancer cells, therapy response, and resistance (Wessels et al. 2022; Kuhl et al. 2016; Rodrigues et al. 2021; Zhao et al. 2021; Yip and Cho

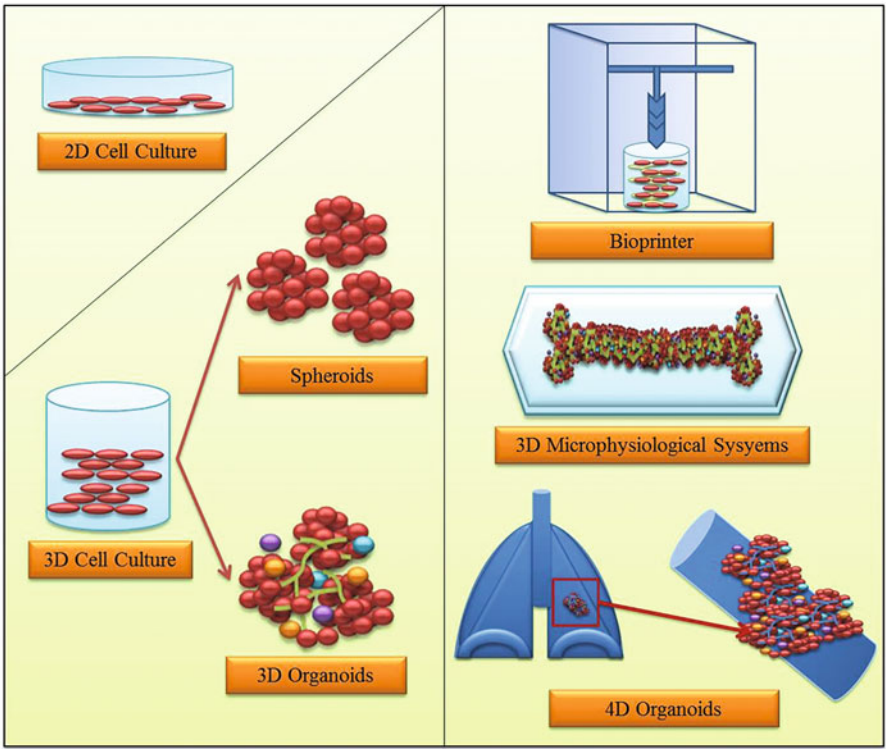

Fig. 2 In vitro cell culture models

2013; Gao et al. 2016). Nevertheless, high-tech 3D and 4D in vitro models still have some disadvantages in comparison to 2D, including methodological difficulties, more expensive infrastructures, time-consuming, and low reproducibility (Bartlett et al. 2014; Kapalczynska et al. 2018; Wan et al. 2020; Tibbits 2014; Gao et al. 2016).

In Vivo Model Systems

Cancer growth and metastasis are dynamic processes, and inside the living organism, numerous factors get involved, including intermediary metabolism and homeostatic and allostatic mechanisms. Animals have been used as experimental models in cancer studies to reveal the tumorigenic mechanisms and treatment options for over more than a century. Subsequent to Rudolf Virchow's chronic irritation and Julius Cohnheim's embryonic rest hypothesis, Johannes Fibiger succeeded in inducing papilloma and carcinoma in wild type piebald rats' esophagus and stomach by *Spiroptera neoplastica* – currently known as *Gongylonema neoplasticum* – in 1907 and was awarded Nobel Physiology and Medicine in 1927 (Nobel 1927). Then,

Yamagiwa and Ichikawa (1977) induced epithelial carcinoma by chronic irritation with coal tar painting for the first time in the laboratory rabbits. Since the discovery of the Rous sarcoma virus (described as an oncogene) in 1910 by Peyton Rous (Nobel Prize in 1966), who identified the cause of malignant chicken sarcoma and triggered new spontaneous cancer models in hens with allogeneic transplantation, research has been carried out on new animal cancer models. Thereafter, Harold Varmus, J. Michael Bishop (Nobel Prize in 1989), Dominique Stehelin, and Peter Vogt found the cellular origin of retroviral oncogenes of avian sarcoma virus, which leads to the new paths for identification of human oncogenes (2021). In spite of the rapid developments on in silico and in vitro models, the in vivo model systems are still essential for translational research including preclinical studies in cancer.

Types of Animal Models

Principally, the animal models could be divided as small and large animals (Ziegler et al. 2016; Mondal et al. 2022; Kandir 2021). Small animals are mostly preferred by researchers due to their well-controlled, easy-handled, cost-efficient, standardized with have a short reproductive cycle and life span advantages. On the other hand, large animals are useful models not only for anatomical or physiological similarities with humans, but also have spontaneous cancer types such as lymphomas, adenocarcinomas, mammary tumors, skin, pancreas, colon, bladder, and prostate cancers, etc. (Pinho et al. 2012; Ziegler et al. 2016; Giuliano 2021; Biller et al. 2016; Hudachek et al. 2010; Schmahl et al. 1978). While the rabbits were selected as experimental model animals initially, the rodent models (mice and rats) are the most preferred animals currently because of their inbred, homogenous and standardized colonies with detailed knowledge about their genetic backgrounds (Mouse Genome Sequencing et al. 2002; Gibbs et al. 2004). As shown in Table 1, the researchers have miscellaneous alternative animal sources and models in order to establish their cancer studies.

Although mouse and rat models are the most preferred animals by cancer researchers due to their highly standardized inbred strains, the large animal models especially pigs and dogs have more similarities to humans with their genetic heterogeneity. Whole listed animals in Table 1 have own genome projects to screen high-throughput sequenced genomes with single nucleotide polymorphism (SNP) datasets (Denoyelle et al. 2021; O'Brien et al. 2002; Ostrander and Kruglyak 2000; Archibald et al. 2010; Alföldi et al. 2009; Howe et al. 2013; Keane et al. 2011; Mouse Genome Sequencing et al. 2002; Wade et al. 2009; Chimpanzee and Analysis 2005; Zorio et al. 2019; Bovine Genome et al. 2009; Romanenko et al. 2015; Adams et al. 2000; Gibbs et al. 2004). This knowledge gathers many advantages for cancer researchers to design their studies. The researchers have to ask the right questions to themselves for the animal experimentation, as "*How will I set up my animal experiment design?*" and "*Would this experiment be translational to humans?*". The aims of the researcher have to be realistic and result oriented for the ultimate patient. Hence, the well-designed, randomized, blinded, controlled experiments

Table 1 Animal models for cancer research (Kemp 2015; Peng et al. 1993; Giuliano 2021; Biller et al. 2016; Raby et al. 2020; Kamdem et al. 2020; Hudachek et al. 2010; Palmarini and Fan 2001; Schachtschneider et al. 2017; Tian et al. 2020; Hicks et al. 2021; Xia and Chen 2011; Schmahl et al. 1978)

Small animal models		Chemically induced	Spontaneous	Tumor transplantation	Genetically engineered	Humanized
	Mice	*	*	*	*	*
	Rat	*	*	*	*	*
	Rabbit	*	*	*	*	
	Gerbil	*	*	*	*	
	Guinea pig	*	*	*	*	
	Zebrafish	*	*	*	*	*
	Chicken	*	*			
	Drosophila	*		*	*	
Large animal models	Pig	*	*	*	*	*
	Dog	*	*			
	Cat	*	*			
	Cattle		*			
	Sheep		*			
	Goat		*			
	Horse	*	*			
	Nonhuman primates	*	*	*	*	

provide a powerful tool for gaining new knowledge. By this aim, chemically induced, spontaneous, syngeneic, xenograft, genetically engineered (GE), and nowadays, humanized animal models are used to establish the in vivo setup of cancer studies.

Chemical agents, toxic substances and their intermediate products are involved in every aspect of our lives. Environmental exposures to these agents induce carcinogenesis, especially in the epithelial tissue. Basically, chemical carcinogens are classified as genotoxic (polyaromatic hydrocarbons, alkylating agents, aromatic amines and amides, etc.), which are driven by DNA damage directly or indirectly, and non-genotoxic (cytotoxic, receptor-mediated, hormonal disruptors, oxidants), which act for prolonged periods with indirectly altered cellular homeostasis, hence leading to spontaneous tumors.

"*Why and when should researchers choose chemically induced models?*" To address this question: chemically induced models contribute to the characterization of toxic mutagens, to screen DNA damage and repair mechanisms, chemoprevention, or the early diagnostic approach. The mice and rats are major models for that type of research due to their well-known genetic backgrounds and ensured genetic homogeneity with inbred strains. Moreover, canine and feline models are suitable for chemical-induced carcinogenesis because of long time exposure to the same environmental pollutants and genetic heterogeneity similar to humans (Yuspa and Poirier 1988; Takashima-Uebelhoer et al. 2012; Hayes et al. 1991; Schmahl et al. 1978).

Each animal have their spontaneous cancer traits. The main question in spontaneous cancer research is that "*What is the similarity rate of cancer compared to humans?*". For example, urothelial carcinoma known as transitional cell carcinoma (TCC) occurs in both humans and dogs with the same origins such as chemical exposure to smoking, organochlorine pesticides, arsenic-contaminated or chlorination by-products of the water that are associated with polymorphisms on glutathione S-transferases (GSTs) genes (G > A in *GSTT1* or 6 bp deletion in *GSTT5* exon4) which eliminates GST enzyme activity (Luethcke et al. 2019; Craun et al. 2020). In this view, the researcher might use the animal-specific genome and SNPs databases by bioinformatics tools to match selected cancer types in animals versus humans.

Tumor transplantation is another option for understanding cancer cells' behavior, mechanism of tumorigenesis, metastatic and invasive features, and alternative therapeutics in preclinical research. Herewith, patient-derived tumors are transplanted either to selected athymic or genetically engineered severe combined immunodeficient (SCID), immunocompromised or pharmacologically immunosuppressed or immunocompetent humanized animals – to keep from graft versus host reaction – named as patient-derived xenograft models (PDX) by orthotropic implantation which provides site localization similarity as humans or subcutaneous, intraperitoneal, or intravenous inoculation (Bosma and Carroll 1991; Fujiwara 2018; Koo et al. 2009; Aartsma-Rus and van Putten 2019; Eswaraka and Giddabasappa 2017; Hirenallur-Shanthappa et al. 2017). Mice and rats are mostly preferred animals for this method because of easy handling, standardized, and homogeneity advantages.

The major disadvantages of these models are lacking tumor microenvironment except for orthotropic implantation, and the used animals need specialized environments such as specific-pathogen-free housing procedures both with autoclaved materials owing to their immunosuppressive situations.

"*What are the humanized models?*" Basically, the immunodeficient animals are engrafted with human cells or tissues, and these xenotransplanted parts physiologically act as in the human body. Various humanized mice have been generated up to date. Fundamentally, SCID mice, which lack of T and B lymphocytes, are engrafted with human peripheral blood mononuclear cells, human $CD34^+$ hematopoietic stem cells, or human fetal thymus and liver cells (Bosma and Carroll 1991; Fujiwara 2018; Eswaraka and Giddabasappa 2017; Hirenallur-Shanthappa et al. 2017). Humanized animals are becoming keystones not only for cancer but also in whole biomedical research areas.

To evaluate the immune response in order to advance cancer immunotherapy or immune response research, syngeneic – allograft, GE, or humanized models are suitable models (Li et al. 2017; Koo et al. 2009). Despite syngeneic models are cheaper than GE or humanized models, species-specific differences could give rise to translational failure.

Ethics in Animal Experimentation

Because of increased sensitivity for experimental usage of the animals, the ethical rules and limitations and end points of research were determined. After the introduction of the 3R (**R**eplacement, **R**eduction, and **R**efinement) principles by Russell and Burch in 1959 (Russell and Burch 1959), to date, we discuss the expansion of the "**R**"ules. The "R" concept (Table 2), enhancing 3R principles to 5R, includes "**R**igour" or "**R**obustness," and "**R**eproducibility" (Russell and Burch 1959 (as reprinted 1992); Kitano 2004; Obrink and Rehbinder 2000), and could be prolonged by new rules (e.g., t**R**ansparency, **R**esponsibility, etc.) to 7R (Lee et al. 2020; Tannenbaum and Bennett 2015), establishing the research culture that includes standardization of experimental animal usage. However, humanity is the first thing to keep in mind before handling animals to develop as the experimental model.

The major goal of animal usage in biomedical research is to achieve the translation ability to human and animal medicine. Thus, translational research is a bridge which comprises "*bench-to-bedside*" by means of the application of basic research to clinical utilization of both human and animal medicine (Cohrs et al. 2015). With the aim of the translational research, choosing the best animal model means finding the best matching organism with human (Mak et al. 2014). Related to recent reports, "*translational failure*" is a serious disadvantage in clinical trial phases and waste of the majority (Ledford 2011; Hackam and Redelmeier 2006). Hence, additionally to the 3R principles, rigor and reproducibility rules have to be essential for robust the obtained data (5R) and avoid the researcher-based prevention of negative results publication behavior, transparency and responsibility rules (7R) are indispensable for translational research.

Table 2 The "R" concept in biomedical research

			"**R**"ules	Definitions
7R	5R	3R	**R**eplacement	Primarily, choice alternative methods, e.g., mathematical and computer models (in silico), tissue culture systems (ex vivo), cell culture (in vitro). If you need a living organism (in vivo), choose insentient (nonsentient) primitive models (metazoan endoparasites, plants) or minimize the stress, pain by anesthesia and analgesia, do not harm, and maximize the animal welfare in the higher organisms
			Reduction	Principally, minimize animal usage through statistical limitations by obtaining reliable data to reduce the animal numbers and increase the obtained information. During the planning period, design the experiment in line with state-of-the-art knowledge; choose the right animal to model the research, adjust statistical methods, and determine the minimum sampling size to obtain reliable data
			Refinement	The term "well-being" could be defined as basically unstressed, feeling safe, maintaining normal behavioral and physiological conditions as animal welfare. Biological requirements and husbandry conditions such as eating, drinking, socializing, day/night cycle have to be maintained, and the researchers have to know the physiologic and behavioral requirements of selected model animals. Determine the limitations and cut-off situations to inhibit pain, fear, stress, and prevent inhuman applications
		or	**R**igor	The rigorousness of animal experimentation onsets with the experimental design by using vigorous scientific methods, robust and objective analysis, and detailed result transparency. This includes consulting with experts (veterinarians, biostatisticians, etc.) before the experimental period and sharing the raw data with the editor, reviewer(s), and readers in the publication period. Due to the translational challenge of animal research, the rigor and transparency directions, and new guidelines report officially (Shaffer 2021; Hewitt et al. 2017)
			Robustness	This term is defined as the quality of being strong and healthy. In terms of animal experimentation, robustness could be defined as the strength of the biological systems in the face of disturbing external (environment) or internal (physiological) conditions, and the quality of obtaining data taken from different laboratories with minimum variations, and translatability strength bench-to-bedside (Friggens et al. 2017; ten Napel et al. 2011). Robustness would lead to the clarity of complex systems and network analysis (e.g., signal transduction, disease mechanisms, therapeutic assays, etc.)
			Reproducibility	Rigor and robustness of research are tightly connected with reproducibility. It means repeating the capability of the same research and obtaining the same results during all repetition. This headline is the source of the big crisis among the same scientific experiments in different laboratories (Baker 2016). The origin of the reproducibility crisis in animal experiments is directly related to design methodology, age, sex, strain, and environmental conditions (von Kortzfleisch et al. 2020). The standardization of disease models, colony formation, and the collaboration among animal facilities could improve reproducibility

(continued)

Table 2 (continued)

			T**R**ansparency	Transparency includes detailed descriptions of methodology and evaluated data. To mirror reproducibility, robustness, and rigor, obtaining data in an animal experiment, transparency is the essential part (Aske and Waugh 2017; Hewitt et al. 2017). Sharing the raw data with the scientific community can improve the research methodology and translational capability by reducing the animal numbers and leading to state-of-the-art experiments
			Responsibility	The researchers/scientists have responsibility for using the animals in their experiments to ethics committees, editors, reviewers, as well as the global community. Hence, the researchers/scientists have to consist of the necessary qualifications such as animal usage license, physiological knowledge of model organisms, and high characteristics of ethics, morals, and in particular humanity

The Best Model Decision Algorithms of a Cancer Researcher

Here is the advice of some toolkits for cancer researchers to make the best decisions before taking action in their research.

The NC3Rs (National Centre for the Replacement Refinement & Reduction of Animals in Research) initiative is leading to new alternative methods for the replacement of animal usage in biomedical research (Singh 2012). Hence, the NC3Rs initiative contributes to the researchers by the ARRIVE (Animal Research: Reporting of in vivo experiments) guidelines to ensure the well-planning, rigorousness, and transparency of animal studies from study design, statistical methods to animal experimentation phases with the solidarity of an international working group (Percie du Sert et al. 2020). The ARRIVE guidelines have updated checklists not only for researchers but also for reviewers and journal editors.

Additionally, in cooperation with the Institute of Animal Technology, the animal technicians have supported the web-based training resources for animal research, which could be helpful for junior researchers related to various issues such as ethics, welfare, legislation, handling, and care of animals (RAT 2021).

Last but not least, Norecopa (Norway's National Consensus Platform for the advancement of the 3Rs) provides another web-based tool and guidelines for stakeholders of animal research namely PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) (Smith et al. 2018).

Conclusion

In conclusion, without a doubt, there are no certain models in cancer research and no perfect experimental design. Therefore, researchers must begin with a better plan and design the wisdom of their studies. The situation is serious, but not hopeless because of the researchers' websites, which have some artificial intelligence-based web instruments that are powerful tools for better scientific planning and design.

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Cellular Interactions Networking in Interactive Models of Diseases

4

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Contents

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Abstract

Integromics is necessitated as the complex diseases require to collate the integrated analysis expression, variation, and regulation of genes involved in trigger, prognosis, and establishment of factors creating the complete disease paradigm. The further involvement of the non-genetic and environmental exogenous factors are also designated to formulate the multitude of data for furthering the "integromic" approaches. Identification and validation of interaction networks and network biomarkers have become more critical and important in the development of disease models, which are functionally changed during disease development, progression, or treatment. We represent the requirement of the multi-node analyses that goes beyond the binary relationships to enterprise the structured interactions at the interface of genotype to phenotype correlations in disease biology. The prevalence and sporadic occurrence of endemic and pandemic infectious diseases, as well as the unmanageable burden of the non-communicable diseases, have emerged as the most burgeoning task of scientific investigations. Disease-specific interaction networks, network biomarkers, or Dynamic network biomarkers have great significance in the understanding of molecular pathogenesis, risk assessment, disease classification and monitoring, or evaluations of therapeutic responses and toxicities. The systems level studies have indicated biomolecule to cellular organization requires communication and cross-talk possibilities at the organism levels. Designing newer theranostic regimes, thus is required to focus on disease heterogeneity integrating the knowledge base of dynamic physical or functional interactions of network of networks. The chapter is targeted toward the identification, characterization, and a follow through of the experimental and computational tools for evincing the futuristic plan for modular endeavors in disease biology.

Keywords

Disease network · Interectomics · Network Biomarkers

Chapter Learning

- Disease Biology – Categories, characterization, and challenges of complex diseases
- Physical and Functional Networks – Multi-omics analyses
- Disease Networks and pathways – Expression, variation, and regulatory factors for identifying dysregulation
- Integromics – Constructing network modules on multifactorial data types and integrating non-genetic components modelling genotype to phenotype correlations
- Applications of networks inferring precision and accuracy in disease biology and management

Introduction

"Disease" aka malaise is a result of combination of genetic and non-genetic factors that have varied stages of acquisitions, activation, advancement and institution. The fact that most of the events of cellular welfare is pertaining to its inherent capacity to repair and regenerate the archetypical state. The perturbations in this capacity is mounted through a scheme of dysregulation involving genes, small molecules, proteins, RNA species propagated through the various stages of disease. Thus, disease biology contemplates the role of such networks, conditioned to the nodes of inter-connected genetic modules or sub-networks (Ghadie and Xia 2022). The emergence of diseases is with shared symptomatic patterns are also pinning that the focal causal factors are co-evolved, with cross-talk between interactive networks. A plethora of diseases fall in the category of such complex disorders that relay the importance of creating the niche of network of networks in concerted efforts toward clinical translation of the mega-initiatives toward precision medicine. The chapter is designated to provide the reader facts behind the pathobiology of complex diseases with focus toward curating the types of tools for functional theranostic designs.

Disease Biology: Noncommunicable/Communicable/Metabolic Syndromes

Diseases are complex network involving interactions between genes, environment, and lifestyle associated with self-limiting to life-threatening entities in all underlying classified diseases, e.g., tumors, infectious diseases, and cardiovascular diseases (Chan and Loscalzo 2012a). These diseases are complex, multifactorial diseases with varied outcome. Multiple physiological systems interact throughout the development of a complex disease. Life sciences research has been revolutionized in past decades by a series of technologies, starting with the Human Genome Project in 1990. The speed and scale of genomics analysis increased exponentially and is classified as discovery science, along with other omics such as transcriptomics, miRNAomics, epigenomics, cistromics, proteomics, metabolomics, and microbiomics. The goal of all these sciences is to collect and store data based on all the molecules involved (Manzoni et al. 2018). This helped in generation of enormous amount of biological data, leading to emergence of challenges in term of analysis and interpretation of data. This led to the discovery of the Network science which is involved with the analysis of interactions occurring between biomolecules (proteins, RNA, gene sequences), pathways, cells, organs. Hence, through network analysis, it is possible to identify complex patterns among different components to generate scientific hypotheses regarding the interactions present in health and disease events (Li et al. 2015). Gaining knowledge of the dynamics interactions across physiological systems facilitate the prevention or mitigation of biological damage in term of loss of functions in complex diseases, many of which are used to add on information or targets in developing new interventions (e.g., hypertension) (Abbas

et al. 2019; Zhou et al. 2016). There is a probability that complex biological pathway have low abundant molecular entities (genes and proteins) which interact with other molecules involved in similar pathways. Hence each pathway represents a specific region of an extended network in a given biological system. This led to thought that there is dire need of network analysis methods that can be elucidated to provide an add on biological insights that cannot be obtained from pathway analyses alone in vivo (Joshi et al. 2021).

Biological networks comprise nodes that correspond to genes, proteins, metabolites, or other biological entities, and edges that correspond to molecular interactions and other functional relationships between the biological molecules. In general, biological networks of the same size and connectivity exhibit significant differences in aspects such as: wiring type or presence of topological motifs (groups of interconnected nodes with a given structure). This affects (1) modularity, i.e., the degree of division of the network into subnetworks that comprise densely connected nodes but share few edges outside the module, (2) dissortativity, i.e., the tendency of nodes to connect to other nodes in the network that are associated with different characteristics (e.g., nodes with many connections link to nodes with few connections), and (3) robustness, i.e., the resilience of the network to the removal of nodes or edges. For example, COPD will be one of the top five chronic diseases in terms of global mortality and morbidity by 2030. The present chapter highlight network biomarkers, interaction networks, dynamical network biomarkers in diseases, with an emphasis to integrate bioinformatics-based screening of biomarkers, network biomarker, dynamic network biomarkers with clinical informatics and phenotypes, and establish a systems biomedicine-evidenced dynamic network specific disease models (Barabasi et al. 2011).

Causative Analysis; Etiology of Disease Immunopathogenesis, Molecular Events, Cellular Events

Complex disease conditions characterized by co-morbidities involve pathological dysregulation that evolves across multiple systems over time. Thus, a holistic approach is required to deconvolve the spatiotemporally distributed mechanisms of multifactorial disease pathogenesis at the tissue, cellular, and molecular levels of analysis. A disease's etiology, or cause, generally falls into three main categories; intrinsic, extrinsic, and idiopathic. The intrinsic etiologies are part of internal system, e.g., inherited disease, metabolic and endocrine disorders, neoplastic disorders and immunity, while the extrinsic etiologies are associated with infectious agents, animal bites, chemical agents. There are certain unknown etiologies are also called idiopathic (Fig. 1).

The development and progression of the disease involves complex etiology associated with interplay of a group of correlated molecules or a network, rather than from the malfunction of the individual gene, protein, or cell. Traditionally, molecular pathology analyzed well-characterized individual genes, proteins, or other molecules. Subsequently, this strategy was expanded to more elaborately

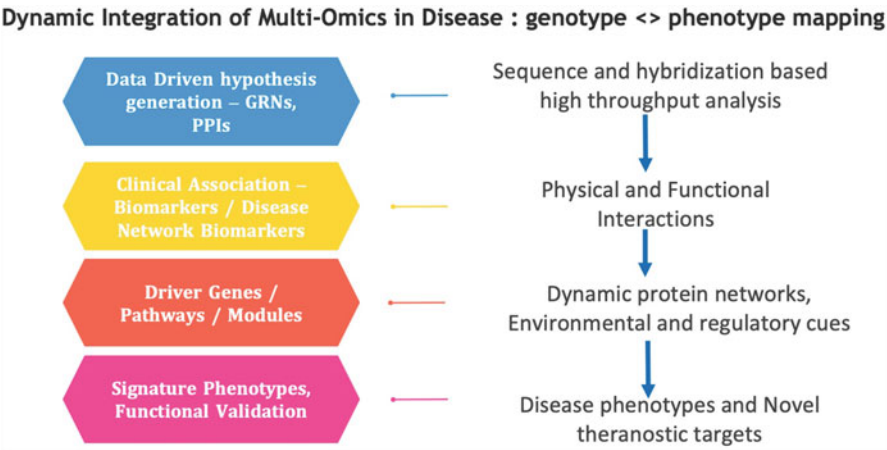

Fig. 1 Hierarchical generation of clinical interventions related to disease pathobiology and structured disease phenotype analyses – Contributions of the biomolecular interactions that modulate the cellular/tissue behavior and the studies conducted to unravel disease progression and establishment

deciphering the systematic alterations in the expression of mRNA using gene expression microarrays. Further, this technology has been advanced with full-genome, deep- and transcriptome-sequencing platforms, for example, mRNA or non-coding RNA quantitation, detection of gene copy-numbers, and genomic sequencing. Similarly, recent advances in mass spectrometry-based analysis now enable detection and quantitation of selected small compounds, proteins, and other biomolecules. Hence, identify and assess individual molecular entities (proteins, in particular) to ongoing molecular pathology toward higher-throughput and clinical applications, using technologies such as serum mass spectrometry. Cell signaling molecules are highly dynamic, potent, and specific in both structure and utilization and in both cell- and tissue-specific manner (Wang 2011) (Fig. 2). In the last decade, network-based approaches have been successfully applied to a broad range of diseases, with examples ranging from rare Mendelian disorders, cancer or metabolic diseases, to identifying basic strategies by which viruses hijack the host interactome, to name but a few. The important aspects of molecular networks such as simultaneous input cues should be processed and integrated to determine alterations in cellular behavior such as migration, proliferation, apoptosis, differentiation, etc. in order to design biomarker-based assays. An important aspect of cellular signaling networks is that at any given time in a given state impacts directly on the cellular response to an environmental stimulation. This multivariate nature enables cells to respond to multiple input events in an integrative and quantitative manner (Winslow et al. 2012) (Fig. 2). Hence there is a probability that failing to describe network states and biological context for molecular biomarkers can have potentially damaging consequences for the patient. It is believed that these potent alternations of complexes will represent and influence the responses of cells or organs to real-time changed microenvironment. Therefore,

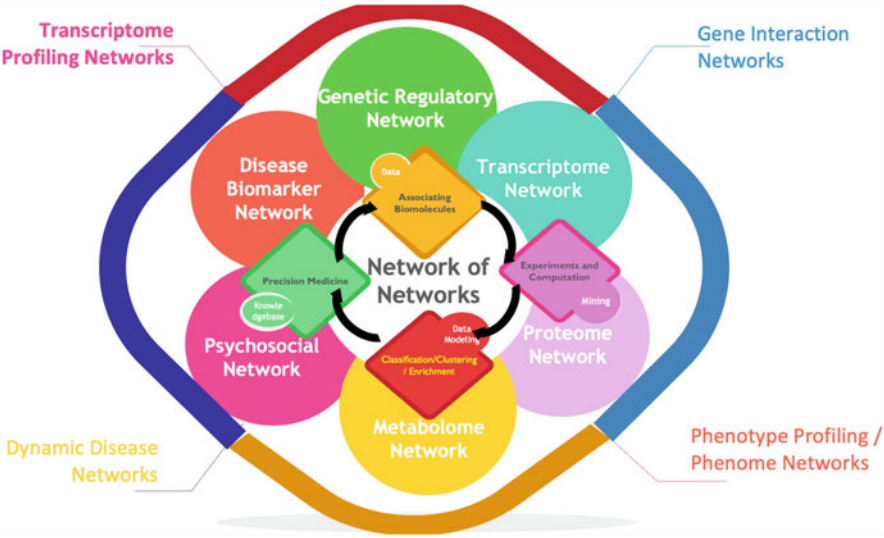

Fig. 2 Biological Networks – Disease biology profiling and the control networks which are integrated to allow dynamic programming and information processing through multitude of interaction networks. The interplay of these integrative models layered and connected to generate the network of networks as the focal high throughput disease models, converging disease states, phenotypes and regulatory patterns in disease biology

identification and validation of interaction networks and network biomarkers, especially at the protein level, become critical to develop disease-specific biomarkers for monitoring disease occurrence, progression, or treatment efficacy. In order to study clinical manifestations, interaction networks between immunologic, molecular, and cellular events should be envisioned. The new insights generated by extracting information from biological events depending on varied patterns of interconnections between these events during the initiation, progression, and extension of disease should be analyzed to understand the clinical sequalae and development of target specific therapies to treat the disease.

One of the major challenges in the medicine is the lack of disease-specific biomarkers for disease diagnosis, illness monitoring, therapy evaluation, and prognosis prediction. Hence, there is a dire need to identify biomarkers that should be a measurable indicator of normal physiological and pathological events. The disease specific biomarkers should be a guiding element in clinical manifestations, intervention, risk assessment, early diagnosis, and prognosis of disease. Disease-specific biomarkers are also expected to demonstrate the disease-associated specificity, sensitivity, traceability, stability, repeatability, and reliability. For example, somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor was shown to be a predictive marker in many lung cancer. However, only a few have been found to be useful clinically, although numbers of discovered and identified biomarkers are generated from preclinical research.

Profiling and Diagnostic Approaches in Disease Biology: Experimental/Computational/Mathematical

The culmination of the high-throughput studies of live images at cellular, and molecular levels have created stimulating possibilities for systematically investigating disease biology. The cataloguing of the diagnostic approaches followed by traditional toward integration of both in vitro and in vivo investigations with molecular basis will allow the clinical decisions based on such exploratory understanding of the disease. A perspective futuristic diagnostic and therapy regimen would thus be mapping functional details of disease progression and hence profiling in depth clinico-molecular aspects. The varied targets of disease biology includes the Experimental analyses, computational simulations, and mathematical modeling (Miles Macleod 2021; Scholl et al. 2018; Trapotsi et al. 2021). Here, we present the profiling approaches based on all these aspects.

Experimental Profiling

Disease research had extensive work performed in creating the experimental niche for demarcating the live cell imaging, cellular and tissue analyses, followed with molecular analysis. These studies have enabled us to update, improve, and facilitate empirical designs of disease biology. The results of experimental profiling is incorporated in the corresponding computational models. The computational studies including drug efficacy mechanistic studies, simulations of the genetic variants, predictive analyses, and dynamic profiling further makes the mathematical models that perceives to theranostic analysis. Together, these convey how experimental standardization, improvized parametric optimization in simulations, model refinements, inverse engineering can lead to inculcation of futuristic use of deep learning and machine learning while designing AI-based disease management strategies.

Computational Profiling

The recent years have seen a heightened interest in generating computational models for predictive and potential disease pathophysiology and drug targets. The computational models mostly range from score-function-based, network algorithm-based, machine learning-based, and experimental analysis-based models (Saiker 2021).

Multifactorial/Combinatorial Designs in Disease Diagnostics/Prognosis and Interventions

Disease biology involves the layered biological components that include the genes, regulatory components, proteins, metabolites, and epigenomes. Singular level omics approaches have been prevalent to be undertaken in disease pathobiological analysis

interrogating pools of genomes, trancriptomes, epigenomes, proteomes, metabolomes, and microbiomes using the ever diversifying workflows of high-throughput technologies. The mechanistic details of each such work-pipelines have paved way from hypothesis driven targeted approach to discovery driven untargeted analyses in disease biology. The data derived from the single level omics is enormous, though still not sufficing the need to resolve the causal relationships between molecular alterations at each level to the phenotypic manifestations in totality. This directs the systems level integration that allows multi-disciplinary data information to be processed through studying physical and functional interactions holistically. Integration of such systematic studies would also require adding up the regulatory window of information that is suitable to dissect the aberrant cellular functions behind complex diseases (Hood et al. 2004).

Multi-omics data generates the clusters of biologically relevant groups, enabling aspects of genetic variants and environment and interaction parameters between them. Thus, predictive models of prognostic and therapy have been devised, that now needs to be processed into integrative disease models to assist clinical translation of the research findings.

Interactome: Molecular Niche

Interactome refers to the inter-connected networks housing the physical and functional interactions, with physical interactions involving direct contact between participating biomolecules while functional interactions catering to biologically relevant relationships. In expression networks where genes are co-expressed and regulated, there are expression patterns that maybe connected, while functional interactions provide for genetic interactions, where genes are linked if simultaneous changes occur in genes involved. The functional and physical interaction networks provide for important insights into disease mechanisms. We process some details of these interaction networks for further analysis.

Physical and Functional Interactions

Diversifications and adaptations in the fields of chemistry, physics, mathematics, biology, critically engineering, the field of diagnostics, and therapy of diseases have paved its way from immunotherapies, radiotherapies, chemotherapies, tissue engineering, and realms of personalized medicine (Krzyszczyk et al. 2018). The genera of innovative technologies have seen the clinical translation and varying levels of success with a research stronghold that involves novel interventions using the "omics" toolkit, mathematical modelling, pharmacokinetics (PK)/pharmacodynamics (PD), and computational simulations and models [Docking and molecular dynamics (MD)] (Kitano 2002).

Cellular activities governed by physical and functional interactions to create biological pathways, with interactions between biomolecules or gene products.

Identification of the systems level cellular process description as a pre-requisite of meaningful derivations of biological state. Disease biology is similarly being addressed, where the advent of high throughput data and sequence details pave way toward elucidation of diverging descriptions of interactomes. The meaningful and accurate procedures deriving binary interactions and gauging their influence in disease development require convergence and comparative assessment of interactome descriptions. These include the core genomic, transcriptomic, and proteomic data inputs deepening the efficacy of theranostics obtained through the comprehensive interplay of bio-interactions.

Network biology scores in coordinating the conserved relations between the immune pathways, effects of pathogens on immunity, diversity and heterogeneity of immune cellular responses, mechanistic details of bio-interactions at the system's level. The integrative networks are being explored to provide for innovative information using a tri-partite approach that involves experimental data, advanced mathematical and computational modelling with validations to ensure the generation of high throughput reliable data (Lim et al. 2013; Voit 2000).

Network of Networks

Human disease is a constant aspect of life and consistently being studied using latest techniques, increasing our knowledge dramatically, through molecular basis, taxonomic and phenotypic screening, and creating therapeutic screens. The associations between diseases using these aspects has further helped in re-categorization and structuring of existing knowledge through the data driven analyses. This is a strong indication of the complex biological and cellular networks underlying the genotype to phenotype correlations. The demarcation of the network biology discussing the relevant types of interactome networks, their mapping, and integration into interactome network models is important for functional theranostic designs in most of communicable and non-communicable diseases.

The interactome networks are suggesting unique inter-connected nodes which represent the biomolecules that are possibly perturbed in disease conditions. These are sometimes referred to as modules or sub-networks that comprehensively depict the coordinated role of the molecular players at the cellular system. The genetic factors that are responsible or modulated can be caused by different genetic directives but have many overlapping factors involved (Vidal et al. 2011). Scientific deliberations are now focusing increasingly on the study of the patterns of interaction networks to comprehend the underlying causal effects in disease development. The complex diseases have inherent heterogeneity that spices up the utmost need of generating the discreet data that relays quantitative details as per the patient based phenotypic characteristics (Vidal et al. 2011).

The complex disease investigations have fundamental difficulties in ascertaining the details of the factors, their levels and role due to the shared combinatorial effects of the genetic and non-genetic influences. The demarcation and discovery of such elements and also the broad spectrum impact of the findings have led to altering the

specified complex disorders mostly to be grouped under the "group of – or spectrum disorders or part of syndrome." Examples of such exist in genetically inherited diseases (e.g., autism); infectious diseases (e.g., Leishmaniases); non-communicable diseases (Cancer) (Pujana et al. 2007). The individual contributions of genetic changes, rare mutations, expression level modulations are challenging to be deciphered for such complex disease settings, making the need of network of networks, more pertinent and prominent in near future.

The identification, characterization, and validation of the interaction networks and network based biomarkers is critical to organize the disease-specific functional biomarker details that modulate during the progression, development, or treatment of the disease. Precision medicine tethers to the concept of network medicine that is crucial to extricate the interplay and cross-talk between clinico-molecular features associated with disease utilizing the multiplexed network of genetic and non-genetic factors.

Drafting Interactome Networks

The dissection of the cellular interactomes is a bottom up approach which simplify the complex systems as components or nodes and interactions as edges. This is how the usual interaction networks are structured, where nodes as mostly biomolecules like proteins, RNA, gene sequences, or metabolites, while the edges are the physical, biochemical, or functional interactions between them that have been demarcated either through experimental means or by prediction algorithms (Allore and Murphy 2008). The "interactome networks" are prepared through systematic, empirical, and standardized assays, serving as scaffold information to create graph theory property or neural networks scaled at either local or global levels between the interacting components or nodes. The unbiased and statistically different outputs from randomized networks have led to potential true estimations of biological processes, though powerful details of dynamic and logical features that connect the structural alterations with functional outputs of the gene products, e.g., alternate splice variants, allosteric changes, and post-translational modifications are mostly not included in the simplified models (Licatalosi and Darnell 2010). To create the modelling details that can scale at the level of complete cells, such molecular transitions also need to be added to the layered interactome networks that might overcome the lacunae of individual interactome networks.

Network Biomarkers

Biomarkers are quantifiable indicators of differential aspects of normal biological processes in comparison to the pathological conditions and therapeutic responses that allow for early diagnosis, predicting, and monitoring outcomes. The disease-related biomarkers are critically important for demonstrating specificity, sensitivity, stability, reliability, repeatability, traceability, as well as levels of treatment efficacy

that can be conferred while banking on their application in disease biology (Simon 2005; Liu et al. 2012). It has been well established that the physiological alterations in the cellular systems is conveyed by mechanistic changes of varied players and their interactions at different stages of disease progression and establishment is critical to estimate dynamical networks and the associated network biomarkers that have inherent all types of interaction networks including the experimental, computation, or bioinformatics based, mathematical model based clinically relevant modules.

The high throughput data mining from the various omics technologies at the level of genome, transcriptome, proteome, epigenomes, and metabolomes all contribute toward the multi-dimensional data that form the systems clinical medicine exploring the untangled realms of functional modules in complex diseases. The categorical heterogeneity of such complex diseases, that confers the severity and progression differences and also the drug responses of being sensitive to resistance, have been deciphered for cardiovascular-related network and respiratory networks. These networks are widely used in studying the complex interactions, InSyBio BioNets, which is a cloud-based web platform offering a unique biomarker discovery pipeline, which combines differential expression analysis and a method for comparing biological networks to identify biomarkers efficiently. As a case study, InSyBio BioNets was applied to a Parkinson dataset of gene expression measurements and outperformed a standard statistical approach by recovering a more compact and informative set Biological of biomarkers (Theofilatos et al. 2016).

The sensitive detections of such network biomarkers have led to categorization of the clinical details in terms of patient stratification in terms of their biological molecular interaction, perturbed under specific therapeutic conditions leading to improved outcomes of patients. The candidate network biomarkers have been intensively utilized in cancer diagnostics, prognosis, efficacy prediction studies that includes microarray analysis as well as protein-protein interaction networks as layered information that were combined to reach accurate molecular interpretations and classification of tumors (Wu et al. 2012; Liu et al. 2013). The network biomarker studies have provided details of disease-related molecular interactions that are altered under the dysfunctional processes triggered under specific conditions relaying diseased phenotypes, e.g., expression profiling studies in combination with functional genomics and proteomics data found potential functional associations in breast cancer studies (Marcotte et al. 2016). Similarly, network biomarkers have been utilized in other complex disorders that target both non communicable as well as infectious diseases.

Interaction Networks and Dynamic Network Biomarkers

Interaction networks embrace the biomolecular factors creating gene regulatory networks (GRNs), RNA network that includes mRNA – miRNA networks, signaling networks, protein-protein interactions (PPI), and metabolite networks. The high-throughput collections of the large heterogenous datasets build using such

interaction networks have been collated for various cancer studies that include breast cancer, prostate cancer, bladder cancer, colorectal cancers, hepatocellular carcinoma (Nibbe et al. 2010; Chan et al. 2012b; Debmalya et al. 2020; Green et al. 2018).

The experimental scale interactome network mapping has been created using proteome scale analysis (Loscalzo et al. 2017; Finley and Brent 1994; Bartel et al. 1996; Fromont-Racine et al. 1997; Vidal 1997), while metabolic pathways at cellular scales and signaling pathways have given detailed cross-talk between biomolecules involving physical as well as functional web of interactions (Leiserson et al. 2015). Similarly, identification of interactions between transcription factors and DNA regulatory sequences are being captured to estimate the expression regulation and its global organization within the cells (Chen et al. 2008). The interactome networks have been compiled using three major strategies, i.e., (i) curating the data from literature studies or text mining, usually obtained from few types of physical and biochemical interactions (Roberts 2006); (ii) Computational simulations and predictions that is structured on "orthogonal" information in addition to the physical and functional interactions that involves sequence, gene order conservations, co-occurrence of genes, as well as protein structural information (Marcotte and Date 2001); and (iii) experimental mapping using high throughput systematic data using the whole genome or proteome analyses (Walhout and Vidal 2001). The interactome networks thus created are complimentary but still have different possible interpretations. Thus, network of networks could probably bridge the gap that exists between the varied literature-based interactome data that lacks systematic analysis, to the efficiency of computational predictions, that handle large data sets though on indirect information to the detailed experimental interactomics describing unbiased, systematic, and controlled data. Such a thorough interactome studies have been conducted on model organisms that have proven a milestone of information and provided support to the conceptual integration through pioneering technologies and improvement in the algorithms thereby.

The largely incomplete and sometimes overrepresented networks that may confer missing nodes, i.e., biomolecules, complexes, or phenotypes and edges, e.g., associations due to co-localization, reactions, or influences; sometime false positives that have any conclusive contextual information in cellular processes. The dynamic structures of the networks also represent changes that necessitate the development of scale free networks (Albert 2005) that allows node connectivity distribution to follow a power-law, having small world networks such that the distance between nodes have proportional increment to the logarithm of the network size (Albert 2005). This leads to few nodes as highly "connected hubs" with majority of these nodes having low degree of connectivity. These dynamical networks have also proven to be valuable in representing the complex biological processes. The dynamic interactions of disease biology is also linked to designing these dynamic interactome networks, as the gene co-expression data, stoichiometry, and kinetic parameters are required to have accurate characterization for knowledge of the underlying mechanisms in disease progression. These are to be further integrated with drug and phenotype networks that could correlate the dysfunctional biological perturbations in disease, to provide comprehensive and concise details for effectual

medical interventions (Pichlmair et al. 2012). Identification of disease micro-biomarkers requires effective computational and statistical methods for determiningfrom a very large number of candidate biomarkers a minimal subset of biomarkers that can accurately discriminate between two or more phenotypes. The various resources, e.g., SparCC: Sparse Correlations for Compositional data (SparCC) infers a network of associations between the microbial species based on the linear Pearson correlations between the log-transformed components (e.g., OTUs). SparCC makes two main underlying assumptions: (i) the number of nodes (e.g., OTUs) is large; and (ii) the underlying network is sparse. Implementation of SparCC included as part of the SPIEC-EASI tool is recommended (Hood et al. 2004). The Meinshausen and Bühlmann (MB) method is another technique for estimating sparse networks based on estimation of the conditional independence restrictions of each individual node in the graph and can also be implemented in SPICE EASI tool (Manazalwy et al. 2019).

Interactome Network Types

Gene Regulatory Networks

The gene regulatory networks (GRNs) or the transcriptional networks involve the transcription factor or putative regulatory biomolecules that act as nodes, and edges represent the physical interaction of these transcriptional factors (TFs) with DNA regulatory elements. The edges are considered as incoming (TFs binding to regulatory DNA) or outgoing (regulatory DNA bound by TFs), that have been deciphered using either in vivo yeast one hybrid or in vitro ChIP approaches for large scale mapping. The yeast one hybrid, utilizes a cis-regulatory DNA element as bait that uses genes and captures associated proteins (gene-centric), while in chromatin immunoprecipitation antibodies are raised against TFs, or against peptide tags fused with TFs, making it as protein centric approach. The techniques can unravel novel regulatory motifs if accurate predictions of TFs are made for applying either of these to demarcate gene regulatory interactions (Zhang and Horvath 2005; Reece-Hoyes et al. 2005; Vaquerizas et al. 2009). Model organisms including yeast, C. elegans, as well as cultured mammalian cells have been used for creating interactome networks using Y1H and ChIP (Vermeirssen et al. 2007; Grove et al. 2009; Lee et al. 2002; Cawley et al. 2004).

The regulatory RNAs including miRNAs or short non-coding RNAs that also sometimes part of the GRNs as they bind to complementary cis-regulatory RNA elements located in 3′ UTRs of target mRNAs. miRNAs form complex networks, interactions with its targets, where nodes are either these miRNAs or target 3′ UTRs, with similar incoming and outgoing interactions possible as edges. The non-coding RNAs are not master regulatory molecules, as they mostly attune to post-transcriptional regulation of gene expression, while mostly computational predictions of miRNA interactions as well as experimental methods are now focusing toward these miRNAs/3′ UTRs as part of GRNs as studies performed as large-scale

miRNA network in C. elegans. These studies need to be also appended in the other known genomes for a comprehensive look into the interactome networks.

Metabolic or Protein: Protein Interactome Networks

The functional protein-protein interaction networks represent the physical association between proteins, its signature peptides, or motifs/domains of the complete proteins as nodes while edges that are non-directed as the interaction module itself. There are various in vitro and in vivo technologies that have been utilized to create the experimental PPI maps as binary interactions, e.g., yeast two hybrid, or as indirect associations using TAP-Tags or Affinity or immune-precipitation for mapping multitude of interacting proteins in a complex, or directly using affinity associated MS analysis for the same (Rolland et al. 2014; Bonder et al. 2017). These interactions create differential maps of interactions due to their direct or indirect analyses patterns, serving as gene essentiality relationships with the number of interacting proteins. The interaction maps have been prepared using the comprehensive Y2H technologies with model organisms (S.cerevisiae, D.melanogaster and C.elegans), and also mapping of co-complex high throughput protein interactions using the AP/MS efforts (Sun et al. 2016).

The cumulative efforts of protein-protein interactions require accurate and sensitive mapping utilizing the empirical framework favoring critical parameters of completeness (most or all of protein physical interactions allowed in given search space), precision (true biophysical interactors), and assay or sampling sensitivity (number of interactions detected by particular assay or fraction of all detectable interactions in a single assay) (van Leeuwen et al. 2016). The interactome proteome maps could pave way for a roadmap toward comprehensive functional maps addressing the biological processes (Srivas et al. 2016). NetworkAnalyst 3.0 its key need for interpreting gene expression data within the context of protein-protein interaction (PPI) networks.

Metabolic Networks

Networks comprising all plausible biochemical reactions, in particular, cellular system or organism, where metabolites act as nodes and reactions or enzyme catalyzing these reactions occur as edges. Like the PPIs, the edges here are non-directions either directed or undirected, depending upon whether reactions is reversible or not (Motter et al. 2008). In some metabolic network models, the opposite situation can also be true, as per the representation of the nodes and edges, with enzyme as nodes and edges belonging to “adjacent” enzyme pairs with interdependent substrate and products among them. Classically metabolic networks have been represented as large metabolic pathways that have been completed with additional gene annotation data from the sequenced genomes. The metabolic networks have been constructed manually with computation prowess

added through a thorough curation of literature or text mining of published reports describing experimental evidences of metabolic reactions characterized from reconstituted or purified enzymes. There is also additionally compilation of orthologous enzyme reactions as part of the computational layer added that are experimentally characterized and show sequence conservation across species. Metabolic reconstructions involve the base of these elaborate proteome scale metabolic network maps demarcated for many prokaryotes and unicellular eukaryotes, as these are the most comprehensive maps of all biological processes occurring inside a cell and representing validated experimental evidences. The gaps that exists in such maps need also direct experimental analysis to generate more robust metabolic network systems or reconstructions simulated on existing networks (Ghiassian et al. 2015).

Designing Interactome Networks with Cellular Networks

The three major types of interactome networks discussed so far based on both physical and biochemical interactions need to be extrapolated to design the "scaffold" that could be used to overlay complete information of cellular systems, with additional "functional" layers to be appended to fine tune representation of biological processes and actual quantitative estimations. These networks that have the functional links represent the conceptual interactions where links between genes and gene products are reported based on functional interactome integrations taking cues from the existing interactome networks, though not requiring always the physical macromolecular interactions (Dezs et al. 2009; Greene et al. 2015). These designs are possible due to the complementary data made available genome scale analyses and predictions that interrogate the complexities or heterogeneities of the genotype to phenotype relationships. This has been mainly branched from the realization of the dysbiotic physiological modulations that affect the functional aspects on the existing interactome network maps (Menche et al. 2015; Corradin et al. 2016; Greene et al. 2015; Xenarios et al. 2002).

These have been further grouped as discussed in brief here so that these accrue the graph properties of interactome networks that can be simulated to generate the most, unbiased profile of cellular status and its correlation to the physiological conditions. These include

(i) Transcriptome – Interactome Profiling Networks
(ii) Phenotypic Profiling Networks
(iii) Genetic Interactions Networks

Transcriptome: Interactome Profiling Networks

Macromolecules or biomolecules are known to coordinate and act together in a biological process, not just individual entities. This cooperativity is tended to be captured at varied interaction networks including the Protein – DNA/RNA/protein

interactions, conveniently represented as networks or graphs with the molecules addressed as nodes or vertices and links or edges denoting the interactions between them. These networks have topological characteristics that include scale free property in a network, that confers in highly connected nodes, called "hubs" have a sub-network of sparsely connected nodes. The contextual application of these networks in disease biology refers to the topological properties of the interacting networks using connectivity or modularity of the participating genes or gene products that rely on generating both the physical and functional correlation. The gene products and complexes in common signaling cascades or similarly in disease biology are expected to show patterns of expression with higher similarities, and such a situation from either using transcriptome or proteome data need to be correlated globally with interactome networks. The vast majority of the transcriptome profiles generated from microarray, RNAseq data, that have been detailed for different species residing across multitude of diverse genetic and environmental conditions (Vidal 2001, 2011). The genes may be co-localized, co-regulated, co-expressed punched in matrices of genes of an organism against all conditions that the organism is exposed toward to generate the expression compendium. Discriminant and correlation analysis are statistically tested on the nodes and edges in the co-expression networks above a set threshold (Kim et al. 2001; Stuart et al. 2003) so as to agree to titration procedures applied thereof in such transcriptome interaction networks. The transcriptome co-expression network profiles created have higher degree of confidentiality about the regulatory network operations (Amit et al. 2009). The profiling networks have been combined using similarly co-expression profiles with the protein interaction maps in yeast revealing the significant overlaps between the interaction edges in interactome networks with the one found in transcription profiling networks. These studies have to be additionally linked to biologically relevant protein interactions who are not a part of such co-expressed systems or are rather segregated as never correlated, to generate a true functional transcriptome interaction profile.

Phenomics: Phenotype Profiling Networks

The need for linking gene modulations that relay functionally or phenotypic detectable changes is quite pertinent to disease biology. Genes encoding functionally related products are linked in networks contributing to similar phenotypic alterations. In the transcriptional profiling networks these are the genes that are grouped under the matrices with all genes of an organism and the phenotypes that are profiled in a same phenotypic compendium. Studies in model organisms (yeast, C. *elegan*, Drosophila) and even in humans (Giaever et al. 2002; Mohr et al. 2010) using gene knock-out/down techniques have shown all genes amenable to perturbations leading to variety of standardized phenotypes. The phenomics or "phenome" networks that are investigated through systematic gene-phenotype analyses is targeted to show these linked genes as nodes, and edges linking pair of genes depicting correlated

phenotypes tested above a set threshold. These efforts require titrations and decisions for the threshold properties of the phenotypic similarity or dissimilarity.

The phenome profiling networks have been shown to be associated with protein-protein interactome networks where overlapping and integrating the binary interactions, co -expression, or transcriptional networks and protein-protein interactions are overlayed to create very robust integrated networks with precise prediction patterns and power (Piano et al. 2002; Walhout et al. 2002; Gunsalus et al. 2005; Grove et al. 2009). The efforts to design these genome-wide phenome networks are underway in most of the model organisms after the proof of concept detailing in yeast model.

Gene Interaction Networks

The systematic mapping of the functionally related genes also points toward them exhibiting genetic interactions through gene mutations studies. The studies include comparison of phenotypes generated by double mutants (mutations in pair of genes) to single mutants (mutation in either pair of genes). These are also termed as synthetic lethals or negative, when phenotype conferred by the double mutant is aggressively worse than single mutant, or as positive or alleviating/suppressive if the phenotype of double mutant is significantly better compared to single mutant (Mani et al. 2008). These gene interaction or linkage studies have been utilized traditionally by genetics, while their inclusion in the functional genome analyses using systematic high throughput mapping has given rise to large-scale gene interaction networks (Boone et al. 2007). The pattern of genetic interactions here would confer similar details as the transcriptional and phenotypic profiling networks, with the gene or the nodes in genetic interaction networks representing the matrices of genes exhibiting the positive or negative features in the interaction and the edges functionally linking such genes based on their high similarities. The genetic interaction networks here provide an additive layer of predictive models of biological processes for its power and robustness along with the other interactome networks. The nature of the genetic interaction maps, derived using various methodologies like high density arrays or synthetic genetic arrays, barcoding microarray using deletion mutants in yeast clearly depicts that these interactions may not correspond to physical interaction of the corresponding gene products (Boone et al. 2007; Mani et al. 2008; Costanzo et al. 2010). This leads to detailing of unique patterns of the interactions from the different datasets, as these increase probability to reveal pair of genes in parallel pathways or in different molecular machines. The negative mode of interactions here would not correlate with the protein-protein interactions in either binary or multi-complex protein modes, while the positive mode genetic interactions provide more probable physical interactions between these genes (Beltrao et al. 2010; Costanzo et al. 2010). These details of positive interactions is studied as loss of either one or two gene products coordinating to provide similar effects in a molecular complex.

Conclusion and Perspectives

The interactome networks confer that discreet detailing and inter-connection of the normal biological processes as well as the disease-specific insights would have a greater impact in understanding and establishing the molecular transitions that are related to the mechanistic details, early diagnosis, risk assessment, classification of the stage or grade, as well as monitoring and therapy regimes. These intricate details would step up the efforts toward targeted directive or combinatorial therapeutics, with highest degrees of sensitivities and specificities. The disease networks could point toward the differential states of normal or pre-disease analyses, optimization of the effectiveness of direct assessment many-fold to allow designing theranostics with impactful effectiveness and finesse. We hope to usher into the era of utilizing and optimizing the upcoming machine learning and artificial intelligence approaches (Sniecinski et al. 2018; Moingeon et al. 2021) further to swiftly turn from reactive to preventive medicine strategies and designs.

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Mice as Experimental Models for Cancer Research

5

Anisur Rahman Khuda-Bukhsh, Jayeeta Das, and Asmita Samadder

Contents

Abstract

Cancer is a devastating disease affecting a large number of people worldwide. There has been relentless effort to learn various aspects of the development of the disease as well as how to combat it in various ways. In that process, ideal models

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S. Pathak et al. (eds.), *Handbook of Animal Models and its Uses in Cancer Research*,
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for cancer research were first in harness. Because of several advantages, inbred mouse strain (*Mus musculus*) has proved to be an ideal animal model to investigate changes in different tissues during and after development of cancer induced by different carcinogens or by genetic manipulation, yielding possibilities of intercepting it at specific steps preventing advancement of the disease by modulating physiological and tissue-specific regulation of genes. Carcinogen-induced cancer models are most suited and generally used to assess the therapeutic properties of drugs, explore precautionary actions for carcinogenicity, and understand molecular mechanisms involved in various types of cancer. Initial manipulation of gene expression in mouse genome resulted in production of transgenic mice categorized by the constitutive expression of a particular gene and knockout mice characterized by the ablation of a specific gene. These planned mouse models are projected to serve as a foundation for additional exploration of the molecular basis of human diseases. The present chapter is mainly focused on the principles, advancement, and application of currently conducted researches deployed in the conditional regulation of gene expression in the investigation of cancer.

Keywords

Oncogene · Transgenic · Knockout · Gene switch · Nude mice · Xenograft

Introduction

Extensive researches for over several decades have enabled researchers to develop mouse models of cancer that serve as a critical tool for studying cancer biology along with their development and management using novel cancer therapeutics. Formerly, researchers studied about cancer using cell lines from human tumors, but though this *in vitro* cell culture system has certain advantages, it also has some limitations in understanding possible physiological interactions that may take place between the tumor cells and their immediate surroundings *in vivo*.

Mouse models of cancer are preferred for biomedical exploration as they are vital in interpreting anatomical, physiological, and genomic similarity to humans. The advantages of mouse model include the following: their small body size, low-cost maintenance, shorter life cycle, and ample genetic possessions. Scientists and investigators have used mouse models to investigate the precise mechanism of cancer initiation, the role of oncogenes and tumor suppressor genes in carcinogenesis, and therapeutic response during cancer treatment (Das et al. 2012, 2013; Paul et al. 2013a, b).

Cancer is a very systematized disease; it is primarily present on local manifestation and later progresses in a multistep course with several hallmarks including fast proliferation, resisting cell death, angiogenesis, local invasion, metastasis, etc. (Hanahan and Weinberg 2011).

Due to unlike pathologies, genetic variations, and patients ending in different clinical signs of oncology, no discrete animal model can entirely imitate this

multifaceted process. In order to generate different human cancer pathologies and address several queries, there have been numerous animal cancer models including tumor transplant, genetically engineered cancer models, and carcinogen-/mutagen-induced cancers.

Strategies Adopted for Developing Different Types of Cancer in Mouse Model

Several strategies are generally adopted for generating mouse models of cancer. Selection of an appropriate strategy has to be made after determining the pre-conceived purposes for which the specific mouse models will be utilized to yield important information in cancer research (Cheon and Orsulic 2011).

The Transplanted Cancer Models

These models are produced by transferring of live cancer cells, *in vitro* or directly from tumors of donor animals to recipient animal. In xenograft models, donor and recipient belong to different species but can share genomic resemblance. The recipients are immune-compromised before cancer cells are transplanted, so that the recipient does not reject donor cells. In allograft models, transplantation is done between same species. In both the models, transplantation can be orthotropic, subcutaneous, or intravascular to examine the specific stages of cancer progression (Vargo-Gogola and Rosen 2007) (Fig. 1).

The Genetically Engineered Models (GEM)

These models for cancer studies utilize specific animal strains with manipulated genomic sequences, having either overexpressed oncogene or loss of a tumor suppressor gene. The GEMs can be subdivided into transgenic and endogenous ones (Frese and Tuveson 2007). These GEMs help us to carry out investigation of certain genes associated with consequences of clinical cancer development from initiation, through progression and clinical onset until then, when it can be ideally used to explore the etiology, prevention, diagnosis, and treatment of cancer.

Induced Cancer Model

Induced cancer mice models refer to particular cancer types developed in mice that have been exposed to certain environmentally hazardous factors such as carcinogenic chemicals, radiation, viruses, microbial flora, or even physical stimuli. The synthetic chemical compounds are exposed to the body by forced feeding,

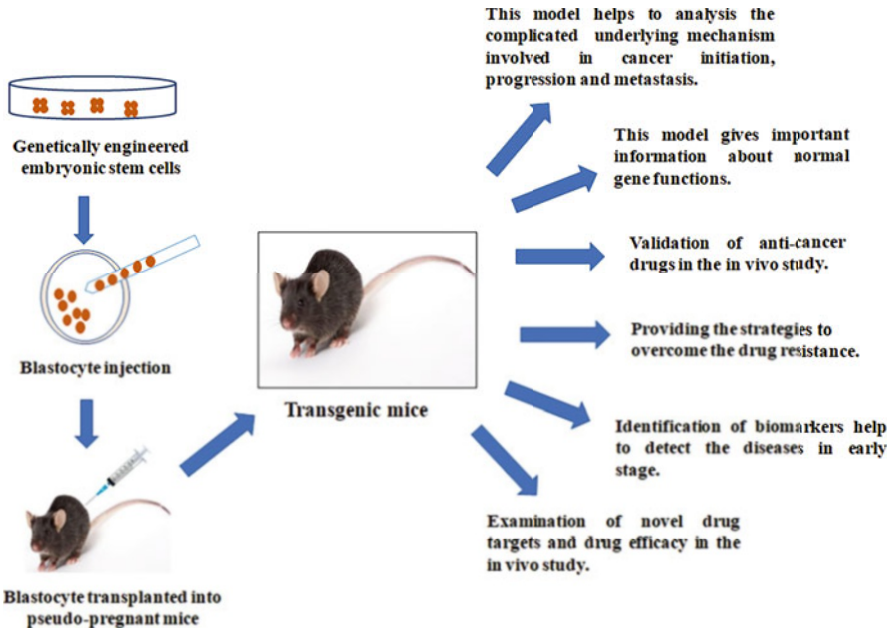

Fig. 1 Representation of formation of transgenic mice model

forced inhalation, injection, dermal absorption, etc. Furthermore, due to analogy in carcinogenesis progression, induced cancer in mice resembles that of human cancer.

Gene-Targeting Technology

Another breakthrough was the development of gene-targeting technology that introduces the transfer of specific mutations in mammalian genes to the mouse germline. This technology results in two different approaches of gene manipulation, either knockout of a specific coding sequence of a gene (by deletion) or knock-in to a specific region of genome (introduction of exogenous sequences). These knockout studies help to establish the functions of tumor suppressor genes by disrupting their expression (Donehower et al. 1992). Likewise, introduction of knock-in mutation of genes into a specific locus of mouse gene helps to understand the effect of gene mutations on physiology for cancer development (De Vries et al. 2002).

RNA Silencing Techniques with RNA Interference (RNAi) or Artificial MicroRNAs (amiRNA)

Mouse model is also used for targeting specific genes for their downregulation or disruption involving RNA silencing techniques with RNA interference (RNAi) or artificial microRNAs (amiRNA) useful for studying gene function, particularly

where the recessive mutations are lethal; however, this technology is limited by nonspecific targeting and silencing of the RNA constructs. Again the chromosomally engineered mouse model serves as a necessary tool of study for bringing back the effect of human chromosomal abnormalities. Thus, using different breeding strategies or other manipulations, different combinations of mouse models can be created to study molecule interplay, oncogene-tumor suppressor gene interactions, and impact of different genetic backgrounds on tumor spectrum (Zhang et al. 2011).

In the following, a little more information has been given about the use of different strategies for inducing different types of cancer in mouse system.

Different Carcinogens Used for Inducing Different Types of Cancer

There are many naturally occurring as well as synthetic chemical carcinogens which are used in mouse models *in vivo* to induce several types of cancers for experimental purposes. Some common carcinogens and their target organ/tissue are mentioned in Table 1.

Primary Cancer Models for Studying Cancer of Different Organs

The primary cancer models for studying cancer of different organs can be broadly categorized into the following subsections (Fig. 2).

Brain Cancer

Use of different types of animal models has helped in the advancement of scientific understanding of human brain tumors. Several years of investigations based on the inciting factors of brain tumor development have enabled researchers to use virus-induced, carcinogen-induced, and transplant-based as well as transgenic models for understanding brain tumor initiation, development, progression, and metastasis. The mouse model serves as a convenient experimental system for discovering novel drug targets in brain as well as chemotherapeutic agents for reliable testing. The mouse models that are being used on a large scale due to convenient maintenance and experimentation are as follows:

(a) **Xenograft Tumor (Cell or Tissue Transplantation) Models**: With advancement in the transplantation techniques, intracerebral tumor growth has resulted in 90–100% yield with reduced extracranial metastases to 0–5% and thus makes this *in vivo* cancer modeling technique a frequently used technique for cancer biologists (Kobayashi et al. 1980). Transplant models include two different types depending upon the origin of cell or tissue:
   (i) Syngeneic Transplantation Models: In these models, tumors are induced by chemicals, viruses, or obtained from transgenic mouse, and these cells are

Table 1 Classification of a few chemical carcinogens in experimental mice models

Category of carcinogen	Name of carcinogen	Target organs/tissues
N-nitroso compound	*N*-Nitrosodiethylamine (DENA)	Lung, Liver, Kidney (Vargas-Olvera et al. 2012)
	N-Nitrosodimethylamine (NDMA)	Liver, Lung (Anderson 1988)
	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Lung (Kreisel et al. 2012)
	N-Butyl-*N*-(4 hydroxybutyl) nitrosamine (BBN)	Bladder (Kim et al. 2002)
	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)	Lymph, Intestine (Ochiai et al. 2002)
Heterocyclic amine	2-Amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx)	Lung (Takeuchi et al. 2010)
	2-Amino-3-methylimidazo [4,5-f] Quinolone (IQ)	Colon (Andreassen et al. 2002)
Polycyclic aromatic hydrocarbon	2-Acetylamino-fluorene (2-AAF)	Liver (Hoogervorst et al. 2005)
	7,12-Dimethylbenzanthracene (DMBA)	Skin, Breast (Siddiqui et al. 2013)
	Benzo[a]pyrene	Lung (Das et al. 2012), Colon, Stomach (Goyal et al. 2010)
	3-Methylcholanthrene (MCA)	Skin, Lung (Brown et al. 1999)
Food additive	Potassium bromate ($KBrO_3$)	Kidney (Giri et al. 1999)
Polychlorinated biphenyl	2,3,7,8-Tetrachlorodibenzo-pdioxin (TCDD)	Liver, Lung (Chen et al. 2014)
Antineoplastic agent	*N*-Methyl-*N*-nitrosurea (MNU)	Breast, Stomach (Leung et al. 2008)
Naturally occurring compound	Aflatoxin B1(AFB1)	Liver (Sivanesan and Begum 2007)
	Asbestos	Lung (Kamp 2009)
	Aristolochic acid (AA)	Liver, Kidney, Fore stomach (Wang et al. 2012)
Synthetic carcinogen	1,2-Dimethylhydrazine Dihydrochloride (DMH)	Colorectrum (Sánchez Negrette et al. 2007),
	Azoxymethane (AOM)	Colorectrum (Hernández-Salazar et al. 2013)
	Methylazoxymethanol	Colorectrum (Pandurangan et al. 2014)
	4-Nitroquinoline 1-oxide (4NQO)	Oral cavity (Kanojia and Vaidya 2006), Esophagus (Yang et al. 2013)
	Ethylnitrosourea (ENU)	Central nervous system (Morrison et al. 2007)
	Phenobarbital	Liver (Lee 2000)

maintained via cell culture and are transplanted intracranially after subcutaneous passage. An example of such syngeneic model is 4C8 mouse glioma mouse model where 4C8 cell line derived from a transgenic mouse glioma-

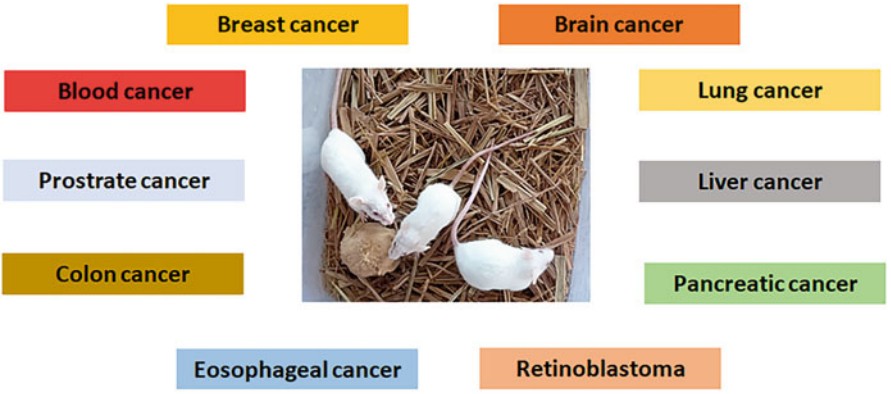

Fig. 2 Mice as experimental model for different types of cancer

tumor is injected intracranially into an immune-competent mouse. This system serves as excellent model for testing antitumor therapy for human use.

(ii) Hetero Transplantation Models: Hetero transplantation of human brain tumors involves transplantation of tumor from different species or different strains of same species; therefore, this process was highly unsuccessful previously due to immune-rejection of brain tumors. Since, the knowledge of an immune-competence came to light, hetero transplantation of brain tumors into either an immune-incompetent nude mouse (genetically thymus deficient) or an immunologically privileged site was performed during experimentation with variable success rates (Jones et al. 1981).

(b) **Genetically Modified Mouse Models (GEMMs)**: GEMMs characterize in providing time-dependent and genetic control to mutant allele expression. Since xenograft tumor models cannot faithfully recapitulate complex processes of etiology, tumor-host interactions, pathobiology, and biochemistry of preformed tumors, GEMMs function as essential experimental tools that can provide invaluable insights on the drug effects on the tumors as well as present high tumor penetrance (Weiss and Shannon 2004), for example, the mouse models for inducing astrocytoma-involved transgenic expression of v-src kinase (Weissenberger et al. 1997) and V12H-Ras both under GFAP (glial fibrillary acidic protein) promoter. Again activation of p21-RAS can activate the signal pathway and thus mimic the effect of EFGRvIII mutation resulting in mouse glioma model.

Retinoblastoma Cancer

Retinoblastoma (*Rb*) is a frequently occurring primary intraocular tumor that leads to death if left untreated, but due to the lack of viable human samples, study of origin of retinoblastoma is quite difficult. Therefore, preclinical research is essential for studying the course of tumorigenesis and searching the diagnostic as well as treatment modalities. For understanding the recognizable pattern of

mutations in the *RB1* alleles, the genetic inheritance and the specific location of the tumorigenesis require combination of both xenograft and transgenic models which can further help in investigating retinoblastoma tumor biology; this also helps in designing effective diagnosis and chemotherapeutic agents (Friend et al. 1986).

(a) **Transgenic models**: Transgenic murine models have been used to show variable similarity with human form of tumor. The lack of one copy of *Rb*1 gene could cause bilateral retinoblastoma in children; however, the same experimental condition in murine model cannot develop retinoblastoma.
(b) **Retinoblastoma knockout models**: Recent study with chimeric mice having retinoblastoma gene knockouts along with loss or inactivation of p107, p130 using promoters of Chx10, Pax6, and Nestin genes reveals that in case of postnatal mouse retinal development, upregulated p107 and p130 have a compensatory role on the inactive *Rb*1 gene and thus prevent deregulated retinal development in mice models. In this way, Cre-Lox transgenic technology helped in selective knocking out of *Rb* genes under the control of Chx10, Nestin, and Pax-6 promoters in retinal progenitor cells to create breedable *Rb* knockout models (Zhang et al. 2004).
(c) **LH-beta T-Ag models**: These models have been invaluable tools in conducting preclinical studies for evaluating better therapeutics such as cryotherapy, radiation therapy, and vascular targeting therapies. However, the use of viral SV40 onco-proteins (Windle 1990) in inducing the tumor is a major disadvantage here as the interactions of the onco-proteins are still not clear. This model also cannot mimic tumor characteristic of human retinoblastoma. The ensuing limitations of this model asserted the development of conditional knockout mouse models.
(d) **Xenograft Models**: The aim of using xenograft mouse model lies in the close histological resemblance of the model for *Rb* gene with that of the human for evaluating the efficacy of existing therapies and developing new treatments. Xenograft models are based on intravitreal injection of *Rb* −/− retinoblastoma cells of human cell line Y79, into the eyes of immune-deficient (athymic nude) mice. Induced growth and spread of tumor were characterized by advanced imaging technologies like scanning laser ophthalmoscopy (SLO), fluorescein angiography (FA), magnetic resonance imaging (MRI), microcomputed tomography (micro CT), and positron emission tomography (PET). Since *Rb* xenograft models can facilitate rapid presentation of symptoms *in vivo* by better tumor engraftment of cell lines and tumor tissues in immune-compromised animals, xenograft models are preferred over the genetic models for *Rb* cancer studies. However, a major drawback of this model is the altered tumor microenvironment seen in murine when human cells are implanted into the eyes. Depending upon the use of cell or tissue implantation, xenograft models are either heterotopic (subcutaneous graft) or orthotopic (Cassoux et al. 2015).

Blood Cancer or Leukemia

Leukemia is a cancer of blood-forming tissues of bone marrows. There are many types, namely, acute lymphoblastic leukemia, acute myeloid leukemia, and chronic lymphocytic leukemia.

(a) **Xenograft model**: Xenograft models focus to study on tumor development because of relative simplicity, high production, and quick result to better understand tumor biology. Xenograft models can be divided into the two following groups based on the source of tumor cells:
   (i) **Cell line-derived xenograft model**: Cultured blood cancer cells are implanted into the immune-deficient mice varying from athymic nude mice to NOD/SCID mice, and after the formation of blood tumor in immune-deficient mice, they are transplanted to other mice to show the biologic behavior, and activities of biomarker, to investigate different drug response against blood cancer (Li and Gu 2021). However, this procedure has some drawbacks like the immune system does not significantly interact with tumors, and culture cells do not always resemble the origin of the parental tumor.
   (ii) **Patient-derived xenograft model**: Tumor cells are obtained from clinically dragonized blood cancer patients, which are implanted into immune-deficient mouse where a tumor is formed, which can again be transferred to other mice to investigate the tumor biology and drug response.

(b) **Humanized mouse model**: In this model, mice are engrafted with cell lines derived from patient's tumor cells, or any genetically manipulated cells to examine tumor pathology. This model develops a competent immune system against blood tumor cells (Jia et al. 2021). Nevertheless, some disadvantages are encountered while using this model for an experiment. These are: It is very expensive, it is time-consuming, and it necessitates cluster breeding structure.

(c) **Genetically engineered mouse model (GEMM)**: This mouse model mimics the human tumor through genetic manipulation of the desired gene. Further, this model is subdivided into the two following groups:
   (i) **Germ-line transgenic mouse model**: The gene of interest is manipulated as a form of vector which is implanted into the fertilized egg. The offspring are born with supplementary copies of the transgenic gene. This model is also known as the knock-in model (Silva et al. 2021).
   (ii) **Conditional transgenic mouse model**: To produce conditional models, the crossing is done between a mouse having recombinase effector genes with a mouse containing target gene, so that the resultant mice show temporally and spatially controlled mutation.

(d) **Multiallelic transgenic mouse model**: In this mouse model, multiple genes are manipulated closely resembling human tumor complexity. Mice group carry

multiple gene mutations by using (CRISPR)-Cas9 genome-editing methods wherein CRISPR-Cas9 is transmitted directly to the zygote of the mouse model (Barghout et al. 2021). Sometimes, this method can potentially mutate off-target genes.

Lung Cancer

Lung cancer can be divided into four major, histologically identifiable subtypes: adenocarcinoma, squamous cell carcinoma, small cell carcinoma, and large cell carcinoma.

(a) **Conventional transgenic animals**: Conventional transgenic animals have reformed our ability to study gene function *in vivo* (Gordon 1989). The first oncogene studied specifically to the lung was the Simian virus large T antigen (Tag). Tag was targeted with both the *SP-C* and *CCSP* promoters.

The outstanding experimental animal model for lung cancer was *rtTA*-induced activated K-*ras* oncogene which caused development of pulmonary adenocarcinoma. The triple transgenic model was generated with mice bearing the *rtTA* regulator transgene and the *tTS* silencer transgene under the regulator of the *CCSP* promoter (Zhu et al. 2001).

(b) **Mifepristone Gene Switch**: The GLVP and GLp65 are mifepristone controllers which were targeted to the lung in transgenic mice using the *SP-C* promoter. Mice expressing the *SPC-GLVP* regulator were then reared to mice having the human growth hormone target transgene (*UASGal4-hGH*).

(c) **Conventional Knockout Mouse Model**: Endogenous genes can be ablated *in vivo* by using homologous recombination in embryonic stem (ES) cells. The use of homologous recombination in ES cells is used to delete a region of chromosome 9F1 as this region is semantic with human chromosome 3p21.2 and comprises of several tumor suppressor genes that are lost in human lung cancer (Lerman and Minna 2000). The phenotype of this mutation is lethal. One tactic to overcome this limitation was to target an event in which somatic recombination would activate a mutant oncogene. This method was used to express a mutant K-*ras* gene containing one K-*ras* allele with oncogenic mutation, and the other allele was wild-type.

(d) **Conditional Knockout Mouse Models**: Conditional knockout models use recombinases to ablate genes in a tissue- and cell-specific fashion. The recombinase system used to induce gene ablation in mice is the Cre–loxP system. Cre recombinase activity eliminates the sequences between the loxP site letting expression of the gene of interest. Defective (replication) human adenovirus having the gene for the Cre recombinase under regulation of the human cytomegalovirus promoter (Ad-Cre) has been constructed and used for a gene modification system in mammalian cells. Administration of adeno-associated viral (AAV) vectors having a Cre recombinase (AAV-Cre), having all viral coding sequences removed to avoid toxicity, can facilitate recombination in mice (Ueno et al. 2000).

Breast Cancer

Breast cancer is the second prominent reason of cancer deaths among women. Breast cancer can be sectioned into at least five main subtypes based on broad gene expression reporting: luminal A, luminal B, basal-like, ERBB2-positive, and normal-like breast cancer.

(a) **Knockout models**: Brca1 knockout mouse model revealed the indispensable nature of the breast cancer gene, as homozygous mutant embryos carrying truncation at *Brca*1 exon 11 (*Brca*1^{11}−/−) are more prone to chromosomal aberrations, alterations, and DNA damage and possess an inability to repair double-stranded breaks. Histological examination of mammary glands after conditional knockout of *Brca*1 (Brca1Ko/Co; MMTV-Cre and Brca1Ko/Co; WAP-Cre) confirmed the formation of tumors after a definite period, but the tumor induction mainly arises due to genomic instability, increased mutation rate, and chromosomal alterations caused due to loss of Brca1 in that specific tissue pointing to the fact that Brca1 indirectly trigger the initiation of the tumor.

Homozygous deletion of the Pten gene (Pten −/−) in mice embryo resulted in abnormal cell growth and proliferation of cells of the mammary gland. The heterozygous model (Pten+/−) also led to the occurrence of neoplasia and mammary tumor formation. Thus, Pten knockouts resulted in a high incidence of breast cancer.

Several strains of mouse model such as 129, C57B6 with mutant p53, or heterozygous p53 gene (p53+/−) are resistant to breast tumor formation but develop tumors in other tissues later. In BALB/c mice, homozygous p53−/− deletion causes early death and onset of lymphomas as well as the genesis of carcinoma in mammary tissue. Therefore, the genetic background of the mice model in the case of p53 knockouts played an important role in the development of a tumor, BALB/c mice being the ideal model to induce mammary carcinoma by knocking out the p53 gene (Kuperwasser et al. 2000).

(b) **Xenograft model**: The technique involves implantation of patient-derived xenograft (PDX) into an immune-deficient mice model. In this process, breast tumor tissues from a patient following removal by surgical resection and digestion into segments of single cells are implanted into the mice either heterotopically or orthotopically. Though heterotopic mode of injection appears to be easier, however, to study the incidence of breast cancer, implanting into mammary fat tissue is preferred orthotopically as tumor samples could directly communicate with their microenvironment and the degree of cancer metastasis is escalated at a higher rate in this mode of implantation.

Esophageal Cancer

The cancer of the food pipe that runs from the throat to the stomach (esophagus) involves smoking and ill controlled acid reflux as significant risk factors.

(a) **Subcutaneous xenograft mouse model**: In esophageal cancer (EC) research, the subcutaneous xenograft mouse model has been utilized broadly, and it is made by cultured cells derived from human esophageal tumor which is transplanted into immune-deficient mice (Fang et al. 2021).
(b) **Orthotopic xenograft model**: Subcutaneous xenograft mouse models are generated by cancer cell line which has limitation, so orthotopic xenograft model was utilized by scientists to understand metastasis of EC properly. Intact human cancer fragments are histologically transplanted by using surgery technique to develop orthotopic xenograft model, and bioluminescent imaging is a modern diagnostic technique that is for analyzing tumor growth in this model.
(c) **Genetically engineered mouse model**: GEMM technologies have been used for genetic modulation of the mice to permit regulation of gene expression level in esophageal tissue at any time like any other tissue.
(d) **Patient-derived xenograft model**: In this model, tumor biopsy cells obtained from clinical cases of human esophageal cancer are injected into immune-deficient mice which closely resemble subcutaneous xenograft model. The benefit from this model is that the heterogeneity of the tumor is sustained.
(e) **Chemical- or diet-induced mouse models**: Chemical- or diet-induced mouse models have been developed by the treatment of carcinogenic agents or diets which display similar phenotypes of human esophageal cancer mouse model.

Pancreatic Cancer

The incidence of pancreatic cancer involves multigene and multistep complex process, its prognosis is highly poor, and so obtaining clinical specimens at different stages of cancer has become almost impossible. Therefore, an induced preclinical pancreatic cancer model serves as an important tool for exploring the incidence, development, maintenance, and metastasis mechanism of the cancer, to find new therapeutic targets at different levels of cancer (Ryan et al. 2014).

(a) **Spontaneous tumor mouse models**: This model system is not used in general for pancreatic cancer as chemically induced mouse models are difficult to engineer, but pancreatic cancer can be induced in mice with the help of genetic engineering.
(b) **Genetically engineered mouse model**: In most of the studies, *Kras* proto-oncogenes are being used for developing GEMMs for research based on recapitulation of the cancer. The Cre/loxP recombination system and the Tet-On-inducible systems are widely used gene knockout strategies (Schonig et al. 2002). Use of GEMMs of pancreatic cancer is advantageous since their nature is similar to that of humans.
(c) **Establishment of mouse model based on cell line-induced tumor**: In order to understand the facets of human pancreatic tumors and its progression biology, researchers have opted for mouse models with transplanted cell line samples

from humans. To accomplish such research, the mouse models that are preferred are as follows:

(i) **Athymic (Nude) mouse model**: This involves mutant mouse that is hairless because of the presence of two copies of the gene "nu" (for nude). These mice lack thymus and therefore lack T cells in body. Thus, nude mice are immune-comprised models that cannot reject tumors or transplants of cell-lines from humans or other animals (Schmied et al. 2000).

(ii) **Patient-Derived Tumor Xenografts (PDTX)**: PDTX serve as crucial tools for coclinical trials of cancer therapies and drug development and are frequently used by researchers to develop targeted delivery vehicles improving the pancreatic patient outcomes.

Liver Cancer

The most common type of liver cancer is hepatocellular carcinoma, which begins in hepatocyte. Other types of liver cancer, such as intrahepatic cholangio carcinoma and hepatoblastoma, are much less common.

(a) **Swiss albino mouse model**: To study the effects of different naturally occurring phytochemicals that can be used as an alternative to anticancer drugs, the Swiss albino strain of mouse (*Mus musculus*) serves as a highly preferred mammalian animal model. They also resemble human being genetically quite closely. Swiss albino mice are fed with respective natural derived product to observe the toxic effects, mortality rate, and abnormal phenotypes adopting multiparametric approaches which could then be used to analyze the potential role of the compounds to negate the toxicity caused by using synthetic drugs (Paul et al. 2013a).

(b) **Xenograft model**: Xenograft model mainly focuses on preclinical testing of anticancer drugs within a limited period. Biopsies of the human liver with HCC when grafted in mice also maintain their original characteristics (Blumer et al. 2019). Xenograft models can be of three different categories:

(i) **Ectopic model**: In the ectopic model, human hepatoma cells are administered subcutaneously in the mice to develop liver cirrhosis and fibrosis.

(ii) **Orthotopic model**: In the orthotopic model, development of HCC is being done directly by intrahepatic administration of tumor cell lines.

(iii) **Hollow fiber model**: The hollow fiber model is the most efficient among the others as it includes a limited use of mice model to examine different cell lines. This technique includes deposition of tumor cell lines into tubes followed by inoculation, culturing *in vitro*, and implanting subcutaneously or intraperitoneally within mice (Hollingshead et al. 1995).

(c) **Knockout model**: Knocking out of tumor suppressor gene TP53 involved in preventing tumor growth by inducing apoptosis in mouse models demonstrated a rise in hepatic proliferation and change in hepatocyte morphology. Deletion of both p53 genes in mouse liver leading to homozygous loss of p53 also displayed

significant HCC formation at a very early age. Thus, as p53 is known to act as a cellular gatekeeper, its absence reduces apoptosis and increases the multiplicative nature and growth of liver progenitor cells along with the expression of the bilineal phenotype of tumor cells.

PTEN (tumor suppressor gene) knocked out mouse, suppressing growth-promoting signals by inhibiting phosphatidylinositol-3-kinases (PI3K), was obtained by a crossing experiment between albumin promoter Cre mice and PTEN-floxed mice. PTEN knocked out model could thus potentially give rise to hepatic inflammation, hepatomegaly and prevalence of hepatic adenomas, and finally development of HCC (Horie et al. 2004).

Colon Cancer

The cancer of the colon or rectum, located at the lower end of digestive tract is often called colorectal cancer.

(a) **Chemical or diet-induced model**: Mice when treated with chemical carcinogen for specific time period to form colon cancer gets evaluated to identify the ability of the chemical agent to induce colon cancer in them (Hung et al. 2010).
(b) **Patient-derived xenograft model**: Colon tumor cells collected from clinically diagnosed patients of colon cancer are injected into immune-deficient mice to form patient-derived xenograft mouse model for study of colon cancer (Rivera et al. 2021).
(c) **Subcutaneous derived xenograft model**: Tumor cell lines or suspensions are injected subcutaneously into immune-deficient or nude mice (enabled to produce T cell) to develop subcutaneous derived xenograft model; these mice, however, show limitations of low metastasis capability (Sudha et al. 2020).
(d) **Orthotopic model**: Orthotopic models are implemented to overcome the disadvantage of xenograft models. In orthotopic model, colon tumor cell line or patients-derived colon tumor cells are implemented into immunodeficient mouse model. After tumor formation in the colon, the colon is cut into fragments and surgically transplanted into another immunodeficient mouse model (Hollandsworth et al. 2020).
(e) **Genetically engineered mouse model**: This model is capable of replicating genetic abnormalities of colon cancer which makes it easy to understand the genetic involvement in human colon cancer (Neufert et al. 2021).

Prostate Cancer

It is one of the most common types of cancer in males, occurring in the prostate gland. Many prostate cancers grow slowly and remain confined to the prostate gland

and may need minimal or even no treatment, other types are aggressive and can spread quickly.

(a) **Transgenic T antigen model**: Male C3(1)-Tag mouse model is known to be used in the development of cancer in the prostate gland by using 5′ flanking region of the C3(1) gene to drive the expression of the SV40 large T-antigen (Tag) (Valkenburg and Williams 2011). Development of hyperplasia in the epithelial lining is followed by the appearance of adenocarcinoma in C3(1)-Tag mice. However, C3(1)-Tag mice are not restricted to develop cancer in the prostate gland but can also be used to develop tumors in the mammary gland of female mice as well as in the salivary gland.

TRAMP (transgenic adenocarcinoma of the mouse prostate) is one of the effective mouse models that can be characterized to induce neoplasia followed by lymph node metastasis and pulmonary metastasis at around 28 weeks (Valkenburg and Williams 2011). In this model, construct constituting 426 bp prostate-specific rat probasin promoter (PB) along with 28 bp 5′ untranslated region (UTR) induced the expression of large and small SV40 tumor antigens (T/tag). The ability of TRAMP-derived prostate tumor cell lines to introduce prostate tumors after injecting into genetically identical male nontransgenic C57BL/6 hosts showed its multifaceted nature in the induction of prostate cancer.

Another model known as the LADY model having similarities to the TRAMP model but possessing slight alteration concerning the design of construct has been made by inserting a larger fragment of the PB promoter (LPB) and 28 bp 5' UTR that could be used to induce transgene expression at a much higher rate. The deletion resulting in the expression of only SV40 Large T-antigen, preventing t antigen expression, is another moderation that has been made. Increased level of transgene expression in the prostate made this model more specific and an ideal one (Valkenburg and Williams 2011).

(b) **Knocked out model**: Knocked out of Nkx3.1, a tumor-suppressing transcription factor led to the hyperproliferative nature of prostate epithelial cells, prostate hyperplasia, and dysplasia. Further, knocking out of Nkx3.1 hampers differentiation and development of the prostate, which induces mutations leading to DNA damage, thus causing genomic instability. The severity of development of PIN (prostatic intraepithelial neoplasia) is low indicating the haplo-insufficient nature of the gene.

Stat5a transcription factor whose absence mainly affects prostate growth and development when knocked out from mouse does not play a role in generating tumors but results in prostate epithelial deformity, disorganization, and occurrence of cysts (Valkenburg and Williams 2011).

PTEN (phosphatase and tensin homolog) is one of the major tumor suppressor genes whose absence is directly correlated with many types of cancer, including

prostate cancer. Different techniques of knocking out of mice *Pten* gene by either introducing a null mutation or a combination of deleting a portion of the gene and inactivating another region had been established, both of which efficiently resulted in hyperplastic prostates along with PIN (prostatic intraepithelial neoplasia) lesions development (Valkenburg and Williams 2011). The inability to induce metastasis by mice *Pten* knocked out gene resulted in further knocking out of another gene in addition to *Pten*, termed as double knockouts. Pten and p27 double knockouts in male mouse model lead to development and induction of prostate cancer at a much higher rate with abnormal phenotype. The second knocked-out model includes loss of both Pten and Nkx3.1 resulting in multifocal prostate cancer lesions in mice along with the formation of adenocarcinoma. The most important model leading to deleterious effects was seen in Pten and p53 knocked out model, as both are principal tumor suppressor agents. Loss of both genes speeds up the rate of prostate tumorigenesis and leads to a more aggressive phenotype (Couto et al. 2009).

Cervical Cancer

Most of the human cervical cancers are associated with human papillomavirus (HPV) types, particularly HPV16 in women. Since new infections by the high-risk HPVs are not targeted by the vaccines, these measures cannot eliminate the risk of cervical cancer (Roden and Wu 2006). Again the traditional therapeutic strategies are of limited outcome in case of patients with advanced or recurrent cervical cancer. Thus a potent and new therapeutic approach is crucial treating preexisting cervical cancer as well as preventing their onset in a mouse model.

(a) **Genetically engineered mouse model**: For elucidating the mechanisms of progression of cervical cancer by active participation of HPV oncogenes and cofactors that contribute to cervical carcinogenesis, transgenic mouse models have been of great use. These transgenic mouse models express HPV16 E6 and/or E7 under human keratin 14 promoter that targets the site of HPV infection. This mouse model recapitulates the histopathological nature, expression patterns of biomarkers, and other aspects of the progressive disease that resembles these preclinical models to cervical disease in women. These mouse models have demonstrated that estrogen is required for development of cervical cancer. But when mouse model of double transgenic expression of HPV16 *E6* and *E7* oncogenes was treated continuously for 6 months with exogenous estrogen (E2) at a physiological level, it implicated the induction of cervical cancer at high penetrance (Brake and Lambert 2005; Riley et al. 2003).

(b) **Patient-Derived Xenografts (PDX)**: Despite new therapies, there is still a need for models to further study the progression of cervical dysplasia toward advanced cancer, and test new therapies. Although transgenic mouse models have been developed to study oncogenic contributions of various HPV genes *in vivo*, these models offer several limitations (Larmour et al. 2018) where the transgenic model-induced cervical cancers are estrogen dependent and also the

tumor behavior is affected due to differences between human and mouse metabolism. Since patient-derived xenograft (PDX) models maintain better gene expression patterns of the parent tumor, cervical cancer PDX models have been reportedly using the subcutaneous model with engraftment rate of 70% and orthotopic (cervical) model with engraftment rate of 48–75% (Hiroshima et al. 2015).

Skin Cancer

According to American Cancer Society, the most prevalent of all cancers is skin cancer; non-melanoma skin cancer (NMSC) is a major health concern worldwide with over 2 million cases each year. Some mice models have been a useful tool for identifying and understanding the underlying molecular mechanisms involved in different skin cancers like basal cell carcinoma, squamous cell carcinoma (NMSCs), and melanoma and developing targeted cancer therapies (Nowotarski et al. 2015).

(a) **Genetically engineered mouse model**: Developing mouse models to study Cutaneous basal cell carcinoma (BCC) has been a challenging process due to the ineffective effort of inducing BCC on murine skin (mimicking human skin cancer) using UV light or chemical carcinogens. Therefore, genetically modified BCC mouse models represent an excellent tool for the study of pathogenesis of human basal cell carcinoma and to evaluate therapeutic interventions as these mice can produce numerous genetically defined tumors in a short span of time; however, the process is cumbersome and expensive for preclinical drug testing. Since the mutations in Patched 1 gene (PTCH1) as well as other genes in the Hedgehog (Hh) signaling pathway play crucial role in the development of BCC in patients as well as spontaneously forming BCCs, the current mouse models for BBCs induction involve overexpression of Sonic hedgehog (Shh) under keratin 14 (K14) promoter and a mutant variant of Smoothened (SMO-M2) under K5 promoter (Xie et al. 1998).

The development of mouse models that recapitulate human cutaneous squamous cell carcinoma (cSCC) is also necessary for understanding the molecular pathogenesis of these tumors. Although mutations in p53 (important tumor suppressor) lead to p53 inactivation which has been implicated in a variety of tumors (Nakazawa et al. 1994), inactivating mutations of p53 leads to loss of cell cycle regulation, thereby increasing the propensity of development of additional mutations in oncogenes that drive carcinogenesis. Therefore, in presence of chronic UV exposure, p53−/− mice (p53 null mice) can serve as mouse model for understanding the role of UV in the pathogenesis of cSCC.

Although melanoma is less common compared to other nonmelanoma skin cancers, it has a record of highest mortality rate. Therefore, development of mouse models that closely mimic human melanoma is important for understanding the pathogenesis of tumor and developing new therapeutics. Recent genetic analysis of

human melanoma indicates that mutations involving activation of B-RAF (most commonly B-RAFV600E) lead to MEK/ERK activation that contributes to over 50% of melanoma. Total 100% penetrance of metastatic melanoma is resulted from conditional expression of B-RAFV600E and conditional silencing of PTEN by deleting its phosphatase catalytic domains.

(b) **Xenograft mouse models**: For studying and analyzing the biology of melanoma, xenograft *in vivo* models involve culture of tumor cell lines on plastic plates and then transferring of the cell-lines into immunocompromised mice (Rebecca et al. 2020). Although, the artifacts that arise due to passaging of these melanoma cells by *ex vivo* method selects subpopulations that adapt to 2-D culture condition, they do not reflect the most active genes involved therein in *in vivo*. This can be a limitation of xenograft approach.
(c) **Patient-Derived Xenografts (PDX)**: Due to the level of heterogeneity maintained in the *in vivo* setting, PDX models are demonstrated to be superior for tumor biology studies. This heterogeneity of PDX models depends on the number of tumor sources, which can be similar to cell-line-based xenograft models. A recent study found significant differences in hypoxia-regulated gene expression and altered fitness of cells (implanted *in vivo*) when compared to cell-line-derived xenografts (CDX) to PDX (Harris et al. 2016).

Conclusion

Despite tremendous progress made in understanding various aspects of cancer through extensive and intensive research around the world, cancer still remains to be one of the burning issues that has catastrophic effects in the lives of many people. Researchers are therefore still exploring newer ways to get a more thorough understanding of the origin of the cancerous cells, their invasive potential, limitless replicative nature, and abnormal behavior to find out a decisive and foolproof way to eliminate the altered cells while safeguarding the normal healthy cells. Mice are considered as one of the elite human disease models to study the effects of different types of cancer and find out possible steps to intercept the progress at strategic points. Different mouse cancer models such as genetically engineered xenograft models, knockout gene models, biopsy, and transgenic models can contribute largely toward understanding multiple aspects of cancer biology. The protective role of different tumor-suppressing genes, pleiotropic expression of oncogenes contributing to neoplasia, and tumor development, carcinogenic effects of different compounds, initiating factors of angiogenesis, the anomalous signaling cascade, and the response of certain drugs to cancer treatments can be evaluated by utilizing and inducing mice known to mimic the human system. The similarities of mice and humans based on cellular, anatomical, and molecular characteristics in addition to similar homology of both genomes enable the researchers to employ mice as an effective cancer-induced tool for gaining better knowledge about the anticipated response to treatment as well as the effects of drugs on normal healthy cells. This could help to pave a way to

develop a therapeutic approach to improve human life expectancy. Thus, *in vivo* models facilitate identifying and characterizing the scientific and experimental data which would lead to a better diagnosis that is lacking in *in vitro* system (Wallace 2000). Therefore, experiments on different cancer-induced mouse models should be undertaken to gain insights into more advanced, painless treatment procedures to ameliorate cancer pains and help patients to lead a relatively more peaceful life. These mouse models can also help in the assessment of the possible benevolent roles played by different naturally occurring compounds on combating cancer, thereby giving a direction to lead a relatively healthy life by incorporating those compounds in our daily diets.

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Use of Stem Cells on Animal Model of Cancer Research

6

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Contents

Abstract

Cancer is the culmination of many complex disease states affecting multiple organs. Primary diagnosis involves the utilization of prognostic cancer markers to evaluate the likelihood of systemic disease progression. However, the establishment of animal models for aid in cancer research has exploded in recent years.

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Multiomics analysis unveiled the sequence of cancer development, while animal model research established the pathophysiology of cancer. Recently, the application of stromal and pluripotent stem cells in cancer development has garnered considerable interest. This chapter contributes an overview of the animal models currently employed for determining the role of stem cells derived from various sources in cancer studies. We also address how a better knowledge of stem cell activity can lead to novel cancer detection and treatment strategies and the chapter also discusses on some of the most current animal models and concepts in cancer research.

Keywords

Mesenchymal stem cells · Cancer stem cells · *In vivo* models · Immunomodulatory agents · Exosomes

Introduction

Stem cell therapy has emanated as a promising approach for combating a spectrum of ailments. The capacity of circulating stem cells or exogenously supplied stem cells to locate and enter an environmental niche is called stem cell homing. The homing ability of stem cells has already been employed to deliver drugs and in targeted gene delivery. A stem cell may migrate across niches throughout its life cycle, both during embryonic and adult development. The therapeutic potency of bioengineered stem cells has been observed in a variety of disorders including cancer. Likewise, studies have demonstrated that stem cells possess intrinsic tumoritropic homing potential (Corsten and Shah 2008).

A recent study has documented the homing ability of Mesenchymal Stem Cells (MSCs) and Stromal Cells (SCs) in the mobilization to the sites of injury and regeneration and identified a relationship among various transcription factors with the homing potential of normal and malignant stem cells (Liesveld et al. 2020). The most extensively studied in vivo model of stem cells homing is stem cells transplantation. Moreover, MSCs homing and cellular accommodation may be tissue-specific in specific cancers. For example, bone marrow MSCs colonize hypopharyngeal malignancies but not primary prostate tumors (Wang et al. 2019). However, the likelihood of generating cancers through incorporated oncogenes, site-directed mutagenesis, or altered tumor suppressor genes holds a big question in clinical applications of induced Pluripotent Stem Cells (iPSCs).

We explore various modes of action for stem cells' therapeutic benefits and experimental models that unravel stem cells' tissue altering functionality and approaches for detecting their impacts *in vivo* by combining cancer biomarkers with niche-specific activity in this chapter.

Types of Stem Cells in Current Research

Stem cells are a type of cell that has the ability to self-renew indefinitely, generate clonal cell populations from a single cell, and specialize into many cell types. The ability of resident stem cell population to self-renew is vital for tissue regeneration and homeostasis. Embryonic Stem Cells [ESCs] and Somatic Stem cCells [SSCs] are the two primary types of stem cells. Adult Stem Cells [ASCs] are composed primarily of mesenchymal stem cells, hematopoietic stem cells, neural stem cells, and endothelial progenitor cells. Likewise, ASCs, notably human neural stem cells, have been effectively employed to investigate fragile X syndrome. Human ESCs, derived from the blastocyst, the inner cell mass of the embryos possess the possibility to proliferate indefinitely and the stemness to specialize to various cell types.

Embryonic Stem Cells [ESCs] with Induced Pluripotent Stem Cells (iPSCs)

Despite the fact that human ESCs have been generated from embryos with mutations for multiple ailments, only a few diseases can be detected by pre-implantation genetic diagnostics. ESCs are pluripotent cells that can develop into any cell type of the body except the placenta which is the gold standard for assessing all *in vitro* cultured pluripotent cells. Even yet, the employment of ESCs in research and human clinical trials is constrained by ethical issues. Therefore, iPSCs reprogrammed from adult somatic cells (for example, skin fibroblasts) have replaced the utilization of ESCs in this process by enforcing the expression of pluripotency factors in the adult somatic cells. iPSCs do not require the destruction of an embryo, like ESCs but do not have ethical constraints, and may be more therapeutically useful than ESCs cells. Moreover, novel treatments, particularly synthetic drugs, and drug toxicity, are being identified and evaluated using iPSC-based models. Furthermore, these models may be used to stratify patients into groups of drug-responsive and non-responsive individuals. However, a significant challenge is differentiating the required cell type from iPSCs for disease modeling (Sterneckert et al. 2014).

Neural Stem Cells (NSCs)

The finding of neural stem cells (NSCs) in the human nervous system has raised many expectations since these individual cells can mimic various neurological disorders, including brain cancers, both functionally and molecularly. NSCs are produced through embryonic tissue differentiation and have the ability to replenish neurons, glial cells in the adult brain over life. NSCs are typified by the expression of Nestin, SRY-box 2 (SOX2), and other traditional markers and proliferate in epidermal and fibroblast growth factor-rich medium. NSCs have been commonly used to

treat brain, breast, prostate, and lung malignancies due to their ability to self-renew and specialize as neurons, astrocytes. This capacity to differentaite has sparked a lot of interest in employing NSCs in stem cell therapy to heal central nervous system damage caused by physical trauma (Rodriguez et al. 2016).

Mesenchymal Stem Cells (MSCs)

Mesenchymal Stem Cells (MSCs) are multipotent stem cells which can give rise to osteoblasts, adipocytes, chondrocytes, and myocytes, among other mesenchymal lineages. The differentiation ability and suppression of the immune system to shelter tumors renders the MSCs as critical regulators of tumor fate. Multiple tumor-supporting pathways are activated by mesenchymal stem cells, including drug resistance, immunosuppresion, metastasis, and angiogenesis. MSCs secrete pro-angiogenic factors that recruit circulating endothelial progenitor cells and differentiate into smooth muscle cells and pericytes, promoting revascularization, neovascularization, and vascular stability. MSCs secrete chemicals that trigger EMT (epithelial-to-mesenchymal transition) in cancer cells. MSCs are prone to develop into cancer stem cells which help tumor cells to become more invasive and leads to metastasis. MSCs suppress B cells, Th1 (T helper type 1) cells, and NK (Natural Killer) cells, activate Regulatory T (Treg) cells and Th2 cells, and significantly alter macrophages toward the M2 (Macrophage2) phenotype and decrease the antitumor immunity. Cancer cells are protected from the cytotoxic effects of chemotherapeutic drugs by MSCs. After therapy, MSCs can enrich the resistant Cancer Stem Cells (CSCs) population, or they can directly differentiate into Cancer Stem Cells (Timaner et al. 2020).

According to several independent investigations, MSCs are recruited for different types of carcinomas including colon, breast, ovarian, melanomas, Kaposi's sarcomas, and gliomas. MSCs appear to suppress the PI3K/AKT pathway in Kaposi's sarcomas in an E-cadherin-independent manner. However, endogenous MSCs' antitumor activities are still unknown, and additional studies are required. According to Qiao L. et al., human MSCs can reduce the proliferation and colony-forming capability of human tumor cell lines, through downregulating the anti-apoptotic regulator protein B-cell lymphoma 2 (Bcl 2). Also, human MSCs were shown to transit 11 times more towards conditioned medium than non-cancer cells in human breast cancer (Stagg 2008). As per reports by Karnoub et al., human MSCs are highly crucial in boosting the metastatic ability of human tumor xenografts. Moreover, the cancer properties of MSCs have stimulated investigations into their prospects as therapeutic gene targeted delivery agents.

Hematopoietic Stem Cells (HSCs)

The property of HSCs in the bone marrow to self-renew and specialize in all kinds of adult blood cells differentiates them from other stem cells. In 1963, Becker and

colleagues were the first to discover the clonal genesis of hematopoietic cells. Progeny from transplanted mouse fetal liver cells labeled with retroviruses was later tracked *in vivo* to confirm the existence of HSCs. HSCs have the potential to assist in the restoration of the hematopoietic system in conditions where illnesses cause loss of the bone marrow or aplasia of the bone marrow. The use of hematopoietic cytokines to mobilize HSC cells from the bone marrow into the bloodstream has made them readily available for clinical transplantation (Gunsilius et al. 2001).

Cancer Stem Cells (CSCs) and Animal Models in Cancer Research

In the past century, several mechanisms for transforming normal cells into precancerous cells have been proposed. It is assumed that cancer originates from a single cell that has lost its differentiated state through successive mutations. Moreover, John Dick and colleagues' groundbreaking work on human Acute Myeloid Leukemia (AML) cells has proven that only a tiny proportion of human AML cells may transmit the disease when implanted into immune-deficient mice (Lapidot et al. 1994). This led to the realization that just a few cells inside the tumor mass were capable of causing tumors. Cancer Stem Cells (CSCs) are cells inside a tumor that can self-renew and produce a variety of cancer cell generations that make up the tumor. They exhibit both cancer cell and stem cell characteristics and have been found in different organs including head and neck, prostrate, pancreas, lung, brain, and colon (Kreso and Dick 2014). Notably, a very few cancer stem cells were able to form tumors in NOD/SCID (non-obese diabetic/severe combined immunodeficient) mice (Biology et al. 2012). CSCs were previously assumed to constitute only a small proportion of a solid tumor's total cell population; nevertheless, it has been reported that up to 25% of cancer cells may exhibit CSC-like characteristics (Cemazar 2018). However, CSCs are linked to chemo- and radio-resistance, causing traditional therapies to fail. The great majority of therapies target the rapidly growing tumor mass rather than the cancer stem cells, which divide slowly. Removing CSCs from the roots, which are the source of cancer's formation and recurrence, has been touted as a hopeful way to extend cancer patients' lives or possibly cure them. Understanding the characteristics of cancer stem cells can therefore, contribute in the production of revolutionary medications to totally eradicate the cancer stem cells that caused cancer, as well as key patents on cancer therapy utilizing cancer stem cells (Hu and Fu 2012).

Animal models are essential tools in cancer research because they give vital information that might lead to therapeutically effective treatments. With the advancement of pharmacogenomics and targeted therapy, researchers are seeking more human-like personalized cancer models. The use of animal models in research helps bridge the gap between in vitro and human clinical trials. The use of animal models to study cancer (e.g., mice, rats, zebrafish, Drosophila) has significantly improved our understanding of the mechanisms regulating cancer origin, development, and metastasis. It has also enabled the development and preclinical testing of new cancer therapies to mimic tumor development in a more pathophysiologic

condition. Concurrently, an enormous number of animal models are being developed and employed in cancer research. The technologies for establishing cancer animal models are evolving, involving chemical induction, xenotransplantation, gene manipulation, and so forth. The patient-derived xenograft model has become a scientific breakthrough in recent years due to its capacity to retain the microenvironment and essential biological features of the primary tumors. Also, it is possible to examine the physiological processes that occur and develop in an experimental live model of cancer, the screening of cancer medications, and gene therapy research using animal models (Zheng and Fang 2021).

Cancer Stem Cell Models

Over the last decade, the CSC models have gained popularity as a valuable research tool in cancer biology. It helped us grasp intratumor heterogeneity as well as cancer tissue hierarchical structure better. According to this hypothesis, the cellular heterogeneity observed inside malignancies results from cross differentiation and epigenetic variation processes found in normal stem cell models. Traditionally, a random or "stochastic" model has been used to explain the cellular heterogeneity observed within an individual tumor (Dalerba et al. 2007). The cancer stem cells idea states that certain malignancies are hierarchically structured into undifferentiated cells and differentiated cells. The undifferentiated cells initiate disease development, whereas differentiated cells have reduced potential to initiate disease progression (Magee et al. 2012).

Drosophila Models

Drosophila melanogaster, the fruit fly, was proven to be a successful model for investigating human cancers during the past several decades. Drosophila has earned a considerable interest as a cancer model, allowing researchers to assess the molecular mechanisms underlying cancer-related gene identification, tumor progression, development, and metastasis. However, epithelial tumors, organotypic models, and liquid tumors are only a few Drosophila tumor models currently available. Many signaling pathways initially identified in Drosophila, such as Decapentaplegic (Dpp), Hippo, Hedgehog, Notch, and Wnt which remain conserved in humans, play critical roles during tumorigenesis. Similarly, research in Drosophila has aided in developing the idea of "tumor hotspots" inside tissues (Yang et al. 2019). The term "tumor hotspots" refers to the tissue-intrinsic features, such as cytoarchitecture and endogenous growth-promoting signals that are more receptive to oncogenic stimuli or mutations. Currently, numerous human cancer models have been established in Drosophila employing oncogenic RAS (Rat Sarcoma Virus) and SRC (SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase) activation, providing an ideal *in vivo* model for studying RAS/SRC-driven tumor growth. The advantages of Drosophila tumors are that they are genetically stable and homogeneous and may

be generated by altering a single gene activity rather than many genetic defects or the deletion of tumor protein-p53, thus enabling convenient analysis (Bilder et al. 2021). A recent study has identified a unique therapeutic approach of developing peptide-based therapeutics (TONDU peptide) for targeting adult intestinal stem cell tumors in the Drosophila tumor models (Bajpai et al. 2020). Similarly, complex human tumors like glioblastoma multiforme, a malignant brain tumor linked with adverse outcomes in patients owing to inadequate medication absorption, low treatment response, and drug resistance, have also been successfully modeled in Drosophila (Chen and Read 2019).

Stem Cells from Large Animals

Soon after the isolation of mouse ESCs, efforts to generate swine, cow, and sheep ESCs cells began. Most significantly, mouse ESCs cells easily integrate into the blastocyst's inner cell mass, allowing the genetic alteration to be studied in later generations. The promise of performing similar targeted investigations in a large animal to increase animal health and output was a primary factor for creating ESCs cells from farm animals. Over the last decade, swine genetic modifications for biomedical research has progressed at a rapid pace. Genetically engineered pigs will be are valuable for understanding illnesses like cystic fibrosis rather than mice model. Mice do not acquire the relevant pathologies associated with cystic fibrosis, and in retinitis pigmentosa, the pig eye architecture is relatively comparable to that of humans. Because genetic alteration reduces hyperacute rejection, genetically modified pigs are being studied as generators of tissues and organs for human xeno-transplantation. However, hyperacute rejection occurs when a wild porcine organ is implanted into a person, and the graft is quickly destroyed, generally within minutes to hours. Hyperacute rejection is unquestionably the most severe and aggressive immunological response a patient may have.

Mouse Models

The use of mouse models to study tumor genesis, growth, and metastasis has shown to be highly promising in understanding cancer biology. Two types of mouse models have shown to be very effective in studying metastasis: genetically modified models and xenograft model systems. In recent years, the emergence of new mouse models of human cancer has mitigated the disadvantages by expediting the discovery of additional oncogenes, which may aid in elucidating the processes of carcinogenesis. Therefore, developing mouse cancer models that are more representative of human diseases and investigating innovative ways of cancer therapy might improve care for individuals with cancer. In future, it can be expected that mouse transgenic cancer modeling research and applications may primarily focus on two areas: "CRISPR/Cas9 system" and "humanized mouse genome." CRISPR-Cas is a bacterial adaptive immune system that uses RNA-guided nucleases to cut foreign genomic material.

The utilization of the CRISPR-Cas9 system will be one such area of present and future intensive study. The CRISPR-Cas9 system has established itself as the most flexible and adaptable transgenic technology available to date, enabling researchers to construct transgenic mice that reflect the precise stages of human carcinogenesis. *In vitro* and *in vivo* CRISPR-Cas9 transgenic mice models may be used to validate gene sequencing analysis for a single patient's needs, which is now more affordable. These new mouse cancer models may facilitate the identification of additional genotype-specific susceptibilities of specific human cancer types, opening the path for developing more individualized, genotype-based cancer therapeutics (Cemazar 2018).

Zebrafish Models

In recent years, the zebrafish cancer model has garnered considerable attention, and it is currently one of the most promising non-vertebrate model species for studying tumor growth. Zebrafish genomes are homologous and similar to humans, making them a suitable model for studying cancer formation. In contrast to the most often used mouse models, zebrafish models have sparked the interest of cancer researchers because of their tiny size, rapid reproduction, and low cost. Zebrafishes have transparent embryos that allow real-time observation and tracking of cancer cell growth, dissemination, and metastasis. Because zebrafish are fertilized *in vitro*, gene modification is straightforward, and a transgenic animal model can be created rapidly. Genetic modifications such as reverse genetic approaches, transgenic technology, xenotransplantation, live imaging, high-throughput drug screening, and toxicity assays are some of the strategies that have been employed to model cancer dynamics and metastasis in the zebrafish organism. Furthermore, Gabellini et al. discovered that the aberrant expression of CXCL8 and BCL-xL in melanoma patients related to unexpected results in zebrafish models, indicating that the autocrine CXCL8/CXCR2 signaling pathway might exacerbate aggressive melanoma behavior in human melanoma xenotransplantation (Gabellini et al. 2018). With recent advancements in xenotransplantation and therapeutic assessment, zebrafish-cancer models may effectively aid in studying patient-derived xenograft cell invasiveness in various types of cancer.

Dog Models

According to several studies, canine genomes appear to be more associated with humans than rodents (Carvalho et al. 2012; Gardner et al. 2016). Canines can develop malignancies with clinical, molecular, and histological characteristics identical to human cancers. Uva et al. studied the expression of genes in dog and human breast cancer tissues as well as normal breast tissues and discovered aberrant gene expression in human breast cancers that was also present in breast cancer tissues of canine. Ressel et al. found similarities in the suppression of gene expression PTEN

(Phosphatase and Tensin Homolog Deleted on Chromosome 10) in human and canine breast cancers, thus revealing the use of canine cancer models in human cancer research.

Pig Models

The human genome and the pig genome are remarkably similar, with profoundly conserved epigenetic regulation patterns. Pigs are suitable animal models for cancer research because their anatomical, physiological, and genetic features are comparable to humans. Mitchell et al. used Diethylnitrosamine to develop hepatocellular cancer in pigs. Diethylnitrosamine is a commonly used synthetic carcinogenic reagent that has the potential to cause cancers in a number of organs, such as skin, respiratory system, liver, and gastrointestinal tract. It was reported that partial hepatic embolism might improve model designing (Mitchell et al. 2016). Gene editing pigs have also emerged as a novel method for studying genes linked to cancer. Likewise, Wang et al. created a lung cancer pig model using CRISPR-cas9 gene editing tool by simultaneously activating one oncogene KRAS (Kirsten rat sarcoma virus) and five tumor suppressor genes such as PTEN, TP53 [Tumor protein p53], BRCA-1, BRCA [breast cancer genes], APC [Adenomatous polyposis coli] (Wang et al. 2017). Indeed, studies have demonstrated that young pigs may help anticipate pharmacokinetics in infants, paving the way for developing anticancer medicines using piglet models (Zheng and Fang 2021). A recent study by Smith et al. suggested porcine as a possible model employing mammary development cues to decipher breast cancer biology based on the similar gene expression patterns in the pig mammary gland and known genes in human breast cancer profiles (Smith et al. 2022).

Factors Influencing Novel Stem Cell Therapeutic Approach in Targeting Tumor Cells

Numerous cancer types get influenced by a group of cells known as CSCs which demonstrates tumor initiation and growth while facilitating tumor metastasis, therapeutic resistance, and recurrence (Batlle and Clevers 2017). CSCs originate from normal stem cells that endure complex genetic alterations. Thus, understanding and targeting cancer stem cells may be a viable avenue for treating a variety of tumor forms.

Various factors play a critical role in regulating stem cell activity and tissue regeneration viz. “apoptosis-induced compensatory proliferation.” Numerous studies have shown that apoptotic factors actively engage with their environment throughout the process of aging, whereas non-apoptotic role of caspases initiate a cascade of signals that promote cellular proliferation and regenerate lost tissues (Ryoo and Bergmann 2012). Also, prolonged apoptosis-induced proliferation (AiP) results in tissue overgrowth, leading to tumor progression and resistance (Fogarty and Bergmann 2017). For example, caspase-induced prostaglandin E2

production, critical for liver regeneration, can stimulate tumor growth in mice and human cancer cells following radiation therapy (Huang et al. 2011). Similarly, suppression of prostaglandin E2 impairs apoptosis-induced proliferation and sensitizes bladder cancer cells to therapy (Kurtova et al. 2015).

Moreover, the interaction of CSCs with their surrounding tumor stroma induces an "active state," marked by enhanced production of pro-inflammatory cytokines and growth factors establishing an active extracellular matrix that promotes tumor cell invasion. In addition, tumor cell defense and malignant cell heterogeneity can be addressed with targeted cytokine immunotherapy while minimizing systemic side effects. Cytokines are pleiotropic regulating proteins that operate as systemic or local agent regulators (Berraondo et al. 2019). Systemic administration of immune-stimulating cytokines (interleukins (IL-2, IL-6, IL-12) and interferons (IFN-α)) have led to significant toxicities that have limited their use in cancer treatment. Cell-based techniques that employ dendritic cells, fibroblasts, or MSCs as "tumor-associated stromal cells" to permanently eliminate aggressive malignant cells, aid in minimizing the downsides of systemic cytokine delivery (Wang et al. 2020).

Studies have shown that tumors impact the recruitment of MSCs to tumor locations by acting as chemoattractants, where the CXCL12 C-X-C Motif Chemokine Ligand 12 (CXCL12)/C-X-C chemokine receptor type 4 (CXCR-4) axis signaling mechanisms play an essential role in recruiting MSCs to tumors (Wobus et al. 2015; Kalimuthu et al. 2020). Likewise, endothelial CXCL12-expressing cells may contribute significantly to the formation of perivascular stem cell niches and play a critical role in regulating MSC activity. In an *in vivo* model of chronic myeloid leukemia, targeted deletion of CXCL12 from MSCs mitigated the hematopoietic stem cells population while allowing CSCs development through Enhancer of zeste homolog 2 (EZH2) activity, but CXCL12 depletion in bone marrow endothelial cells was shown to limit CSCs (Agarwal et al. 2019). Recently, Pal and colleagues developed a human pluripotent stem cell-engineered co-culture method to promote the *ex vivo* growth of patient-derived leukemia cells in adult solid tumor Phase I trials. They demonstrated the *in vivo* effectiveness of ADH-1 (N-cadherin antagonist)/Dexamethasone in treating blood malignancies (Pal et al. 2021).

Similarly, various studies have demonstrated that genetically altered stem cells can deliver therapeutic proteins to tumors and other areas of inflammation with excellent efficiency (Kumar et al. 2008) (Table 1). As MSCs may migrate to injured tissue and tumors, they represent a potential delivery approach for cancer therapy. Besides, the application of tumor-suppressive agents in MSCs programming for cancer therapy has expanded beyond cytokines to include a variety of other proteins. However, the engagement of MSCs in the tumor microenvironment holds contradictions. They may either promote tumorigenesis or inhibit tumor cell proliferation in the tumor microenvironment. Evidences suggest that MSCs play a critical role in modulating the tumor microenvironment since they can regulate and direct the fate of tumor cells. Furthermore, the mobilization of MSCs into breast and prostate cancers was shown to enhance the angiogenic factors such as transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and Interleukin 6 (IL-6), thereby triggering tumor angiogenesis and expansion (Zhang et al. 2013).

Table 1 Experimental models involving MSCs infused with immunostimulatory agents/drugs directed against cancer cells

S. No.	Source of stem cells	Infused genes/agents	Target cancer cells/tissues	Expression in cancer cells/ tissues	Animal models used in the study	References
1.	Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs)	CXC chemokine receptor 1 (CXCR1)	Glioma cells	Inhibition of interleukin-8 (IL-8) or its receptor, CXCR1 in glioma cells suppresses the migration of MSCs toward glioma cells that facilitates their homing to glioma tumors in mice	Mouse tumor models	(Kim et al. 2011)
2.	Human bone marrow-derived mesenchymal stem cells (hBMSCs)	Macrophage migration inhibitory factor (MIF) and CXC chemokine receptor 4 (CXCR4)	Pulmonary metastatic cell line	MIF released by tumor cells is required to attract MSCs, which requires activation of ERK and JNK pathways predominantly *via* CXCR4, thus facilitating homing in the tumor cells	*In vivo* mice lung metastasis models	(Lourenco et al. 2015)
3.	Mesenchymal stem cells derived from the mouse adipose tissues	Doxorubicin (DOX) containing poly (d, l-lactic-co-glycolic acid) (PLGA) nanoparticles	Lung melanoma	MSCs carrying PLGA-DOX nanoparticles effectively abolished melanoma cells *in vitro* and *in vivo* in a dose dependent manner	Mouse tumor models	(Zhao et al. 2017)
4.	Primary bone marrow-derived human mesenchymal stromal cells (hMSCs)	Platelet-Derived Growth Factor AA (PDGF-AA)	Head and Neck Squamous Cell Carcinoma (HNSCC)	The enhanced phenotypic plasticity of MSCs to the tumor site may result from HNSCC tumor cells producing PDGF-AA and IL-6	Mouse tumor models	(Watts et al. 2016)
5.	Human placental derived MSCs	Docetaxel (DTX) infused in nanoparticles (NPs)	Lung cancer	MSCs/NPs/DTX drug delivery system exerted primary tumor inhibition efficiency in lung cancer	Nude mice models	(Wang et al. 2019)

Possible Stem Cell Therapies in Cancer Disease Models

The therapeutic advantages of MSCs have proposed their incorporation into cell-based techniques for managing various disorders, including cancer (Table 2). Similarly, drug-loaded nanoparticles can be delivered to primary target tissues by MSCs, enabling nanoparticles to penetrate the tumor core (Caplan and Correa 2011). Factors such as the cell proliferation rate, exposure time, and MSC culture conditions facilitate receptor-mediated nanoparticle uptake in MSCs. Cancer therapeutics are precisely administered to the tumor location *via* MSCs homing capabilities, and drug-infused MSCs are delivered by exocytosis and diffusion. Sadhukhan et al. revealed that transporting poly(d,l-lactide-co-glycolide) (PLGA) nanoparticles primed with Paclitaxel were an efficient tumor-targeting method in engineering MSCs. This work demonstrated that MSCs absorbed nanoparticles in a concentration and time dependent manner, with no effect on MSCs characteristics and mediated dose-dependent cytotoxicity in lung and ovarian cancer cells *in vitro* and *in vivo* (Sadhukha et al. 2014). A similar study by Wang et al. demonstrated that MSCs loaded with Paclitaxel-encapsulated PLGA nanoparticles for orthotopic glioma therapy could provide an ideal approach encouraging targeted chemotherapeutic strategy for the drug delivery (Wang et al. 2018). In order to overcome the constraints of existing therapies, such as short drug half-lives and inadequate delivery, tumor homing qualities will be critical in MSCs-based cancer treatments.

Furthermore, based on the generation of a bioactive biomolecule in the tumor site, MSCs expressing transgenes maintained long-term expression *in vivo* compared to the systemic administration of immune-stimulating cytokines (Meyerrose et al. 2010). According to a recent study by Wang et al., the dynamic homing profile of MSCs in the peripheral blood was determined by employing *in vivo* flow cytometry to monitor the dynamics of fluorescence-labeled cells noninvasively. According to the findings, MSC clearance kinetics differed between healthy and tumor mice models in the research, showing that MSCs actively inhibit tumor microenvironments (Xie et al. 2017). Likewise, several factors such as local irradiation, overexpression of CXCR4, epidermal growth factor receptor (EGFR), or synthetic receptors targeting tumor-specific molecules implicated in MSCs homing enhance the population of MSCs in the tumor microenvironment (Kalimuthu et al. 2020; Golinelli et al. 2020). Furthermore, MSCs are crucial in "theranostics,''a current approach that integrates cancer screening and therapy with an individual chemotherapeutic drug. In colon cancer models, genetically engineered MSCs expressing the sodium iodide symporter protein, encoded by a theranostic gene under the Chemokine (C-C motif) ligand 5 (CCL5) promoter, displayed a substantial decrease in metastatic tumor growth (Knoop et al. 2015).

Numerous researches in recent years have shown that exosomes promote the interchange of biological material between MSCs and human tumor cells. Exosomes (~30–100 nm in size) are membrane-bound extracellular vesicles that encapsulate various molecules, including proteins, DNA, mitochondrial RNA (mRNA), and microRNAs (miRNAs). Current findings have shown that exosomes may hinder tumor growth (Bruno et al. 2013). Evidences suggest that MSCs secrete extracellular

Table 2 List of mesenchymal stem cells derived from various sources and its anti-cancerous effect on tumor

S. No.	Stem cells	Sources	Target cancers	Mechanism	Animal models used in the study	References
1.	Human umbilical cord matrix stem cells (hUCMSCs)	Wharton's jelly	Breast cancer	By suppressing the Akt and MAPK pathways, non-engineered naïve hUCMSCs inhibit the proliferation of human breast cancer cells *via* restoring the intrinsic apoptosis mechanism	Female CB-17 SCID mice	(Ayuzawa et al. 2009)
2.	Rat umbilical cord matrix stem cells (rUCMSCs)	Wharton's jelly	Mammary adenocarcinoma	Both contact-independent and contact-dependent inhibition of tumor cell growth has been observed to be a feature of rUCMS cells. It enhances the phosphorylation of p38 while decreasing phosphorylation of ERK1/2, suggesting contact-independent Mat B III tumor cell growth inhibition	Female 4- to 5-mo-old F344 rats	(Ganta et al. 2009)
3.	Rat umbilical cord matrix stem cells (rUCMSCs)	Wharton's jelly	Lewis lung carcinoma	Rat UCMSCs results G0/G1 arrest followed by decrease of cyclin A and CDK2 expression in lung cancer cells thus attenuates tumor growth	Mouse syngeneic lung carcinoma models	(Maurya et al. 2010)
4	Human adipose tissue-derived mesenchymal stem cells (hAT-MSCs)	Abdominal subcutaneous fat	Melanoma	hAT-MSCs-conditioned medium (AT-MSC-CM) suppresses melanoma proliferation and can significantly induce cell-cycle arrest and apoptosis in the melanoma cells where hAT-MSCs migrate efficiently to the tumor tissues	Female, 6-week-old BALB/c nude mice models	(Ahn et al. 2015)
5	Human Wharton's jelly stem cells (hWJSCs)	Umbilical cord	Human Osteosarcoma and Mammary Carcinoma	hWJSCs possess paradoxical anti-tumorigenic properties that inhibit mammary carcinoma and osteosarcoma cells *via* apoptosis and autophagy	Severely combined immunodeficient (SCID) mice models	(Gauthaman et al. 2013)

exosomes that act as paracrine mediators transmitting signaling molecules, thereby regulating tumor cell proliferation, angiogenesis, and metastasis (Phinney and Pittenger 2017). Exosomes derived from drug-loaded MSCs are efficient in eliminating cancer cells (Table 3). For instance, exosomes derived from Paclitaxel-loaded MSCs hinder the progression of pancreatic adenocarcinoma cells *in vitro* (Pascucci et al. 2014). Recently, CRISPR/Cas9 technology has made it possible to explore *Xenopus tropicalis* in the area of cancer research as it has been observed to be an efficient and suitable organism for genetic manipulation approach. CRISPR/Cas9-mediated deletion of Retinoblastoma 1 (RB1) and Retinoblastoma-like 1 (RL1) in *Xenopus tropicalis* resulted in the first accelerated retinoblastoma model (Naert et al. 2016). Likewise, novel human cancer models are being established due to the recent improvements in *in vitro* 3D cultivation technologies such as organoids (Yan et al. 2018). In addition, cancer initiation and progression may be modeled and studied using organoid-based systems. Notably, several studies have demonstrated that tumor organoids retain the histopathological traits of the original tumors not only *in vitro*, but also after transplantation into immunodeficient mice, highlighting its application to assess *in vitro* drug treatments in a more dynamic *in vivo* environment (Fujii et al. 2016; Schütte et al. 2017). Besides that, organoids may be generated with incredible efficiency from patient-derived healthy and tumor tissues, possibly allowing patient-specific formulation of personalized treatment regimens for each patient (Yao et al. 2020).

Challenges in Usage/Implantation of Animal Models in Cancer Research

To be more precise, every cancer model has inherent limits when it comes to simulating patient-specific tumors; therefore, using a suitable model is critical to a study's success. Exosomes derived from MSCs may have various benefits, but it should always be remembered that the dosage of infused MSCs is capable of not only circulating rapidly, but, can also be eliminated easily after post-transplantation. This may assist in deciding a higher dose that distributes more widely and evenly. Also, in contrast to transplanting biologically derived cells, employing MSC-derived vesicles is limited to being static and unable to be replenished *in vivo*. Similarly, the CRISPR/Cas9 gene-editing method now allows for the simulation of mutations in patients' tumors and their functional evaluation using animal cancer models. Moreover, the limitations of CRISPR/Cas9 in terms of editing accuracy, delivery methods, possible off-target consequences, clinical implications, and ethical issues requires additional investigations and development. Thanks to organotypic tumor models, we are learning more about cancer heterogeneity and its implications for personalized therapy. However, organoid cultures yet lack the components of the tumor microenvironment and other physiological factors. The absence of stroma, blood arteries, and immune cells are one of the inherent constraints of organoid cultivation. Therefore, there is a need to develop reproducible platforms that speed translational insights into patient treatment, minimizing unwanted technical variability associated

Table 3 List of mesenchymal stem cells derived exosomes and its inhibitory role in cancer

S. No.	Source of exosomes	In vivo study models	Target cancer cells/tissues	Modes of action	References
1.	Bone marrow mesenchymal stem cell-derived exosomal MicroRNA-126-3p	Male BALB/c nude mice models	Pancreatic Cancer	Overexpressed miR-126-3p derived from BMSCs exosomes inhibits the development of pancreatic cancer through the downregulation of a disintegrin and a metalloproteinase-9 (ADAM9)	(Wu et al. 2019)
2.	Bone marrow mesenchymal stem cell-derived exosomal miR-206	BALB/c female athymic nude mice	Osteosarcoma	BMSCs derived exosomal miR-206 can migrate into osteosarcoma cells and inhibit tumor progression by targeting the oncogene transformer 2β (TRA2B)	(Zhang et al. 2020)
3.	miR-145 derived from adipose-derived stromal cells (ASCs)	Male athymic nude mice	Prostate cancer	Both *in vitro* and *in vivo* experiments exhibited the inhibitory effect of ASCs on tumor cell proliferation inducing apoptosis and controlling tumor growth	(Takahara et al. 2016)
4.	Exosomes derived from miRNA-584-5p transfected MSCs	U87 xenograft nude mouse models	Malignant Glioma	MiRNA-584 suppresses the activity of glioma cells by binding to the 3′-UTR of CYP2J2. Exosomal miRNA-584 migrates from MSCs to U87, and affects the activities of U87 *in vitro* and the tumor progress *in vivo*	(Kim et al. 2018)
5.	Mesenchymal stem cells delivered miR-199a	Female Balb/c nude mice	Glioma	MSCs-derived exosomes deliver miR-199a to glioma cells and thus prevents the	(Yu et al. 2019)

(continued)

Table 3 (continued)

S. No.	Source of exosomes	In vivo study models	Target cancer cells/tissues	Modes of action	References
				tumorigenic progression of glioma *via* the downregulation of onco-protein Arf GTPase-activating protein-2 (AGAP2)	

with cancer organoid cultures. Furthermore, organoids have become valuable *in vitro* models for cancer research despite these drawbacks. Future adoption of these strategies to enhance the cancer model system will need multidisciplinary collaboration efforts involving clinicians, biologists, and genetic engineers.

Conclusion and Future Directions

During the last decade, MSCs have grown in popularity in clinical applications as an alternative to ES cells and iPSCs due to their lack of ethical constraints and teratoma development. Researchers may now study the migration, engraftment, and differentiation of MSCs, therapeutic or diagnostic cellular markers, in cancers employing complex animal models. Cancer cell-intrinsic and cell-extrinsic processes of tumor growth, metastasis, therapeutic responsiveness, and successful treatment for many cancer types have all been developed from animal cancer models. New 3D culture methods have generated innovative and better functional healthy tissue and cancer models, which are already being utilized in research. Engineered stem cells are being explored more and more for their potential in several biological interventions where they are known to interact and impact tumor cells at different phases of development. Despite the promising preclinical results, only few clinical trials using stem cells as delivery vehicles for anticancer medicines have been in practice (Table 4). The achievements and advantages of each animal model in experimental and clinical settings has been covered in this book chapter. Therefore, there is a compelling need for more rigorous research on stem cell-based treatments' to increase oncological efficacy so that they may be employed in clinical applications to their full potential. Additionally, to develop innovative therapeutics with enhanced effectiveness and safety profiles, it is necessary to conduct preclinical investigations involving effective *in vivo* models. Considering the potential therapeutic ability of stem cells, some difficulties must be addressed promptly, such as the *in vitro* growth of these cells without compromising their stem cell characteristics and the long-term outcomes of the implanted stem cells *in vivo* with *ex vivo* variation. However, more studies are warranted to overcome some significant obstacles, such as the need to improve the

Table 4 Clinical studies of mesenchymal stem cells (MSCs) in cancer tumors

S. No:	Clinical trials accession No.	Therapeutic interventions	Cancer targets	Clinical trials – phase	References
1	NCT02530047	Human mesenchymal stem cells with interferon beta (MSC-INFβ)	Ovarian cancer	Phase I, completed	(Andreeff et al. 2018)
2	NCT03106662	Human mesenchymal stem cells infused with Cyclophosphamide drug	Hematological malignancies	Phase III, completed	(Arslan et al. 2018)
3	NCT03096782	Umbilical cord blood stem cells with added sugar chemotherapy and radiation therapy	Leukemia and lymphoma	Phase II, ongoing	(de Lima et al. 2012)
4	NCT01460901	Allogeneic hematopoietic stem cells transplantation (HSCTs)	Neuroblastoma	Phase I, completed	(Cruz et al. 2013)
5	NCT03407040	Cancer antigen-specific T-cells from human induced pluripotent stem cells (iPSCs)	Gastrointestinal cancers, breast cancer, pancreatic cancer, melanoma, lung cancer	Cohort, completed	(Nishimura and Nakauchi 2019)

innovations being used to make protocol settings more consistent when generating these different models.

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Role of Animal Models in the Development of Bacteria-Based Live Therapeutics to Fight Cancer

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Contents

Abstract

Cancer is the most prevalent cause of death worldwide. Cancer affects the lifestyle of a person both mentally and physically. Chemotherapy and radiation therapy have been the major treatments that kill the cancerous cells along with the surrounding healthy cells. As these therapies suffer many side effects, researchers are concentrating on the therapies which could combat cancer with minimal side effects and higher efficiency. Bacteria-based therapies have come into the limelight with their myriad health benefits as well as their potential to cure cancer. Due to the large versatility in the strains of bacteria especially probiotics, there has been increasing

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research on the bacteria-derived treatment strategies which could specifically target cancer cells while maintaining homeostasis and leaving healthy neighboring cells unaffected. This chapter is aimed at understanding the beneficial effect of various bacterial strains on cancerous cells as well as the improved beneficial effects of bioengineered bacterial systems that leave hope for promising future treatment regimens for cancer.

Keywords

Cancer · Bacterial therapy · Designer bacteria for cancer therapy · Probiotics-based cancer theranostic

Introduction

Cancer is the common name that refers to a large group of diseases having the ability to affect any part of the human body. It is one of the most serious and fatal diseases, accounting for most deaths from a single disorder throughout the world. As per WHO, nearly 10 million deaths were reported to be associated with cancer in 2021 ranking the second leading cause of death (WHO Report 2020). The global cancer burden has been predicted to rise by 47% in 2040 (Sung et al. 2021). In females, breast, cervical, uterus, and thyroid cancers are more prevalent, while lung, liver, prostate, and colon cancers are common in males (WHO Report 2020). Surgery, chemotherapy, and radiotherapy are the conventional antitumor treatment strategies shown to be efficient in relieving symptoms of several types of cancers. However, these therapies remain ineffective for certain types of cancers; moreover, these interventions exhibit serious limitations such as off-target toxicity, insufficient availability of the drug at the target site, emergence of drug-/radiation-resistant cancer cells, limited tumor penetrance, increased rate of tumor recurrence, etc. (Baskar et al. 2012). These limitations urged the scientific groups to develop an alternative targeted therapy for cancer treatment, and as a result of rapid development in the field of science and technology, many advanced and innovative treatment strategies are being developed, such as: (i) immunotherapy is a novel treatment strategy wherein the body's immune system will be stimulated or boosted either by injecting the person's own immune system components or by introducing laboratory-prepared antigenic components to fight against tumor cells (Zhang and Chen 2018); (ii) one another emerging strategy to treat cancer is gene therapy where target gene will be delivered into the body using a vector. In this method, the inserted gene may replace the defective gene or can enhance/repress the function of the existing gene. Employment of viral vector as the delivery vehicle limits its applicability (Das et al. 2015); and (iii) thermal ablation or hyperthermia is an innovative treatment where a series of procedures that exploit heat or cold are given to neoplastic tumor cells. Long exposure to higher temperatures or lower temperatures could destroy the cell membrane of the highly

dividing cancerous cells which automatically leads to the death of the cell. However, the technology to control the temperature and their delivery to specific tumor cells is not well understood and developed (Crezee et al. 2021). Stem cell therapy involves the use of stem cells especially the hematopoietic stem cells (HSC) for the treatment of blood-associated cancers, namely leukemia, lymphoma, and multiple myeloma. This therapy does not directly kill or eliminate cancer cells instead it helps in the recovery of the patients after the conventional procedures of cancer treatment. It has been shown to have many side effects such as rejection, autoimmunity, inability to reach the target site, transformation of normal stem cells into cancerous, etc. (Chu et al. 2020). Photodynamic therapy is a recently developed alternative treatment strategy to control malignant cancer. This technique utilizes a photosensitizer molecule that gets excited upon exposure to light of a particular wavelength. This reaction triggers the generation of oxygen free radicals in the target tissue which ultimately results in cell death. Although PDT is considered a relatively safer and efficient intervention, the detailed knowledge of the molecular mechanism of target selection and drug delivery is still lacking (dos Santos et al. 2019). Active research is being carried out to develop a safer and effective treatment that can overcome the shortcomings of the abovementioned techniques. Bacterial therapy is one such promising development having great potential in the treatment of cancer. Cancer-specific colonizing ability of the bacterial strains has labeled them as better live biotherapeutics. Moreover, the genetic manipulation of bacteria is relatively easy so that they can be converted into nonpathogenic forms, attenuated forms, and target-specific delivery vehicles. By way of administering antibiotics, one can have complete control over the action of bacterial agents. However, genomic instability, antigenicity, cytotoxicity, and inability to completely eliminate cancer cells are the limitations of this therapy (Rommasi 2021). This chapter gives details of bacterial therapy for the diagnosis and treatment of cancer and also highlights the recent advancements in bacterial therapy making it a promising strategy to fight cancer successfully.

Bacteria in Cancer

First report on the involvement of bacteria in improving cancer disease dates back nearly 100 years. *Streptococcus pyogenes* and *Bacillus prodigiosus* are the initially identified bacterial strains that were shown to heal cancer experimentally. Certain anaerobic bacterial species, namely *Clostridium* spp., were found to create a hypoxic environment in cancer tissue that eventually killed the cancer cells (Coley 1912). *Mycobacterium bovis* strain was able to activate Toll-like receptor 7 (TLR7) through the caspase 8 signaling pathway thereby causing cancer cell apoptosis. Growing research on this aspect revealed the tumoricidal effect of many other bacterial species, namely *Clostridia, Shigella*, *Lactococcus*, *Bifidobacteria*, *Listeria*, *Salmonella*, *Vibrio*, and *Escherichia coli* (Rommasi 2021).

Bacteria in Cancer Diagnostics

Bacteria as a Prognostic Marker in Detecting Cancer

Physical examination, blood test, biopsy, and imaging are the common methods of cancer diagnosis. Recent developments have introduced improved imaging technologies and biomarkers of the circulating tumor cells which have enhanced the precision in the diagnosis of cancer to a greater extent. However, the accuracy and efficacy of these techniques are still a subject of debate. Therefore, search for improved cancer screening techniques remains as a valuable research area. Association of bacteria with cancer provoked scientists to investigate the possible role of the human microbiome to fight cancer (Shirazi et al. 2020). Strains such as *Fusobacterium nucleatum* (Fn) (upregulated), *Clostridium hathewayi* (Ch) (upregulated), and *Bacteroides clarus* (Bc) (downregulated) can be identified in the fecal samples of patients for colorectal cancer diagnosis. *nusG*, butyryl-CoA dehydrogenase gene targets for qPCR detection of Fn species can provide more sensitivity in detecting colorectal cancer. Lachnoclostridium species strain m3 can also provide better diagnosis marker property for adenoma and colorectal cancer diagnosis (Liang et al. 2020). The *pks*$^{+}$ *E. coli* can be useful in diagnosis but become difficult to distinguish between colorectal cancer (CRC) and colorectal adenomatous polyposis (CAP) (Liu et al. 2021). Diagnosis of colorectal cancer can also be done with colonic tissues by isolating DNA from the tissues and followed by a qPCR analysis of bacterial biomarkers such as *Fusobacterium nucleatum* (Tunsjø et al. 2019). Non-small cell lung cancer is also associated with microorganisms and its detection or diagnosis for squamous cell carcinoma can be done using sputum, lung tissues for *Acidovorax* (high) in SCC, *Capnocytophaga* (high) for AC, and *Haemophilus* (low) and *Fusobacterium* (low) for AC, with sputum *Acidovorax* (high), *Streptococcus* (high), *Veillonella* (high), *Capnocytophaga* (high), and *Helicobacter* (low) (Leng et al. 2021) (Table 1).

Bacteria as Biosensor

Microorganism-based biosensors are an innovative technique that was developed by Divies in 1975. In this technique, microorganisms act as the recognition agents, and an oxygen electrode is used as the physical transducer, and such properties have a high potential for analytical applications (Riedel 1998). Recent developments in bacterial biosensors have further enhanced the specificity and efficiency. The key particulars of bacterial biosensors are: (1) biosensors that detect temporary or localized signals; (2) processing circuits that turn the stimulus into a specified downstream output; and (3) operators or DNA-based storage mediums that report on the stimulus' presence. Effective bacterial sensor should reach the cancer microenvironment and thus requires sensing modules to endure the perturbations as well as the genetic controllers that optically disperse over time without imposing fitness burdens on the bacterial chassis (Tanna et al. 2021). For instance, engineered *E. coli*

Table 1 List of bacteria associated with different types of cancers and their application in the diagnosis of cancer

Type of cancer	Identifiable bacteria	Type of sample	Regulation	References
Bladder	*Fusobacterium* *Campylobacter* *Jonquetella*	Urine	Up	Bučević Popović et al. (2018)
Colorectal	*Fusobacterium* *Campylobacter* species	Tissue	Up	Bučević Popović et al. (2018), Liang et al. (2020), and Liu et al. (2021)
	Fusobacterium nucleatum *Clostridium hathewayi, m7,* m3, pks^{+} *E. coli*	Fecal/ tissue	Up Up Up Up Up	
	Bacteroides clarus	Fecal	Down	
Esophageal	*Fusobacterium nucleatum*	Tissue	Up	Bučević Popović et al. (2018) and Yamamura et al. (2016)
Laryngeal	*Fusobacterium nucleatum* *Gemella* *Capnocytophaga* *Parvimonas* *Aggregatibacter* *Peptostreptococcus*	Tissue	Up	Gong et al. (2017) and Bučević Popović et al. (2018)
	Streptococcus *Firmicutes* *Actinobacteria*	Tissue	Down	
Urothelial carcinoma	*Streptococcus*		Up	Xu et al. (2014) and Bučević Popović et al. (2018)
Lung squamous cell carcinoma	*Acidovorax*	Sputum	Up	Leng et al. (2021)
Lung adenocarcinoma	*Acidovorax* *Streptococcus* *Veillonella* *Capnocytophaga* *Helicobacter*	Sputum	Up Up Up Up Down	Leng et al. (2021)
	Capnocytophaga *Haemophilus* *fusobacterium*	Tissue	Up Down Down	

were tested in mouse models to detect liver metastasis. PROP-Z (programmable probiotics with lacZ) are orally administered into the immunocompetent host mice bearing colorectal cell line (MC26-LucF). A conjugate of luciferin and galactose were used in order to track the presence of tumor. The activity of LacZ converts the substrate to luciferin which can be detected in urine. This report demonstrated the

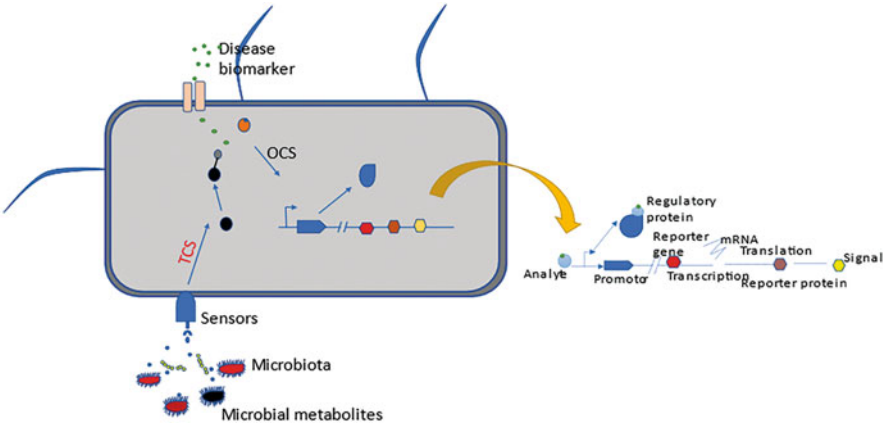

Fig. 1 Components of bacterial biosensors one-component systems (OCSs) or two-component systems (TCSs), processing circuits for transducing inputs into outputs, and actuator elements such as reporter proteins

potential of engineered probiotics in the diagnosis of liver metastases; furthermore, this helps in the imaging of complete liver in a noninvasive mode (Danino et al. 2015).

Bacteria-based heme biosensor has been designed to detect gastrointestinal bleeding events via heme dispersed by the lysed red blood cells. This technique involves probiotics combined with an ingestible microbioelectronic device (IMBED). It was built using a synthetic promoter (PL(HrtO)) which was controlled by the *Lactococcus lactis* heme-responsive transcriptional repressor HrtR and ChuA, an outer membrane transporter from *E. coli* O157:H7 that allows extracellular heme to pass through the cell envelope. The genetic circuit's output was *Photorhabdus luminescence luxCDABE*, which acts at body temperature and encodes intracellular production of substrate. It detects heme in blood and generates a fluorescent signal, subsequently activating the adjacent electronic circuit to generate a wireless signal. This approach led to a new model for investigating disease diagnosis and health management. The mechanism works on the understanding that bacteria produce light when they sense target biomarkers through the help of photodetectors that emit electric signals. These electrical signals are then processed by a bioluminescence detection circuit (Mimee et al. 2018) (Fig. 1).

Engineered Bacteria to Detect Inflammation

Besides the bacterial prevalence as a biomarker, genetically modified bacteria are being exploited for the precise detection of tumors. For instance, the ability of certain bacterial strains such as *Salmonella* to specifically accumulate and proliferate in tumors has made its way into the development of programmable bacteria for the diagnosis of cancer (Zheng et al. 2000). The bioengineered *Salmonella* strain

VNP20009 has been able to particularly colonize within murine tumor models and effectively sequestered a radiolabeled nucleoside analog, 20-fluoro-1- b-D-arabino-furanosyl-5-iodouracil, which can be detected by positron emission tomography (Panteli et al. 2015). Commensal *E.coli* strain is constructed with two different genetic circuits, in which one of the circuits acts as a promoter and the other acts as a memory molecule. The circuit which acts as a promoter has been made to trigger the Cro expression and Cro-inducible CI/Cro transcriptional switch to function as a memory unit for the detection of tetracycline in the human gut. Reactive oxygen species (ROS) create tetrathionate, which is a transitory consequence of inflammation and its level can be determined. Interestingly, the predesigned strain maintained memory for 7 days in detecting the tetracycline derivative and stopped after the inhibition of signal in the in vivo mouse gut (Riglar et al. 2017). This study ascertained the functional advantages of commensal bacteria that are predesigned to act as a sensor for the detection of inflammation or infection. This generally allowed the inflammatory state to be spotted even after the tetrathionate signal had vanished.

Bacteria in Imaging of Cancer

Tumor-targeting bacteria like *Salmonella* or *E. coli* produce the enzyme beta-lactamase, which could be enough to split the beta-lactam ring in Bluco (luciferin, released by bacterial β-lactamase hydrolysis) and release D-luciferin. FLuc (firefly luciferase) could then oxidize free D-luciferin to emit light, which might be used to detect malignancies and metastasis in vivo (Cronin et al. 2012). Jiang et al. have reported the construction and use of a live, EcN-based scannable vehicle functionally co-expressed firefly luciferase (Fluc) and luciferin-regenerating enzyme (LRE), an enzyme that contributes to steady bioluminescence. In vitro and in vivo, the recombinant EcN strain expressing the genomically integrated Fluc-LRE cassette was a beneficial tool for generating bioluminescence for bacterial tracking, providing an optical tumor-targeting system for the in vivo study of bacteria-assisted cancer imaging. Moreover, in vivo imaging of the recombinant EcN strain in the mouse intestinal tract revealed its ability for gut investigation (Jiang et al. 2021).

A recent trend is the use of bacteria in imaging to increase the early detection and diagnosis via bacteria-mediated targeted delivery of imaging agents. Furthermore, bacterial-based imaging systems show considerable potential for imaging micro-sized metastatic nodules that are difficult to detect using traditional imaging techniques in metastatic illnesses (Li et al. 2021). OMVs are being used to create a biomimetic system (Li et al. 2021). A nanoparticle (OMVMel) was developed for tumor theranostics. *Escherichia coli* strains are engineered largely considering the fact that they are less endotoxic by inactivating the *msbB* gene (to yield OMVmsbB) and overexpress tyrosinase, which creates melanin that is passively integrated into the cytosol and membrane of OMVmsbB (to give OMVMel). The modified bacteria would release OMVs with a high photothermal conversion efficiency, allowing for photoacoustic (PA) imaging and photothermal therapy of 4T1 breast tumors.

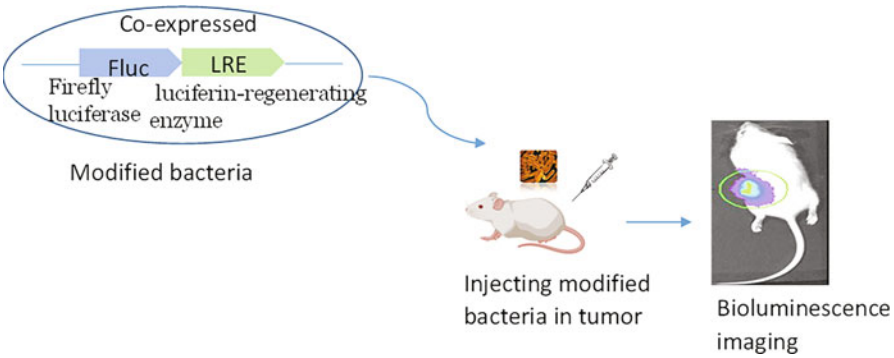

Fig. 2 Schematic illustration showing the potential ability of bioluminescent bacteria in imaging the cancer

Furthermore, the photoacoustic signal created by OMVMel may endure for up to 24 h, allowing for long-term tumor monitoring. The photoacoustic signal strength was shown to be favorably linked with the amount of melanin in the tumor tissue. Optoacoustic may provide longitudinal imaging of tumors in vivo, which has various advantages over fluorescence approaches for cancer surveillance, using melanin-containing OMVs as a biocompatible contrast agent (Gujrati et al. 2019).

Genetically modified bacteria have been introduced to disperse a novel fluromarker to colonize and detect tumor location. *Salmonella* was transformed with pDF02 to express ZsGreen gene that comes under the control of pBAD promoter, after induction of L-arabinose. An important advantage of this detection is that they possess high specificity and directly target malignant cells. Because of its specificity to human tumors, detecting bacteria could be used as a primary screen for metastatic recurrence. In a microfluidic system, the modified bacteria colonized small tumor measuring about 0.12 mm^3 can be detected using ZsGreen fluromarker produced by tumor-targeting *Salmonella*. These results show that *Salmonella* produces measurable levels of ZsGreen when injected into tumor-bearing mice (4T1breast carcinoma cells) and had no adverse effects. Therefore, such detection using fluromarker releasing bacteria would be helpful in early prognosis in cancer detection (Panteli et al. 2020) (Fig. 2).

Diagnosis of Cancer Using Microbial DNA and RNA Signatures

Microbial DNA and RNA markers are unique diagnostic agents that complement current tumor diagnostics. Poore et al. presented a masterful study aimed at filling this gap in translational research by using data from The Cancer Genome Atlas' whole-genome and whole-transcriptome sequencing studies (TCGA); the researchers used trained artificial intelligence (AI) algorithms applied to whole-genome and whole-transcriptome sequencing investigations (TCGA). Filtering and precise classification of microbial nucleic acids produced from samples were made

possible by AI models. They looked at data from over 17,000 samples taken from 10,000 individuals, which includes primary tumors and recurring tumors resulting from metastatic dissemination. This study revealed the fact that nonhuman organisms accounted for 7.2% of the total, with bacteria, archaea, and viruses accounting for more than a third of the total. A 12.6% single genus level categorization has been achieved due to more precise classification. When it came to differentiating between cancer patients and healthy controls, the model performed the best. The model, on the other hand, had varying degrees of success in discriminating between stage I and stage IV tumors (Poore et al. 2020). According to the study, predictions based on microbial DNA in blood could distinguish CRC patients from healthy, cancer-free people.

Bacteria for Cancer Therapy

Bacteria are an excellent choice for immunotherapy due to their significant hypoxia localization potential as well as selective colonization at the tumor location, and also their immunogenicity and programmability. LPS, which is located on the outer membranes of gram-negative bacterium, is a strong immunogenic microbial-associated molecular pattern (MAMP) that gets mostly paired with Toll-like receptors (TLRs) on immune cell membranes. The intricacy of bacteria as a living organism dictates the challenges and liabilities of changing them into tumor-fighting weapons (Huang et al. 2021a). Certain bacteria prefer to congregate at tumor sites because the TME provides an appropriate environment, and such germs can reach this site via flagellar motility. Because the TME is a nutrient-rich environment, both obligatory and facultative anaerobic bacteria may thrive there. As facultative anaerobes, *S. typhimurium* and *E. coli* may sense a nutrient-rich and favorable environment via their chemoreceptors and aggregate in the tumor's periphery and core. Several bacteria, such as *Clostridium* spp., *Listeria*, and *Salmonella*, have inherent tumor-targeting capabilities that allow them to target, penetrate, multiply, and decrease solid tumors through various processes. *Clostridium* genus bacteria, such as *C. butyricum* and *C. novyi*-NT, can live in the hypoxic environment created by the tumor mass. To decrease nonspecific pharmacological effects on normal tissues, probiotics can be administered directly to the TME. Tumor-targeting bacteria and probiotics have several limitations in their usage as anticancer medicines since it is difficult to balance bacterial doses for therapeutic applications (Allemailem 2021). *S. typhimurium* A1 can expand quickly if it obtains enough adequate nutrition from tumor tissue.

Tumor Elimination by Live Bacteria

Bacteria possess inherent anticancer effects, however various strains of bacteria may use different ways to eliminate solid tumors. Aside from the intrinsic anticancer effects, the bacterial infection produces both innate and adaptive immunity targeting

bacteria and cancerous cells (Zhou et al. 2018). In animal tumor models, live tumor-targeting bacteria can colonize tumors or tumor-driven lymphatic systems, decrease tumor development, and prolong longevity following systemic infection. The utilization of tumor-targeting bacteria as a framework for sustainability can surmount permeability constraints and maximize chemotherapeutic drug activity while lowering systemic toxicity to the host (Duong et al. 2019). Bifidobacterium has been shown to transport effector genes to tumors. Numerous recent reports have revealed that genetically altered living bacteria can be used as tumor-targeting vectors in human cancer treatment. Bifidobacteria were also utilized to encode cytosine deaminase (CD), allowing cancer site–specific transformation of 5-FC to 5-FU, which resulted in a strong anticancer impact in rat malignant cells and melanomas after intratumoral or systemic injection of altered *B. longum* or *B. infantis*. *Salmonella typhimurium* has indeed been genetically modified to generate a range of therapeutic medicines, again along with effectiveness in animal models, achieving specific tumor curative expression of genes (Morrissey et al. 2010). Despite their ability to develop in aerobic circumstances, *S. typhimurium*, *E. coli*, and *S. flexneri* are limited to the purportedly hypoxic zones of necrosis within a solid tumor.

Recombinant Bacteria as Cancer Theranostics

By integrating the selectivity of the tumor-focusing bacterium with the accuracy of biological marker identification, a direct process capable of detecting tiny lesions and metastases might be developed. A screening approach that combines the advantages of microbial tumor targeting, as well as biomarker analysis, would be a valuable addition to conventional testing protocols since it would be both selective and precise (Panteli et al. 2020). *Salmonella* invades tumors at frequencies 10,000 orders of magnitude greater than healthy tissue after initial therapy to tumor-bearing mice. Several ways of detecting tumors using microorganisms have been reported. Ferritin-expressing *Escherichia coli* improves magnetic resonance imaging (MRI) by boosting iron uptake and enhancing the signal-to-background ratio. Through innate absorption of FDG, *Escherichia coli* has also been utilized to improve positron emission tomography (PET). These bacteria enhanced FDG absorption when paired with the right uptake of malignant tissue, resulting in a stronger radiologic signal. The production of bioluminescent proteins by *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholera*, and *Listeria monocytogenes* has also been utilized to observe the multiplication of bacteria of several tumor types (Panteli et al. 2015).

The detection of chronic disorders such as osteomyelitis, wherein *S. aureus* is a common infection, can be significantly obtained by integrating the specificity offered by MRI probe with infinite tissue probing properties (Periyathambi et al. 2021). Bacterial surfaces, cell walls, proteins, nucleic acids, and enzymes are often targeted by these probes. Positively charged AIEgen and conjugated oligomers, for example, target the bacterial surface via electrostatic and hydrophobic forces. Fluorescent probes made from boric acid derivatives and conventional antibiotics like

vancomycin and polymyxin can mark the carbohydrates and lipids in cell walls (Huang et al. 2021a). Naturally occurring substances and tiny substrates were used to generate fluorescent biochemical markers, allowing drug targets to be identified and connections among enzymes and their substrates to be envisioned conventional fluorescence imaging technologies have relied on recombinant protein (e.g., green fluorescent protein [GFP]) or generic fluorescent dyes that extensively stain cellular structures, but chemical probes give vital new tools (Marshall et al. 2020). Furthermore, considerable research has been done on the creation of fluorescent organic probes that can differentiate bacteria by exploiting the physical and chemical features of bacterial cell walls. Fluorescent organic probes provide real-time bacterial imaging and quantification in vitro or in vivo.

Previous research has shown that various bacterial species, including *Salmonella*, *Clostridia*, and *Bifidobacteria*, may enter solid tumors and colonize hypoxic areas. As a result, these bacteria can be regarded as suitable tumor-targeting vectors for tumor medication delivery. Genetic modification of tumor-colonizing ability of bacterial species such as *Salmonella* sp., *Clostridium* sp., and *Escherichia coli* allows them to express antitumor agents, cytotoxic proteins, reporter genes, and tumor-specific antigens, as well as targeted delivery of these bioactive agents into tumor cells (Laliani et al. 2020). Engineered bacteria have the capacity to identify cancers from normal tissues while being less harmful. Bacterial MVs were also used to deliver immune-stimulatory substances as vaccines and therapeutic compounds in cancer immunotherapies. According to current research, genetic alteration of bacterial MVs is a potential technique for improving their effectiveness in cancer immunotherapy (Yang et al. 2021) (Fig. 3).

Tumor Colonizing Bacteria

Genetically engineered bacteria used as therapeutic biological agents must be lacking toxicity as well as nonpathogenic than their wild-type counterparts (Zu et al. 2014). The advancement of recombinant DNA technology rekindled the field, allowing genetic enhancement of *Clostridia*'s natural oncolytic capabilities. It offers a potential solution to the problem of employing wild-type strains. Several approaches were used, including genetically modified bacteria to express prodrug converting enzymes or other bioactive molecules or cytokines (Fig. 4 and Table 2).

Probiotics-Based Cancer Therapy

The cancer patients who undergo traditional treatment strategies of cancer such as radiation and chemotherapy often encounter with gastrointestinal disorders especially diarrhea. Application of probiotics for the improvement of gastrointestinal problems in such patients sounded logical and that is how probiotics entered in the field of cancer treatment strategies. In a clinical study, the patients suffering from grade 3 chemotherapy-induced diarrhea were successfully treated using a

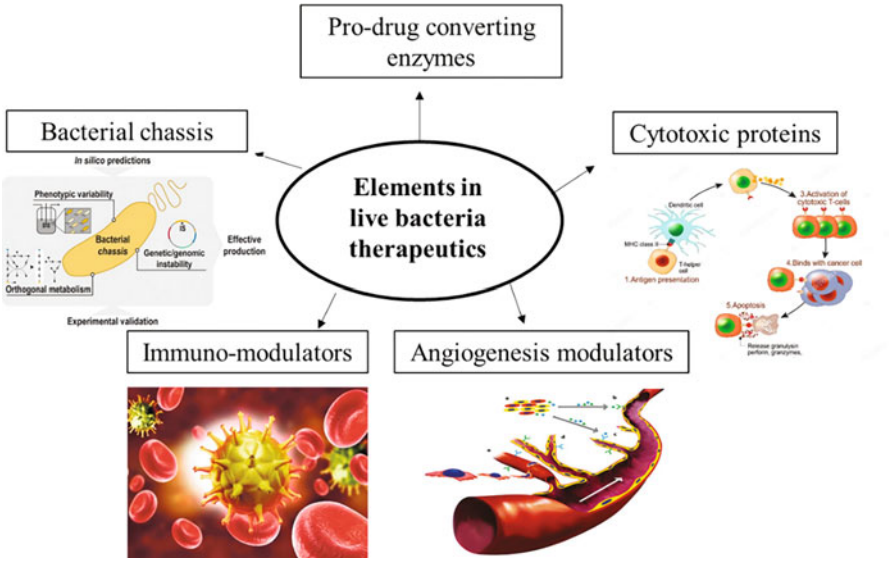

Fig. 3 Picture depicting the role of bacteria as a targeted delivery system for various therapeutic payloads for the treatment of cancer

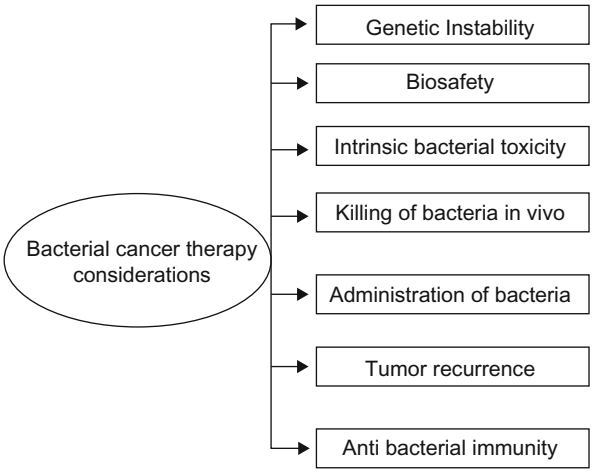

Fig. 4 Schematic illustration showing the concerns associated with bacterial mediated cancer therapy

multispecies mixture of probiotics (El-Atti et al. 2009). Most importantly, probiotics were used to combat chemotherapy-induced vomiting in cancer patients and also the study demonstrates that probiotics must be considered for advanced breast cancer patients with chemotherapy-induced diarrhea (El-Atti et al. 2009). Very interestingly, research in the recent past over probiotics reveal the fact that apart from its influence in the gastrointestinal tract it could also affect the growth and properties of

Table 2 List of bacterial strains and their application in cancer treatment

Bacterial source	Cancer type	Membrane type	Efficacy	Reference
Salmonella	Ehrlich ascites carcinoma (EAC)	OMV	1. Passive accumulation in tumor tissue through EPR effect 2. Amalgamation of chemotherapy and immunotherapy	Aly et al. (2021)
Escherichia coli	Human lung carcinoma A459 cells	Protoplastderived nanovesicles	1. Bioengineering with high expression of the EGF to target tumors 2. Mitigation of systematic toxicity	Kim et al. (2017)
Escherichia coli	B16F10 tumor	DMV	1. Bioengineered with high expression of RGD motifs to target the tumors 2. Targeting neutrophils or monocytes	Gao et al. (2021)
Escherichia coli	CT26 and 4T1 tumors	OMV	1. Surface is functionalized with a calcium phosphate shell to respond to the acidic environment of the tumor 2. Combination of photothermal therapy and immunotherapy	Qing et al. (2020)
Escherichia coli	TC-1 and B16F10 tumors	OMV	1. Use as a cancer vaccine 2. Induction of production of the antibodies that target tumor angiogenesis	Huang et al. (2020)

cancerous cells. Based on these findings, applications of probiotics are being investigated in different aspects, especially their implication in the treatment and prevention of cancer labels them as potential therapeutic agent (Górska et al. 2019). The probiotic namely *Lactobacillus caseiBL23* has been shown to significantly protect the mice against the development of colorectal cancer. Further investigation has revealed the fact that *L. Casei* BL23 induces the downregulation of the IL-22 cytokine on the one hand and upregulates caspase-7, caspase-9, and Bik on the other. Altogether, *L. Casei* BL23 could exhibit immunomodulatory and antiproliferative effects; these findings clearly indicate that *L. Casei* BL23 has a strong potential to develop modern, probiotic-based approaches to combat colorectal cancer (Jacouton et al. 2017). Recently, the small intestine bacterial overgrowth (SIBO) has been shown to be associated with the development of gastrointestinal cancer (Ma et al. 2019). Interestingly, treatment with *Bifidobacterium* triple viable capsule has been reported to be successful in the battle against SIBO, simultaneously it could enhance urinary signs in such patients. These results pave a way for more investigation into the future application of the probiotics in the management of gastric cancer and colon cancer (Markowiak and Śliżewska 2017).

Collection of evidences indicate that the ingestion of probiotics can play a preventive role in colorectal cancer (Markowiak and Śliżewska 2017). Despite a significant number of literature reports, the exact pathways by which probiotics can prevent colorectal cancer remain unknown. Initial efforts to understand the mechanism of action of probiotics suggest that it may act by modification of the intestinal microflora; inactivation of cancerous compounds; interaction with putrefactive and pathogenic microbiota; enhancement of the immune host response; antiproliferative effects by control of apoptosis and cell differentiation; fermentation of undigested food; and disruption of tyrosine kinase signaling pathways (Markowiak and Śliżewska 2017). Furthermore, pH acidification, while not regarded as a distinct mechanism of action, is an essential and basic characteristic through which many probiotics work out their metabolic processes. Significant scientific data are indicating that probiotics and prebiotics can be useful for the prevention and diagnosis of colon cancer and the modification of intestinal microflora by increasing levels of good bacteria such as *Lactobacillus* and *Bifidobacteria* and by decreasing rates of pathogenic microbes (Markowiak and Śliżewska 2017). Reports indicate that *Lactobacillus* administration results in higher activity of antioxidative enzymes and modulates circulatory oxidative stress that protects cells from carcinogen-induced damage (Wang et al. 2017). Nevertheless, there is no clear scientific proof for the reduction of cancer in human volunteers as a result of the ingestion of probiotic cultures in fermented or unfermented dairy goods, although there is a variety of preliminary data focused primarily on laboratory research. Specific probiotic strains have also been found to have antiapoptotic effects.

A study involving a combination of microbial dysbiosis and breast cancer confirmed that healthy breast tissue was enriched with *Sphingomonas yanoikuyae* relative to the diseased tissue, indicating its possible function as a probiotic (Xuan et al. 2014). *Lactobacillus crispatus* and *Lactobacillus acidophilus* have been reported to have antiproliferative action against breast cancer cells. *Lactobacilli* can decrease the transcriptional activity of several specific cancer-testis antigens (Azam et al. 2014). Research in Japanese women indicated that *L. casei* when orally taken daily has been strongly linked with a reduced incidence of breast cancer. This study also suggested that long-term exposure was thus necessary to create a chemopreventive impact of probiotics on the growth of cancer (Toi et al. 2013). Probiotic strains such as *L. acidophilus*, *Bifidobacterium infantis*, *Lactobacillus paracasei*, and *Bifidobacterium bifidum* were observed to reduce cancer cell growth in MCF7 cells. The tests revealed that the *L. Plantarum* I-UL4 strain had decreased the number of breast cancer cells in the media supplemented by Tween 80 (Nazir et al. 2018). Probiotic strains have exhibited antineoplastic behavior inhibiting mammary tumors in animal models, convincing the ubiquitous efficacy of probiotics as anticarcinogenic agents (Malik et al. 2018). Probiotics have been shown to reduce the mutagen-induced DNA damage or DNA deposition in the colonic epithelium (Garcia-Gonzalez et al. 2020). In vitro experiments using rat intestinal epithelial cells demonstrated the preventive function of probiotics against enterocyte apoptosis induced by 5-fluorouracil (5-FU) when an in vivo analysis with rats revealed a mixture of resistant starch and *B. Lactis* allowed the apoptotic reaction to

carcinogen-induced DNA damage to rat colorectal cells. This shows that probiotics have related roles to the tumor suppressor protein p53 when DNA damages occur at higher levels (Garcia-Gonzalez et al. 2020).

Significant number of studies have shown that the administration of probiotics greatly decreased the incidence of colon cancer in animal models (Drago 2019). Animal tests have repeatedly demonstrated a decrease in the occurrence of chemically mediated colorectal tumors and the aberrant crypt development correlated with probiotic administration (Panebianco et al. 2020). In vitro experiments can offer proof of safety and have a deeper explanation of the active substances involved and the pathways behind their anticarcinogenic activity. Probiotics can beneficially modulate many vital intestinal functions: detoxification, colonic fermentation, movement, and immune status that may follow the development of colon cancer (Saikali et al. 2004). Recently, 20 patients with colonic adenomas participated in a three-month study, in which *L. acidophilus* was administered together with *B. bifidurn* where decreased symptoms of colonic cancer were noted (Panebianco et al. 2020). Reports have also shown that thermally killed *Enterococcus faecalis* fraction activates the gastrointestinal immune system toward vancomycin-resistant enterococci, whereas heat-killed *Bifidobacteria* induce major increases in tumor necrosis factor (TNF)-5-007 and interleukin (IL)-6 levels (Saikali et al. 2004). Many reports have confirmed that probiotics not only prevent the growth of acute gastric mucosal lesions but also improve the healing process of triggered gastric ulcers. The impact of probiotics on gastric ulcers is due to many cellular and molecular pathways. Karakazu have shown that pretreatment of rats with LG21 yogurt containing *Lactobacillus gasseri* OLL2716 greatly prevented the development of acetic acid–induced gastric ulcers on a dose-dependent basis. This influence was caused by a rise in the development of mucosal prostaglandin E2/I2, thus ultimately reducing the risk of gastric cancer (Khoder et al. 2016). Probiotics could inhibit infection of *H. pylori* through nonimmunological and immunological pathways. Several in vitro experiments have demonstrated the inhibition or even killing of *H. pylori* in preclinical and clinical studies. These trials demonstrated only modest effectiveness of probiotics against *H. pylori* when given alone. Increased effectiveness and/or elimination of side effects were shown when probiotics were administered in conjunction with normal triple standard treatment (Mathipa and Thantsha 2017) (Table 3).

Designer Probiotics

It is becoming evident that probiotics offer a broad variety of health benefits by their inherent ability to modify different components of molecular pathways of the human system with which they interact. Although the beneficial effects of probiotics are well established, in terms of commercialization they still lag far beyond because of the following limitations: (i) inability to target particular pathogen(s); (ii) the cocktail intake of probiotic strains to target specific group of pathogenesis is not reproducible and changes from person to person; and (iii) generally, these probiotics are taken

Table 3 List of probiotics that have been shown to be effective against certain types of cancer

Name of the probiotic strain	Type of the cancer	Source
Lactobacillus plantarum	Breast cancer	Drago (2019)
Lactobacillus acidophilus	Breast cancer	Toi et al. (2013)
Lactobacillus casei	Breast cancer	Xuan et al. (2014)
Bifidobacterium infantis	Breast cancer	Azam et al. (2014)
Lactobacillus paracasei	Breast cancer	Azam et al. (2014)
Bifidobacterium bifidum	Breast cancer	Azam et al. (2014)
Lactobacillus salivarus	Gastric cancer	Garcia-Gonzalez et al. (2020)
E. coli Nissle	Melanomas	Khoder et al. (2016)

orally either through food or in the form of capsules where it passes through the gastrointestinal tract which needs to overcome technical and gastrointestinal stress factors. These limitations have further paved a way for the discovery of more novel and innovative probiotics called either bioengineered, designer, or recombinant probiotics (Amalaradjou and Bhunia 2013). These bioengineered probiotics are genetically modified to specifically produce the protein and to target the specific pathogen. Further they are tailored to have improved tolerance to stress conditions such as extreme temperature, pH, oxygen alterations, and also has improved survival rate than the traditional probiotics in the gastrointestinal tract, kill particular infections or pathogens, improve immune response, and imitate cell surface receptors. In a study undertaken by Desmond and his group, it was observed that recombinant probiotics survived 10–54 times better than conventional recombinant probiotics in the gut (Amalaradjou and Bhunia 2013; Cláudio Lima de Jesus et al. 2020). Bioengineered probiotics have also been shown to increase stress resistance, development of antimicrobial peptides, enhancement of anti-inflammatory reaction, enhancement of colonization exclusion, receptor mimicry, and toxin neutralization. Recent studies investigating the targeted drug delivering ability of probiotics have taken the probiotics research to another level. Recombinant probiotics are being developed for the delivery of molecules such as RNA, peptides, single-chain vector fragments, cytokines, enzymes, and allergens, contributing to the idea of "bio drug" for the treatment and control of different diseases (Amalaradjou and Bhunia 2013; Cláudio Lima de Jesus et al. 2020).

A recombinant strain of *L. lactis* expressing IL-17 was constructed and showed a lower tumor incidence and reduced tumor size compared with nonrecombinant LAB strain when administered. The antitumor effect of recombinant *Lactococci* secreting biologically active IL-17 could be more effective than traditional probiotics because of the immune response resulting in partial protection against TC-1-induced tumors in mice (Jacouton et al. 2019). Novel probiotic-derived protein, p8, has been shown to have anticolon cancer benefits. Bioengineered probiotics in particular expressing this protein has been shown to kill tumor cells and decrease tumor-related behavior. The antiproliferation behavior of p8 was regulated by inhibition of the signal pathway p53-p21-cycline B1/Cdk1, leading to growth arrest during the cell cycle. Taken together, these findings indicate p8 is detrimental to cancer cells and also

demonstrates stable cell expression and displays clear cancer-suppressive action by causing cell cycle arrest (Charrier-Savournin et al. 2004). Nonpathogenic strains such as *E. coli Nissle* can target, kill, and replicate in tumor cells as well as necrotic tissues. This strategy could effectively be used to combat both predominant and metastatic melanomas by the usage of cancer colonizing facultative and compulsory anaerobes such as *Shigella, E. coli, Clostridia, Bifidobacterium, Salmonella*, and other oncolytic viruses. *E.coli Nissle* (EcN) tumor-targeting properties were studied using luciferase *luxCDABE* operon, and the findings revealed that EcN could directly accumulate in the solid tumor areas of SMMC-7721 tumor-bearing BALB/c nude mice. The EcN (Tum 5-p53) group tumor inhibition rate was also as high as 69.47% ($P < 0.05$) in the tumor volume and as high as 62.5% ($P < 0.05$) in the tumor mass. In the research, the Tum 5-p53 bifunctional proteins were initially designed and then delivered to solid tumor regions utilizing the guided transporter EcN for cancer therapy and suggested that the gene delivery of Tum 5-p53 bifunctional proteins to solid tumors may be a successful cancer therapy technique (He et al. 2019,). Genetically engineered *C. acetobutylicum* expresses and secretes *E. coli* cytosine deaminase (CDase). These findings show the possible application of clostridium-based therapeutic protein transfer to tumors in anticancer therapy. Recombinant *Bifidobacterium* showing WT1 protein was developed and can effectively distribute WT1 protein to the intestinal immune system when extracted, and demonstrated an important antitumor impact in the C1498-WT1 murine leukemia syngeneic tumor model (Shirakawa and Kitagawa 2017). Tumor-targeting ability of EcN for inhibiting mouse melanoma B16 and breast tumors 4T1 by expression of azurine protein has been demonstrated. Recombinant azurine-expressing EcN was constructed and found to be remarkable in restraining 16 melanoma and orthotopic 4T1 breast tumor growth as well as to prevent pulmonary metastasis in immune-competent mice (Zhang et al. 2012).

Probiotics-Conjugated Nanoparticles

Probiotics have gained attention for their anticancer potentiality as well as their myriad health benefits; however, their bioavailability when administered into the body restricts their biological impact. This issue could be solved through nanotechnology where probiotics-conjugated nanoparticles would directly target cells and increase their availability (Date et al. 2016). Experiments involving mice indicate that modified citrus pectin probiotic microbeads improve bioactivity and chemopreventive action against precancer colonic tumors and adenocarcinoma by inhibition of GAL-3 and VEGF in the Balb/c in colonic carcinogenesis model (Odun-Ayo et al. 2016). *E.coli*-conjugated doxorubicin enhances antitumor efficacy in inhibiting tumor development, prolonging animal life, and inducing apoptosis of tumor cells. In fact, following antimicrobial treatment, it was removed from tumors and other tissues. The acid-labile *E.coli* conjugates thus offer a secure and succinct technique to improve the temporal and spatial controllability of anticancer treatments. It is reported that autonomously formed doxorubicin and sorafenib (DOX/SOR/

Spore-DA) nanoparticles can enhance the stability of drugs in GIT and overcome the multibiological barriers of intestinal epithelium in colon cells suitable for anticancer treatments (Xie et al. 2017). By hitchhiking nanoparticles to the surface of bacteria, several approaches to improve nanoparticle delivery to tumors or other tissues can be an alternative approach. Such polymeric nanoparticles conjugated with probiotics have great advantages in overcoming the cellular barriers of mammalian cells and help in enhancing the biological activity as a delivery carrier of antibiotics, chemotherapeutics, etc. (Song et al. 2019).

Conclusions

Taken together, the current study emphasizes the potential role of bacteria for the treatment of cancer. Bacterial strains have become extremely prevalent in the research to combat cancer and the usage of engineered bacterial strains have become more popular for targeted delivery of therapeutics to cancerous cells. Though the exact mechanism of action by which bacterial cells induce apoptosis of cancerous cells is not well understood but it is expected to be significant because of the production of bacteriocins or by modulating the immune responses. Various studies on probiotics with their myriad health benefits have centered on a common conclusion that daily consumption in day-to-day food activities could definitely improve the overall health of the individual and also the specificity of the probiotics could pave a path for the cancer treatment. It has been noticed that most of the scientific research had focused on the development of bioengineered bacteria because of their ability to specifically target cancerous cells and also have shown increased efficacy and bioavailability, but still there is a lot of space in research which needs to be focused on the risk assessment of their intake, mechanism of action to increase its overall beneficial effects on human life.

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Repurposing of Drug: Utility of Animal Models

8

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Contents

Abstract

Drug repurposing is an up-and-coming concept in the world of medicine. It is an efficient way to use already existing drug formulations in the treatment of diseases besides the ones which were initially intended for. This circumvents

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the tedious process of drug development and approval and helps to conserve resources. While multiple drugs have already been repurposed, their utility and reintroduction into the market is still a new concept. Even though it optimizes the use of resources, lab testing and animal model trials form a crucial aspect of determining the efficacy of these drugs in different diseases. This review encompasses the methods and trends in the repurposing of drugs while highlighting the use of animal models and the benefits of repurposing drugs during the COVID-19 pandemic.

Keywords

Reposition · Data mining · Computational repositioning · Animal model

Introduction

As Eroom's law states, even though there are major advancements in scientific and technological areas, there is a decline in the count of novel drugs acquiring approval. It takes approximately 5–7 years to introduce a drug into the market since the information used for synthesis is based on data on receptors, also called de novo synthesis. The rate of approval and further application is limited to 2.01% (Pease et al. 2017). This causes a backlog and delays in drug-related research output, which is required for developing novel drugs, resulting in continual lacunae in accessible therapies. This builds up to an unmet demand for more effective drugs, resulting in a growing interest in drug repurposing. Drug repositioning aims to find alternative uses for already approved drugs unrelated to their initial indication.

This shortens the long process of new drug delivery into the market. A total of 94 repositioned drugs have made it to the market till 2018 (Xue et al. 2018). This gives an incentive for further research and new treatment alternatives which extend to even orphan diseases. The repositioned drugs have been successfully used in various fields such as from symptomatic treatment of hypertension to malignancies. Drug repurposing, in conjunction with novel approaches to drug validation and appropriate animal models like mice and zebrafish, has significantly contributed to the identification of therapeutic targets (Kari et al. 2007). This technique is considered superior as it uses compounds with documented risks and known pharmacodynamic, pharmacokinetic, and preclinical characteristics that can bypass phases I and II of clinical trials, thereby making the process of drug development less tedious and relatively quick. As a result, the World Health Organization (WHO) and other health agencies have turned to re-evaluating the efficacy of licensed and experimental drugs to treat emerging health problems. Drug repositioning, also known as retasking and drug rescue or repurposing, has saved us from emerging infections such as COVID-19, where the time required to develop a vaccine or alternative drug was limited. As a result of repurposing old drugs, a global pandemic was controlled symptomatically (Shende et al. 2021).

Purpose of Drug Repositioning

Animal models are useful in scientific studies since in many diseases human models are inaccessible and it involves ethical issues in conducting trials in human models for chronic diseases like cancer, and Alzheimer's disease, among others (Nykamp et al. 2022). For studying alcohol abuse and dependence and administering the addictive drug, psychotropic, and narcotic substances, humans pose a high risk due to their psychological effects. Therefore, repurposing not only reduces the cost of trials but makes it very easily accessible. It can be bred in suitable conditions synthetically, in comparison to human models. The conditions can be altered, and their response can be recorded in comparison to the difficult follow-up required in human models. In nearly 71% of cases, human drug toxicity can be elicited and tested on animal models. In comparison to cell lines, animal models help in studying the effect on the complexities of immune and multitissue responses as well as other side effects and reactions associated with drug trials on the model. Research shows major similarities in human cells and animal cells, for example, human brain cells with mice brain cells. This is due to orthologous genes being preserved in organisms and corresponding gene expressions being similar (Iskar et al. 2013). It simplifies the process of introduction of a drug into the market since it is previously approved and hence safety and toxicity can be checked on animal models, which speeds up the initial process. The knowledge of the possible adverse drug reactions of such a drug allows filtering of the indications and to prescribe accordingly, significantly decreasing the risk during and after the trials. It proves to be more cost-effective for the reintroduction of a new drug since existing formulations have already passed the pharmacokinetic and clinical trials (Sleigh and Barton 2010). The availability of the drug into the market is easier as it is already indicated, and the cost of latency reduces with the cost of production. It competes with the rising cost and attrition of a newly introduced drug. It is a huge initiative to meet the needs and demands of a large population and also the number of orphans and difficult diseases (de Oliveira and Lang 2018).

Methods of Drug Repositioning

Drug repositioning is the reusing of an already existing active ingredient on the market for a different disease or indication. It excludes any modification done to the drug in terms of dosage, formulation, and ingredients. It majorly acts on the principle that an already approved drug, with known formulation, dosage, and side effects, can be used in new indications to treat a variety of diseases.

It requires two approaches, the discovery and knowledge of factors that can influence a disease itself and progression which will help recognize common and exploitable factors (Zhu et al. 2020). Data mining is a method that helps in understanding these properties (Masoudi-Sobhanzadeh et al. 2020). Along with pleiotropic drugs, which include drugs having more than one receptor or action site all of which act in synergy thereby increasing efficacy and ultimately proving to be beneficial in the case of comorbidities as well (Hu et al. 2016). This also includes

phenotypically similar drugs. After the selection of drugs to be repositioned, animal model needs to be chosen on the basis of similarity to the targeted human cells. All drugs are tested in a series of microarrays of cell lines or tissues showing similarity, at least to a considerable extent. Nevertheless, there are intrinsic differences noticed in the animal models as opposed to human diseases due to normal pathobiological differences between the two species (Chan and Lascalzo 2012). Drugs can be induced in animal models surgically, genetically, or through pharmacological manipulations. Pharmacological induction can be intravenous, intramuscular, or intraperitoneal (Satoskar and Bhandarkar 2020). In some cases, the animal model is prepared by adjusting the conditions of the animal to adapt to and then the study progresses. For example, in the case of alcohol abuse studies, animals are allowed to voluntarily consume alcohol in the form of sweetened solutions. Surgical techniques require a high level of skill which is why it is limited in usage. In genetic manipulations, the animals are allowed to inbreed or selectively breed thereby helping study the progression of the genetic aspect of the disease. The repositioned drugs are then studied, and the results are meticulously documented (Table 1 and 2).

Table 1 Table representing repurposing of medical drugs

Drug	Indication	Repositioned/Marketed for
Hydrochloroquine and chloroquine	Malaria	COVID-19
Sildenafil	Vasodilatory properties in case of hypertensive patients	1. Penile erection was a side effect hence was marketed as "viagra" by Pfizer 2. It is also used as an antianginal drug and has been indicated as "Revatio" for pulmonary artery hypertension
Aspirin	Analgesic	1. It is used in cardiovascular disease due to its effects on the cyclooxygenase pathways which inhibit prostaglandins 2. It is protective against colorectal cancer
Thalidomide	Antiemetic	It is banned now due to its teratogenic properties. The discovery of its antiangiogenic property and hence the usage in oncology and its wide usage in erythema nodosum leprosum in leprosy led to its popularity

Table 2 Table representing the repurposing of nonmedical drugs

Drug	Indication	Repositioned/Marketed
Dimethyl fumarate	Mold inhibitor for leather companies	1. Treatment for psoriasis and autoimmune diseases like multiple sclerosis 2. It also decreases the risk of multiple sclerosis (MS) patients for developing progressive multifocal leukoencephalopathy
Methylene blue	Staining	1. Treatment for methemoglobinemia 2. Malaria 3. Antiseptic for urinary tract infection (UTI) 4. Noninfectious conjunctivitis

Drug Repositioning in Animal Models

Every drug introduced into the market or for approval has to be tested on either animal models or cell lines after which it is put through clinical trials on humans. Animal models have been used for various trials. In ophthalmology, for acute optic nerve crush injury, in the testing of intraocular injection of N-methyl D-aspartate receptor excitotoxic agents and glaucoma, animal models have been used extensively. It has been used in chemotherapy-induced peripheral neuropathy as well (Harada et al. 2019). Many studies have shown multiple events occurring after chemotherapy in the dorsal root ganglion and spinal cord in rodents (Sisignano et al. 2016; Yeh et al. 2020). Especially, taxanes lead to serious inflammatory reactions in comparison to platinum derivatives. Although the phenotypes are the same, every chemotherapeutic drug causes chemotherapy-induced peripheral neuropathy (CIPN) through an alternative mechanism. Therefore, it is vital to understand the pathology specific to drugs and develop solutions to each variant of CIPN (Sałat 2020).

Oxaliplatin treatment has been shown to activate nuclear factor erythroblast-related factor 2 (Nrf2) signaling, which is essential for mitochondrial function, and Nrf2 knockout mice reported increased neuropathic symptoms after administration of oxaliplatin (Yang et al. 2018).

Studies done recently show that organic cation transporter-2 (OCT2) is mainly expressed in satellite glial cells (SGCs), suggesting important interactions between peripheral glial cells and sensory neurons. The cell type mediated by oxaliplatin should be corroborated by cell-type-specific regulation of OCT2 expression employing recombination tools used for neuronal damage (Yamamoto and Egashira 2021). Additionally, studies show that carnitine transporter, organic cation, toxic compounds, and extruded multidrug are involved in the reactions of oxaliplatin in the diagnosis-related group (DRG). Intake of oxaliplatin in the DRG causes nerve damage by oxidation, then axonal degeneration of the rodent's sciatic nerve (Hu et al. 2021; Sałat 2020). The changes in the miR15b, β-secretase-1, and truncated neuregulin-1 pathways have been shown associated with myelin sheath hypoplasia of the sciatic nerve (Kaiser et al. 2015). However, the disadvantage associated with the advancement of older drugs may be the lack of recently generated data reports. One study demonstrated that genes associated with inflammatory bowel disease (IBD) are targets for approved therapies for IBD and identified drugs that may be rearranged or further developed for the treatment of IBD and primary sclerosing cholangitis (PSC) (Jiang and Karlsen 2017).

Repositioning of drugs has helped wherein an analysis identified tricyclic antidepressants (TCAs) and related molecules as potential inhibitors of small cell lung cancer. These compounds have apoptotic cell death properties in chemotherapy-resistant tumor cells and are chemically conserved in culture, genetically engineered mouse models, and human small cell lung cancer (SCLC) tumor cells transplanted into immunocompromised mice (Jahchan et al. 2013; Riess et al. 2020). Imipramine and promethazine are two major drugs, activating stress pathways and promoting SCLC cell apoptosis and necrosis by blocking cell survival

autocrine signals involving the receptors of neurotransmitters and also neurotransmitters. These have been shown to suppress the spread of neuroendocrine tumors, including pancreatic tumors. These experiments highlight the power of a bioinformatics-based drug repositioning approach. When repositioned, tricyclic antidepressants escitalopram dose-response showed that rutin was one among the main compounds with a positive connectivity score (Sage 2014). Rutin, a citrus flavonoid, is available in abundance in citrus fruits. Many reports show that rutin has properties acting as an antidepressant in the mouse. The substance with the highest positive connectivity score with nortriptyline was loxapine. Loxapine is a typical antipsychotic drug used in schizophrenia. Similar to nortriptyline, loxapine on N-demethylation is metabolized to amoxapine, a TCA. Therefore, loxapine represents a compound with plausible repositioning potential for major depressive disorder (MDD). In a series of experiments, each replication represents a subculture of cells got from different passages (Powell et al. 2017).

Escitalopram and nortriptyline have the two genes insulinoma-associated *1 INSM1* (gene helping increase neural progenitor cells in the neocortex) and oxidation resistance 1 gene *OXR 1* which generates a protein effective against oxidative stress and are protective to MDD patients' brain cells. These data were collected when drugs were purposed individually. The research showed that drugs like bafilomycin have a reverse effect on antidepressants drugs like clomipramine, showing similar effects on mRNA; therefore, it can be potential antidepressants.

Repositioning in cosmetology is done in animals including zebrafish and mice to try antidandruff, antiaging, and other cosmetic products. Neuromuscular junction agonists can be used as novel antiwrinkle agents. Botulinum toxin (Botox) obtained from *C. botulinum* is an exotoxin which is a prototype drug that acts on the neuromuscular junction and helps decrease wrinkles or in antiaging. Argireline is a synthetic hexapeptide that displays antiwrinkle activity. 14waglerin-1, a protein isolated from the poison of temple viper (*Tropidolaemus wagleri*), inhibits wrinkling (Sotiropoulou et al. 2021).

Bioactive compounds such as adenosine are endogenous nucleosides used as cardiac medication, are also accepted for the treatment of paroxysmal supraventricular tachycardia (PSVT), and when repositioned for use on the skin, are potent antiwrinkle drug, and are active ingredient in creams. Repositioning of cosmetological drugs in other fields such as epidermal growth factor as supportive therapy for the treatment of diabetic foot ulcers is a proven alternative. It also improves osteogenic differentiation of dental pulp stem cells in vitro and increases calcium deposits suggesting a role in biomineralization (George et al. 2008).

A clinical study suggested that the application of the plant-derived epidermal growth factor (EGF) improved the appearance of rhytids, skin texture, pore size, and various dyschromatic conditions within the initial months of use. The growth factor (GF) derivative COL17A1 is used for cell division of epidermal stem cells to help in the proliferation of epidermal stem cells for skin antiaging. Interferons (IFN) are small proteins that give innate and adaptive immunity (Domaszewska-Szostek et al. 2021). IL15 is a cytokine expressed mostly in muscles. Polymorphisms in IL15 have

shown effective endurance training in women, which was tested on Tg IL15 mice that showed running endurance. Recombinant IL10 was used as a drug to treat inflammatory bowel disease, including Crohn's disease. It stimulates adult fibroblasts to synthesize hyaluronic acid. This effect shows the method of fetal regenerative wound healing with decreased fibrosis. IL10 improves scar-free wound healing and can be used in new cosmeceuticals for improved wound healing (Sotiropoulou et al. 2021).

Even tobacco dependence has been successfully treated by repositioning. Zebrafish larvae, cultivated in the Mayo Clinic Zebrafish Core Facility according to the National Institute of Health (NIH) Guide for the care and use of Laboratory Animals and approved with the aid of Mayo Clinic's Institutional Animal Care and Use Committee, can be used to test repositioning for substance use. Larval zebrafish on stimulation to nicotine show increased locomotion at once stimulated (Morales-Rosado et al. 2015). Apomorphine, clonazepam, betaxolol, topiramate, carisoprodol, diazepam, lorazepam, and zolpidem weakened the nicotine effect not impacting the locomotor reaction to peripheral-appearing stimuli (cinnamon and mustard oil). Mixed treatment with varenicline and bupropion attains a progressive reaction over monotherapy, at half of the dose, recommending feasible synergism. Combination treatment of varenicline with both topiramate and apomorphine additionally found out a progressed weakening reaction to nicotine, even though at a lesser value to varenicline with bupropion mixture, diazepam-maintained efficacy, betaxolol becomes near an additive reaction, meanwhile, carisoprodol, zolpidem, and the alternative benzodiazepines confirmed a much less-than-expected reaction. These medicinal drugs can be used for application in mixture remedy for smoking cessation or possibly 50% of the effective dose as a subthreshold degree to show a useful reaction (Cousin et al. 2014).

Betaxolol, beta1-adrenergic antagonism authorized to tackle increased blood pressure, inhibits cocaine-prompted conditioned vicinity desire at high doses; however, it is now no longer used at low doses and also blocks cocaine and opiate withdrawal-prompted phenotypes. This indicates beta-1 adrenergic receptors are used in drug-prompted phenotypes and has guided the findings that betaxolol can weaken nicotine-prompted locomotor activation in larvae of zebrafish. Betaxolol and different adrenergic antagonists can also additionally have results on comorbid high blood pressure and tobacco dependence, in cardiovascular diseases (Cousin et al. 2014).

Apomorphine D1, and D2 dopamine receptor agonist authorized for anti-Parkinsonism treatment, was substantially studied for dopamine receptor sensitivity and function and to treat alcohol dependence (Morales-Rosado et al. 2015). The blunting impact of apomorphine on acute nicotine-prompted locomotor reaction with its capacity to reverse neuroadaptations of the dopamine machine following persistent nicotine publicity proves apomorphine good for treating tobacco dependence. Topiramate is an authorized anticonvulsant and used in the treatment for migraines, added with phentermine used for obesity. Topiramate weakens nicotine and ethanol locomotor reaction in the larva of zebrafish.

Amyotrophic lateral sclerosis (ALS), motor neuron disease in adults, has important metabolic factors involved in disease progression. Ambroxol, a chaperone molecule that inhibits GBA2, has been shown to have a profitable effect in delaying the onset of SOD1G86R mice. Glucocerebrosidase (GBA) 2, a glucosylceramide-degrading enzyme, was shown to be abnormally elevated in the spinal cord of the ALS SOD1G86R mouse model (Shah et al. 2021).

Repositioning ivermectin in gastric cancer (GC) provides data indicating an alternative mechanism of action for ivermectin. Instead of acting on the glutamate chloride channels commonly found in nematodes, insects, and ticks, it blocks the mammalian WNT/β-catenin signaling pathway, thereby paralyzing the pharyngeal muscles and somatic cells. Since ivermectin is very safe for mammals due to the blood-brain barrier, further preclinical and clinical studies are needed to rearrange ivermectin for GC (Rabben et al. 2021; Juarez et al. 2018).

Recent Developments in Drug Repurposing

Drug repurposing can be grouped into three categories which are repurposing strategies, repurposing approaches, and validation techniques.

Repurposing Strategies

Genome

A lot of genomic and transcriptomic data have been gathered from the latest advances in genomics, ranging from cell lines, normal tissue samples, disease samples, and animal models. Although gene expression data are largely used, other sources are being used for drug repositioning as well. Along with the phenotypic and clinical database, it has become easier to identify and elaborate upon different disease mechanisms and therefore to identify newer uses of drugs that already exist. Genetic mutations can also be used in the repurposing of drugs. A wide variety of gene expression profiles were created using the Connectivity Map (CMap) project and the Library of Integrated Network-Based Cellular Signatures (LINCS) where human cancer cell lines were treated with various drug compounds under varying circumstances (Gns et al. 2019). The aim of Cmap is to establish a functional connection between drug-induced gene expression profiles and a set of genes representing a biological state of interest by creating an integrated database. Gene expression signatures are compared to compound signatures in reference databases such as CMap (Musa et al. 2018). This query signature can be produced from a disorder, a drug variation, or a genetic variation that can be used to compare drugs-drugs, drugs-genes, or disease drugs. Gene Expression Omnibus (GEO) microarray data, for example, were used to create disease signatures for 100 diseases, and each disease signature was mapped to 164 drug signatures in CMap (Qu and Rajpal 2012). Many

known diseases-drug correlations as well as new ones were discovered among the strongly anticorrelated disease-drug pairs, including cimetidine for lung carcinoma treatment. A common assumption in these studies is that if there is a significant anticorrelation between disease and drug signatures, then the medicine may have a therapeutic effect on the disease. Noncoding RNAs (ncRNAs), particularly microRNAs (miRNAs), regulate many cellular processes acting as potentially enticing therapeutic targets for drug repurposing (Martinez-Hernandez et al. 2021; Kato 2018).

Phenome

In recent years, the phenome-wide association study (PheWAS) has gained popularity as a comprehensive approach to identifying important genetic associations with diseases (Roden 2017). Novel drug indications were discovered on the basis that drugs with similar adverse effects have similar therapeutic benefits. This requires a rigorous understanding of the underlying molecular/pathological pathways. The phenomenon can be integrated with other types of data like the genome for drug repositioning (Bush et al. 2016; Rastegar-Mojarad et al. 2015). For instance, in ovarian cancer patients, the *BRCA1* or *BRCA2* mutation has been linked to an increased risk of breast cancer (Li et al. 2016). However, there is an urgent need to better understand these interrelations and to use them for disease management or individualized health care.

Chemical Structures of Drugs

Drug chemical structures can act as an indicator of potential repositioning avenues. Integration of publicly accessible databases of chemical structures, screening data, and literature-derived biochemical data can be used for repositioning and is a booming field of research. An extremely efficient, support vector machine (SVM) classifier integrates drug side effects, chemical structure, and molecular activity, thereby making drug repurposing more efficacious (Wang et al. 2013; Pinzi and Ristelli 2019).

Drug Combinations

Current combination strategies are in popular demand because it depends on clinical and empirical data, along with computational prediction. The Drug Combination Database (DCDB) integrates gene expression profiles from multiple drugs to predict effective drug combinations as well as look for adverse effects, genes, or disease pathways that may be affected by the drugs in the combination (Cui et al. 2018; Ryall and Tan 2015). These traits were then used to train a machine-learning classifier to predict effective drug combinations. To identify new drug combinations, experimental drug efficiency characterizations, for instance, library screening and cell viability assays, were used in addition to the computational approach. Eleven antileukemic drugs that could be combined with imatinib to overcome drug resistance in BCR-ABL leukemia were identified by screening databases, collecting literature, and using comparative analysis (Singh et al. 2017).

Repurposing Approaches

Machine Learning

A machine learning framework that assimilates and categorizes data based on a similarity approach PREDICT uses drug-protein interactions, sequence, and gene-profile to integrate drug-drug similarities as well as uses disease-phenotype and human phenotype to integrate disease-disease similarity. This has attained AUC = 0.9 in drug indication prediction (Huang 2014). Similarity-based methods have been used for predicting novel drug indications. Deep learning, comprising of multiple processing layers, uses raw data, identifies finely detailed structure in huge data sets, as well as permits changes in the internal parameters. Deep learning models outperform the existing selective vector machine (SVM) by using gene expression data, suggesting its use in pharmaceutical research. It enables multitask learning which aids to predict toxicity, thereby, being superior to conventional learning (Ekins 2016) (Fig. 1).

Network Models

The network-based analysis is another popular approach for in silico drug repurposing. Many such networks are either extrapolated computationally or based on knowledge accumulated by screening libraries or literature. This model is made

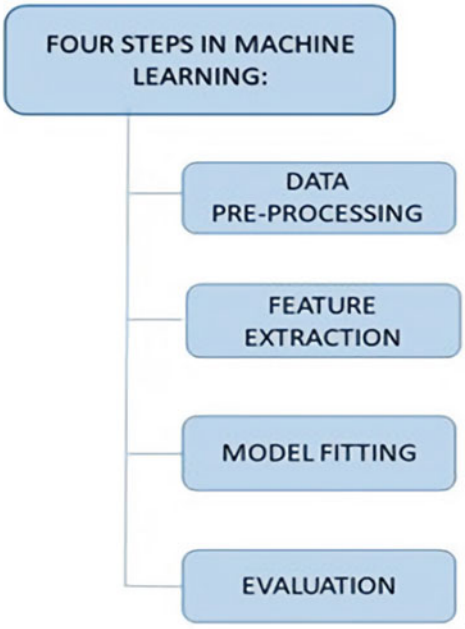

Fig. 1 Diagrammatic representation of the steps that are involved in machine learning

up of nodes that represent diseases, drugs, or gene profiles and edges representing the connections between them. The relation between the nodes and edges is weighed using the Jaccard scoring (Hodos et al. 2016). Many homogeneous networks are either disease-centric or drug-centric. Soon, a bipartite network was built using drug-target interactions and it was discovered that the network-based interpretation method achieved the best performance, with a mean ROC AUC of 0.96 (Durán et al. 2021). Many similar bipartite or homogeneous models have been set up using phenotypic data like drug-disease, adverse effects. According to the guilt-by-association principle, many undiscovered drug-disease relationships can be explored by integrating a heterogeneous network using a compilation of genetic data. Bayesian factor regression model recognized drug off-target effects on signaling proteins using a network component called cancer-signaling bridges. This may impact important cancer signaling pathways and has proved to be a breakthrough in cancer therapeutics (Jin et al. 2012).

Validation Approaches

Computational data need to be confirmed for the purpose of drug repositioning by clinical trials to have a tangible impact on patients. This calls for validation by experiment, and notwithstanding the limitations in the laboratory and in vivo models, it can prove to be helpful for drug evaluation prior to clinical trials. For instance, mouse models were used to demonstrate pentamidine sensitivity for clear cell renal cell carcinoma (ccRCC) as it induces apoptosis in cells (Zerbini et al. 2014).

Another approach for validation is data mining of patients clinical records. The theory of a model's performance could be assessed using the Precision-Recall curve along with the AUCROC curve. Cross-validation has been recommended to bypass an increase of false positives over true values (Winne and Baker 2013).

Possible Repositioning Alternatives

In repositioning in pulmonary arterial hypertension (PAH), single-cell RNA sequences were performed in the lungs of monocrotaline (MCT), sugen-hypoxia (SuHx), and control rats in association with human PAH using the integration of patient lung histology with human PAH loci and known disease genes. The study reveals clear and common dysregulation of genes and pathways in two commonly used PAH models for the first time in single-cell resolution and their association with human PAH and drug repositioning (Hong et al. 2021).

Fibrodysplasia ossificans progressiva (FOP) is a rare congenital disease that progresses through intermittent episodes of bone formation at ectopic sites. FOP patients carry heterozygous gene point mutations. The mutant *ALK2* displays neo-functional responses to activin, a bone morphogenetic protein (BMP) cytokine that inhibits regular bone formation. Moreover, the mutant *ALK2* becomes

hypersensitive to BMPs. Both these activities contribute to enhanced *ALK2* signaling and bone formation in connective tissue. Being a receptor with an extracellular ligand-binding domain, the mutant *ALK2* is a druggable target. The molecular mechanisms underlying signaling and ectopic bone formation are caused by *ALK2* mutations. This opens up pathways to drug repositioning for FOP (Ventura et al. 2021).

Cell Culture: An Alternative to an Animal Model

3D cell culture techniques mimic in vivo microenvironmental characteristics, yielding data with a stronger prognostic value for treatment outcomes, and are hence becoming increasingly popular in drug development and toxicity screening. It allows to simulate the cell culture environment and thereby encourages desired cell behavior which enables drug discovery by targeting specific cell behavior more accurately (Breslin and Driscoll 2013). Preclinical screening of a personalized panel of drug candidates may be performed by integrating chemotherapeutic agents with 3D cell culture techniques derived from molecular profiling of datasets and tumor cells from primary patients, which enhances the result and reduces the adverse effects of cancer therapy (Santo et al. 2017). The fundamental challenge in this technique is to create physiologically meaningful 3D cultures that mimic in vivo tissue and disease pathophysiology. Cell lines are useful when there is less availability of tissues from patients or primary cells for research. Immortalized cell lines have the advantage of being easy to culture making them suitable for use in higher levels of drug screening. The variants can be introduced easily into cell lines, which is faster than in animal models. It can be useful to assess the response of extremely rare mutations, where animal models are not available, or primary cells cannot be obtained (Ravi et al. 2015). Cell lines have limitations since they are commonly cancer-derived or are immortalized conventionally resulting in genome instability and altered gene expression. Immortalized cell lines lack mechanical and biochemical indicators and as a consequence can lose expression of differentiation; therefore, they poorly represent the in vivo situation. This is overcome by improvements in cell expansion techniques like conditional reprogramming, allowing cells to be passed numerous times while maintaining a normal phenotype. Specific epithelial cell lines can also be purchased readily from commercial suppliers or biobank repositories, avoiding the process of ethical clearances and procedures for patient tissue collection. Organoids are now an extremely useful tool in research. It is derived from stem cells which, when under the right culturing conditions, automatically form in vivo like organs with differentiated and functioning cells retaining the genotype and phenotype of the parent tissue. 3D culture lines are derived from resident tissue adult stem cells. Induced pluripotent stem cells which are now used as alternatives can be generated

from patient cells like fibroblasts or blood cells. Protocols for differentiation of the induced pluripotent stem cells into relevant epithelia are used to generate further cultures. Apart from the expense and ethical conundrums, authentic 3D cell culture models employing human cells can avoid the disadvantages of mouse models, which are not always able to effectively reproduce human illnesses or capture adverse effects of medications such as hepatotoxicity (Langhans 2018). A huge disadvantage of these cell lines is the fast tissue deterioration rates which make long-term studies difficult. The lack of vasculature and inability to recruit nonresident immune cells and the missing interactions between different cells are factors limiting their use as a replacement for in vivo experiments on animal models. Therefore, in vitro and ex vivo models obtained from animals and humans build a foundation for in vivo experiments and clinical application. It is essential for the preclinical pathogenetic study of infectious diseases with or without zoonotic potential.

Challenges with Repositioning

The major challenge in drug repositioning in animal models would be to mimic the human diseases in the model chosen. Animal models are criticized due to the limitation in the prediction of new chemical entities, toxicity, and safety in humans. Due to difference in species, clinical presentation of certain features and side effects is not reliable or possible. The selection of the animal model is a tedious and time taking process defeating one of the advantages of drug repositioning. Another ethical challenge in repositioning on animal models is concluding a method with animal suffering minimized after the trial. Hence, when research is done on cases such as sepsis, the animal models should only be chosen if in vitro, in vivo, in silico, and all other clinical approaches are ruled out. For studying specific biological responses, in vitro cell cultures are a better alternative to animal models. The cell culture lines have evolved since it gives a more focused approach and might better translate to the human condition rather than a generalized approach and thereby increasing sensitivity (Novac 2013). The other challenges faced in the implementation of repositioned drugs would be the use of an already approved drug, in which the formulation, dosage, route of administration, and features cannot be altered since it will cause ethical and patency-related issues. Furthermore, the regulation of such drugs poses a challenge due to its availability which increases the risk of over-the-counter drug distribution leading to tolerance and addiction in certain classes of drugs. Hence, if introduced in the market it has to be regulated. While being inclusive, it is also necessary to be released with a restriction. There are many orphan diseases that have no available treatment, yet which makes this the need of the hour. Some already approved over-the-counter drugs are not patentable and have the additional benefit of facilitating the drug reuse process from an intellectual property management perspective (Pushpakom et al. 2019; Talevi and Bellera 2020).

Drugs Repositioned During the COVID Era

SAveRUNNER is built on the concept of proximity between targets (drug component) and disease-specific associated genes (disease component) which enables to find alternative uses for already approved drugs unrelated to its initial indication. Network module separation is based on the concept of disease module wherein diseases with similar modules can be treated with similar drugs. For instance, similarity found between COVID-19 and diseases such as Crohn's disease, H1N1 flu, and septic shock leads to the usage of drugs such as corticosteroids, adrenaline, and norepinephrine in the management of COVID-19. The RWR algorithm identifies diseases whose drugs would interfere with the COVID-19 module (Fiscon et al. 2021) (Fig. 2).

Furthermore, COVID-19 is not more common in thyroid cancer patients than in the general population, despite a greater incidence in thyroid cancer patients treated with multikinase inhibitors. Olverembatinib's kinase activity profile revealed that it inhibits 11 of the 13 kinases that are thought to be essential for N-terminus domain-mediated chemokine and cytokine production. Analysis has documented that olverembatinib, a clinical-grade multispecific kinase inhibitor, inhibits the activity of a number of kinases that are associated with cytokine signaling, reducing Omicron N-terminus domain-mediated cytokine release. Therefore, it was also thought that medicines such as ponatinib and olverembatinib, which target numerous kinases involved in SARSCoV-2-mediated cytokine production, might be novel treatment options for COVID-19 patients with moderate to severe disease (Prete et al. 2021; Chan et al. 2022).

Certain medications with proven pharmacotherapeutic benefits and drawbacks, such as Thioridazine, Primaquine, Prochlorperazine, Tamoxifen, and Imatinib, were validated using animal models. The key pathway miner was used to select drugs with the highest efficacy against COVID-19 with the highest peak value on the RHR

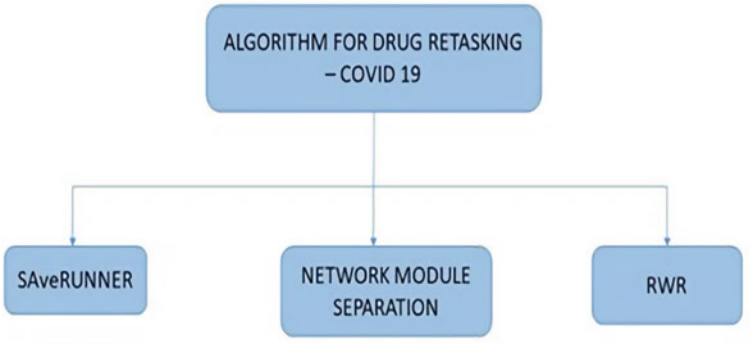

Fig. 2 Schematic representation of how drugs repositioned for COVID-19 were done using algorithms

Table 3 Table demonstrating drugs retasking COVID-19

Drugs	Indication	Repurposed in COVID-19
Heparin	Anticoagulant Antiplatelet Lipemia clearing	To prevent thrombosis
Chondroitin sulfate Pentosan polysulphate	Anticoagulant Antiplatelet Lipemia clearing	Interacts with spike proteins, thereby decreasing virion internalization, preventing inflammation and cytokine storm
Tranexamic acid	Antifibrinolytic	Binds to lysine binding site on plasminogen which reduces circulating plasminogen. Plasminogen is necessary for spike protein cleavage
Diphenylpyraline Chlorpheniramine	Antihistamine	Prevents the early phase of the cytokine storm
Entrectinib Larotrectinib (tyrosine kinase inhibitors)	Anticancer	Prevents cytokine storm
Ofloxacin Ciprofloxacin (fluoroquinolones)	Antibiotic agent	Prevents cytokine storm. Added cover over superadded bacterial infections
Clonidine Prazosin	Antihypertensives	Blocks alpha-adrenergic signaling pathway which helps in early-stage infection
[a]SNRI & [b]SARI	Antidepressants	Prevents the production of TNF-alpha, IFN-gamma prevents inflammation and cytokine storms
Propafenone	Antiarrythmic	Inhibits spike protein cleavage
Obeticholic acid	Primary biliary cholangitis	Prevents ligation of the virion and human ACE2

[a]SNRI – Serotonin and norepinephrine reuptake inhibitors
[b]SARI – Serotonin antagonist and reuptake inhibitors

scale. The repositioned drugs are then thoroughly studied, and the outcomes were meticulously reported (Lucchetta and Pellegrini 2021) (Table 3).

Conclusion

Drug repositioning is a relatively quick and cost-effective method of using available drugs for multiple purposes. The extensive use of animal models has its own set of advantages, but not limited to closely mimicking human tissues. It allows researchers to collect data for repurposing drugs and aids in determining its applicability in human diseases. Animal models have a distinct disadvantage of being a generalized approach and sometimes following genetic transmission in animal models poses a challenge. Despite ethical issues surrounding the use of animal models, it cannot be ignored that it has aided to achieve outstanding results, even in cases of rare or orphan diseases. Therefore, animal models on the whole form a crucial cog in the machinery of drug repurposing.

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Zebrafish as an Animal Model for Cancer Research

9

Stephen Adeniyi Adefegha, Olorunfemi Raphael Molehin, Omowunmi Monisola Adefegha, and Aderonke Elizabeth Fakayode

Contents

Abstract

Zebrafish has emerged as a veritable animal tool used for modeling human cancers. It is currently used to study the development of tumors in several organs in relation to human cancers. This model studies the similarity in the morphology, histology, physiology, and genetic composition of zebrafish cancer model and human malignancies. This model gives room for cancer therapeutic research, drug design, and modification. With the zebrafish as an animal model for cancer, research tools can have relatively short duration and little amount of drug can be

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easily tested, easily manipulated, transparent, and highly predictable. It is of note that the zebrafish cancer model provides suitable underlying and unique insights into mechanisms of cancers. It may provide possible cancer chemopreventive actions based on the underlying mechanisms. This chapter compiles the biology of zebrafish and its suitability as an animal model for cancer.

Keywords

Zebrafish · Cancer · Mechanism · Animal model · Veritable tool

Introduction

The usage of *Danio rerio* (Zebrafish) as an animal model has grown significantly across universities and research institutes throughout the years. The use of this animal model has thrown fresh light on a variety of study disciplines, since it has been discovered to be less complex than mice and to be an excellent experimental model for a variety of biological diseases. George Streisinger of the University of Oregon was the first scientist to use zebrafish to study the nervous system (Gut et al. 2017). The glucose metabolism genes in zebrafish embryos and larvae are comparable to those in humans. Zebrafish larvae in addition have adipocytes, which store lipids, as well as other lipid-related cells (Howarth et al. 2013). The endocrine and exocrine pancreas of zebrafish are comparable to those of mammals. Just like mammals, insulin, somatostatin, and glucagon are released by the endocrine cells, respectively (Teittinen et al. 2012). There are three kinds of cells in the exocrine gland: centroacinar, ductal, and acinar cells.

One of the most well-known species of fish is the Zebrafish. Because of its fully sequenced genome, simplicity of genetic manipulation, high fecundity, small size, external fertilization, and quick maturation, it has become an essential and unique model for cancer model biomedical research, including studies of biological processes and human diseases (Howarth et al. 2013). The zebrafish (Danio rerio) has developed a method for studying the genetic foundations of vertebrate development (Figs. 1 and 2). This model may be used to accomplish a wide range of experiments,

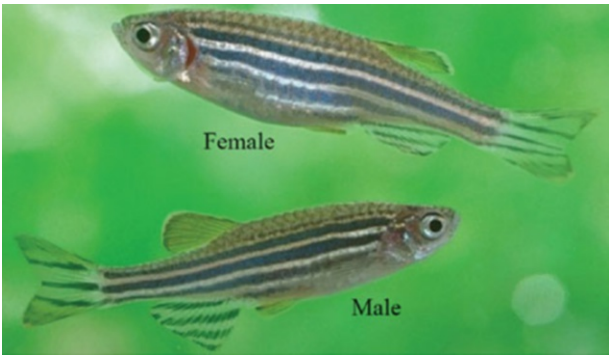

Fig. 1 Zebrafish

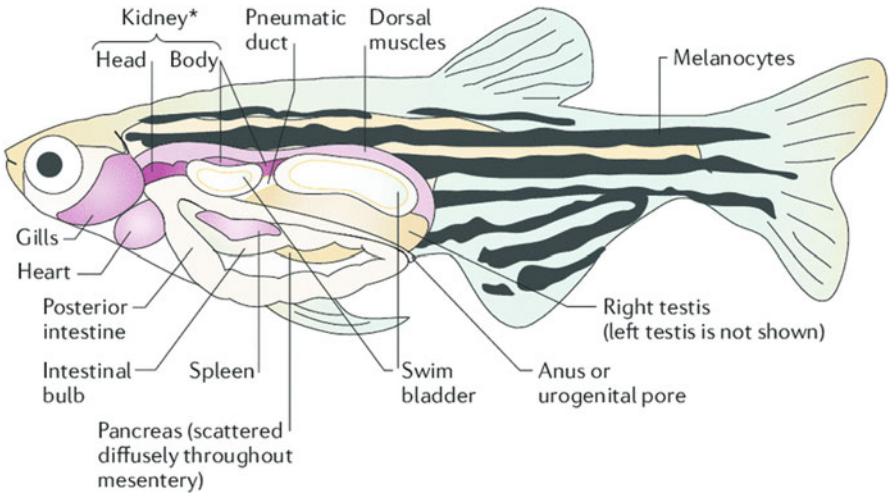

Fig. 2 A well-labeled diagram of a zebrafish

including target identification, target validation, and toxicological investigations, as well as the production of tumors for in vivo effectiveness testing (Ribas and Francesc 2014). The zebrafish model has a number of distinct advantages that make it a versatile research tool, including the ability to produce large numbers of progeny, a high degree of human protein similarity (a higher percentage of human proteins have an ortholog in zebrafish), low maintenance costs, higher absorption of water soluble molecules, allowing for drug administration, and transparent embryos and larvae that allow for easier visualization (Tavares and Santos Lopes 2013). In comparison to other animal models, housing is less of an issue, allowing for cost savings. This tiny organism's fast egg production and early developmental characteristics is the reason why it is one of the most popular animal models for drug testing. Several illnesses have been mimicked thanks to the creation of transgenic and mutant lines in zebrafish. These characteristics make the zebrafish a good model for human disease, and they have increased its use in genetic research and molecular foundation of developmental pathways (Ribas and Francesc 2014). Invertebrate models are typically unable to reproduce the pathophysiology of many human illnesses, which has positioned the zebrafish as a notable embryological model and led to its great therapeutic application potential (Gut et al. 2017).

Using zebrafish, on the other hand, allows for forward genetic methods in a vertebrate that may mimic human disease pathologies, bridging the gap between mammalian and invertebrate model systems. In one night, just a mating process can release up to 300 eggs in each conjugation (Beekhuijzen et al. 2015). After 3 days postfertilization (dpf), the zebrafish embryo reaches the early larval stage and matures into an adult after 3 months. If the eggs are placed in the wells of the plates, they can survive for up to 7 days without any food or water change. These qualities are advantageous because a large number of medicines may be tested in a short amount of time. Because the animals are tiny, the amount of drug/plant extract

examined is reduced, allowing for the testing of a little amount of chemical (s). Zebrafish genes are 70% related to mammalian illness genes and 84% comparable to mammalian disease genes. Zebrafish are used as a model for the study of genetic functions and diseases in mammals, such as metabolic syndromes, because of their genetic similarity.

Zebrafish: A Metabolic Diseases Model

The prevalence of major metabolic disorders such as type 2 diabetes (T2D), nonalcoholic steatohepatitis (NASH), obesity, and atherosclerosis has grown over the last three decades, posing a danger to longevity in our modern era. In order to appropriately balance metabolic rate, consumption, and storage, whole-body energy homeostasis demands dynamic interactions between various organs and endocrine signals. Because in vitro research cannot replicate all of the complexities that occur in vivo, whole-animal methods are more suited to studying metabolism in a multicellular setting. Zebrafish is an excellent model for studying metabolism since they have all of the crucial organs essential for metabolic regulation in humans, from the hypothalamus' hunger circuits through the pancreas and insulin-sensitive tissues.

In the zebrafish pancreas, the endocrine and exocrine segments are the two primary divisions. The exocrine segment is composed of ductal, acinar, and centroacinar cells, the three cell types mentioned above. There are three types of cells in the endocrine system, which are divided into three islets: beta (insulin producing), alpha (glucagon releasing), and delta (Menke et al. 2011). This suggests that humans and zebrafish have similar physical characteristics. Zebrafish blood glucose levels (50–75 mg/dl) and human blood glucose levels (70–120 mg/dl) are both in the normal range.

Similar genes controlled by carbohydrates were found in mammals after PCR study of zebrafish (larval and adult). In glucose homeostasis, the zebrafish pancreas is mostly constituted of exocrine tissue arranged in acini, which is similar in function and structure to the mammalian counterpart (Kimmel and Meyer 2016). They can also transcribe all of the genes involved in gluconeogenesis (glycogen synthase, cytosolic phosphoenol, glycogen phosphorylase, pyruvate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and pyruvate carboxykinase) and lipolysis (fatty acid synthase, acetyl-CoA carboxylase) after 4 (days postfertilization) dpf. Insulin resistance and obesity are closely linked with metabolic syndrome, and zebrafish nonalcoholic fatty liver shares striking similarities with human non-alcoholic fatty liver. Researchers could use fish as models because of their propensity to accumulate enormous amounts of fat in their bodies, which makes zebrafish perfect. Lipids are stored in nonspecialized cells in Drosophila and *Caenorhabditis elegans*, which fulfill a number of functions in addition to lipid storage (Gesta et al. 2007).

The zebrafish gene nostrin has been indirectly related to high blood pressure. Deficiency of this gene can cause glomerular endothelial cells and the glomerular

basement membrane to be damaged, resulting in the disruption of retinal blood vessels, which can lead to blindness. This injury also affects salt absorption in the circulatory system (Lai et al. 2014). To their surprise, they found that in zebrafish, silencing of the WNK1 gene, which is responsible for angiogenesis, had negative effects on the fish's ability to grow new blood vessels. Overexpression of this gene in humans has been associated to an increased risk of cardiovascular disease. The gene linked to pulmonary arterial hypertension (CCDC80) was recently discovered, and it was detected in zebrafish genetic material. Studies on the pancreas have made considerable use of zebrafish. Like mammals, the zebrafish pancreas is divided into exocrine and endocrine compartments, which are linked to the digestive tract by ducts (Wertman et al. 2016). Glucagon-producing cells surround an insulin-producing core in zebrafish pancreatic islets. As early as 24 h after fertilization, the first islet can be seen forming. Five days later (dpf), more are beginning to appear (Hesselson et al. 2009). A large portion of the adult pancreas is composed of the latter. Several genes have been found to influence -cell formation in zebrafish, resulting in a phenotype that in some cases closely mirrors that of established human illnesses. Mice with a Hnf1 mutation show signs of pancreatic underdevelopment and the development of renal cysts, which is similar to human Maturity-onset diabetes of the young type 5 (MODY). Using whole-blood analysis in mature zebrafish, it is possible to quantitatively analyze glucose homeostasis (Moss et al. 2009; Fajans et al. 2001). For high-throughput research, its use is limited due to the tiny size of embryos and juvenile fish.

Small chemicals capable of inducing cell neogenesis were discovered via a chemical screen in which Zebrafish were employed (Rovira et al. 2011). Small compounds that could stimulate the development of secondary pancreatic islets in the pancreas of zebrafish were sought out by Rovira and colleagues in this investigation. Three of the six new hits were found to be associated with the differentiation of -cells for the first time. The zebrafish model has various advantages for diabetes research, including the ability to adjust the number of -cells, see -cell genesis and replication, and investigate -cell activity using the glucose assay. High-throughput screening of small-molecule libraries with the zebrafish could provide a new tool for the discovery of new diabetic therapies. Because of their small size, zebrafish are an ideal model for studying fatty liver disease. The excess of lipids in the liver can lead to inflammation, malignancy, and fibrosis. All of these conditions are collectively referred to as NAFLD disease. Hepatocytes and cholangiocytes differentiate within 48 h of zebrafish fertilization, and the liver primordium has developed (Chu and Sadler 2009). Several liver metabolic pathways and genes, including uncoupling protein 2, pck1, and carnitine palmitoyltransferase 1A, appear to be stimulated between days 4 and 6 when the yolk is depleted and independent feeding begins. Using quantitative reverse transcriptase PCR (qRT-PCR), it is possible to identify these genes (qRT-PCR). These findings show that the zebrafish liver is metabolically mature by day 6, at least in contrast to adult mouse livers (Gut et al. 2013). The Oil Red O whole-mount staining method can be used to identify fatty liver in zebrafish (Sadler et al. 2005). There are a lot of interesting prospects for using zebrafish to mimic human metabolic illness in the future. The zebrafish system's unique tools are

expected to enhance our understanding of metabolic illness genesis and development, as well as discover previously identified targets for disease therapy.

Cancer Model Establishment in Zebrafish

The study of cancer metastasis and brain orthotopic tumor models is vital. This method compares the growth of a zebrafish tumor to that of a human tumor. Patients are most at risk from cancer recurrence and metastasis. By the time the patient detects the clinical signs of these tumors, such as a palpable lump, distant metastases are likely to be in place. In order to assess cancer cell migration and the efficacy of anticancer drugs, metastatic animal models must be developed. Using zebrafish models, researchers can predict the efficacy of tailored cancer treatments and investigate the pathways that underlie tumor growth, metastasis, and treatment response (Wong et al. 2019). Zebrafish have been widely used in numerous tumor investigations as a promising in vivo tool for cancer research. This low-cost and high-throughput cancer research tool may be quickly set up and only requires a small sample size for researchers to use. Clinical trials using the zPDX model have shown promising results for short-term therapy.

Leukemia and lymphoma, as well as other diseases stemming from the lymphatic system, are only some of the examples of cancer cells that have been successfully transplanted into zebrafish (i.e., liver, gastrointestinal tract, colon, pancreas, prostate, lung, ovary, and breast).

Researchers can use the zebrafish model to better study tumor angiogenesis, cell invasiveness, and treatment responses. For individualized cancer treatment, it can be employed in real time in the vivo. According to the zebrafish genome project, 82% of the genes associated with human disease have been shown to be orthologues for the zebrafish genes. As a model for cancer research, it has a number of limitations, including irregularities with the creation of models, a high mortality following injection, variances in procedures among laboratories, and differences in body temperatures between fish and humans. Zebrafish, despite their flaws, continue to be a valuable model for cancer research (Fig. 3).

Mechanisms for Developing Cancer Models in Zebrafish

According to studies, using zebrafish in cancer research has a number of benefits over standard cell culture assays, including the capacity to examine a larger range of phenotypes. The tumor development mechanisms in zebrafish and humans are similar. Furthermore, over 130 genes found in zebrafish liver tumors have been shown to be expressed similarly in the liver of humans with cancer profiles, corresponding with histologic tumor type, grade, and stage. Transgenic zebrafish models have been shown to have molecular markers with human hepatocellular cancer (Zhang et al. 2016).

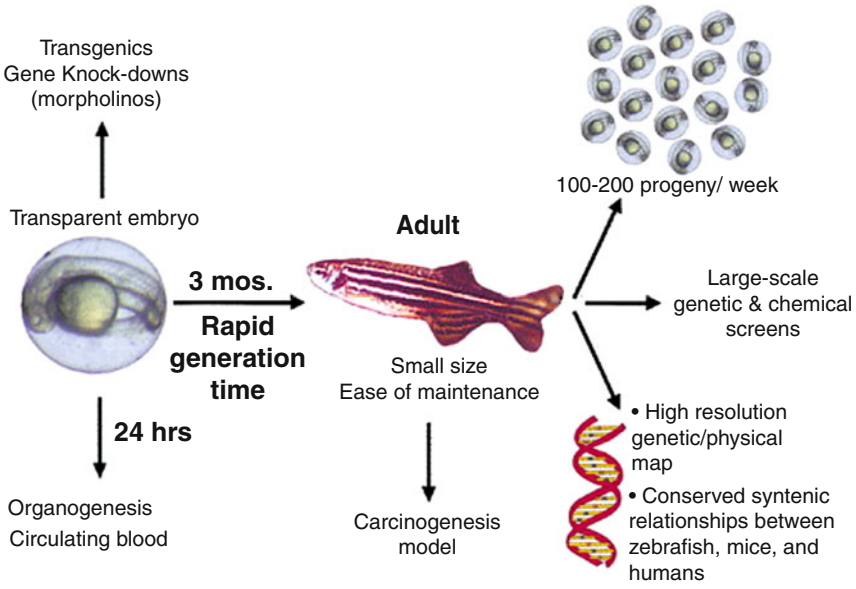

Fig. 3 Establishment of zebrafish as a cancer model

A variety of strategies have been used to generate zebrafish cancer models. The creation of mutant and transgenic lines and the implantation of tumor cells are all methods used to induce human cancer in zebrafish (Fig. 3). The use of any of the zebrafish stages in an experiment is typically determined by the study's goal, as each developmental stage has its own set of advantages. Because the tumor process in zebrafish can be visualized, the embryos of zebrafish may be employed because of their transparency, which allows for microscope observation. Furthermore, cancer grows more quickly in embryos, with tumor development visible 2 days after induction. As a result, they might be used in initiatives that need speed, such as cancer imaging or screening programs. Adults, on the other hand, provide a more realistic in vivo model due to their fast organ and immune system development.

Cancer modeling in zebrafish has grown to include a wide range of mutant and transgenic lines, despite the fact that early research relied on chemical carcinogens. The effectiveness of various chemical carcinogens in fish, simulations of human carcinogen exposure, and assessments of environmental pollutants have all been studied in fish species (Kumar et al. 2016). Among zebrafish, the most common methods of carcinogen exposure are ingestion of aqueous or food carcinogens. Aside from injecting embryos and administering topical medications, fish species also use these methods. A number of carcinogens have a direct effect on the liver in both fish and rodents, despite differences in how these carcinogens are absorbed into the body (Astone et al. 2017).

Cancer modeling in zebrafish has developed from relying on chemical carcinogens to include a wide range of mutant and transgenic lines. Different chemicals

have been tested on fish to see if they can cause cancer and whether they can imitate human carcinogen exposure (Kumar et al. 2016). Zebrafish are most commonly exposed to carcinogens through aqueous or nutritional means. Embryo injection and topical administration are two other methods that are used in fish species to administer medication. Many carcinogens affect the liver in both fish and rodents, despite the fact that the mechanisms of exposure may differ between fish and mammalian species in carcinogenicity studies.

Animals' genes can also be manipulated to produce cancer models. In the absence of commercially available zebrafish embryonic stem cell technology, reverse genetic screenings of mutagenized zebrafish have been used to produce zebrafish lines with mutations in genes of interest. Large populations of zebrafish are commonly generated via chemical or retroviral mutagenesis methods, with each fish possessing a unique collection of randomly dispersed mutations. The offspring of each person who has been modified can be used to create a mutant line by finding mutations in desired genes. Major cancer susceptibility genes, including zebrafish orthologues of human tumor suppressor genes TP53, APC, PTEN, and BRCA2, have been discovered in zebrafish mutants (Huiting et al. 2015). By using zebrafish embryos, which are optically transparent, and a variety of zebrafish lines that express fluorescent proteins in normal tissues, it is possible to conduct in vivo studies on the interactions between cells and the stroma to better understand how tumor cells migrate and invade. Transplanted zebrafish embryos provide a high-throughput platform for testing new pharmacological treatments in the presence of living cancer patients in a variety of experimental settings (Wu et al. 2017).

Transplantation of Tumor Cells in Zebrafish

Cell transplantation is the act of transferring cells from one individual to another of the same species, and it has become an important tool in the study of development, immunology, regeneration, and cancer biology (Lilljebjorn and Fioretos 2017). Allogeneic transplantation has greatly benefited the rapid development of the zebrafish as a cancer-genetic model. After radiation or chemical destruction of the immune system, cells can be transplanted into larval or adult zebrafish that have not yet formed an immune system and can be used to induce engraftment. Clonal syngeneic strains or newly generated immune-compromised zebrafish models with abnormalities in genes necessary for proper immune cell activity can be used to complete the transplantation in adult fishes. Tumor cell malignancy and migratory abilities, as well as the presence of cancer stem cells, can be determined via transplantation. Transplantation has already been used to define zebrafish models of leukemia, rhabdomyosarcoma, and melanoma. A better understanding of tumor cell biology will be gained by the use of zebrafish transplantation of tumor cells. The angiogenesis, tumor cell extravasation, migration, and metastasis processes can all be better understood using this model of cancer formation as a starting point.

Zebrafish have innate immune cells but no adaptive immune system until 30 days after fertilization, when they acquire a working adaptive immune system.

Xenotransplantation of human cancer cells can be performed without the necessity for immunosuppressive medicines or immunocompromised variations because of this favorable feature. The zebrafish model's ability to rapidly produce and survive large numbers of fish is one feature that significantly boosts the transplant experiment's throughput. It is possible to produce a large number of tumor-bearing fish, as well as hundreds of recipient fish. We have already learned a lot about the biology of zebrafish tumor cells through tumor transplantation (Avdesh et al. 2012). Transplant recipient tumor growth in zebrafish has demonstrated tumor aggressiveness in numerous tumor models. Fluorescently tagged transgenics were used to find cancer stem cell groups in rhabdomyosarcoma that were particularly good at regenerating the tumor. Metastasis has been shown to be possible when zebrafish models of melanoma are implanted into transparent fish. Human tumor cells can activate vasculogenesis in the embryo of the zebrafish. The use of zebrafish as a model system allowed these research to be carried out. In fish, transparent adult fish have substantially improved transplanting as well as the ability to keep huge numbers of fish and generate a wide diversity of transgenic lines. Transparent fish. Zebrafish tumor transplanting will become increasingly important to our understanding of tumor biology as this tumor test develops. A commercially accessible cell line cannot supply the tumor's heterogeneity and genetic mutation. Tumor cell microinjection sites also differ based on the zebrafish embryonic stage. Smaller injection sites like the heart or the Cuvier caudal vain are less popular in zebrafish because they do not provide as much surface area for transplanted cells and so are more difficult to perform by hand. It is necessary to apply stains for cell membranes to cells prior before injecting them. Embryos injected with the correct volume of cells are frequently investigated these days because it is difficult to establish a repeatable volume of cell administration (Fig. 4).

Immune rejection of the implanted tumor cells is one of the major disadvantages of transplantation. This is due to the fact that Zebrafish embryos do not fully develop their innate and adaptive immune systems until they are 21 days old. Furthermore, certain chemicals and irradiation have the ability to depress the immune system. Immature T and B cells enter the thymus at this point, completing the immune maturation process. Immunosuppression is unnecessary because there is no immune response until 3–4 days after fertilization (dpf), making an embryo model excellent for transplantation investigations (Fig. 4).

Adults, on the other hand, require immune system ablation to prevent engraftment rejection. The stage at which the tests are performed is an essential consideration in zebrafish cancer models. Immunosuppression in adult zebrafish is achieved using methods similar to those used in mouse models.

Twelve days after radiation, the body's ability to produce red blood cells is restarted. Then, 20 days later, the marrow's ability to regenerate is complete.

The use of clonal or syngeneic fish, which transplant tumor cells from a donor fish to a genetically similar recipient, eliminates the need for immunosuppression. Because the immune system has been completely engaged in this situation, research into the interaction of immune cells with the tumor, as well as long-term engraftment, is possible.

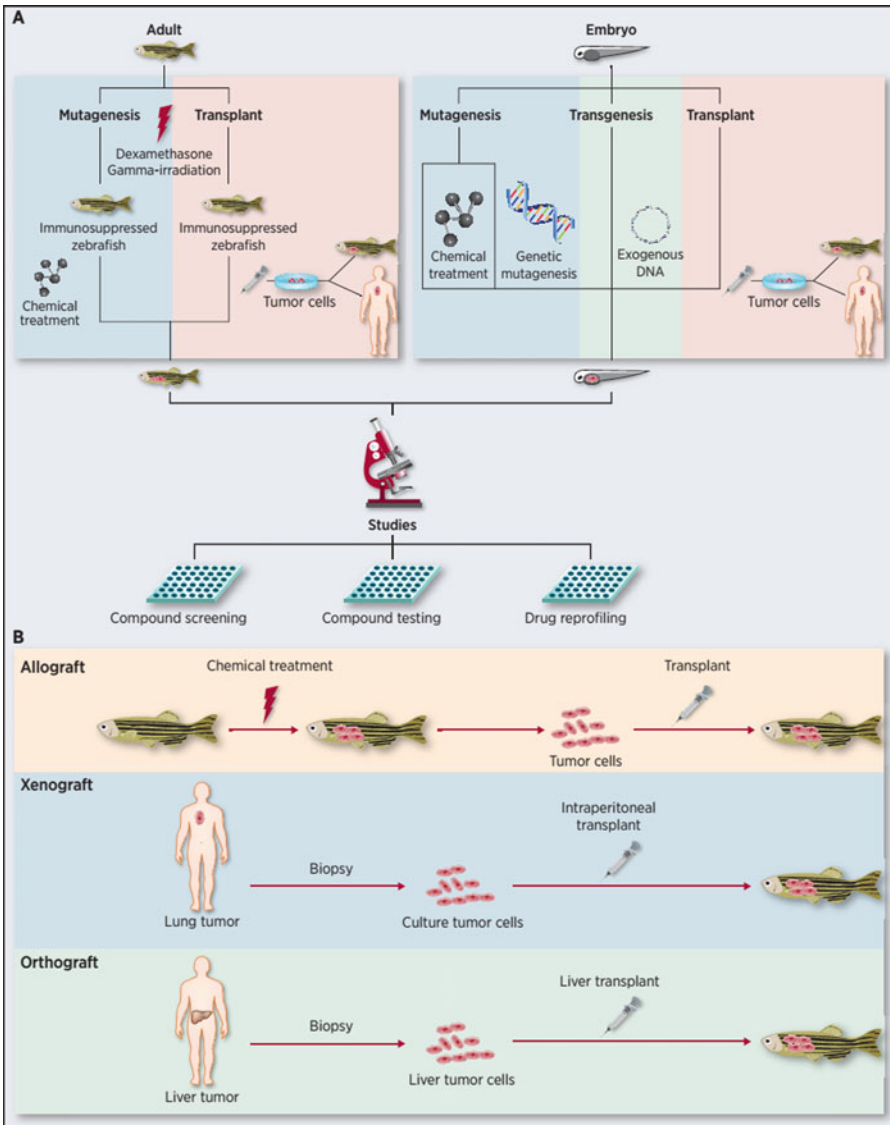

Fig. 4 Transplantation of tumor in zebrafish

Tumor Angiogenesis in Zebrafish

When it comes to researching tumor-induced angiogenesis in the wild, these zebrafish cancer models are ideal. This study shows that zebrafish tumor models can be utilized to examine tumor angiogenesis and even invasion processes in

complete vertebrate animals, as tumor cells can generate neoangiogenesis from existing arteries (Mirbahai et al. 2011).

Angiogenesis is thought to play a role in tumor development and metastasis. Tumor arteries are responsible for delivering oxygen and nutrients to tumor cells, allowing them to proliferate. As a result, the tumor's capacity to produce blood vessels impacts both the cancer's aggressiveness and its treatment effects and prognosis. Angiogenesis inhibitors used in conjunction with chemotherapy have been shown to enhance cancer patient outcomes in both research and clinical studies. Traditional mammalian models, on the other hand, make it difficult to detect the initial vascularization since they only allow for the collection of static pictures, which are likely to reflect the tumor's late stage (Feitsma and Cuppen 2008). In vitro studies of angiogenic processes frequently employ human umbilical vein endothelial cells (HUVEC). As a result, in vitro studies may not be appropriate for simulating tumor angiogenesis. Zebrafish are an excellent in vivo model for studying tumor angiogenesis. Angiogenesis in zebrafish mimics the human disease process because the zebrafish tumor microenvironment is quite similar to that in humans. To better understand angiogenesis and develop new antiangiogenesis drugs, finding additional genes is essential (Huang et al. 2012). In order to evaluate the angiogenesis system, HUVECs' biological responses can be employed for cell proliferation, cell cycle, tubular development, migration, and adherence to matrix proteins (Huang et al. 2012). It is possible to continuously monitor the angiogenic response using additional quantitative angiogenesis assays including matrix implant tests and microcirculation preparations like chicken chorioallantic membrane and corneal micropocket assays (Lee et al. 2005). However, in cancer research, angiogenesis may be in a completely different state. Angiogenesis in the tumor microenvironment differs significantly in morphology and function from that of normal vasculogenesis because it relies on a different signaling pathway.

Tumor Metastasis in Zebrafish

Tumor metastasis is a multistage process in which primary tumor cells diffuse from their origin location, intravasate, and subsequently exit the bloodstream to infect distant organs (Salgado-Pabón et al. 2014). The metastasis process is a dynamic, intricate, and multistep process in which tumor cells enter the circulation, spread to distant tissues, engraft in the parenchyma, and proliferate in the graft site. Understanding how tumors spread can benefit in the development of antitumor drugs and the improvement of therapeutic methods (Rocker et al. 2015). In vitro studies of metastasis ran into serious issues because the complete metastatic process cannot be isolated from its surrounding in vivo environment and circulation system.

Transgenic zebrafish expressing the green fluorescent protein (fli1:EGFP) in their vascular endothelial cells allow researchers to study tumor cell dissemination and modifications in the vascular system throughout the body (Mantilla Galindo et al. 2019). The cancer stem cells in mammalian models are too few to be transplanted, whereas zebrafish are tiny enough for xenografting and can exhibit quick metastases

within 2 days after injection. Metastasis suppressing and enhancement factors can be discovered in Zebrafish, a simple animal model. The zebrafish model was used to examine the metastatic signaling pathway. The use of pharmacological inhibition or short interfering RNA to target proteins in the zebrafish signaling system is not new (MacRae and Peterson 2015). Human breast cancer metastasis in zebrafish can be influenced by TGF-beta signaling. Zebrafish invasion and metastases were dramatically delayed after treatment with a TGF-beta-signaling pathway inhibitor.

Zebrafish Cancer Model in Drug Discovery

In the drug development process, the zebrafish animal model has lately stood out for:

(i) Discovering compounds that particularly alleviate a disease phenotype
(ii) Performing extensive characterization studies on optimized compounds, concentrating not only on efficacy (dose-response), but also on toxicity and/or mechanism of action (Ji et al. 2019)

Oncology drug discovery has benefited greatly from the use of zebrafish in the identification of new compounds or existing medications with antitumor potential, as well as the full therapeutic evaluation of optimized molecules (dose-response and/or toxicity studies). Biochemical and cell-based drug screens have been critical in the discovery of novel active compounds from vast chemical libraries (Olsen et al. 2012). But as an emerging technology, whole organism screens have emerged as an alternative to testing thousands of compounds at a time. An intriguing tool for this reason, the zebrafish provides a reliable, inexpensive, and fast method for screening large libraries and establishing their rapid therapeutic efficacy (Fang and Miller 2012). In zebrafish screening, libraries of any size can be used, ranging from modest collections of well-defined compounds to enormous collections including dozens or even hundreds of different compounds. The screening test's drug library is selected in accordance with the study's objectives. Novel compound libraries are utilized to discover new chemistries and/or mechanisms of action. Repositioning FDA-approved medications may lead to a new drug development effort; on the other hand, initial hits may lead to the creation of new drugs (Clark and Ekker 2015).

Compound Administration and Pharmacokinetics

Dissolving the chemical directly in the fish water is the traditional method of medication delivery. Solubilized chemicals may be absorbed by zebrafish embryos, making administration possible (Brugman 2016). However, this technique has its own set of difficulties to solve, such as molecule solubility fluctuation, potential precipitation, and compound permeability. Because zebrafish can survive in 1% concentrations, dimethyl sulfoxide can be used as a carrier if the drug is not soluble in water. When drugs are delivered directly into the body, long-term testing may be

impossible because of the discomfort of intraperitoneal or retro-orbital injections (Chin et al. 2018; Chen et al. 2018). Researchers have devised a method for cancer preclinical research in adult zebrafish that includes oral gavage and anesthesia. Phospholipid-based artificial oil bodies have also been developed for noninvasive medicine delivery, as have synthetic oil bodies. Adult zebrafish can now be administered medication via an oral route that eliminates the uncertainty that happens when pharmaceuticals are injected directly into the tank water. A micropipette with a tiny tip is inserted into the mouth and throat of adult zebrafish.

Furthermore, zebrafish has lately emerged as an instrument for developing and testing novel medication delivery methods, such as nanoparticles. As a result, predicting how much medication will be absorbed is challenging. Small molecule entry sites differ depending on the developmental stage of the fish, and as a result, the screening findings might vary. Other factors, such as genetic penetrance, in vivo chemical alteration, or pharmacokinetics (a crucial issue, discussed below), might affect the findings in addition to utilizing a complete organism.

Since the zebrafish genome contains many orthologues of human proteins, medications that target these zebrafish orthologues may have varying effects on patients. Phase I and Phase II metabolic activities can be carried out by zebrafish larvae, according to previous studies (Elo et al. 2007). Since zebrafish have the full complement of human cytochrome P45O enzymes necessary for drug metabolism, it is possible to mimic these processes in zebrafish. Scientists have found that the blood–brain barrier (BBB) permeability in both zebrafish and higher vertebrates is comparable. However, there have been just a few recorded cases of this animal model being used to study the pharmacokinetic characteristics of novel drugs.

However, high-throughput screening of anticancer drugs using the zebrafish model has been successful. Simply putting the medicine in the water makes it possible for drug treatments to be carried out. Noninvasive monitoring of antitumor and pharmaceutical toxicity is made possible by the zebrafish's transparent body (Capiotti et al. 2016).

The antitumor effects of chemotherapy medications can also be tested using zebrafish, as they can model most types of cancer. Affective effects include the growth and spread of cancer cells throughout the body. There have been several significant chemical screenings of zebrafish in the last 5 years.

Zebrafish's hematopoietic system is very similar to that of humans, with nearly all adult blood lineages having homologous cell lines in zebrafish (Capiotti et al. 2014). To put it another way, zebrafish hematopoietic treatments may also be effective in humans. An antileukemia chemical known as LDK was found in this pharmacological screen to be effective in treating adult zebrafish with T-cell acute lymphoblastic leukemia for an extended period of time. For whatever reason, it was given the name lenaldekar (LDK) (T-ALL). According to following investigations, LDK's antileukemia impact was not only confined to T-ALL, but also B-ALL and CML (Beekhuijzen et al. 2015). Zebrafish were used to test antiangiogenic medicines in the laboratory. Antiangiogenic effects were found in two kinase inhibitors, and the kinase target was identified as PhKG1 (Beer et al. 2016). A similar pattern of angiogenesis inhibition in humans has been observed in developing zebrafish

embryos when rosuvastatin is administered. Pharmacological compounds that inhibit lymphatic drainage were found in zebrafish. Antilymphoma medicines formerly used in humans have been found in zebrafish (Kucinska et al. 2017). For large-scale screening and efficacy testing of antineoplastic medications, zebrafish may be a suitable platform. Using zebrafish, researchers were able to identify compounds that function in pathways of genetic signaling during carcinogenesis (Cagan et al. 2019).

Conclusion

Overall, this chapter has described the concept of using zebrafish as a veritable tool and animal model for a better understanding of cancer pathogenesis, mechanisms, and treatment. The use of zebrafish in cancer research has added to the pool of knowledge as it provides additional information to the available in vitro cell model and in vivo murine model. Zebrafish model in cancer research has been able to compete favorably with other models in that it is cheap to maintain, encourages feasible research design, provides the opportunity to easily manipulate the genome to rapidly generate transgenic animals, and allows for easily studying tumorigenesis and development of novel therapeutics for cancer.

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Contribution of Zebrafish in Cancer Research: Tiny but Not Trivial

10

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Contents

Abstract

The zebrafish successfully established as a vertebrate model in the study of developmental biology is nowadays adding immense insights in the field of cancer research in spite of the in vitro cell lines and mammalian animal models. Owing to effortlessness cancer generation in zebrafish by means of gene-specific mutagenesis to suppress tumor suppressor genes and generation of specific transgene to overexpress oncogenes makes it an appropriate model in the cancer research field. The ease of transplantation of cancer cells in zebrafish via allograft and xenograft from cancer subjects and their resemblance to that of human cancers like T-cell leukemia, prostate and hepatocellular carcinoma, melanoma, and myelomas confers to its reliability as a model in the field of cancer research. Finally, the zebrafish model is paving its path as a trending cancer research model due to the on-site cancer detection, in vivo imaging of cancer in living fish by

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fluorescence; molecular and genetic screening of markers as well as characterization of changes in the signal transduction cascade associated with cancer offer great advantages in physiological and genetic revealing of tumors and cancer. Ultimately the devastating and extensive use of zebrafish in the development and screening of chemical and therapeutic approaches to eliminate cancer has led to the development of novel anticancer drugs, which adds to its advantages as a model in cancer research.

Keywords

Zebrafish · Cancer model · Mutagenesis · Transgenesis · Allograft · Xenograft · Genetic screening

Introduction

The relevance of zebrafish as a model organism is not a latest trend in the field of biology. But surely it is not the most ventured one till today in this versatile field of cancer research. Almost over a decade ago, zebrafish had been introduced as a cancer research model (Rennekamp and Peterson 2015). Among various emergent vertebrate models, fish models have been upcoming into this mainstream of cancer research. Zebrafish is considered as the most advantageous model over multiple fish families. They share 70% genetic similarity with humans and have some conserved molecular signaling cascades as well (Howe et al. 2013a). Another advantage is the availability and low maintenance cost of zebrafish. The spawning rate of zebrafish is much higher which helps to conduct the study for several generations and gain multiple piece of information to increase the statistical level of significance (Zhao et al. 2015; Chávez et al. 2016). It is easy to expose zebrafish to the target drug just through exposure via its habitat environment (Rennekamp and Peterson 2015). The in vivo studies get further dimensions using zebrafish as a cancer model, and studying embryos in ex utero conditions is a further advantage because of the transparency and ability to clearly observe the developmental stages of the zebrafish embryo. The very short developmental period of zebrafish makes it easier to conduct the study in a short period of time (Teraoka et al. 2003). In the field of cancer research, scientists have to greatly focus on the identification of the detailed molecular interaction of the cancer causative agents in the body of living organisms, and when the model organism employed for studying such molecular interaction is as tiny and diverse as zebrafish, the study of these intense molecular network becomes more straightforward and less troublesome. Studying the genetic basis associated with the cancer, the scientists can rely on zebrafish because of its 80% genetic similarity based on human genetic orthologs (Howe et al. 2013a). Genetic modifications and alterations can also be deciphered elaborately, and its inheritance can be studied more elaborately on the zebrafish model as it has a higher spawning rate and several generations can be investigated. Making transgenic model with zebrafish as well as tissue grafting in the zebrafish model frequently occurs with great success. The zebrafish model is also nowadays

extensively used in the cancer research and drug development laboratory for drug discovery and advancement of new drugs in the treatment of cancer.

In this chapter, the various implications of zebrafish in cancer research and its advancement as a model organism in the field of cancer research and novel drug discovery for the treatment and eradication of cancer will be elaborately discussed. Lastly, like every model organism, several drawbacks and shortcomings of employing zebrafish as model organism in cancer research biology are also discussed.

Screening of Cancer Using Zebrafish Model

Zebrafish had turned out to be one of the most suitable targets and living model for screening of cancers. The interaction between the tumor and the blood vessels should be assessed thoroughly through cancer screening to gather idea regarding the nature of cancer and its progression. The understanding of such interactions is made possible and uncomplicated using the xenograft and transgenic zebrafish models which are nowadays playing a crucial role because of their tiny size, transparency, and tons of other advantages as a model organism. Such models have made it reliable to understand the way a tumor or cancer is developing and how proper prognosis can be done through screening cancer. Here we will discuss some of the most ventured cancer screening processes that have been done using the zebrafish model (Fig. 1).

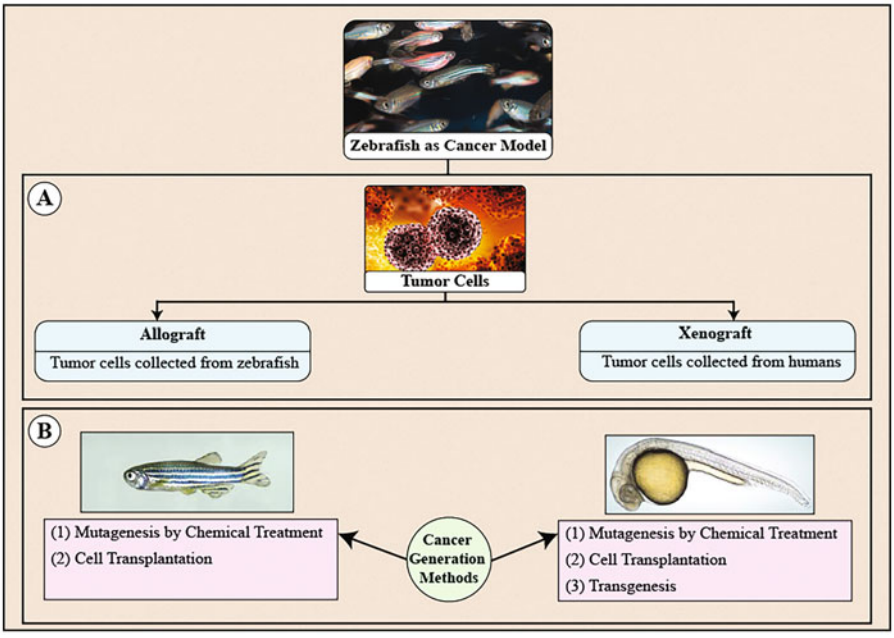

Fig. 1 (**a**) Generation of cancer in zebrafish via allograft and xenograft procedure of in vitro cancer cells. (**b**) Generation of cancer in zebrafish via transgenesis and mutagenesis techniques

Transplantation of Cancer Cell Lines Via Allograft and Xenograft

The zebrafish transgenic model is highly informative in the field of cancer research whether it is an allograft or xenograft. Zebrafish as a cancer xenograft model first was established in the year 2005 where metastatic myeloma cells were successfully injected and established in zebrafish embryos (Lee et al. 2005). Since then, xenograft modeling with zebrafish has attained wider dimensions in the field of cancer research and is still improving. Using embryonic zebrafish as a cancer xenograft model has exceptional advantages (Gutiérrez-Lovera et al. 2017). Larval zebrafish are the best model for xenotransplantation because 2–3 days post-fertilization (dpf) larvae have a developed and complete circulatory system, metabolic organ and organ system, and the yolk system which serves as a 3D medium ideal for drug screening (Wang et al. 2019). Zebrafish larva and the embryonic stages are the most powerful tools employed for noninvasive cancer imaging and drug screening (He et al. 2012; Drabsch et al. 2013). Producing xenograft model of cancer in zebrafish is done aided with the cell lines or patient-derived cancer stem cells transplanted at different point-of-life stages at various sites. It also may be transplanted by injecting directly into the blastomere stage or to the yolk and even directly in circulation in the embryonic stage of zebrafish (Konantz et al. 2012). Currently, a novel transparent mutant zebrafish model named "Casper" has been introduced that is helping in the in vivo imaging of cancer (Stoletov et al. 2007; White et al. 2008).

Angiogenesis and metastasis which play vital role in the progression of cancer can also be examined by producing xenograft and allograft zebrafish models. Tumor angiogenesis can be better studied in zebrafish because it offers striking similarity between these two tumors' microenvironments (Tobia et al. 2011). Apart from this, patient-derived xenograft transplantations are more successful in zebrafish because this procedure requires only a very small amount of cancer stem cells from human and other host to be transplanted and successfully develop and grow into a cancerous tumor in zebrafish. Owing to its tiny size and ease of availability, zebrafish allows a huge number of allograft or xenograft transplantation and studying of cancer in fish models. In addition to this, in vivo screening is the most advantageous component, while using the former as a model in cancer research and anticancer drug development (Hidalgo et al. 2014). Zebrafish have nowadays become one of the potent hosts for cell-derived xenograft models, employed for cancer research and drug development and screening for the former.

Diverse types of cancerous cell lines like pancreatic cancer, ovarian carcinoma, prostate cancer, leukemia cell lines, etc. can be successfully xenotransplanted from human and other mammalian cell lines in zebrafish. Patient-derived xenograft is also happening with great success in zebrafish easily because of the deficiency of adaptive immune system in the embryonic and larval stages of the zebrafish which set aside the demerit of transplanted cancer cells from graft rejection (Pascoal et al. 2020; Jung et al. 2018). The method of suppressing immune response in adult zebrafish is achieved by constant and easy exposure of dexamethasone which in turn suppresses the immune responses to foreign cells and hence lowers the chance

of graft rejection (Smith et al. 2010). This is one of the positive aspects of using zebrafish as successful model organism in cancer research.

It is also observed that different critical as well as essential factors are associated with hepatocellular carcinoma in human beings. So, it is important to identify these target molecules or the genes associated with the former for the successful prognosis (Schlaeger et al. 2008). Zebrafish diversely share a similar range of essential factors as well as the molecular pathways of hepatocellular carcinoma with humans, making it applicable for the screening of liver cancer (Huynh et al. 2006).

Recently, scientists have been able to generate the ache^{sb55} mutant xenograft zebrafish model which explores the interaction between AChE (acetylcholinesterase) and liver cancer in vivo more vividly (Avci et al. 2018). The xenograft model of zebrafish is produced with few tumor-initiating cells from prostate cancer primary cell lines to look into the tumor development, metastasis, and effects in a noninvasive way (Bansal et al. 2014). Taking zebrafish as a vertebrate xenograft model, interaction between the genes of colon cancer, circadian clock, and the cancer phenotype has been assessed with one specific gene NR1D1, which adds a new dimension to the field of cancer research (Basti et al. 2020). Another model has suggested cGAMP to be a potential target for colorectal cancer and liver metastasis (Jiang et al. 2019). BPIQ-based chemotherapeutic mechanisms in the treatment of non-small cell lung cancer have been examined through the zebrafish xenograft model which has become one of the most successful therapeutic approaches in cancer treatment (Chiu et al. 2015). Role of BMP signaling in the early micrometastasis of breast cancer has been examined by performing duct of Cuvier implantation of breast cancer cell line in the zebrafish embryo and through in vivo imaging; it is possible to explore the BMP signal transduction cascade role in the expansion and progression of breast tumor and cancer (de Boeck et al. 2016). Some well-recognized xenograft zebrafish models employed in different aspects of cancer research are discussed (Table 1).

Transgenic and Mutant Zebrafish as Cancer Research Model

Studies reported that it is comparatively easier to generate a transgenic zebrafish model to observe different forms of malignancies. The transgenic fish model proficient in the expression of vascular endothelial cell-specific fluorescent protein helps in the proper understanding of tumor development and vascular angiogenesis (Guerra et al. 2020). Generation of transgenic zebrafish model is efficient because it is reliable to obtain a stable gene expression; either it expresses a protein or a fluorescent marker gene. The first invented transgenic lines of zebrafish for cancer research were developed in the year 2003. A particular oncogene was tagged with a green fluorescent protein and observed for changes in its expression level (Langenau et al. 2003). Generations of tissue-specific cancers in zebrafish model with different promoter oncogene constructs are well developed. Tissue-specific promoters are

Table 1 Zebrafish xenograft models employed in cancer research

Cancer donor cells	Number of cells transplanted	Recipient zebrafish developmental stage	Site of transplantation	Observations	References
Lung adenocarcinoma cell line – PC 9 and H1975	200–300	Wild-type zebrafish embryo at 48 hpf	Yolk sac	Exploring the drug therapy for osimertinib drug-resistant lung cancer	Li et al. 2019
Human pancreatic cancer tissue	20–30	Wild-type zebrafish embryo at 48 hpf	Yolk sac	Studying the cancer cell viability in different cell composition and population and their drug responses	Wang et al. 2019
Nalm-6 cells, CAR T cells	200	Mitfab692/b692;ednrbab140/b140 embryos Nalm-6 – At 48 hpf CAR T – At 50 hpf	Circulation	Investigating CAR T mediated in vivo target cell killing	Pascoal et al. 2020
Hep3B, SKHep1, Huh7-GFP human liver cancer cell line	300	*achesb55* mutant and AB lines (within 5 dpf)	Yolk sac	Discovering new cholinergic drug target in the treatment of liver cancer	Avci et al. 2018
Two colon cancer cell line – HCT116 and SW480 cells	1500	Zebrafish Tg(fli:eGFP) at 2 dpf	Perivitelline space	Presenting the involvement of circadian core-clock genes	Basti et al. 2020
MCF 10A M2 cells of breast cancer cell line	400	Zebrafish Tg(fli:eGFP) at 2 dpf	Duct of Cuvier	Revealing involvement of Smad6 and BMP signaling in breast cancer cell invasion	de Boeck et al. 2016
Human leukemic monocyte lymphoma cell line (U-937),	Embryo: 300–500	Embryo: 48 hpf Adult: Casper strain	Embryo: Yolk sac near duct of Cuvier	Cancer compartmentalization and identification with their	Khan et al. 2019

Human hepatocellular carcinoma cell line (Huh7)	Adult: 1×10^5		Adult: Near dorsum aorta	chemotherapeutic-resistant and relapsing character as well	
MC38 colorectal cancer cells	4×10^6	Wild-type AB strain or STING-deficient zebrafish ($STING^{gt/gt}$)	Intraperitoneal cavity	Discovering the inhibitory role of cGMP on colorectal cancer cell metastasis via STING/STAT3 axis	Jiang et al. 2019
Prostate cancer cell line PC3 – CTR	500	Zebrafish embryo, 48 hpf	Subcutaneous Above the yolk	Tumorigenicity and aggressiveness of the prostate cancer cell line in vivo	Xu et al. 207
MDA-MB-231 – a triple-negative human breast cancer cell Line MCF7 – a hormone receptor-positive breast cancer cell line	50–400	Zebrafish transgenic lines Tg(kdrl: mCherry) and Tg(fli1:GFP) larva at 2 dpf	Duct of Cuvier	Studying the cell behavior during bone metastasis	Mercatali et al. 2016

used for the overexpression of specific oncogenes in the tissue; for example, a myeloid-specific promoter spi1 is capable of inducing acute myeloid lymphoma in zebrafish (Lu et al. 2016). The heat-shock promoter is another inducible promoter of the same use where the oncogenic expression is in tight control with a heat-inducible promoter (Santoriello et al. 2009). The yeast binary system – GAL4/UAS – is another approach for having a controlled expression of desired genes, which is a great advantage in the study of cancer succession. Another way to control the particular transgene expression is through Cre/loxP recombination-mediated system (Raby et al. 2020). All these transgenic zebrafish approaches have diverse implications in the field of cancer research and novel drug screening for cancer treatment. However, these transgenic zebrafish lines face several drawbacks, one of which is that these are more prone to death even before attaining their sexual maturity. Owing to their limited life span, these transgenic zebrafish lines need to be maintained with care through tightly controlled in vitro fertilization (Langenau et al. 2005). Several transgenic zebrafish lines have been designed with the expression of *wnt*, *myc*, and *ras* genes, and many of these oncogenes are fluorescently tagged to study the different molecular pathways associated with hepatocellular carcinoma (Yao et al. 2018).

Transgenic zebrafish systems described till now are capable of overexpressing oncogenes and induce cancer in the zebrafish model. Also, there are few approaches, with the aid of which the expression of distinct tumor suppressor genes in specific tissues can be downregulated or even knocked out. Some chemical mutagens are reported to induce random mutations. For example, N-ethyl-N-nitrosourea which is an alkylating agent and its repeated use can cause point mutation in zebrafish genome (Weyden et al. 2016). Such agents are in great use to develop transgenic zebrafish lines capable of controlled expression of specific oncogenes and tumor suppressor genes for the study of cancer.

Zinc finger nuclease-based methods and transcription effector-like activator nuclease (TALEN) are reported to be another two most efficient methods for targeted gene knockout. These two gene knockout approaches have similar architecture but differences in their DNA binding domain moiety, and both are parallel laborious methods. These approaches are nowadays employed for inducing mutations in particular genes and causing distinct cancers. Studies reported that mutated neurofibromatosis-1 and mutated tumor suppressor retinoblastoma1 have been generated in zebrafish through the gene knockout strategies, zinc finger nuclease-based method, and TALEN, respectively (Kirchberger et al. 2017). One of the latest and most effective methods, CRISPER/CAS9, is a current strategy employed for gene editing and takes an important measure in targeted gene knockout in the zebrafish model, thus adding to their significance in cancer research (Ablain et al. 2018). Another strategy is the electroporation-based approach, TEAZ (transgene electroporation in adult zebrafish) which has been applied for expressing transgene and inducing particular mutations in the somatic cells (Callahan et al. 2018). All these strategies for the development of transgenic and mutant zebrafish as cancer

model have opened up new dimensions in the field of cancer research; few are discussed here (Table 2).

Identification and Screening of Novel Cancer Molecular Markers and Therapeutic Approaches Using Zebrafish Model

Cancer heterogeneity arises from the idea of personalized medicine. Depending on the next-generation sequencing data only, outcomes of the application of particular medicines or therapeutic drugs on cancerous cells cannot be predicted without proper in vivo exposure. Zebrafish serves as a superior model for such in vivo drug screening. Using zebrafish as a model which aids in integrating the genomic data with drug screening helps in the generation and validation of highly effective personalized medicine in cancer treatment (Tsoli et al. 2018). In the year 2005, melanoma development was first established concerning the interaction between BRAF and p53 pathways by generating a transgenic zebrafish model for human $BRAF^{V600E}$ (Patton et al. 2005). Since 2015, zebrafish xenograft models have been widely used to screen and study the drug efficacy for acute T-lymphocyte leukemia and multiple myelomas (Bentley et al. 2015; Lin et al. 2016). By efficient transplantation of different lung cancer cell lines in zebrafish, the dose-dependent therapeutic effects of two drugs – osimertinib and gefitinib – have been screened for their ability to regulate angiogenesis in vivo which can be readily observed and checked with the aid of confocal microscopy. The effects of these chemotherapeutic drugs have been widely established in the epidermal growth factor receptor and tyrosine kinase inhibitor drug-resistant xenograft model (Li et al. 2019).

Screening of zebrafish in vivo model with the aid of cell transplantation strategy has successfully established the clonal variation within T-ALL cells and also revealed their functional roles in leukemia propagation. Studies with this type of zebrafish model have also revealed the role of AKT in activating mTORC1 in increasing the number of T-ALL cells with the clonal variation which is the result of their development of spontaneous resistance against chemotherapies (Blackburn et al. 2014). The NHA9 transgenic zebrafish line has been able to identify the diverse number of genes and molecular pathways that plays an important role in the human acute myeloid lymphoma (AML) and, in turn, also helped in the identification and screening of effective therapeutic drugs and personalized medicine as well (Deveau et al. 2015). A novel CXCR4-dependent metastatic pathway of highly aggressive triple-negative breast carcinoma has come into light with its probable therapeutic blocker (Tulotta et al. 2016). With the ease of oral gavaging and anesthesia methods, zebrafish turns out to be a novel model for effective cancer preclinical studies (Dang et al. 2016). Two multiple tyrosine kinase inhibitors, 419S1 and 420S1, were reported to have better therapeutic effects than sorafenib in the treatment of hepatocellular carcinoma, which is made easier and possible through in vivo screening in the zebrafish cancer model (Lin et al. 2019).

Table 2 Transgenic and mutant zebrafish model employed in cancer research

Cancer type	Transgenic and mutant cancer lines	Recipient zebrafish developmental stage	Observations	References
Malignant melanoma	Casper Triple line (*mitfa*:BRAFV600E; *p53*−/−;*mitfa*−/−)	Adult zebrafish	Studying tumor progression and metastasis in a fully immunocompetent zebrafish	Callahan et al. 2018
Hepatocellular carcinoma	Twist1a- ERT2 transgenic zebrafish line	Zebrafish embryo at 1–2 cell stage	Chemical screening for identification of anti-metastasis drug to target metastatic dissemination of cancer cells	Nakayama et al. 2020
Germline mutation in von Hippel-Lindau (*VHL*) – a tumor suppressor gene	Zebrafish *hif1ab* and *epas1b* mutants	3 dpf	Exploring strategy of improving VHL-related phenotype and treating VHL disease	Metelo et al. 2015
Liver autophagy	Tg(fabp10: EGFP-Lc3) transgenic zebrafish	One cell stage embryo	Investigating the relationship between autophagy and liver cancer	Cui et al. 2012
Hepatocellular carcinoma	Tg(cryB:mCherry; LexA:EGFP-myca) and Tg(cryB:mCherry; LexA:EGFP-mycb)	One–two cell stage	Physiological functions of two myc paralogs are identified. Signaling pathways other than Tp53 involved in tumor regression are studied	Sun et al. 2015
Hepatocellular carcinoma	*krasV12* transgenic zebrafish *Tg(fabp10:rtTA2s-M2; TRE2:EGFP-krasG12V)*	One cell stage embryo	Discovering new avenues of hormone therapy in liver cancers	Li et al. 2017
Alveolar rhabdomyosarcoma	PAX3-FOXO1 transgenic zebrafish	One cell stage embryo	Cancer biomarkers helping in disease targeting and making avenues for treatment	Kendall et al. 2018

Embryonal rhabdomyosarcoma	Complete loss-of-function *tp53* deletion allele in syngeneic CG1-strain zebrafish $tp53^{del/del}$	One cell stage CG1 syngeneic embryo	Discovering novel roles of Tp53 in embryonal rhabdomyosarcoma progression	Ignatius et al. 2018
Mucosal melanoma	Transgenic and mutated zebrafish model for several target gene expression	Embryonic stage	Genomic analysis of human cancers and finding out same major drivers in cancer alteration	Ablain et al. 2018
Myeloid malignancy	*asxl1* mutants of zebrafish $asxl1^{+/+}$ and $asxl1^{-/-}$	Single-cell wild-type embryo	Studying the inflammation and risk of myeloid malignancy progression in mutated model	Fang et al. 2021

Demerits of Zebrafish as a Cancer Research Model

Alongside various mammalian in vitro cell lines and animal models like mice, rats, guineapig, etc., zebrafish are also gaining worldwide significance as a valuable model for studying tumor and cancer biology and molecular as well as therapeutic approaches in cancer treatment. Although a nonmammalian vertebrate model, zebrafish is securing a robust position to be accepted as a cancer research model because of diverse reasons as previously discussed and ease of maintenance as well, though still the zebrafish model is facing few drawbacks and insufficiencies to be established as a model organism in cancer research. Being a fish model, the different organ-associated cancers like breast, prostate, and also lung-like tissue-specific cancers cannot be properly studied and explained. On the other hand, the genetic makeup of zebrafish and its molecular networks and signal transduction cascades are far simpler than human being, which makes it unable to justify and compare the molecular basis of cancer as in humans up to the mark. Zebrafish being a poikilothermic animal cannot tolerate a vast range of temperature variation, and this feature may possess a huge problem at the moment of mimicking the homeostatic feature of human body (Spence et al. 2008). However, studies of molecular genetics associated with cancer can also be hampered in zebrafish because of the occurrence of genome duplication events which lead to the generation of paralogs (Taylor et al. 2003). These drawbacks of zebrafish as a model organism in the field of cancer research cannot be neglected.

Conclusion

The zebrafish is suitably recognized as the "whole-organism test tube" because it has also proven its strength and novelty in the field of cancer research owing to its capacity to generate any type of cancer by simpler mutagenesis and transgenesis techniques. Successful allografting and xenografting of cancer cells in zebrafish and development of cancer with similar phenotype as that of humans have led to the rapid recognition and identification of novel cancer molecular markers, easier assessment of changes in the expression of oncogenes and tumor suppressor genes, and study of associated host responses and have proved its reliability as cancer research model. Zebrafish also provides novel insights in the development of therapeutic approaches and anticancer drugs.

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Bioreactor-Based Tissue Models as an Alternative Approach in Cancer Research

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Atil Bisgin and Cem Mujde

Contents

Abstract

Integration of new technologies in cancer research in medicine provides an opportunity to do more comprehensive studies on more specified areas. One of the major study fields of clinical cancer research is the drug design and targeting studies together with the microenvironment in which most of them are performed on experimental animals. However, there are distinct differences when it comes between animal models and humans: metabolic, immunologic, and genetic. Thus, traditional (2D) cell culture techniques are not insufficient

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enough in cancer research due to the limitations of not showing the characteristic features as in vivo. Therefore, 3D cell culture techniques were developed to overcome these problems. In the comparison of 3D to 2D cell cultures, the most important advantage is that the 3D culture of cells/tissues presents more likely real in vivo patterns such as drug metabolism, proliferation, and responses to the stimuli.

According to the most recent developments in 3D cell culture techniques bioreactor-based 3D cell technologies have been designed. Moreover, these methodologies can also be utilized in combination with biopolymers, microcarriers, spheroids, and organoids. Among these 3D cell culture techniques, the most remarkable one is the rotary cell culture systems (RCCS), because providing microgravity reduces the shear stress caused by the mixing force. In addition to bioreactors, microfluidic chips were also developed as a result of the most innovative approach. Microfluidic chips can also be used in any cancer study depending on the researchers' needs, providing a study-specific modification and 3D cell culturing with accessible manipulations. Hence, improvements in bioreactors lead to the population and acceleration of cancer-related drug response, immunological and genetic studies as an alternative to experimental animal models.

Keywords

Bioreactor · Alternative model · Cancer study · 3D cell culture

Introduction

Cancer is the most common leading cause of death accounting for approximately ten million deaths in 2020 (Pilleron et al. 2021). According to World Health Organization (WHO), cancers impose the largest global burden. In 2018, 18.08 million new cancer cases have been reported. Lung cancer (2.09 million), breast cancer (2.09 million), and prostate cancer (1.28 million) was the three most frequent cancer types, while lung (1.37 million) and prostate (1.28 million) cancers are still in the first and second positions in men followed by stomach cancer (0.68 million) and liver cancer (0.60 million). On the other hand, breast cancer is the most frequent (2.09 million) in women, followed by lung (0.72 million cases), cervix uteri (0.57 million), and colon cancers (0.58 million) (Mattiuzzi and Lippi 2019). A wide variety of drug studies are conducted for each type of these cancers. These drug studies are first performed on cells and then on experimental animals to investigate their suitability for human use. However, results obtained by using experimental animals are often not consistent with the results obtained from human studies. This is due to the differences in metabolism and genetic structure of experimental animals and humans. On the other hand, most recent developments in tissue engineering are promising to prevent these problems.

Main Text

Cell culture studies are considered the basis of all studies in biotechnology, health science, and genetics. The cancer research studies can be performed by using both 2D (Dimension) and 3D cell cultures. 2D cell culture studies are depending on the principle of attachment on plate surface (glass/polystyrene) (Setioko et al. 2007). That culture medium needs to be changed every 2–3 days for cell proliferation. However, in 3D cell culture, the main principle is that the cells create and mimic the extracellular matrix (ECM) where the cells can proliferate like real-world tissue to better show the characteristic features as in in vivo.

The design of the cell culture carries great importance for artificial tissue studies. In this sense, the closer the processes related to the growth and development of cells are to reality, the more successful the study will be. In 2D cultures, only a part of the cell membrane is in contact with the ECM, while the other parts are not in contact with the culture medium. Overtime, conventional, 2D artificial tissue studies have been replaced by 3D cell culture studies. The most important reason for this conversion was the tissues obtained by 3D cell culture showing real-world tissue characteristics. The development of 3D cell culture systems, allows the researchers to overcome the obstacles in 2D cell culture and more successful results can be obtained in the studies. The main advantages of 3D cell culture compared to 2D cell culture are the resemblance of in vivo system and morphology together with being able to track differentiation, drug metabolism, expression patterns (gene, protein), cell activity, proliferation, viability, and responses to the stimuli.

Artificial tissue studies have accelerated with the development of 3D printers and 3D cell culture systems. In this sense, 3D cell culture studies, which have been carried out statically, become available to be performed dynamically by the development of bioreactors. Dynamic culture systems (bioreactors) have some advantages over static culture systems. The most important advantages are:

1. Supporting the 3D environment existence
2. Appropriate mixing in the culture medium to reduce tension
3. Diffusing the nutrients simpler to the cells
4. Accelerating matrix synthesis

The primary bioreactor systems used in clinical cancer research are: spinner flask bioreactor systems, perfusion bioreactor systems, and rotary cell culture systems (RCCS).

Spinner Flask Bioreactor Systems

Spinner flask bioreactor systems are one of the simplest bioreactor models to use. The biopolymer in which the cells are seeded is attached to the hanging sticks from the lid of the bioreactor. Then, the culture medium is added to cover the whole

biopolymer and mixed with a magnetic stirrer (Fig. 1). The culture medium is changed every 2–3 days.

Perfusion Bioreactor Systems

Cells are seeded on biopolymers and placed in the culture chamber of the bioreactor in perfusion systems while the culture medium has been sent to the chamber via a pump (Fig. 2). Depending on the amount of medium, that the pump sends into the chamber, the same amount of medium has also been removed from the chamber to be

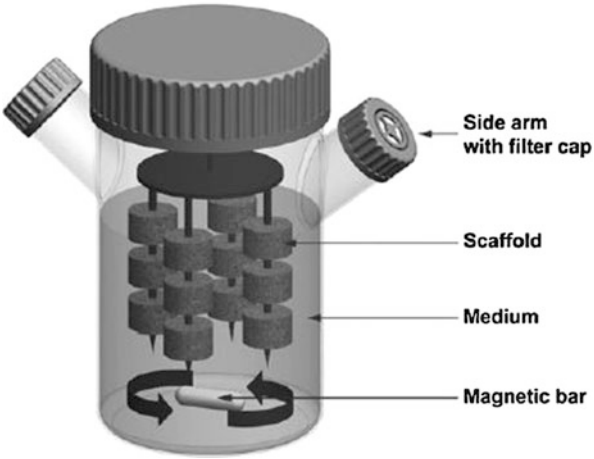

Fig. 1 Spinner flask bioreactor system and components (Rauh et al. 2011)

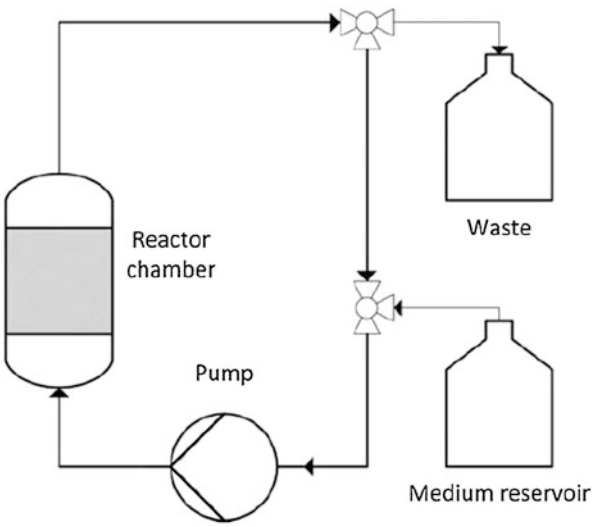

Fig. 2 The schema of perfusion bioreactor system and its components (Rauh et al. 2011)

connected to the waste unit (Rauh et al. 2011). In this way, the adverse effect of mixing speed can also be eliminated. And, the culture medium can be changed at desired times and amounts.

Rotary Cell Culture Systems (RCCS)

Rotary cell culture systems (RCCS) is 3D bioreactors with microcarriers or biopolymers which enable culturing without any mechanical mixing (Rauh et al. 2011). These bioreactors are designed by the National Aeronautics and Space Administration (NASA) to minimize the effect of gravity (Freed and Vunjak-Novakovic 1997). The bioreactor system consists of two concentric cylinders with a membrane in the fixed inner cylinder that will provide gas exchange. The outer cylinder of the bioreactor is not permeable while having a rotatable structure (Fig. 3). Tissue or cell proliferation is performed via the placement of biopolymers or microcarriers in the space between two cylinders (Grande et al. 1997).

The RCCS bioreactor system provides microgravity that positions it in a special place among all bioreactor systems. In this system, the medium mixing occurs by rotation of the structure which helps to reduce the shear stress caused by the mixing force (Unsworth and Lelkes 1998; Weiss et al. 2017). The bioreactor system is designed to have zero force on the structure (biopolymer/microcarrier) letting a gravity-free environment in this way.

Another important issue in artificial tissue studies is the biopolymers. The biopolymers used in the area of artificial tissue can be investigated under four headings: synthetic, natural (non-synthetic), hydrogels, and biomimetic biopolymers. These

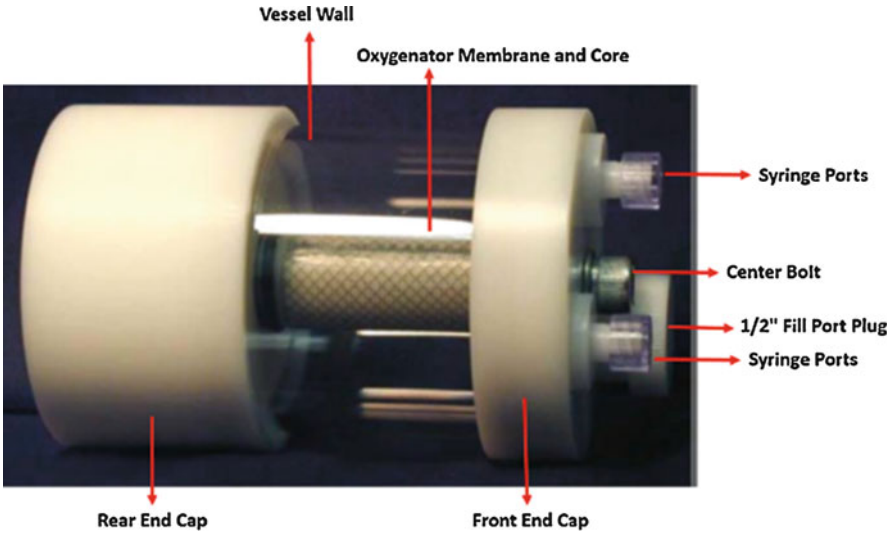

Fig. 3 Rotary cell culture systems (RCCS) to 110 ml

biopolymers can be used alone or in combination with each other. The shaping processes of these biopolymers can be carried out via 3D printers.

1. **Synthetic biopolymers**: After the degradation of the biopolymers, the products can be reabsorbed (Sittinger et al. 1996). Examples of such materials used in health science are polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA), polylactic acid (PLA), Dacron, Vicryl, and some polyurethanes that all can be used as an absorbable suture (Freed et al. 1994; Sithole et al. 2017).
2. **Natural (non-synthetic) biopolymers**: The naturally synthesized polymers in the body of a living organism are consisting of this group of biopolymers. Because of this, they do not cause problems such as rejection, degradation, or toxic effects after transplantation. The most used natural polymers are collagen-gelatin, and collagen-GAG (glycosaminoglycan), and collagen-hyaluronic acid composites (Grande et al. 1997; Gu et al. 2019).
3. **Hydrogels**: Polymers containing a high percentage of water in their structures are called hydrogels, which have high hydrogen and Van der Waals bonds between the main chains of the polymer, giving them to be insoluble (Risbud et al. 2001; Huang et al. 2017).
4. **Biomimetic biopolymers**: Biomimetic polymers are defined as imitation structures of natural tissues. These biopolymers occur as a result of interaction between some signaling proteins and ECM (Furukawa et al. 2003). To attach cells to biopolymers, arginyl-glycyl-aspartic acid (RGD) peptides are used to be recognized as the adhesion motif of various ECM proteins (Tahlawi et al. 2019).

Additional to these, some 3D printers have been used to shape the biopolymers to make them usable in cancer research studies. However, there are certain properties they have to have:

- Biodegradability
- Biocompatibility
- Mechanical strength
- Suitable surface chemistry
- High porosity

Apart from the artificial tissue studies mentioned above, the developments in 3D culturing result in spheroid/organoid terms. Technical advances allow the researchers to mimic the complex cultures for better tumor microenvironment representation. There are various 3D tissue models based on spheroid, organoid, and microfluidic chip technologies.

Spheroids

Spheroids have been considered as the gold standard model for the 3D in vitro models in the past 50 years. These are the cells aggregating and growing in the suspension of with/ without an ECM. The size of the spheroid usually used in cancer

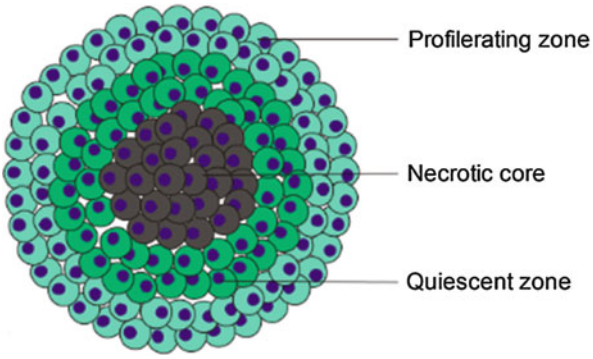

Fig. 4 Spheroid structure (Vadivelu et al. 2017)

research is between 0.5 mm^3 and 1 mm^3. A spheroid (>500um) is divided into three parts (Groebe and Mueller-Klieser 1991). First, there is the necrotic/apoptotic core, then a quiescent cell layer, and finally the proliferative cells are positioned at the periphery (Vadivelu et al. 2017) (Fig. 4). Spheroids are capable of reproducing the structure and metabolism of the original tissues to certain extents. These models are used for both normal tissues as well as tumor tissues. But mostly the spheroid 3D cell cultures are used to examine and better understand the immunological response and drug studies (Boucherit et al. 2020).

Organoids

Organoids are mini-organs that are reconstituted and embedded in an extracellular matrix which is usually procured from primary tissue/tumor, and arise from the stem or differentiated cells. Organoids reproduce the structure with various cellular compartments (Lou and Leung 2018). Most recently, patient-derived organoid studies have started to be in cancer research. This methodology allows the 3D culture of cancer cells isolated from primary cancer tissue, and leads to the loss of stromal and immune compartments. After the growth of organoids and their subculture, immune cells may also be added in the culture to mimic tumor microenvironment. Thus, the organoids become crucial for the cancer studies to carry out drug studies with immunological response analysis.

Microfluidic Chips

Microfluidic chips combine the advantages of 3D culture in two major areas: a dynamic and more controllable environment. These chips make it possible to position 3D models by position in the cell lines/spheroids/organoids in a physiologically dynamic environment. In addition, carcinogenesis, drug screening, and prediction response to therapies can be investigated by these systems (Boucherit et al. 2020). Briefly, the cells/spheroids/organoids formed and placed in a microfluidic

chips are perfused with or without the therapeutics agents via the culture medium (Bhatia and Ingber 2014; Sontheimer-Phelps et al. 2019). For the cancer studies, different models of microchips exist and all can be conditioned to the design of studies. An example of a microfluidic chip is shown in Fig. 5.

To enlighten the basic artificial tissue workflow, it is summarized as:

- Cell isolation and/or cell line preparation
- 2D cell culture
- Biopolymer and/or spheroid
- Bioreactor system (3D cell culture) culture

The most recent bioreactor-based cancer research studies are summarized in the following paragraphs.

Zheng-Yang Chen et al. performed a bioreactor-based cancer study on human HGC-27 gastric cancer cells in the RCCS to better understand the major effect of lipid metabolism on HGC-27 gastric cancer cells (Chen et al. 2020).

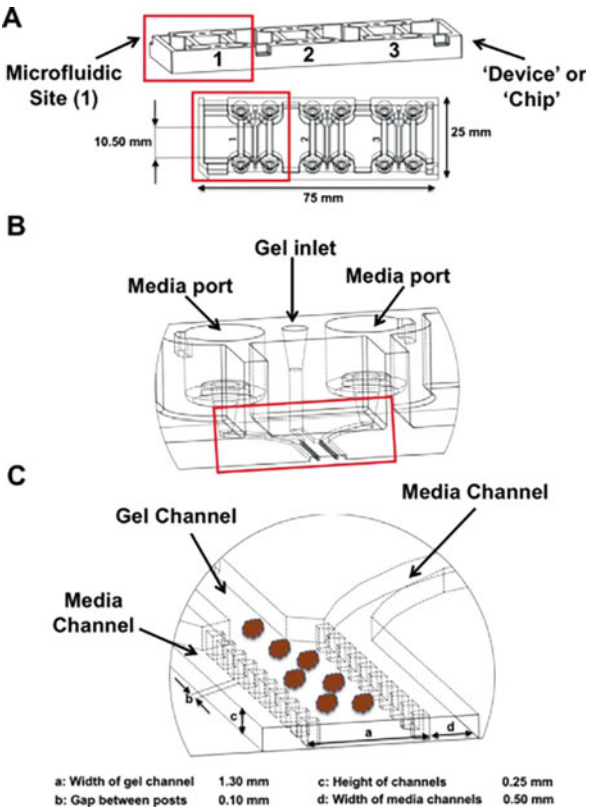

Fig. 5 Microfluidic chip. (**a**) The 3D cell culture chip with three independent microfluidic chambers per chip. (**b** and **c**) The single microfluidic chamber in the 3D cell culture chip (indicated in red rectangle). Each device has a center gel region with posts separating the gel region from the antiparallel side channels. Gel loading port and media ports (**b**); center and side channels (**c**). (Aref et al. 2018)

In another study, E. P. McNeill et al. used the RCCS bioreactor for modeling bone–tumor interactions to provide its advantages over the standard 2D culture including substantially increased cell yields and a closer mimic of bone tissue topology. At the same time, this study stated how a study could be carried out without the need of experimental animals for drug trials with an advantage obtained from 3D culture resembling the tumor tissue structure (McNeill et al. 2018).

Most recently, Nguyen et al. reconstituted a HER2+ breast tumor along with its microenvironment, culturing four different cell lines. They combined breast cancer, endothelial cell, CAF (cancer-associated fibroblast) and fibroblastic cell lines via culturing them on a microfluidic chip. This model was used to investigate the effects of trastuzumab, an anti-HER2 mAb that showed the highly reduced ADCC (antibody-dependent cellular cytotoxicity) effect of trastuzumab in the chamber containing the cancer-associated fibroblasts. Thus, the authors indicated that CAFs were modulating the functions of immune cells by reducing the contact time of tumor cell lines (Nguyen et al. 2018).

Cell Cultures and Genome Editing in Cancer

Integrating genome editing into the 2D and 3D cell culture techniques brought a new era in regenerative medicine and enabled to design of new therapeutic options for cancers. Gene editing fundamentally relies on cell culture. Therefore, genome editing techniques are improving simultaneously with recent developments in cell culture techniques. Especially 3D cultures may help to overcome some of the main issues in genome editing by presenting cell and tissue layers as in vivo experiments.

Since CRISPR/Cas9 technique used in genome editing, development of genome engineering accelerates enormously. Traditionally, genome editing technologies require high cost and highly qualified scientific labor. Moreover, the accuracy and precision of these techniques are relatively low according to CRISPR/Cas9 methodology. Previously, zinc finger and TALENs were the most frequent options for genome editing which are based on protein alteration. However, CRISPR/Cas9, based on protein/RNA combination and target sequence, may simply alternate by designing a RNA strand instead of a protein. Thus, required scientific labor and costs are decreased while the accuracy and specificity of the process are increased.

CRISPR (clustered regularly interspaced palindromic repeats) and Cas (CRISPR-associated) protein complex is originally an adaptive immune response system in bacteria. Bacteria cut a small portion of a pathogen's genetic material via specific cas proteins and place it in its own genome, which is called the CRISPR locus. Then, when the second encounter occurs, bacteria recognize the phage and viral genome is easily degraded by expression of the CRISPR locus to form a complex with nucleases in bacterial cell. Thus, in the following infections, the viral genome is destroyed without harming the host (Fig. 6) (Barrangou and Marraffini 2014).

In 2012, this defense system was modified and integrated into the genome editing research areas and brought the Nobel Prize to Jennifer Dounda and Emmanuelle Charpentier in 2020 (Doudna and Charpentier 2014). Similarly, to the immune

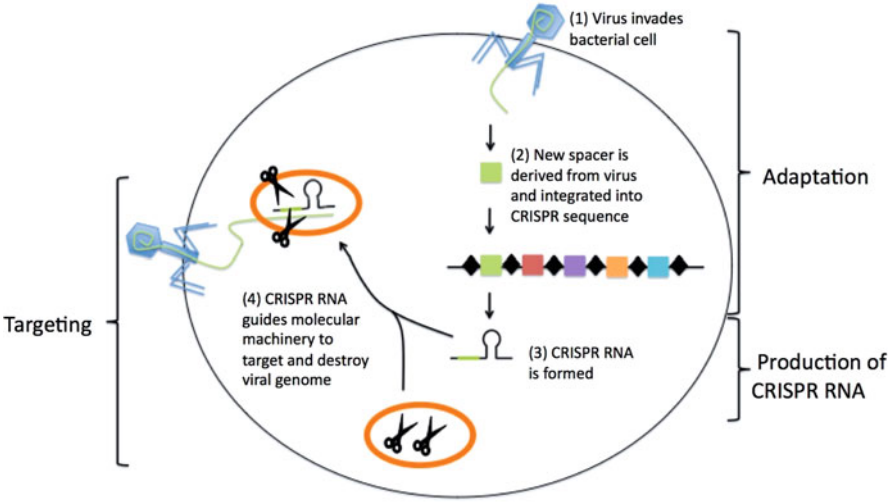

Fig. 6 CRISPR immunity in bacteria. CRISPR loci have clusters of repeats (shown as black diamonds) and spacers (shown as colored boxes adjacent to black diamonds). Production of crRNA: a primary CRISPR transcript is processed by Cas endoribonucleases (Brouns et al. 2008). Targeting: the crRNA spacer and target sequences (complementary protospacer) match and specify the nucleolytic cleavage of the invading nucleic acid (Garneau et al. 2010; Gasiunas et al. 2012; Jinek et al. 2012). (Adapted from Molecular Cell 54, April 24, 2014)

system of a bacteria, RNA molecules that would match a specific DNA sequence on the target genome were designed and synthesized. This "guide RNA" forms a complex with a suitable cas [a ribonucleoprotein (RNP)] which can create double-strand breaks in DNA. After the break is generated, the cell itself initiates either NHEJ (nonhomologous end joining) or HDR (homology-directed repair) mechanism according to the presence of a homolog DNA template. At the end of the NHEJ mechanism, usually, an indel occurs, so the gene will be knocked out or partially disturbed. Controversially, if the HDR mechanism takes place, a specific sequence can be implemented to related region, so a knock in will occur in order to repair the original sequence or insert a desired one.

In recent years, this gene-editing machinery was more improved to cause single-strand breaks or even regulate the gene expression without causing any breaks in the target sequence via synthetically producing different RNP complexes with modified enzymes (Zhan et al. 2019). Types of the applications are shown in Fig. 7.

The main obstacle of using CRISPR/Cas9 systems in genome editing is the in vivo and in vitro delivery of cargo molecules. Most popular delivery methods in use divide into three main categories: viral, nonviral, and physical CRISPR delivery methods. Viral delivery consists of adenoviruses, adeno-associated viral vectors (AAVs), lentiviruses, and retroviruses as CRISPR/Cas9 packaging systems for mostly in vivo experiments, and less frequently in vitro and ex vivo studies (Xu et al. 2019). Secondly, liposomes and lipid nanoparticles, cationic lipids (lipoplexes) or cationic polymers (polyplexes), inorganic nanoparticles (such as

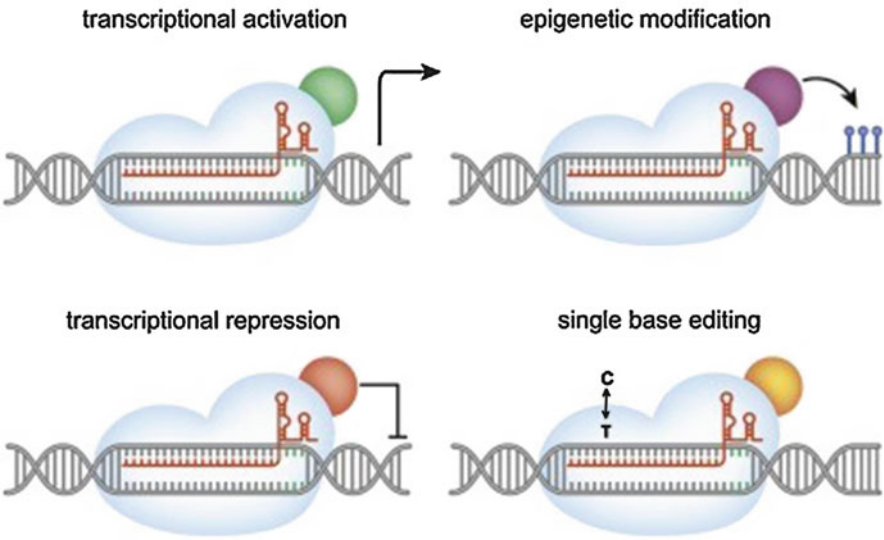

Fig. 7 Different types of CRISPR-based applications. (Adapted from: Zhan et al. 2019)

gold nanoparticles), and cell-penetrating peptides are utilized as nonviral delivery systems which can be considered better choices for in vitro and ex vivo studies (Li et al. 2018). Lastly, physical CRISPR delivery techniques include methods such as electroporation, microinjection, and microfluidics. The advantages and disadvantages of these delivery methods are summed up in Table 1 (Yip 2020).

One of the most important applications of this so-called "gene scissors" technology is to enable making cell cultures and animal models with desired genetic changes for diagnostics, gene therapy, target discovery, and also crop and animal modification studies.

Almost all genome-editing procedures rely on cell culture. However, there are some points to consider due to achieving successful genome editing. To study experimental genome editing, whole workflow must be finely tuned for cell culture and the fundamentals of editing tool. It may require additional evaluation of all modifications and factors to the most efficient study design. It starts with the selection of cell lines. In the genome editing experiments, researchers should compare the final editing products to parental clones. Also, the type of cell line is crucial for the experiment. For instance, a study consisting of stem cell lines may be different from a cancer study plan and this also should be considered while study results are assessed. In addition, it should not be forgotten that ploidy is another important factor that might directly affect study results. So, researchers should pay attention to the genome and its genomic profile (replication period, ploidy, etc.) regarding the cell line that has been preferred (5).

When experiment was designed for genome editing, type and impact of editing (knock out, knock in) must be fore seeded by the investigator. Timing of editing must be decided accordingly considering cell cycle, and changes in gene expression and

Table 1 Summary of advantages and disadvantages of the most frequently used (CRISPR)/Cas9 delivery strategies. (Adapted from Yip 2020)

	Viral delivery				Nonviral delivery				
Strategy	LV	AAV	AV	EV	Microinjection	Electroporation	Cell-penetrating peptide	Lipid-based nanoparticle	Gold nanoparticle
Cas9 delivery	DNA	DNA	DNA	Protein	DNA, mRNA, or protein	DNA, mRNA, or protein	Protein	DNA, mRNA, or protein	Protein
Efficiency	+++	++	++	++	+	+++	+	+	++
Safety concerns	+++	+	++	+	+	+	+	+	+
Cost	+	++	++	+	+++	+++	+	+	++
Technical requirement	+	++	+++	+	+++	+	++	+	++
Advantages	Efficient delivery, large cloning capacity	Non-integrating		Non-integrating, transient exposure, multiplexible, all-in one format	Direct delivery, more controllable dosage	Efficient delivery, easy to operate	No risk of virus	FDA-approved, low stress to the cells	No risk of virus
Limitations	Random integration, insertional mutagenesis	Limited cloning capacity	Immune response	Limited quantification method	Technical challenging, in vivo work not feasible	Cell viability issue, in vivo work difficult	Variable efficiency depends on cell types, requires extensive optimization		
Applications	In vitro and ex vivo	In vivo	In vivo	In vitro, ex vivo, and in vivo	In vitro and ex vivo	In vitro and ex vivo	In vitro and in vivo	In vitro and in vivo	In vitro and in vivo

AV, adenovirus; AAV, adeno-associated virus; EV, extracellular vesicle; LV, lentivirus; + denotes low; ++ denotes medium; +++ denotes high

upregulation/downregulation of specific proteins must be followed-up. Also, the genomic timing of cell lines must be considered while genome editing is occurring. It is advised to create stress-free environment for the control group in order not to induce any mutagenesis mechanism. Finally, an optimal concentration of vector or the editing tool may differentiate between different phases of cell proliferation. So that, both quality and quantity of used must be set up accordingly.

Tracking of the editing in cell culture is possible using different methodologies such as IFS (immunofluorescence staining), flow cytometry (FACS), or NGS (next-generation sequencing). Even though the type of cell culture (2D or 3D) and the purity of the cell lines are substantial for all techniques, it is especially important for sensitive genetic methodologies because the final product may not be affected by genomic background. On the other hand, other factors such as ploidy of cell lines or the type of genomic editing should be considered while choosing techniques such as NGS.

In consideration of all these impressive developments in genome editing, cancer research is one of the most benefitted fields. CRISPR-based techniques enable us to effectively change DNA sequences in vivo and replace the homolog recombination (HR) and random transgene integration, which have been the golden standard for animal modeling in cancer for many years. Recent studies showed the applications of generating models of lung, pancreatic, liver, hematopoietic, and brain tumors on cell lines, tissue, and organoid models (Yang et al. 2013; Chiou et al. 2015; Heckl et al. 2014; Platt et al. 2014; Sanchez-Rivera et al. 2014; Xue et al. 2014; Weber et al. 2015). In addition to cell line/animal modeling in cancer, CRISPR strategies are also utilized in new cancer genes discovery and enlighten the underlying cause of tumorigenesis and cancer evolution (Wang et al. 2013; Li et al. 2016).

One of the great examples of these emerged techniques is the production of chimeric antigen receptor T cell (CAR T-cell). These genetically modified and in vitro produced cells have been used in precise and targeted cancer treatment with very promising results (Watanabe et al. 2021). In the future, CAR T-cells and alternative genetically modified cell therapies will be implemented widely in cancer treatment studies.

Conclusion

Although cancer and drug studies on experimental animals give us a basic idea, they often do not fully reflect the real-world data. The most important reasons for the diversity are the genetic and metabolic differences of the experimental animals used. In order to prevent such situations, transgenic animals such as “Humanized mice” have been produced. However, these animals are transgenic, and their production becomes more expensive than any other experimental animals. Thus, a serious of cell culture and genome editing techniques are needed to be performed to produce these model organisms. Additionally, the “replacement” rule, which is included in the 3R (replacement, reduction, and refinement) rule, is also violated (Schneider and Pincelli 2018). As a solution to all these problems, it is now possible to prevent the

unnecessary use of experimental animals and also reduce the cost of experiments with bioreactor-based 3D culture systems.

To sum up, cell culture and tissue engineering applications gain value along with the recent technology developments in health sciences. Even though cell culture basics have remained constant for decades, advanced techniques have been added to more realistic modeling such as 3D tissue culture. Moreover, the integration of molecular genetic and genome engineering techniques expands the utility of cellular experiments.

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Animal Model of Human Cancer: Malignant Lymphoma/Colon Cancer/Lung Cancer/Liver Cancer/Brain Tumors/Skin Cancer

12

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Contents

Abstract

Considering that the number of people who died from cancer alone in 2020 is more than ten million, the importance of early diagnosis and treatment is once again important. New diagnostic methods and innovative treatments are being developed because the early detection and treatment rates of most types of cancer are insufficient. Selecting a suitable animal model for a given problem is occasionally random and usually a matter of convenience. As in preclinical disease models, selection of the appropriate animal model is very important, since no single animal model can mimic all clinical features in cancer studies. In particular, although mouse models whose genome shares 99% homology with the human genome take the traditional animal model for the molecular biology of cancer, other organisms such as rats, zebrafish, worms, cats, and dogs have been used as research models. Furthermore, although other species (such as dogs and pigs) are more closely related to humans than mice, they are not preferred due to ethical problems and the costs of these species. This chapter is aimed to discuss distinct

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animal models used in cancer studies and specific animal models for particular human cancer types.

Keywords

Cancer · Animal models · Tumour models · Genetically engineered mouse models · Human tumour xenografts · Orthotopic models · Metastatic models

Introduction

Cancer is one of the formidable diseases that all humanity is struggling with, and it is known that there are about ten million deaths from cancer in 2020 (Sung et al. 2021). Since most cancer types' early diagnosis and treatment rates are insufficient, developing new diagnostic methods and innovative treatment options is crucial. However, the 3R rule (Reduction, Refinement, and Replacement) limits the researcher for animal testing, which means *replacing animal experiments whenever possible*, *keeping the number of animal experiments as low as possible*, *and only using the necessary number of animals* (Ghasemi and Dehpour 2009). Therefore, in vitro and in vivo models such as human cancer cell lines and animal models are mainly used to understand cancer progression. However, due to time limitations and ease of handling, in vitro studies are the first step of cancer's cellular pathway, proliferation, and apoptosis studies.

On the other hand, in vitro studies possess the limitation of tissue, immune system, microenvironment, and microfluid interactions. Hence, the obtained results generally do not provide sufficient information. Therefore, the primary step of clarifying the progression of cancers is choosing appropriate animal models that are valuable tools for developing effective therapeutics against cancer. Different animal models are frequently used in cancer studies, including genetically engineered mouse models (GEMMs), chemically induced models, and xenograft models (Sobczuk et al. 2020). Furthermore, while several animals respond similarly to humans from physiological, pathological, and therapeutic perspectives, notable species-species differences exist. Hence, a well-designed animal model demands a thorough understanding of similarities and differences in the responses between humans and animals and includes that information into the study's goals. In addition, knowing the anatomical and physiological characters of the species to be used will be very advantageous. Furthermore, the animal to be selected should be easily cultured, have a high homology with the human genome, be suitable for genetic analysis, be ethically unproblematic, and be economical and readily available (Table 1).

In particular, although mouse models whose genome shares 99% homology with the human genome take the traditional animal model for the molecular biology of cancer, other organisms such as rats, zebrafish, worms, cats, and dogs have been used as research models. Furthermore, although other species (such as dogs and pigs) are more closely related to humans than mice, they are not preferred due to ethical problems and the costs of these species. This chapter is aimed to discuss

Table 1 Comparison of the types of animal models and the advantages/disadvantages

Model	Advantages	Disadvantages
Subcutaneous (heterotopic implantation)	Effective and low cost, short time tumor tissue generation, good external monitoring	The tumor microenvironment bears little resemblance to humans, with limited metastasis to distant tissues in case of metastasis
Orthotopic implantation (xenograft and syngeneic)	It provides good reproducibility for parameters such as target validation and candidate selection. Therefore, syngeneic models mimic the clinical tumor microenvironment	The predictive relationship to clinical development is weak
Patient-derived xenograft (Hidalgo et al. 2014)	This model gives much more effective results. It can be used as a clinical trial model in preclinical studies (the data obtained creates a heterogeneous distribution in the population)	In vitro cell migration causes unidentified tumor structure and heterogeneity
Organoid xenograft	Allows for ex vivo tumor growth. It is suitable for high drug yield screening	In organoid models, immunosuppressed mice should be preferred
Genetically engineered mouse models (GEMMs)	Some disease states can be summarized over a more extended period, including cell types and development in the organ of origin	Tumor tissue formation time is 2–10 months. Therefore, colony improvement needs to be studied with different methods
Somatic tumor models	Allows monitoring of implant cells by simultaneous transduction	It cannot fully model the early stages of the disease due to potent cancer agents

distinct animal models used in cancer studies and specific animal models for particular human cancer types.

Fundamental Animal Models in Cancer Research

1. **Mouse Model**

Mice are one of the most preferred animals in the studies. Their size, short tumor formation time, genetic modification, and ease of handling make them favorable during cancer research. Besides therapeutic agent studies, mouse models are valuable for genetic studies. Animal models used in cancer studies are chemically induced model, cell line-derived xenograft model (CDX), patient-derived xenograft model (Hidalgo et al. 2014), and genetically engineered mouse model (GEMM).

The chemical induction model is used to explain the molecular mechanism in the initiation and progression of experimental tumors induced by chemical carcinogens. This model possesses advantages in modeling various cancer types, straightforward procedures, and efficient tumor generation. However, in addition to the time-consuming process, the difficulty of mutation screening is the disadvantage of the

method (De Minicis et al. 2013). The CDX model is the subcutaneous or orthotopic transplantation of cells grown in vitro conditions (Bibby 2004). Although it is easy to create this mouse model, the behavior of cells in vitro may differ from the original tumor tissue. Furthermore, since immunodeficient mice must be used, the metastasis rate is low (Richmond and Su 2008). The PDX model consists of implanting cells obtained directly from patients or by mechanical or chemical disruption into mice. Xenograft samples are similar to the original tumor in chromosomal, histological, and gene expression profiles. The GEM model often uses tissue-specific promoters to direct oncogene expression or tissue-specific expression of recombinase enzymes to deletion tumor suppressors (Olson et al. 2018).

2. **Rat Models**

Rats are another rodent used in drug development and preclinical cancer research. Rats are larger than mice, and this feature gives them an advantage in surgical and radiological imaging (Johnson and Fleet 2013). Rat models used in human cancer research are generated spontaneously by mutagenesis of tumor suppressor genes or transgenesis of activated oncogenes. Rats are used for modeling exposure to chemical carcinogens, particularly colon and bone cancers. In addition, rat models, unlike mouse models, are produced by percutaneous injection of cancers into the bone. However, rat models are primarily limited in elucidating the pathophysiology of human cancer.

3. **Zebrafish Models**

Zebrafish is one of the promising models used for the treatment of various diseases, including cancer. The zebrafish model is used for multiple human cancer research due to their genomes' anatomical and physiological homology to humans (Teittinen et al. 2012). The zebrafish model has advantages over other in vivo models: ethical and economic benefits, rapid reproduction, small size, and transparency. In particular, the transparency of the models provides the opportunity to observe the proliferation and metastasis of cancer cells in real time. In addition, the effects of therapeutic agents on the organism occur in a shorter time than other animals used in in vivo experiments.

4. **Other Animal Models**

Small animal models are preferred for their ethical and financial advantages, easy maintenance, and strong reproductive capacities. However, surgical and radiographic interventions are difficult in such models due to their small size. Therefore, models in which large animals such as pigs, monkeys, dogs, and rabbits are frequently used can become advantageous (Li et al. 2021). Besides, large animal models are advantageous in size and are used in preclinical studies because of their longevity and genetic heterogeneity similar to humans. On the other hand, these models have some problems, such as housing and reproduction. In addition to cancer

studies, sheep are often used in gene transfer techniques, and pigs and horses are used in cardiovascular disease research.

Malignant Lymphoma

Cancers that start in the bone marrow, lymph nodes, spleen, intestines, and other areas of the lymphatic system and have the ability to spread are called malignant lymphomas (Matasar and Zelenetz 2008). The morbidity rate (3.37%) of malignant lymphoma is very high in North America, Austria/New Zealand, and Europe, while it is low in Asia and Africa (Huh 2012). Heterogeneous hematologic malignancy of lymphomas is the reason for its widespread effects worldwide. Since malignant lymphomas arise from hematopoietic tissues, the similarity and homogeneity of hematopoietic cells are the critical factors for choosing animal models. In humans, neutrophils are the predominant circulating white blood cell, while lymphocytes are in mice. Besides, in the adult mouse, the red pulp of the spleen's extramedullary hematopoiesis is physiologic and should not be defined as neoplasia. Furthermore, abundant hematopoietic ingredients are preserved in the bone marrow throughout the life of mice, whereas it is decreased in the paucicellular marrow of adult humans. Moreover, ectopic thymic tissue in the mice can be found in several cervical region locations and should not be mixed with infiltrative neoplasia.

The progress in genomic techniques has allowed the development of highly translational mouse models of malignant lymphoma. Spontaneous, xenograft and genetically engineered models are the main class of methods that used the formation of mouse models of human malignant lymphoma (Table 2) (Richmond and Su 2008). Therefore, it is much more possible to achieve a high incidence of malignant lymphoma in immunocompromised mice.

One of the most common hematologic malignancies in humans is β-cell lymphomas, classified as large β-cell lymphoma follicular lymphomas, marginal zone lymphomas, and Burkitt's lymphoma (Donnou et al. 2012). The myc,

Table 2 Animal model of malignant lymphoma

Disease	Model	Therapeutic use
Acute lymphocytic leukemia	Transgenic	They are used for differences between MYC-related pre-B and pre-B acute lymphocytic leukemia treatment
Acute lymphocytic leukemia	Xenograft model	In AML, bone marrow acts as a SOX4 marker
B cell lymphoma	Transgenic model	CD20 immunotherapy and SYK inhibitors
Peripheral T cell lymphoma	Transgenic model	SYK inhibitors
Cutaneous T cell lymphoma	Transgenic model	HDAC inhibitors

myc + ras, and spleen tyrosine kinase (SYK) gene translocations to the β-cell-specific enhancer or promoter region caused the formation of β-cell lymphoma. Besides, myc gene insertion into the IgH locus significantly increased (up to 100%) the incidence of β-cell lymphoma (Park et al. 2005). Chakraborty et al. accomplished to generate acute lymphomagenesis using Moloney murine leukemia virus temperature-sensitive mutant-1 (MoMuLV-ts-1) in BALB/c mice, and the success rate of this model is found as 50% (Chakraborty et al. 2011). This model may clarify the chromosomal properties and cellular abnormalities of human malignant lymphoma. Using the Vav gene regulatory sequences down-regulates BcL-2, an oncogenic protein, and is one way to create follicular lymphoma [FL] in mice to examine the prolonged germinal center reactivity V-gene hypermutation (Egle et al. 2004). Epstein-Barr virus (EBV)-transfected humanized mice have also been used for the development of β-cell lymphoma (Shannon-Lowe et al. 2017). The mice model possesses EBV transfection following the alteration of the genes and is also used to clarify these genes in the progression of malignant lymphoma. Another aggressive form of non-Hodgkin lymphoma is peripheral T cell lymphoma (PTCL), formed with the fusion of ITK and SYK, which *has been studied after developing* a *mice model*-inducible fusion protein crossed with CD4-Cre. In that transgenic mouse model, T cells express the kinase fusion protein, and malignant lymphoma is developed. This lymphoma mouse model is used for the investigation of SYK inhibitors (Young et al. 2009). Besides, intrahepatic injection of cutaneous T cell lymphoma (CTCL)-derived cell lines into NOD/SCID/IL2rγ mice successfully developed CTCL and a good model for investigating CTCL inhibitors (Wu and Hwang 2018). Another transgenic mouse model possesses nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) fusion protein, a constitutively active tyrosine kinase (Gu et al. 2004). Upregulation of ALK transgenic cassettes under hematopoietic promoters can force β- or T cell transformation (Turner and Alexander 2005). NPM-ALK fusion protein plays a significant role in tumor pathogenesis and regulates the expression of FOXO3a, a barrier to hematopoietic transformation. The c-MYc animal is known to increase in the immunoglobulin enhancer/c-myc fusion animal model. Having this property, c-myc preferential mRNA has methylated anti-sparking DNA. It can be considered in c-myc protein in peripheral lymphocytes after treatment (Wickstrom et al. 1992). Non-Hodgkin lymphoma model was created using B cell receptor and myc. It is known that there is a slowdown in tumor development due to spleen tyrosine kinase (SYK) deficiency in vivo. In line with this information, B cells transformed into mice were used. When the in vivo results were evaluated, the transformed B cells were tumor-regulated. It has also been found to inhibit Syk activity (Young et al. 2009). Vargas-Castro et al. used stabilized-gold nanoparticles with L/D-methionine for tumor treatment in BALB/c mice. The study stated that gold nanoparticles stabilized with zwitterions suppress tumor growth and would be a good candidate as an antitumoral Malignant lymphomas agent.

Colon Cancer

Colorectal cancer (CRC) is the third most frequent malignancy among the world's population, accounting for 935,000 of the 1.9 million cancer deaths in 2020 (Sung et al. 2021). In vitro studies developed to investigate CRC have critical limitations to understand the nature of CRC cancer. Cell lines possess complex mutations, which limits them to investigate individual mutations. Besides, lacking microenvironment, e.g., stromal cell signals and matrix interactions, and time limitations are also significant obstacles for the studies. Therefore, developed animal models for improving and testing hypotheses concerning colorectal cancer are critical tools. Herein, in vivo CRC models have been observed as crucial tools needed to investigate the molecular mechanisms of colorectal carcinogenesis, test possible preventative and therapeutic approaches, and translate hypotheses originated from cell models into the complex physiology of the colon (Johnson and Fleet 2013). Although rodents such as mice and rats are the primary animals for the model studies, non-rodent species are also used in colorectal cancer studies due to the similarities between canines and humans. Colon cancer mainly occurs in the large intestine in canines, and pedunculated adenomas are also common in the distal colon/rectum as in humans. The dog's main triggering factor of colorectal carcinogenesis is the WNT signaling pathway, and β-catenin has accumulated in the cytoplasm and nucleus in canine colorectal adenomas that progress to malignancy (Restucci et al. 2009). The other non-rodent species is sheep that displayed a significant incidence (1.6%) of intestinal cancer. Besides, histological similarities and metastatic behaviors make sheep an attractive model for CRC studies. On the other hand, intestinal adenocarcinomas occur in the small intestine. Although diet-induced, chemical-induced, and mutagen-induced models are among the most commonly used animal models in in vivo studies, these models have advantages and disadvantages when compared to each other (Table 3).

Rodents can easily be manipulated via the administration of endogenous compounds (foods or chemicals) to form colorectal carcinogenesis. There is a strong correlation between diet and colorectal carcinoma. Especially, western-type diets, high protein and fat and low carbohydrate, promote the formation of colon adenocarcinoma. Rodents fed with high concentrations of fat (20%) and decreased concentrations of calcium (0.05%) and vitamin D (100 IU/kg) tend to develop CRC in 12 weeks (Newmark et al. 1990). In addition, western-type diets modulate the expression of several genes in signaling pathways. For example, a western-type diet causes loss of the Apc allele; therefore, it inhibits the expression of the APC gene, whose frequency seen in all sporadic colon cancers is >70%, and upregulates the transcripts in WNT signaling in the colon and small intestine (Wang et al. 2011). On the other hand, since the western diet significantly affects the bone and calcium (1/10) metabolism in animals, these physiological alterations do not reflect the etiology of human CRC.

Another method of developing CRC in rat and mice models is with chemical agents. For example, 1,2-dimethylhydrazine (DMH), azoxymethane, 2-amino-1-methyl-6-

Table 3 Advantages and disadvantages of colorectal cancer animal model

Animal model	CRC type	Advantages	Disadvantages
Western diet	Sporadic	Initiation of carcinogenesis in the small intestine, cecum, and proximal colon	Nutritional mutations have not yet been identified. However, a few animal species develop neoplastic lesions, and the time to formation is quite long
1,2-Dimethylhydrazine (DMH)	Sporadic	Potential to cause metastasis Onset of adenomas and adenocarcinomas It is a very special model for the intestine	Indirect inducer required Causes liver toxicity
Azoxymethane (Tago et al. 2013)	Sporadic	Direct inducer It belongs to a fairly high specificity for the intestine	Quite an expensive method from DMH Causes liver toxicity
APC	Sporadic and hereditary	It is a good model for hereditary colorectal cancer studies	In this model, animals die within 4 months
P53	Sporadic	It strengthens the effect of other genes	Inhibition of gene expression cannot induce carcinogenesis
MMR	Hereditary	Rather a good model for hereditary CRC study	Most tumors are in the small intestine

phenylimidazo[4,5-b]pyridine (PhIP), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), and N-methyl-N-nitrosourea (MNU) are frequently used as CRC-forming chemical agents. The strain and dose of the chemicals are the critical factors that determine the colon tumor incidence and latency period. For example, after subcutaneous injection of 20 mg/kg/weekly DMH into Sprague-Dawley and BD rats, the success rate was 100% in both, whereas the colon formation time was determined as 28 weeks and 33 weeks, respectively (Druckrey et al. 1967). Colon tumor incidence rate significantly depends on the injection period of the chemicals. The success rate is almost 0 with one-time SC injection of 21 mg/kg of DMH, while this rate increased to 55.7%, 65%, and 92.6% with 12 weekly, 24 weekly, and 27 weekly, respectively (Kobæk-Larsen et al. 2000). Intraperitoneal or subcutaneous injection of the alkylating agents, DMH and AOM, promotes a colorectal tumor that possesses β-catenin gene mutations that increase WNT signaling to drive tumorigenesis in the distal colon (Yamada et al. 2000). DMH seems much more efficient than the AOM since weekly administration of AOM caused metastasis to other tissues and organs such as 58% ear canal, 52% small bowel, and 14% liver metastases (Nordlinger et al. 1991). Furthermore, the combination of alkylating agent injection with the western diet increases tumor incidence and multiplicity, so this animal model is a valuable tool for the gene-gene and gene-environment studies in colorectal carcinoma.

PhIPs, heterocyclic amines produced during the cooking of meat and fish, solely rarely cause tumorigenesis, but with DSS treatment in rats or treating the Apc^{Min}

mice can enhance the tumor formation (Andreassen et al. 2006). In PhIP-induced tumors, Kras and Tp53 mutations were slightly detected, while Cnntb1and Apc mutations were found in a significant amount. Besides, the metastasis and invasion capacity of the tumor are found low. It was demonstrated that the gender of the rats is also another crucial factor for the formation of colon tumorigenesis. Administration of 400 ppm PhIP to the diet of male rats (F344) causes colon tumor formation in 52 weeks with a 55% success rate, while it was only 7% in female rats (F344) in the same period (Ito et al. 1997).

MNNG and MNU, active carcinogens, cause tumorigenesis in mice and rats. Injection of 2 mg MNNG/rat IR twice a week for two weeks to male Sprague-Dawley rats causes colon tumors in 52 weeks with a 67% success rate. Besides, 16 doses of 1.5 mg/mouse biweekly administration to shrew mice develop colon tumors 31 weeks after the first injection with 100% success (Yang et al. 1996). On the other hand, this animal model has a significant metastasis rate to the other tissues such as prostate or breast. Apart from PhIP-induced tumors, MNNG- or MNU-induced tumors possess Kras (5–30%) and Apc (6%) mutations.

Mutagen-induced models are perhaps the most complex and influential of all cancer animal models. The development and progression of colorectal carcinoma are controlled by several genes, including APC, DCC, TP53, MCC, K-ras, SRC, c-myc, hMSH2, hMSH6, hMLH1, CD44, and COX-2 (ASC and Costa-Casagrande 2018). The most common sporadic CRC in humans are APC $>$ 70%, p53 $>$ 60%, and K-ras$>$40%, respectively. The oldest of the mutagen-induced mouse models is the $Apc^{Min(Multiple\ intestinal\ neoplasms)}$ mouse model, which was first described in C57B1/j mice in the 1990s. It is due to the splitting of the Apc protein at amino acid 850 because of the transversion of thymine to adenine at 2549 nucleotides of the mouse Apc gene, which is an autosomal dominant mutation. Apc^{Min} mice display significant molecular and pathologic similarities with humans; herein, it is preferred for colorectal cancers studies. Apart from human colorectal carcinoma, the colon tumors in mice developed in the small intestine rather than the large intestine, and mutant mice showed severe phenotypic damage and died at 120 days of age (Wang et al. 2021). Despite mice, Pirc (polyposis in the rat colon) rat (male) models in which adenomas are developed in the small and large intestine at a ratio approaching 1:1 (Amos-Landgraf et al. 2007). *Pirc* rat model also reflects the human adenoma's progression to invasive characteristic properties. On the other hand, Tp53 knockout rarely promotes the formation of colorectal tumors, so the association of Apc^{Min} or AOM and p53 knockout mutations increases in aberrant crypt foci number.

Western diet causes sporadic colon cancer. In the study of Harris et al., they fed rats with western diet and standard diet after tumor formation with benzo(a)pyrene. Tumor sizes in the colons of the rats after scarification were examined, and in accordance with the data obtained, it was determined that the tumor size was larger in rats fed with the western diet compared to the control group and standard diet (Harris et al. 2016). In the study of El-Khadragy et al. (El-Khadragy et al. 2018), 1,2-dimethylenehydrazine (DMH)-induced colon cancer model was created in rats. Colon cancer tumors were treated with bone marrow cells. At the end of the histopathological studies performed on the tumors, recovery was observed in the

tumors. In addition, it was determined that the expression of surviving, β-catenin, MDR-1, and cytokeratin 20 genes was decreased in tumors treated with bone marrow cells. This was explained by the reduction of lipid peroxyoxidation and nitric oxide in tumor tissues and the increase in glutathione and superoxide dismutase levels as a result of bone marrow cell treatment. Thus, they found that bone marrow cells have tumor-suppressive properties in colon cancer caused by 1,2-dimethylenehydrazine (DMH) (El-Khadragy et al. 2018). In the study of Sang et al., the effect of wheat bran oil on colon cancer has been tested in $APC^{min/+}$mice. 2% wheat bran oil that treated the $APC^{min/+}$mice tumor formation has inhibited 35.7%. Wheat bran oil sub-fractions has been detected with HPLC methods. Wheat bran oil sub-fraction has treated colon cancer cell line, and it has inhibited cell proliferation. Wheat bran oil has protective activity on colon cancer (Sang et al. 2006) Tsao et al. investigated cancer related to diet and aging in DNA mismatch repair (MMR)-deficient mice. MMR-deficient mice were observed in a controlled manner on a high-fat-low-calcium (HFLC) diet. The number of adenomas increased (2.2-fold) in HFLC-diet MMR mice (Tsao et al. 2002).

Lung Cancer

Lung cancer is the second most common type of cancer and is the leading cause of cancer-related deaths. 1 in 10 cancer diagnoses (11.4%) and 1 in 5 cancer deaths are due to lung cancer (Sung et al. 2021). The main reason for the high mortality rate of lung cancer is the progressive metastasis of the diseases.

Lung cancer can be divided into two main groups depending on the histopathological properties: small-cell lung cancer (SCLC; 20%) and non-SCLC (NSCLC; 80%). SCLC is subdivided into squamous cell carcinoma (30%), adenocarcinoma (50%), and large-cell lung carcinoma (20%).

Since humans and a few species may exhibit spontaneous lung cancer, developing unique and controlled animal models is crucial. Its heterogeneity and aggressive biologic nature (carcinogenesis, proliferation, invasion, angiogenesis, metastasis) do not let the investigators develop a single animal model for lung cancer experiments. Chemically induced lung tumors, transgenic mouse models, and human tumor xenograft models are widely used animal models for lung cancer studies. The researches proved that lung tumors produced in mice and rats are pretty similar in histology, molecular characteristics, and histogenesis to human ones (Balmain 2000). Distinct animal species, including mice, hamsters, rats, cats, and dogs, have been used to develop chemical-induced lung cancer models. These animals' properties have a significant role during the selection step. For instance, the low metastatic rate of hamsters and dogs makes them suitable candidates for early-stage lung cancer studies. Besides, DEN-induced lung adenocarcinoma hamster model and MNU-induced tracheobronchial squamous cell hamster model have also been used for lung carcinoma studies. Half of the male hamsters developed lung tumors in 2 months (Steele et al. 1994). On the other hand, xenograft lung cancer models do not suit the studies of the early events since malignant cells/tissues are straightly

inoculated into the host animal. However, examining new therapeutic agents and developing new strategies can be well studied in this model. Transgenic lung cancer models can determine the molecular issues that provide the pathogenesis and progression of the condition.

Due to the prevalence of human lung SCC, developing a suitable model is critical. Furthermore, this model must reflect the squamous cell lesions of the bronchus for standard phase studies. Animal lung models must be reproducible for scientific studies. Unfortunately, although B[a]P delivered into the trachea successfully produced SCC, its reproducibility rate is low (Kasala et al. 2015). Another method for the development of SCC mice is skin painting of N-nitroso-methyl-bis-chloroethylurea (Tago et al. 2013). But, on the other hand, this method produces toxicity due to the nature of the compound.

The high effectiveness, ease of handling, and rapid timeframes make the chemical-induced animal model favorable for lung cancer research. Since smoking is accepted as one of the most critical factors in the development of lung cancer, it is not surprising that polycyclic aromatic hydrocarbons (PAH) and aromatic amines in cigarettes are used in creating a lung cancer model (Liu et al. 2015). Oral administration of chemicals, including dibenz(a,h)anthracene, isonicotinic acid hydrazide, urethane, nitrosamines, (chloromethyl)benzo(a)pyrene (B[a]P)9 and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosodiethylamine, 3-methylcholanthrene, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and asbestos, stimulates progression of lung adenomas in mice and rats (Liu et al. 2015; Anandakumar et al. 2008).

These chemicals promote the formation of different types of cancerous cells. For instance, while NNK promotes the development of lung adenocarcinoma, N-nitroso-tris-chloroethylurea (NTCU) causes squamous cell lung carcinoma (Wang et al. 2004). Besides, following the single dose of NTCU to female NIH Swiss mice, lung SCC closely resembles that in humans and develops approximately 32 weeks later. NNK-induced lung cancer-promoted A/J mice have been used to investigate the effectiveness of NNK antagonists, which block β-adrenergic receptors (β-AR), and it is suggested that NNK antagonists may be an effective strategy for the treatment of lung cancer (Ge et al. 2015). Moreover, a single i.p. injection of ethyl carbamate developed benign lung adenomas in newborn inbred A/J mice within several months. Strain A mice are good candidates for murine lung tumor bioassay studies. They have been used to evaluate the carcinogenic activity of chemicals and environmental agents, including urethane, benzopyrene, metals, aflatoxin, polyaromatic hydrocarbons, and nitrosamines (constituents of tobacco smoke) (Liu and Johnston 2002).

The development of transgenic lung cancer models via microinjection, retroviral infection, and embryonic stem cell transfer makes these animals excellent candidates for oncogene studies including gene expressions and the possible effect of these oncogenes on the growth and differentiation. Integration of mutated H-ras or p53 genes into the mice genome resulted in lung tumor formation in mice soon after birth. However, these transgenic mice die after a short period, so they are suited for lung cancer progression studies. Still, it is difficult to use them to investigate the early events due to rapid progression and the early onset of cancer. Significantly, the

strains that possess p53 mutation or K-ras knockout spontaneously generate lung adenocarcinomas, although the tumor incidence is relatively low, and the latency is usually long. On the other hand, administration of carcinogens to p53 mutations or K-ras knockout mice increases the incidence. A golden point in lung mice models is the presence of the K-ras oncogene. Although carcinogens and mutations trigger the development of lung cancer, the strain of the animals induces susceptibility. For instance, C3H/J and A/J mice possess low and high lung tumor susceptibility, respectively. Furthermore, their F1 hybrid, C3A F1 (C3H/J (females) to A/J (males)) and AC3 F1 (A/J (females) to C3H/J (males)), has K-ras mutation and develops spontaneous and chemically induced lung carcinoma.

Immunosuppressed rodents are also used to create human lung tumor xenografts that are histologically and biologically mimicking their human counterparts. The xenograft model nowadays is a pretty simple method in which the cells are directly transplanted into the dorsal lateral flank. Although it has advantages in the clinical efficacy of the drug candidates, this method possesses some disadvantages, including low tumor rate, inappropriate microenvironment, and lack of compatible invasion and metastasis. These disadvantages can be eliminated with orthotopic models in which human tumors are implanted straight into the relevant organ/tissue of origin in the rodents. On the other hand, Howard et al. (1991) implanted NSC lung tumors (A549, NCI-H460, and NCIH125) and SC lung tumors (NCI-H345) endobronchially in nude rats. In this model, they observed that the cells metastasized to the mediastinal lymph nodes rather than systemic circulation (Howard et al. 1991). A systemic metastatic model was developed using H640 cells for the orthotopic lung cancer model, the primary tumor take rate increased to 100% in nude rats, and tumor weight increased to 4 grams in a month (Howard et al. 1991). Besides, human lung tumor tissue can also be directly implanted orthotopically. When human SC lung cancer tissue is implanted into the mice lung, it was found that it metastasized to the contralateral lung and mediastinal lymph nodes.

Liver Cancer

Liver cancer ranks sixth worldwide in incidence and third in mortality. Of the 906,000 patients diagnosed with liver cancer, 830,000 died. The main causes of liver cancer are hepatitis C and hepatitis B viruses, foods containing aflatoxin, obesity, and excessive alcohol consumption (Sung et al. 2021). Trials are ongoing to create new therapeutic approaches in the treatment of liver cancer. In the development of new drug candidates, animal models provide the most important data for understanding disease biology. Therefore, many animal models, including mouse models, are used to understand the pathogenesis of liver cancer and to develop new drug candidates (Fig. 1). Genetically modified, chemical, or dietary sources and implantation methods are used in mouse models. Genetic mutations that cause liver cancer are TERT, TP53, CTNNB1, ARIDA1A, and AXIN1, while mutations such as EGFR, PIK3CA, or KRAS are rare. Genetically modified mice are used in human liver cancer. In these mice, genes are activated or inactivated as a result of mutation

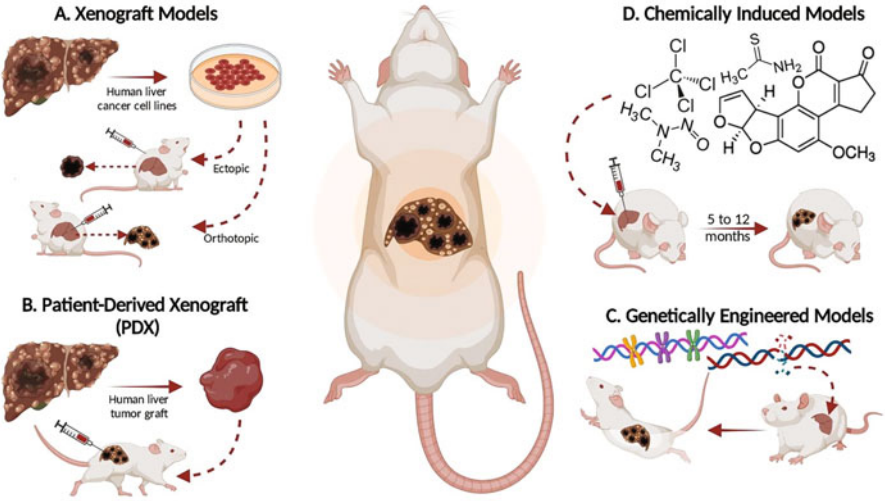

Fig. 1 Animal models in lung cancer

of oncogenes and tumor suppressor genes. Thus, a genetic factor-dependent liver cancer model was created in mice. In addition to these methods, recombinant DNA is created by transfecting the embryo and transferring embryonic stem cells to the host via lentiviral. While the Cre/loxP recombination system is used to create conditional mutagenesis, the necessary genetic modifications are made to create a liver cancer model with RNA interference (siRNA, miRNA, piRNA) and CRISPR/Cas9 method. In addition to modifications that have the potential to create a direct model of liver cancer, obesity mutations that cause liver cancer are also being made. For the TP53 mutation, the tumor suppressor gene is suppressed using the CRISPR/Cas9 method with the vector containing Trp53, and a liver cancer model is created in the absence of TP53 (Bruix et al. 2015).

There are genetically engineered mouse models as well as chemically and nutritionally created models. Models created using chemicals are divided into two groups. These are genotoxic and non-genotoxic chemicals. Genotoxic substances cause DNA damage, while non-genotoxic chemicals can induce the formation of liver cancer by promoting clonal proliferation of preneoplastic cells. DEN (diethylnitrosamine) is the most common genotoxic agent used to establish a liver cancer model. DEN is an alkylating agent that forms mutagenic DNA after being bioactivated by cytochrome P450, which is involved in xenobiotic metabolism (Bakiri and Wagner 2013). Diethylnitrosamine better induces the formation of liver cancer after intraperitoneal injection. Carbon tetrachloride (CCl_4) and thioacetamide (TAA) are the most commonly preferred non-genotoxic chemical agents. Non-genotoxic substances such as CCl_4 and TAA cause genetic differentiation by destroying cellular structures. They act as triggers for poor prognosis of cells by affecting proliferation and apoptosis. CCl_4 is a liver-destroying agent by increasing the amount of ROS in the mitochondria and causing peroxidation of

phospholipids in the membrane (Santos et al. 2017). Alcoholic liver diseases and related liver cancers are seen in animals fed with ethanol in their diets. It is used as a western diet in the feeding of animals with fat, high cholesterol, choline, and methionine deficiency and in models created by feeding. In this animal model, rats first develop nutritional diseases, and then liver cancer develops due to these liver diseases. Xenograft models of liver cancer occur by subcutaneous or direct transfer of human solid tumors to the liver. Cell-derived xenograft (CDX) models are often performed in in vivo studies of drug candidates being studied preclinically for use in liver cancer treatment. Patient-derived xenograft (Hidalgo et al. 2014) animal models are preferred for modeling the disease states of liver cancer patients. In PDX models, tumor tissues from liver cancer patients are subcutaneously inoculated into mice (Macek Jilkova et al. 2019). In Helm et al.'s study, a liver cancer model was created in Sprague-Dawley rats with thioacetamide. These rats were treated with thymoquinone and increased expression of TRAIL/TRAILR2. In addition, it was determined that there was a decrease in TGF-β1 and Bcl-2 gene expression and an increase in Caspase-3 expression. A decrease in hepatic GSH level was also detected (Helmy et al. 2019).

In Huang et al.'s study, the effects of mir-221 on the JAK-STAT3 pathway in liver cancer were investigated. It was determined that SOCS3 expression increased. In tumor samples from mice, mir-221 mimic was found to be larger than the mir-221 inhibitor. In this context, it was concluded that the SOCS3 gene of miR-221 supports tumorigenesis in liver cancer.

Brain Cancer

Brain cancer, which is the leading cause of cancer-related death in men <40 years old and women <20 years of age worldwide, is responsible for 308,102 new cases and 251,329 deaths, with incidence rates 1.29-fold higher in men than in women and mortality rates 1.3-fold higher (Sung et al. 2021). Despite the tremendous identification of brain tumors in the last decade, the main problems it presents such as aggressive and invasive behavior, high heterogeneity, and therapeutic resistance to current therapies, as well as the etiology and molecular mechanism, have not yet been fully elucidated (Reimunde et al. 2021). However, high-grade gliomas, which have a poor prognosis, are far from satisfactory, causing neurological side effects on patients due to their resistance to conventional treatments such as radio- and chemotherapy. In addition, therapeutic goals focus primarily on palliative therapy, given the inadequacy of conventional treatments. Thus, biotechnological advances and laboratory techniques have shed light on the improvement of therapeutic regimens in in vivo animal models that can faithfully reflect the brain tumor, elucidate the underlying mechanisms, and predict the response of the tumor to drug therapy (Reimunde et al. 2021).

A large number of in vivo animal models have been developed to date for brain tumors, which are arguably the most difficult to treat and most devastating of all cancer types. These models provide a promising experimental platform as new

techniques are developed and appropriately reflect tumor behavior in all mammalian organisms. Accordingly, a good animal model should have a high incidence rate, identify the histopathological and molecular mechanisms underlying tumor growth, explain tumor heterogeneity, and predict patient response to drug therapy. A number of in vivo animal models, including xenograft animal models, genetically engineered mouse models (GEMMs), and syngeneic models, have provided unique and valuable information to improve brain tumor simulation (Fig. 2).

Xenograft tumorigenesis, often preferred in in vivo cancer modeling, refers to the transplantation of biopsies from human brain tumors (patient-derived xenograft: PDX) or cultured cell lines (cell-derived xenograft: CDX) into the immunodeficient model animal. This preclinical modeling is widely used by researchers as it allows to study the mechanism of formation, growth, and spread of human tumor cells implanted subcutaneously (s.c.) or orthotopically (natural tumor organ or tissue) in experimental animals and to determine their therapeutic efficacy. Cell-derived xenograft (CDX) models, which are easy to grow in culture and have well-defined biological properties such as rapid tumorigenesis, good reproducibility in terms of engraftment rate, and reliable disease progression, involve direct implantation of the model animal into targeted locations in the brain (Fig. 2a) (Table 4). Commonly used and commercially available glioblastoma cell lines for implantation of this model include U87, U251, T98G, and A172. These cell lines are used to

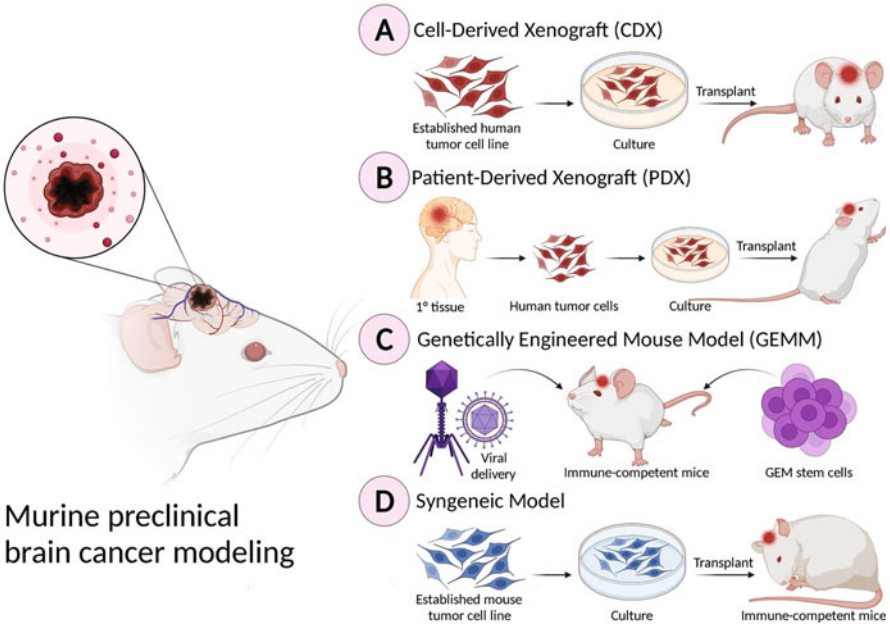

Fig. 2 Murine preclinical brain cancer modeling (Hicks et al. 2021) (**a**) cell-derived xenograft (CDX) (**b**) Patient-derived xenograft (PDX) (**c**) Genetically engineered mouse models (GEMM) (**d**) Syngeneic model

investigate signaling pathways by protecting genetic mutations (Akter et al. 1875). In addition, the cell-derived xenograft model possesses some disadvantages such as i) inability in preclinical use to often mimic key features of brain tumors such as invasive and vascular growths (Wang et al. 2009), ii) pharmacologically limited role of model animals in cancer drug development (Peterson and Houghton 2004) and iii) unsuccessful preservation of the genetic information in the tumorous cells (Martens et al. 2008), Hence, this model is not sufficient for the preclinical studies (Kim et al. 2016). In contrast to CDX models, patient-derived xenograft models (Hidalgo et al. 2014) offer a powerful modality for translational cancer research, consisting of biopsy tissues grafted directly subcutaneously or orthotopically into immunocompromised mice and surgically dissected (Fig. 2b) (Table 5). These models are becoming an integral part of the field of drug development by preserving the biological and

Table 4 Cell-derived xenograft (CDX) murine models

Mouse species	Brain tumor modeled	Tumor cell line	Reference
C57Bl/6 J mice	High-grade glioma	U87MG and GL261 cells	(Hülper et al. 2011)
NOD/SCID mice	GBM	U87MG and T98 cells	(Jandial et al. 2018)
BALB/c nude mice	GBM	U87MG and LN229 cells	(Han et al. 2020)
BALB/c OlaHsd-Foxn1^{nu} (athymic nude) mice	Glioma	BT_4C cell	(Miner et al. 2020)
BALB/c athymic (nu/nu) mice	Glioma	A-172, U343, U87MG, and T98G cells	(Xu et al. 2017)

NOD/SCID nonobese diabetic/severe combined immunodeficiency; *GBM* glioblastoma

Table 5 Patient-derived xenograft (Hidalgo et al. 2014) murine models

Mouse species	Brain tumor modeled	Source of tumor cells	Reference
eGFP NOD/SCID mice	Human oligodendroglioma	Patient-derived tumor cells	(Klink et al. 2013)
NOD-SCID	Glioma	Patient-derived orthotopic glioma tissue	(Xue et al. 2020)
NOD/SCID Il2rg$^{-/-}$ (NOG) mice	GBM	Patient-derived orthotopic GBM cells	(Joo Kyeung et al. 2013)
Athymic nude mice	GBM	Patient-derived orthotopic GBM tissue	(Irtenkauf et al. 2017)
SCID	IDH-mutant glioma	Patient-derived glioma neuro-spheres	(Wakimoto et al. 2014)

eGFP green fluorescent protein positive; *GBM* glioblastoma; *IDH* isocitrate dehydrogenase; *SCID* severe combined immunodeficient mice

histopathological characteristics of tumors and the complex interaction between cancer cells and the tumor microenvironment (Hidalgo et al. 2014). In addition, when the first studies were examined, rat C_6 glioma cell lines were implanted into adult or neonatal mouse brains and used to clarify tumor boundaries in order to establish a reliable murine xenograft glioma model (Kaye et al. 1986). However, key points in PDX research include (i) the cost and resources required that make this approach unfeasible, (Tago et al. 2013) differences in the expression of selected drug targets as there may still be changes in specific genes and drug targets between the PDX model and the original patient tumor, and (iii) tumor grafting and the need to use immunocompromised (SCID) mice to prevent rejection of foreign tissues (Hidalgo et al. 2014). To sum up, athymic nude mice, severely compromised immunocompromised (SCID) mice, or other immunocompromised mice constitute models that will readily accept heterotransplantation of human cancer cells or tumor biopsies (Richmond and Su 2008).

GEM models have gained popularity by revealing the events underlying the genetic changes that occur in response to specific mutations that reflect the histopathology, etiology, and biology of human glioblastoma, defining the molecular events and pathways responsible for tumor initiation and progression (Fig. 2c) *(*Huszthy et al. 2012*)*. Several criteria have recently been presented for GEM models of human cancers: (1) mice and humans as model animals must carry the same mutation; (2) mutated genes must be silent, except in inherited pediatric tumor models; (3) mutations must be within specific target tissues; and (4) mutations must occur in a limited number of cells. However, there are some limitations of GEM models, such as the inability to smoothly mimic the human tumor and the therapeutic response cannot be predicted because the model animal tumor is not the same as the human tumor. Therefore, the response that occurs when a mouse tumor is treated differs from the clinical response (Richmond and Su 2008). For example, telomere dysfunction caused by mice having longer telomeres than humans may be responsible for variations in the molecular mechanisms underlying tumorigenesis in human cells.

Syngeneic mouse models of glioblastoma were first described in the 1970s at the age of 15–18 of murine pregnancy. It is an alkylating agent of ethyl nitrosourea (ENU), a carcinogen, administered by injection into the placenta, and the development of brain tumors with various mutations has been observed in offspring (Akter et al. 1875). These models are used as indispensable tools in the investigation of molecular events such as tumor formation or progression, testing therapeutic strategies, and in glioblastoma research with their affordable cost. In addition, P560 spontaneous mouse glioblastoma models that summarize the histological and biological features of glioblastoma (GBM) were also produced by intracranial injection of the carcinogen 3-methylcholanthrene, resulting in the formation of tumors resembling GBMs by producing chemically induced mouse models such as GL261 and CT-2A (Hicks et al. 2021). Thus, glioblastoma tumor immunology and immunotherapeutic studies are analyzed with these immunocompetent mice used.

Melanoma of Skin Cancer

Skin cancers are an oncological disease that includes cutaneous melanoma and non-melanoma skin cancers that are frequently seen among Caucasians (Hochberg 2013). Among these, basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) are the most common and exhibit relatively lower aggressiveness, metastatic activity, and mortality. Malignant melanoma, which is characterized as the most aggressive and deadly form of skin cancer, is the most common (91.2%) melanoma type according to the National Cancer Database (NCDB-US) (Martinelli et al., 2020). Malignant melanoma is responsible for 324,635 new cases and 57,043 deaths worldwide. Although melanoma is known as the most serious form of skin cancer, it is more comfortable to treat when diagnosed early. Therefore, using animal models (Fig. 3) for melanoma progression is an important strategy for diagnosis and treatment (Le Bras 2020).

The supreme environmental risk factor in the development of malignant melanoma is exposure to sunlight as it causes mutations along with DNA damage. In addition, the number of melanocytic nevi, genetic predisposition, skin, hair, and eye color are among the risk factors. Since it is known that the multiple risk factors contributing to the development of malignant melanoma do not significantly increase the survival rate in conventional treatments, it has become imperative to develop targeted therapies that will provide more effective strategies. Accordingly, revolutionary treatment methods have been developed with in vivo animal models that maximize efficacy; provide a deeper understanding of molecular mechanisms such as tumor formation, development, and spread; and accurately simulate melanoma behavior. Models used in current melanoma research are (i) xenograft models; (ii) models involving genetically modified animals; and (iii) syngeneic models.

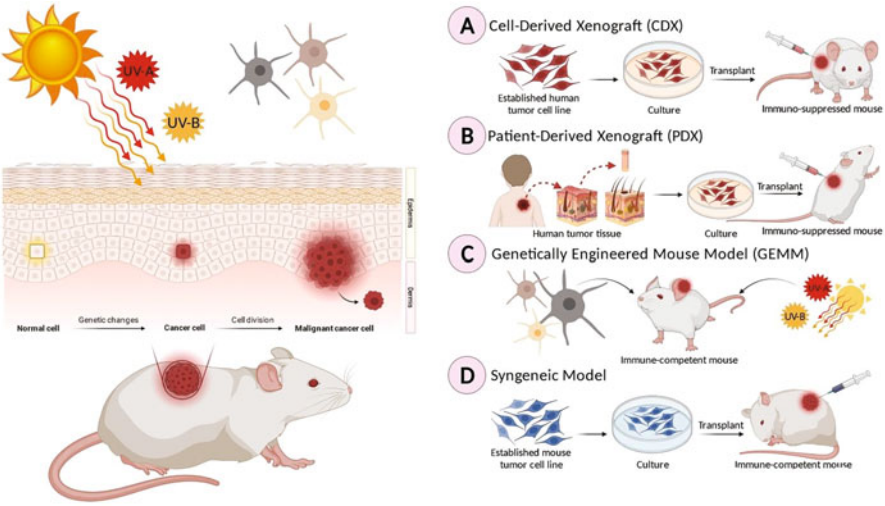

Fig. 3 Murine melanoma models

Genetically modified mouse (GEM) melanoma models have higher physiological growth rates than other models. GEM models elucidate the elusive biological responses in the subtleties of the tumor immune response and microenvironment for spontaneous melanomas. Several genomic loci have been identified that play a role in the formation and progression of familial and sporadic melanomas. The cyclin-dependent kinase inhibitor 2A (CDKN2A) locus located on 9p21 encodes two independent protein products, p16INK4A and p14ARF (p19ARF in mouse). P16INK4a blocks phosphorylation of the pRB protein and inhibits G1/S cell cycle progression. P14ARF stabilizes p53 protein by inhibiting mdm2 and is a promoter of cell cycle arrest in G1 and G2 in response to abnormal stimuli (Patton et al. 2021). Therefore, GEM models are important to demonstrate the dependence of melanomas on developmental lineages. Unlike humans, mouse melanocytes are found in hair follicles. Hepatocyte growth factor-based GEMs show mouse cutaneous melanocytes localized at the epidermal/dermal junction as well as hair follicles.

Syngeneic models have been used for melanoma research for a long time and have some advantages such as low cost, ease of use, and rapid response capability. A variety of cell lines (Table 6) are used for genetic transplantations, including B16 melanoma in C57BL/6 mice, Harding-Passey melanoma in BALB/c DBA/2F1 mice, and Cloudman S91 melanoma in DBA/2 mice (Teicher 2006). B16 melanoma is most commonly used because of its spontaneous tumorigenesis and high proliferation capacity (McKinney and Holmen 2011). B16 melanoma has variants of B16F1 known for its low metastatic feature and B16F10 known for its high metastatic potential. In addition, the Cloudman S91 cell line is preferred as a nanotransporter in anticancer drug delivery systems.

Another model used in melanoma research is patient-derived xenograft (Hidalgo et al. 2014) models. PDX models have been preferred in translational research in recent years because of their easy availability, affordability, and accurate response to targeted therapies. Krepler et al. (2017) created a comprehensive collection of melanoma PDX by deriving 459 PDX models from 384 patients from metastatic lesions representing genetic factors such as BRAF, NRAS, and NF1. Kawaguchi et al. (2016) investigated the effects of vemurafenib (VEM), temozolomide (TEM), trametinib (TRA), and cobimetinib (COB) on tumor growth by creating BRAF-V600E mutant melanoma patient-derived orthotopic xenograft (PDOX). It was

Table 6 Xenograft models of melanoma of skin cancer

Mouse species	Type of melanoma developed	Melanoma cell line	Reference
BALB/c nude male mice	Cutaneous melanoma	A375 achromic human melanoma cells	(Avram et al. 2017)
NOD/SCID mice	Cutaneous melanoma	A375 human melanoma cells (s.c. inoculum)	(Kimpel et al. 2018)
Athymic nude mice	Cutaneous melanoma	A375 human melanoma cells (s.c. inoculum)	(Liang et al. 2017)
Athymic-Foxn1^{nu} nude mice	Melanoma	1205 Lu and UACC 903 melanoma cells	(Gowda et al. 2017)

demonstrated that TRA is a successful candidate by targeting BRAF-V600E mutation in all drugs.

When looking at chemically induced mouse models of melanoma, chemical carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphobol-13-acetate (TPA) have generally been used. DMBA suppresses the immune system (Miyata et al. 2001), and TPA acts as a tumor promoter by activating protein kinase C (Oka et al. 2009). These chemicals are often used in combination with ultraviolet radiation (UV), xenotransplantation, and other genetically engineered modeling techniques. However, this model has some disadvantages such as being clinically unsuitable for human disease, inhomogeneous application of chemical agents, and non-pigmented cells. However, the immune systems of the mice used with this model are mostly functional and thus can be tested in other immunotherapeutic strategies.

Consequently, there is always a need to develop effective and efficient in vivo animal models to understand the biology of human melanomas. Considerable progress has been made in this regard, with increasingly complex models being developed that allow testing of anti-melanoma treatments. With the use of each model described above, we can develop treatment modalities by identifying every challenge necessary to eradicate this disease.

Conclusion

Due to increasing cancer cases and genetic differences, the use of animal models in cancer studies is gaining more and more importance day by day. Rodents are frequently used model animals because of their ease of handling and openness to genetic manipulations. Each selected animal model has its advantages and disadvantages. Therefore, the choice of a model organism is closely related to the type of cancer to be studied.

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Animal Models in Cancer Research: Breast Cancer

13

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Contents

Abstract

This chapter covers the main animal models employed in basic research to study breast cancer, including murine (chemically induced, transplanted, radiation-induced, *in silico,* and *in vitro* models) and feline and canine models.

Keywords

Animal models · Breast cancer · Carcinogenesis · Cancer mechanisms

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Introduction

Breast cancer is the most common malignant neoplasm that affects women worldwide. Despite advances in the last few decades concerning disease diagnosis and treatment, many patients do not present favorable outcomes – a poor prognosis in breast cancer results from a combination of patient, environmental, and tumor features. In practice, it has been observed that some patients undergo very aggressive diseases even when these characteristics support a phenotype for a good prognosis. The mechanisms underlying this context are unclear, and its investigation is only possible by exploring *in vitro* and animal models. Basic research has advanced in this aspect, providing a range of experimental models that subsidize investigating the mechanisms implicated in tumor behavior and pointing out the putative targets for intervention in human tests. This chapter provides detailed information concerning the use of animal tools, overviewing the chemically induced, transplanted, radiation-induced, in silico, and *in vitro* models for breast cancer research in murine, feline, and canine models.

Murine Models

Chemically Induced Models

Animal models have been contributing over the years to understanding breast cancer pathogenesis, and one of the most accepted methods for the induction of breast tumors is through chemical compounds. There have passed many years already since the disclosure that chemical compounds could drive tumor development, helping to evaluate the diagnostic/therapeutic effects of candidate drugs (de Alencar et al. 2019), such as estrogen pathways inhibitors (Clarke 1996), investigate the influence of many biological factors, exploit measures for carcinogenicity prevention, and also to perceive the molecular mechanisms involved in all three phases of tumor development, initiation, promotion, and progression (Nassan et al. 2018). Although it is crucial to keep in mind that each method has its purpose and that the best choice depends on the hypothesis-driving question of the study, concisely, chemically induced models are worth investigating early-stage processes and malignant progression (Russo and Russo 1996; Liu et al. 2015).

As an essential advantage in chemically induced carcinogenesis, it is possible to mention the overall simplicity of procedures, high-yielding tumor generation, and the similarity to clinical human primary cancers. However, there are also some disadvantages, such as the time necessary for the whole process and, even more, the difficulty in noninvasive tumor burden assessment in small animals (Liu et al. 2015).

As for improving the investigation capabilities and decreasing the risk of confounding factors as results, some points need to be considered, such as the type

of tumors likely to be generated. Among the most frequently applied chemical methods are 7,12-dimethylbenz(α)anthracene (DMBA), which, together with 1-methyl-1-nitrosourea (MNU) (Liu et al. 2021) are the two major mammary carcinogens, but 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine (PhIP) can also lead to mammary tumors (Papaioannou et al. 2014), among others as, methylcholanthrene (MCA), 2-acetylamino-fluorene, 3,4- benzopyrene, ethylnitrosourea, and butylnitrosourea (Russo and Russo 1996).

It is necessary to know their time range for induction, which has shown to be around 6–7 weeks in rodent animals, depending on the species, and take around 8–13 weeks for tumor development (Zeng et al. 2020). Another essential point is their mechanisms of action; as described by (Choi et al. 2014), DMBA needs to be activated in the liver, and its metabolites have a long half-life, remaining in the animal for days; on the other hand, NMU carcinogenic properties are more direct, thus not presenting a high risk of confounding factors through chemical interactions.

To investigate DMBA in early and late pregnancy, Zhao et al. (2011) showed that this carcinogen generates the augmented expression of AgNOR, C-erbB-2, PCNA, Ki67, and MCM2 in breast epithelial cells in female Sprague-Dawley (SD) rats. However, other aspects must be taken into consideration. One example is Ras expression, one of the genes usually associated with carcinogenesis with a substantial role in the disease development (Galiè 2019). DMBA-induced tumors have a relatively low detection of Ras expression, around 25%, while NMU-induced tumors have around 75% expression of this gene or more (Clarke 1996). While DMBA yields the expression of hormonal receptors, estrogen, and or progesterone positivity in mammary tumors, NMU has shown low and intermediate estrogen levels in breast tumors (Liu et al. 2021).

In the meanwhile, this is a heterocyclic amine, which causes complete carcinogenicity through damage and mutation of DNA, being an initiator of the development, but also a promoter of carcinogenesis by its activity inducing estrogen receptor alpha (ERα), which affects miRNA in the mammary gland during the initiation and progression of breast cancer (Papaioannou et al. 2014). Accordingly, the most usual methods for breast cancer chemical induction in animals are administered intravenously, subcutaneously, or intraperitoneally (Zeng et al. 2020). Also, no single model can reflect all the characteristics of tumor development, such as initiation, promotion, and progression. Once breast tumors are a very heterogeneous disease, even within the same subtype, there is a range in heterogeneity, making it difficult for a single model to reflect the complexity of the disease entirely (Figs. 1 and 2).

Transplanted Tumors Models: Cell Line-Derived Xenografts/Patient-Derived Xenografts

Cell line-derived xenografts (CDX) correspond to cell lines from tumor patients transplanted to immunodeficient mice to be used to predict treatment response. Researchers insert the tumor cells in the organ or tissue that matches the parental tumor. However, some genetic alterations occur because of the microenvironment of

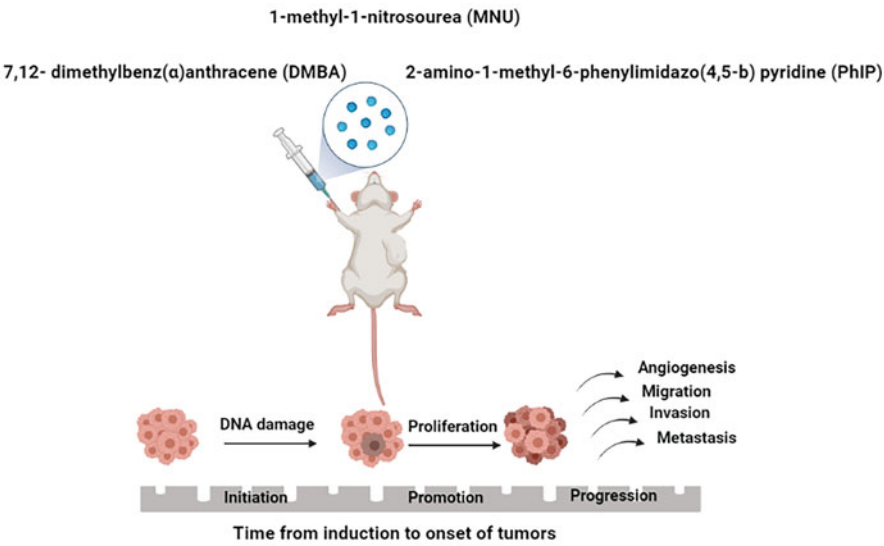

Fig. 1 Overview of the main chemicals used for breast tumor induction in a time space of carcinogenesis development (Liu et al. 2015)

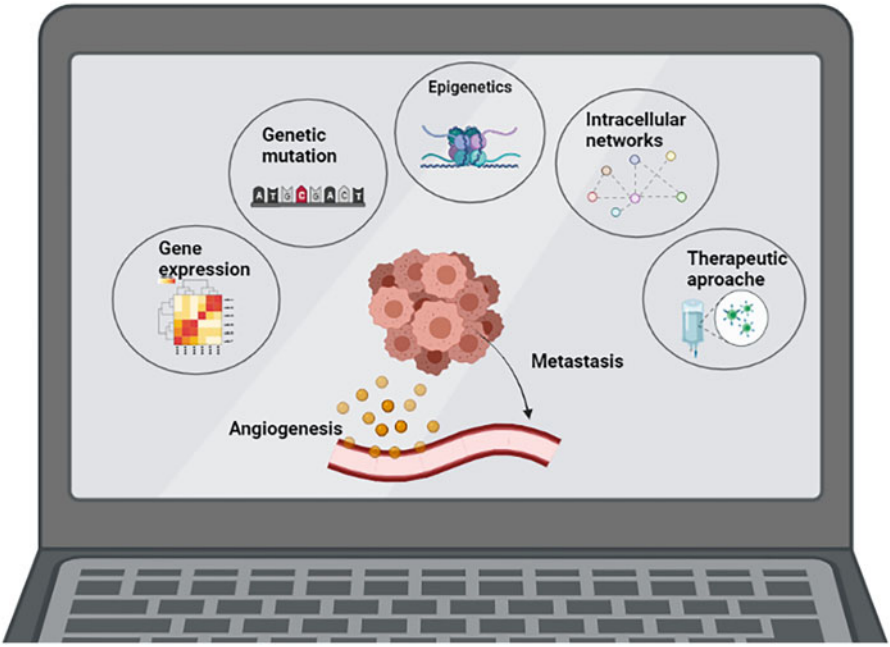

Fig. 2 Overview of cancer biology complexity integrated with computational models

the mice organism and the lack of the tumor's original conditions. Also, if the culture is made in vitro, studies show even more differences in the gene expression compared to nongrowth in vitro cell lines (Daniel et al. 2009).

On the other hand, patient-derived xenografts (PDX) consist of the application of tumor pieces (that are mechanically or chemically digested before insertion) derived from primary surgical resection into immunodeficient mice (Yoshida 2020). Also, researchers choose the organ or tissue of the mice which matches the tumor histotype in a subcutaneous or orthotopic manner, as well as cell lines. This technique has been demonstrated to be more successful in maintaining the original parental tumor organism's characteristics than the CDX technique (Tentler et al. 2012; Whittle et al. 2015). Besides the implantation of tumor tissue, advances have been made to keep the breast cancer (BC) microenvironment even more similar to the original tumor conditions. Thus, orthotopic implantation, estrogen supplementation, and the addition of mesenchymal stem cells have all been employed (Kabos et al. 2012).

Triple-negative tumors have preserved their characteristics in mice PDX, even after several lines (Derose et al. 2013). At the same time, hormone subtypes have not been as successful in reproducing the features observed in the original tumor microenvironment (Marangoni et al. 2007; Derose et al. 2013; Li et al. 2013). Because of this difficulty, hormonal-type tumors remain with CDX as the gold standard (Clarke 2009). The authors observed that allele frequencies were preserved in a study that analyzed the genomic characterization of BC-derived xenografts concerning resistance to hormone treatment. In contrast, at the single nucleotide level, it was possible to observe changes but little functionally significant or even nonfunctional (Li et al. 2013).

Researchers showed that BC PDX could recapitulate the native tumor biology by analyzing 20 PDX and their biological, clinical, and genetic features. The cohort comprised a majority of triple-negative and 25% estrogen hormone receptor-positive tumors. PDX tumors maintained their original molecular classifications; however, in a minority of cases, slight genomic instability (events corresponding to 9% of the genome) was observed (du Manoir et al. 2014). Another study evaluated different generations of PDX and authors observed that the critical aspects of human malignancies at histologic, transcriptomic, proteomic, and genomic levels, and thus adequately predict drug effects as those observed in humans were conserved (Zhang et al. 2013).

The study of the metastatic potential of BC in PDX models showed a direct correlation between the burden of cells with metastatic potential from the tumor and the development of metastasis. Furthermore, it was observed that in PDX models, the metastasis of BC occurs in many of the same organs affected in the original patient (Lawson et al. 2015). The triple-negative tumors grew faster than those of the hormonal type, as is observed in the clinic. In addition, it was possible to observe the development of metastasis in axillary, thoracic, and mesenteric lymph nodes. Other sites were also affected by the tumor, such as the thymus, lungs, bones, and peritoneum, detected grossly and by immunohistochemical staining (Derose et al. 2013).

Box 1: Breast cancer studies that show the effectiveness of using PDX models in predicting tumor behavior

Authors	Main findings
Derose et al. (2011), Kabos et al. (2012)	Hormonal supplementation and the addition of mesenchymal stem cells into the mice contribute to maintaining the tumor behavior found in the parental tumor.
Marangoni et al. (2007), Derose et al. (2011), Li et al. (2013)	Triple-negative tumors have better results than hormonal-dependent subtypes.
Li et al. (2013)	Genomic characterization of BC-PDX showed allele frequencies preserved.
du Manoir et al. (2014)	Biological, clinical, and genetic features were analyzed, and in the minority of the cases, a few genomic instabilities were observed in PDX models.
Zhang et al. (2013)	PDX models were capable of predicting drug effects as those observed in humans.
Lawson et al. (2015)	Metastasis of BC PDX occurs in many of the same organs affected in the original patient

Radiation-Induced and Genetically Engineered Models

Cancer has many etiologic factors that contribute to its carcinogenesis, and ionizing radiation (IR) is one of them, mainly in human breast cancer. Many rodent models have been developed for studying the effects of this radiation on mammary tumor development (Medina 2010).

BALB/c is an albino, laboratory-bred strain from which several common substrains are derived. This strain is known to have a low mammary tumor incidence, although they develop other types of cancers in later life, such as reticular neoplasms, lung tumors, and renal tumors (Okayasu et al. 2000). In this sense, hyperplasias and adenocarcinomas in female BALB/c mice can be stimulated by radiation exposure, γ-rays or neutrons, at low incidence (20–45%) and with long latency.

After the tragic atomic bomb in Japan, several studies focused on cancer and IR exposure. One was based on the atomic bomb survivors and clinically irradiated patients. It established that the female breast is one of the most susceptible organs to radiation-induced cancers, and the risk of breast carcinoma is enhanced compared to all other solids tumors upon exposure to IR (Thompson and Singh 2000). In addition to sparsely ionizing (low linear energy transfer [LET]) radiations such as photon radiation, induction of mammary cancers is observed after administration of densely ionizing (high LET) particle radiations including neutrons, and heavy ions, such as neon, iron, and carbon ions (Imaoka et al. 2007).

Although mouse mammary tumors have some dissimilarities from human breast cancers such as the low frequency of hormone dependence and the progression of carcinoma predominantly from alveolar hyperplasia, they provide a valuable route

for genetic experimentation (Thompson et al. 1994). For example, chromosomal instability, a feature not seen in cells from chemically induced tumors, has shown to be a mechanism in cells derived from tumors induced by radiation exposure. It is also proven that radiation-induced tumors in mice are hormone-independent and metastasize to the lung, similar to the behavior seen in human breast cancer. The protocols used for radiation-induced mammary tumors in mice are highly appropriate for modeling radiation-induced human breast tumors (Welsh 2013).

The low levels of DNA-PKcs, a catalytic subunit of a nuclear DNA-dependent serine/threonine-protein kinase called DNA-PK responsible for DNA repair, partially contribute to BALB/c mice strain radiosensitivity leading to diminished double-strand break repair capacity (Okayasu et al. 2000). In breast cancer, the BALB/c mouse is a very known model that can be induced with the implantation of irradiated tissues into syngeneic mice or full-body irradiation (Medina 2010). In this regard, the models based on BALB/c strain have their relevance to human breast cancer induction once the human ortholog of this same gene (DNA-PK) has a deficient level of differential expression in human breast tissue (Okayasu et al. 2000). In summary, there are two mainly BALB/c models: whole-body exposure and syngeneic transplant.

Studies based on the BALB/c female whole-body irradiation model show an increase in mammary carcinogenesis. This increase in carcinogenesis is from a background frequency of around 8% to about 22% over the mouse's entire lifetime. This induction method consists of irradiating 12-week-old females with a total dose of 2.0 Gy at the dose rate of 0.35Gy/min. The high dose rate seems to be key rather than the total dose once; even a dose of 0.25Gy at 0.35Gy/min is capable of inducing mammary tumors in about 20% of mice, while hyperplastic lesions in the mammary ducts can be detected generally a year after IR exposure, before the appearance of the tumor itself (Ullrich 1983). However, it is notable that this model has a significant drawback, despite its simplicity, is its high rate of concurrent ovarian and lung tumor development, detected in over 90% of autopsied mice (Zheng et al. 2020).

The BALB/c syngeneic transplant model consists of clearing the mammary fat pad from a 3-week-old female virgin mouse and subsequently transplanting a 1 mm duct fragment from a donor mouse with hyperplastic lesions. The most general study in this model is Ethier and Ullrich (Ullrich et al. 1987), which employed a combined in vitro/cell culture model in which 12-week-old virgin donor BALB/c females are whole-body irradiated with a total dose of 1.0 Gy, with mammary tissues removed at 24 h postexposure. After this procedure, a single-cell suspension of 104 cells from these donor animals is injected into 3-week-old virgin BALB/c females with cleared mammary fat pads. Finally, 10 weeks post-procedure, recipient mice are sacrificed and outgrowths removed and analyzed for anomalies in ductal architecture (Kefayat et al. 2019).

Normal duct outgrowths are known as having 2–3 terminal ducts and capped by end buds in the fat pad. When analyzed, the abnormal outgrowths, on the other hand, can have ten or more terminal ducts capped with hyperplastic end buds. These abnormal architectures are classified between Classes I and III, with Class III being the most severe. Moreover, cells harvested from an irradiated donor passaged

in vitro and finally transplanted into unirradiated recipient mice develop into either dysplasia or adenocarcinomas. The degree of dysplasia exhibited in the host mouse depends upon the harvesting time and the number of passages in culture before implantation. Cells harvested 52 weeks post-IR tended to generate dysplastic outgrowths in 75% of mice and develop into whole tumors in 25% of cases. Cells harvested up to 16 weeks of IR only develop into normal outgrowths unless subjected to extensive in vitro passaging. This observation suggests that the irradiated ductal microenvironment plays a critical role in the initiation of oncogenesis (Ullrich et al. 1987; Rivina et al. 2016).

Box 2: Characteristics of breast cancer induction in mice with low-LET IR

Mouse strain	Age	IR dosage	Latency	Induced frequency	Spontaneous frequency
BALB/c	12 weeks	2.0 Gy TBI	2 years	22%	8%
BALB/c orthograft		1.0 Gy TBI of donor cells	10 weeks	Tumor 25% Dysplasia 75%	<1%

[a]TBI, Total body irradiation

Finally, a study was made using BALB/C female rats an injection of specific breast cancer cells to identify treatments capable of stimulating T cell responses. In this way, they injected the BALB/c female rats with breast cancer cells, and then irradiation therapy was given with a 6 Gy dose at a dose rate of 2.77 Gy/min. After 22 days, tumors and tumor-draining lymph nodes were collected for flow cytometry analysis. T-cell tumor-specific responses were stimulated, and tumor-targeted radiotherapy has shown the ability to foster antitumor immunity (De Martino et al. 2021).

A high expression of activin A, a member of the transforming growth factor-beta (TGF-β) family, has been described in other solid malignancies such as esophageal and non-small cell lung cancers (Wamsley et al. 2015; Wang et al. 2015) Considering it, it was revealed that activin A and TGFβ blockade has shown to be the way to unleash radiation-induced antitumor immunity against breast cancer optimally. Also, it is plausible that increased activin A could contribute to limiting the efficacy of TGFβ pathway inhibitors, at least in some patients. However, the clinical relevance of their findings remains to be investigated (De Martino et al. 2021).

Genetically Engineered Mice Models (GEMM)

Breast cancer is a heterogeneous disease. Multiple subtypes vary significantly in their histopathology, hormone relation, and local growth factors, and each has its response to therapy and clinical presentation. Studying these tumor subtypes in vivo requires not just one but many types of genetically defined model systems as research tools. Therefore, there is no perfect model for breast cancer. The single option is to create a unique model representing some particular tumor feature.

Furthermore, advances in cancer studies have given rise to many other types of animal models. It is because discoveries have elucidated many other pathological pathways and different cells participating in carcinogenesis (Sakamoto et al., 2015).

In particular, mouse models have become a highlight tool for cancer research, including breast cancer. Among these tools, genetically engineered mice models (GEMMs), xenograft models, and chemically or radiation-induced models have stood out.

The genetically engineered mice models (GEMMs) provide many studies, including the participation of specific genes in the beginning and progression of cancer, understanding pathological pathways and cellular functions in starting and ending tumor activity (Sakamoto et al. 2015; Webster et al. 2020). The knockout mouse is an example of genetically engineered mice models. This animal has a gene removed or deactivated. The idea of knocking out is to study the function of a considered gene by excluding its activity in the cell (Webster et al. 2020). This technique reached significant utility by making a possible detailed study of oncogenes and tumor suppressor genes (Walrath et al. 2010).

In addition, the GEMMs help with studies of new drugs. The study of Pfefferle et al. using gene expression microarrays defined 27 profiles of murine models of mammary carcinoma and normal mammary tissue. Eight of them resemble human breast cancer subtypes. Furthermore, many pathological pathways were identified and compared to human ones (Pfefferle et al. 2013). The GEMMs show more effectiveness in cancer studies than xenografts (Greenow and Smalley 2015).

As previously discussed, GEMM would help in drug studies. Doxorubicin (Dox), for example, can induce therapy in breast cancer patients. Füredi et al. (2017) assessed a new form of doxorubicin, pegylated liposomal doxorubicin (PLD), in drug resistance using GEMM. The study pointed out that PLD is a promising strategy for treating therapy-resistant breast cancer patients. Right after Hall et al. (1990) found the BRCA, scientists started ongoing research with mice models to investigate the relations between the BRCA gene and breast cancer. The GEMMs significantly contributed to our understanding of the biological and molecular functions of BRCA1 and BRCA2 (Evers and Jonkers 2006).

In cases of breast tissue inflammation, such as epithelial carcinomas, the immune cell macrophage may be an essential factor. This cell is correlated with the promotion and proliferation of cancer cells, facilitating cell invasion and defining a poor prognosis. This function may be related to the expression of CC-chemokine ligand 2 (CCL2), a proinflammatory cytokine that attracts monocytes and macrophages to injured tissue (Sun et al. 2017; Li et al. 2020). There is a possible correlation between tumor cell invasion and high expression of CCL2 in breast tissue. The more CCL2 is expressed, the more macrophages come to the site, inflammation increases and leads to tumor invasion and metastasis. This way, a transgenic mouse was developed to investigate the CCL2 expression and mammary tumor development. The group's genetically modified model (Mmtv-Ccl2) expresses CCL2 in mammary epithelium. The animal showed elevated cancer risk by the perturbing in collagen remodeling, mediated by CCL2 inflammation function and macrophages' local expansion.

Furthermore, a comparison between the mouse and human mammary tissue revealed similar alterations in collagen perturbing (Sun et al. 2017).

These murine-modified models are the most modern models of human breast cancer. Nevertheless, more resources and time are required to create new GEMM lines and incorporate new mutant alleles. CRISPR-Cas9 genome editing seems to be the most technical method of mammary gene editing nowadays (Annunziato et al. 2020). Well described by Emmanuelle Charpentier and Jennifer Doudna, the CRISPR-Cas9 technique enables the addition of any mutation in any part of double-strand DNA with an ease never seen before, including mammary cell DNA, which expands the possibilities of studying breast cancer the way, mammary mouse DNA can be edited. A RNA guides the Cas9 endonuclease to a specific DNA part, then there is the "cut" (Chen et al. 2019; Annunziato et al. 2020).

Although, the application of the method in cancer still demands enhancement, remarkably due to the high mutation potential of cancer. The introduction of the CRISPR-Cas9 method in cancer studies is happening, and there is enormous hope in its promises (Chen et al. 2019).

In Silico Models

Breast cancer is a highly heterogeneous disorder characterized by some gene expression and pathway dysregulations. As cited by Jean-Quartier et al. (2018), improving our understanding of the disease, as such a complex biological system characterized by its interconnected dynamic processes, in different spatial and temporal aspects, requires methods to integrate a diversity of data sets. Meanwhile, in silico analysis can be defined as integrating computational approaches to biological analysis, considering the acquisition and generation of vast amounts of information, being a central point mainly in oncological studies (Tsai et al. 2019). In this way, in silico models have been in development over the years to help detect endogenous and exogenous cancer factors and also to aid in tests and development of new therapeutics, based on the increasing network evidence between genes and proteins that play crucial roles in molecular cancer mechanisms (Wu et al. 2012), as well as to assist in planning future experiments and studies. Thus, according to Wang (2011), with the advancement of omics data and biological discoveries at scales beyond a handful of interacting components, simple analysis techniques can become obsolete in providing comprehensible insights into phenotypic behaviors.

Computational biology is based on integrative approaches to characterize biological systems, in which interactions among all components in a system are described mathematically to establish a computable model, complementing traditional biological models (Edelman et al. 2010). As mentioned by Schwartz et al. (2009), connecting biological experiments with computational analyses and modeling can help reduce the number of experiments required and ameliorate the quality of information generated. So, instead of broad high-throughput screens, a better option is focused screens, which refine sensitivity and clinical validation (Wang 2011). Also, Jean-Quartier et al. (2018) emphasize that there is no perfect model because all of them will lack some aspects of reality, mainly in diseases as heterogeneous and

complex as breast tumors. Thus, the choice and appropriate application of research models are fundamental for advancing specific experiments.

Therefore, in silico models in oncological research embrace a range of techniques, which embraces computational validation and classification, inference and prediction, and the development of mathematical and computational modeling (Carels et al. 2016). As the diversity of cancer models reflects the complexity of the molecular and physiological events of the disease (Edelman et al. 2010), there are several well-maintained databases, which can provide much information about the various aspects of the disease (Mulas et al. 2017), these databases can supply information on various cancers, as well as integrated and annotated data. Some examples of such databases are, accordingly with Weinstein et al. (2013), The Cancer Genome Atlas, and The Cancer Genome Project, which gave an increased number of data on molecular disturbances related to carcinogenesis, and are increasingly being used for the exploitation of predictive models, which in turn will inform and guide biomedical experiments. The most usual approach uses statistical analysis of high-throughput expression data to uncover molecular signatures of cancer phenotypes. These signatures indicate the aberrant function of genes or pathways and can be used for many purposes to improve cancer understanding (Wu et al. 2012). However, Edelman et al. (2010) also refer to the fact that there are much more advanced methods intending to statistically infer the structure and/or quantitative relationships among biomolecules within interaction and regulatory networks of importance in cancer.

Thereby, analyzing molecular disrupts and disclosing gene expression signatures of tumor cells is a significant step for understanding cancer development and progression, leading to new therapeutic discoveries and contributing to an increasingly personalized medicine (Tsai et al. 2019). As it is, such approaches demand state-of-the-art diagnostic and prognostic tools, comprehensive molecular characterization of the tumor, as well as detailed patient health records over the years (Carels et al. 2016). In this way, customizing healthcare, optimizing treatment for the personal requirements of each patient, often based on the genetic signatures or other molecular biomarkers using computational tools, yields the possibility of uncovering new entities in critical signaling pathways and biomarkers, providing promising targets for anticancer therapy (Jean-Quartier et al. 2018).

Therefore, as in tumor cells, genomic aberrations disrupt central signaling pathways encompassing various genes; tracing signaling pathways can help understand the pathogenesis of cancer. So, bioinformatics tools can further help detect essential network interactions between the genes to get a greater biological context and improve the disease prognostic and therapeutics.

In Vivo/in Vitro Models of Metastasis 2D/3D and Chemoresistance

The most common etiology of death in most breast cancer patients is metastasis. Furthermore, this can occur because of the resistance to anticancer drugs (Kim and

Baek 2010). Thus, studies on these themes may involve several techniques, like in vitro and animal models (in vivo).

In vitro models can be classified into 2D (monolayer) and 3D (spheroid). The 2D model, as its name indicates, represents a two-dimensional growth of cells, depending on the adherence to a flat surface, such as a Petri dish, to supply the cells with mechanical support. However, despite its simple, widespread, and efficient use, due to a homogeneous growth of the medium, this monolayer model has limitations in the contact of cells with the environment since it does not reproduce an extracellular environment similar to the reality in vivo (Duval et al. 2017).

The 3D in vitro cell culture model, known as a spheroid, has the advantage of developing a more realistic cell microenvironment, similar to the interactions that occur in an in vivo environment, allowing the migration of cells in all directions, in addition to the possibility to initiate processes related to tissue differentiation, growth, and survival. For example, the spheroid heterogeneously enables exposure to nutrients, growth factors, and gas exchange in the tumor environment. Thus, experiments with in vitro 3D cell culture using spheroids mimic some aspects of tumor complexity (Kunz-Schughart et al. 1998; Griffith and Swartz 2006).

Due to its technique, the monolayer model analyzes more limited aspects of human cells. In contrast, the 3D model, with spheroids, allows analyzing the transendothelial migration of metastatic human breast cells and monitoring their behavior, as in a study of bone micrometastases from breast cancer in a controlled, bone-like environment (Bersini et al. 2014).

These associations of metastatic models are associated mainly with chemoresistance and testing new lines of treatment to understand the success or failure of tumor invasion. In this case, the model with spheroids proved to be more advantageous when compared to the monolayer since the concentration of some drugs used in the 2D model showed considerable discrepancy with the concentrations found in the in vivo model. Thus, the 3D model, as it is competent in reproducing situations of hypoxia, pH variations, and different cell proliferation rates, is capable of showing specific resistance to the drugs used as well as solid tumors, being the in vitro model closer to reality in vivo (Kunz-Schughart et al. 1998; Lin and Chang 2008).

The use of in vivo models, through animal experiments, is critical to assess the effect and response to certain drugs, like chemotherapy, because of more extensive similarity with the anatomical, physical, and pathological characteristics of the human being (Festing and Wilkinson 2007; Zeng et al. 2020).

An expression of tumor aggressiveness is represented by the spread of malignant cells to other locations, called metastasis, whereas therapeutic failure can occur due to resistance to the proposed treatment. Therefore, animal metastasis models can be performed in different ways, according to Ottewell et al. (2006), maybe as spontaneous tumors, chemically induced tumors, orthotopic and syngeneic tumor transplantation, injected tumors, and genetically engineered mice with a predisposition to neoplasia.

A widespread and disclosed spontaneous tumor model is the 4T1 mammary carcinoma that can metastasize on its own from the primary breast tumor to lymph

nodes, blood, liver, bone, lung, and brain (Pulaski and Ostrand-Rosenberg 2000). However, some studies with this model also explore mechanisms of anticancer, like the study produced by Kubatka et al. (2019), who says that using some natural phytochemicals in this model may have a substantial chemopreventive and healing effects against breast carcinoma.

The chemoresistance results from several elements, ranging from genetic variations specific to the individual to intrinsic resistance to malignancy and acquired drug resistance through the expression of one or more energy-dependent transporters that detect and eject anticancer drugs from cells. Other mechanisms of resistance, including insensitivity to drug-induced apoptosis and drug induction, are also related to anticancer drug resistance (Gottesman 2002). The significance of the in vivo study refers to the perspective of evaluating the drug response in animals, as well as the identification of the resistant mechanism, being similar to humans. An in vivo study of chemoresistance induction in spontaneous mammary tumors in mice with deficiency in BRCA1 and p53 genes, to doxorubicin, docetaxel, and cisplatin, in which resistance to the maximum dose of only docetaxel and doxorubicin was noted, which last was justified by the process of positively regulated drug transporters (Rottenberg et al. 2007).

These study models generally aim at the improvement of new therapeutic strategies, as well as at broadening the understanding of the mechanisms that perpetuate metastasis and chemoresistance. Therefore, it is undeniable that the existing animal models of breast cancer induction and progression are more similar to the human being due to physiological, morphological, and pathological attributes, and their study is significant. However, due to bioethical issues, the use of animal models in much research is being discouraged, and the use of cell culture with spheroids is widely encouraged due to the similarity with the in vivo microenvironment (Cannon 2015; Festing and Wilkinson 2007; Duval et al. 2017).

Canine and Feline Models

All mammal species are susceptible to breast cancer. For this reason, comparative oncology has studied it in animal models. Rodents are the models most frequently used, though domestic cats (*Felis catus*) and dogs (*Canis familiaris*) are appropriate and solid models for human breast cancer (HBC) research in those species is still underused (Pastor et al. 2020; Visan et al. 2016).

The current use of cats and dogs in cancer research is primarily in vitro models. Malignant and healthy mammary' cells isolated from canine and feline are a possible way for monitoring cancer molecular biology as cytotoxicity, molecular regulation, and interaction, and are also a safe way to experiment with new antineoplastic drugs. Thus, correctly employed on these species as in vitro and in vivo models can contribute to cancer biology, therapeutics, diagnostic, and clinical trials (Al-Mansour et al. 2018; Howard et al. 2020; Lutful et al. 2015; Visan et al. 2016).

The most substantial reason for having cats or dogs as models, when compared to the murine, are the more significant genomes similarities, especially canine, and

consequently similar hormone receptors, proliferation markers, altered expression of regulatory genes, tumor suppressors and oncogenes, and a large group of noncoding RNAs or microRNAs (miRNAs). Other significant points are that these animal companies are widespread, are exposed to the same environmental factors as humans, have a shorter lifespan than humans, and the epidemiological and clinical aspects of HBC like humans (Gray et al. 2020; Urfer et al. 2020; Visan et al. 2016).

Considering the ethical aspects, researching spontaneous breast cancer in pets could allow access to treatment for a large population of cats and dogs, even if experimental; replace animals used in laboratories; and accelerate clinical trials. Possible adversities when using pets as models can be the lack of information in the literature, the fact that exams and treatments are not currently performed on animals, as in humans, higher costs, and longer time compared to rodents. Other points to be considered in the research are that cats and dogs have a specific life span, lifestyle, breeds, and size variations (Gray 2020; Pastor et al. 2020; Urfer et al. 2020).

Comparative Canine Mammary Tumor (CMT)

CMT, as HBC, is the most common tumor in intact female dogs, accounting for more than 50% of all neoplasms, and often occurs at 8–11 years of age. As with men, male dogs are rarely affected. The risk factors for CMT include higher estrogen exposure (intact females are four times more likely to have CMT than ovariohysterectomized), environmental contaminants, obesity, and advancing age, similar to HBC. CMT, as HBC, is a group of diseases of different types, classified by the World Health Organization (WHO). The course of the disease, clinical stages, and metastasis occurrence are similar in dogs and humans (Gray 2020; Queiroga et al. 2011; Moe 2001).

The CMT is histology classified into malignant (carcinomas, sarcomas, and mixed) and benign tumors (adenoma, fibroadenoma, and mixed). However, molecular subtype classification, specific receptor status, or immunohistochemical are usually not required for CMT or FMT. The malignant tumors occur in almost 50% of the CMT, the majority being carcinomas, which have a spread rate of 50%. The canine mammary carcinoma used as a model for HBC has been classified as the same immunohistochemical breast cancer in women (Goldschmidt et al. 2011; Gray 2020; Lutful et al. 2015; Al-Mansour et al. 2018, Pastor et al. 2020; Visan et al. 2016).

Molecular similarities between CMT and HBC include hormone receptors such as estrogen receptor alpha (ERα), progesterone receptor (PR) expression, the overexpression of human epidermal growth factor receptor 2 (HER2), and EGFR (Al-Mansour et al. 2018; Gray 2020; Lutful et al. 2015; Pastor et al. 2020).

Homology markers of signaling pathways and their expression, including KRAS, PI3K/AKT, MAPK, PTEN, Wnt-b-catenin, P-cadherin, BRCA2, and ESR1, are currently upregulated; at the same time, tumor-suppressive pathways, such as p53, p16/INK4A, PTEN, and E-cadherin, are downregulated in both human and canine breast cancer. Other markers studied are insulin-like growth factor, growth hormone, metalloproteinases, Wnt signaling, mucins, heat shock proteins, CEA, CA 15–3,

VEGF, and cyclooxygenases (Gray 2020; Lutful et al. 2015; Queiroga et al. 2011; Visan et al. 2016).

Comparative Feline Mammary Tumor

Naturally occurring breast tumors in domestic cats are relatively common and tend to occur in older cats (10–14 years), intact or ovariohysterectomized cats; most FMT is malignant, predominantly carcinomas in situ or invasive (Pastor et al. 2020; Queiroga et al. 2011).

Feline mammary tumors tend to show ER- and PR-negative markers as studied by ligand-binding assay and immunohistochemistry in homology with human triple-negative breast cancer (TNBC). Similarly, TNBC is a highly aggressive subtype of breast cancer with a poor prognosis in cats, notwithstanding that no abnormalities on BRCA1 and BRCA2 were detected for FMT. According to histological features,

Table 1 Comparative aspects between human breast cancer, canine, and feline naturally occurring mammary tumors

Author	Features	Humans	Dogs	Cats
Epidemiological data				
Pastor et al. (2020), Queiroga et al. (2011) Cannon (2015), Howard et al. (2020), Wiese et al. (2013)	Median age, in years	62	10	10 to 14
	Estrogen exposure as risk factor	Long exposure to estrogen increases the risk of tumor occurrence	Occurs four times more in intact dogs than spayed	Occurs more in intact cats than ovariohysterectomized
Histopathology and genes				
Pastor et al. (2020)		Carcinoma	Carcinoma	
Howard et al. 2020		TNBC		Mammary basal-like adenocarcinomas
Cannon (2015), Queiroga et al. ((2011)), Lutful et al. (2015)	Signaling pathways and genes	PI3K/AKT, KRAS, PTEN, Wnt-b catenin, MAPK cascade, BRCA1, BRCA2, p53	PI3K/AKT, KRAS, PTEN, Wnt-b catenin, MAPK cascade, BRCA1, BRCA2, p53	CK5/6, Ki-67, EGFR
Pastor et al. (2020), Queiroga et al. (2011), Al-Mansour et al. (2018)	Molecular markers		ER, PR, HER2, EGFR, COX-2 Ki-67	ER, PR, HER2

FMT may be a potential in vivo model for HBC. Despite this, feline comparative mammary cancer is scarce in the literature, and more research is needed (Cannon 2015; Howard et al. 2020; Wiese et al. 2013) (Table 1).

Conclusions

Despite the ethical considerations concerning their practical use, animal models remain essential tools for breast cancer studies. Molecular findings have shown numerous similarities to human disease, which have supported the discovery of new targets for drug development. Although none are perfect, these models will keep providing a valuable overview of breast cancer biology, especially concerning those aspects that are not amenable to testing in humans.

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Animal Models for Bone Metastasis Study

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Contents

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Abstract

Cancer-mediated death is promoted by the process of metastasis. Often, bone is the preferred site of metastasis. The process of metastasis at the bone is mediated by interactions between cancer cells and bone marrow which results in production of factors that promote proliferation and survival. Investigations carried out to study these interactions pointed out that tumor cells in bone marrow can remain dormant for an undefined period of time lasting up to months and years. At the end of this stage, the cancer cells become active and form metastases. There is a need to understand these mechanisms on a real-time basis. In order to accomplish this, animal models of bone metastasis are used. The animal models used for investigation of bone metastasis need to be clinically suitable to mimic the events that occur in human bone metastasis. The results obtained from these model organisms also need to be consistent, so that these results can be used for designing therapeutic regimes. Use of model organisms also gives us the benefit of determining the toxicity profile of the therapeutic agents.

Keywords

Bone metastasis · Osteoblast · Osteoclast · Theraputic intervention

Introduction

Cancer is one of the leading causes of death around the world. The major contributor to cancer-related mortality is metastasis. Metastasis enables tumor cells to spread to other parts of the body to form secondary cancers called metastases. Theses metastases hinder the activity of organs, resulting in death of the patient. Bone metastases are most prevalent in cancer patients and are often observed in cases of breast, prostate, and lung cancer (Simmons et al. 2015). Bone metastases are mainly characterized by either increase in osteolytic or osteoblastic activity. Osteolytic activity is mediated by osteoclast cells which promote bone break down. These metastases display "punched out" lesions due to focal bone destruction. These types of lesions are observed in patients of lung cancer. On the other hand, bone metastases with increased osteoblastic activity are promoted by osteoblast cells that carry out bone formation. Theses lesions appear as dense osteosclerotic lesions and are seen in patients suffering from prostate cancer. Finally, even if one type of activity appears to be dominant, the activities of both the types of cells appear to be sped up in bone metastasis. This leads to formation of "mixed" lesions which are prevalent in case of metastatic breast cancer (Coleman et al. 2020). Irrespective of the type, bone metastases affect bone functioning adversely. The issues associated with bone metastases are collectively termed as skeletal-related events (SREs)

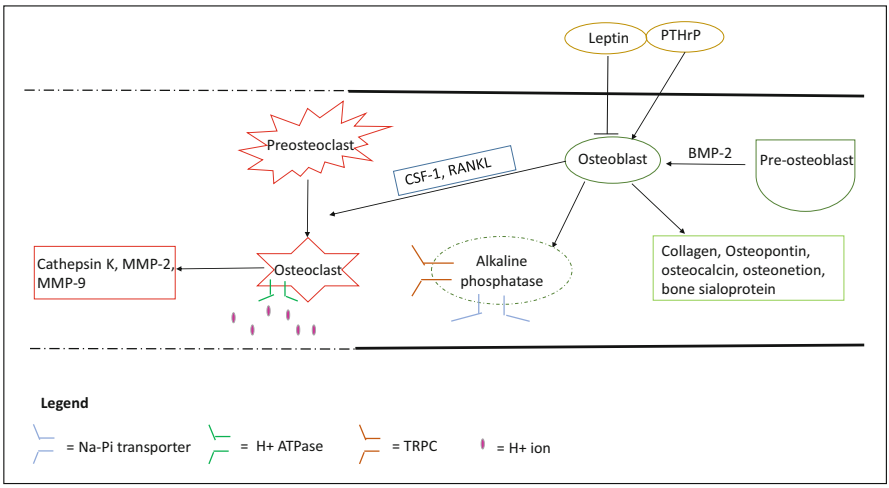

Fig. 1 Bone remodeling is tightly regulated by the action of osteoblasts and osteoclasts. Osteoblasts promote formation of bone; on the other hand, osteoclasts promote breakdown of bones. The function of osteoblasts is carried out with the help extracellular matrix proteins such as collagen, osteocalcin, and osteonectin, and bone sialoprotein, as well as vesicles with Na-Pi transporter and TRPC. On the other hand, osteoclast break down bone by decreasing the pH and secreting cathepsin and metalloproteinases

(von Moos et al. 2019). The major issues faced by patients suffering from bone metastasis are pathological fracture, bone pain, spinal compression, and hypercalcemia (Coleman et al. 2020). The deleterious effects associated with bone metastasis greatly reduce patient life span. For instance, investigation of literature points out that the median survival after detection of bone metastases is approximately 1 year in case of lung cancer patient. On the hand, the average life span after detection in case of those suffering from breast cancer, prostate cancer, or multiple myeloma ranges from 3 to 5 years (Coleman 2006). Taking the widespread nature of bone metastases into account, investigation of methods to cure and arrest the spread of bone metastases in cancer patients is of paramount importance.

From the abovementioned brief introduction, it can be concluded that for the formation of bone metastases, impairment of functioning of cells that maintain bone is essential. The normal functioning of these cells is explained in the following section along with Fig. 1.

Remodeling of Bone Is a Tightly Regulated Process

Bone tissue may appear to be rigid, but it is undergoing a constant process of remodeling. This activity is carried out by the bone resorbing osteoclast cells and the bone forming osteoblast. The osteoblast cells play an important role in the formation of bone. In this context, the differentiated osteoblast cell acts as the hub

of production of a variety of extracellular matrix proteins like collagen, osteocalcin, and osteonectin, and bone sialoprotein. These proteins help in the formation of bone matrix (Mandal 2015). Moreover, vesicles containing alkaline phosphatase, Na-Pi transporter, and calcium ion channel TRPC (transient receptor potential cation channel) pinch off from osteoblast cell membrane. The Na-Pi transporters and calcium ion channels ensure inward movement of calcium and phosphate ions. Moreover, alkaline phosphatase breaks organic phosphate to supply phosphate. The calcium phosphate so formed is unloaded on the collagen fibrils formed by osteoblast cells. This process hardens the soft bone (Sharma et al. 2016).

The osteoblast cells also secrete a variety of osteoclastogenic factors like colony stimulating factor 1 (CSF-1) and RANKL, which promote the differentiation of pre-osteoclasts into mature osteoclasts. The osteoclast cells drive the resorption of bone by lowering the pH of the extracellular matrix via H^+ -ATPase (Kenkre and Bassett 2018). This promotes the disintegration of bone cementing material. Finally, osteoclast cells also secrete proteolytic enzymes like collagenases (cathepsin K) and matrix metalloproteinases (MMP-2, MMP-9) that facilitate the breakdown of bone matrix proteins (Mandal 2015). Other than the osteoblast and osteoclast cells, the bone remodeling process also falls under hormonal regulation. In the course of the literature survey, many instances were seen such as leptin secreted by adipose tissue which prevents osteoblast differentiation (Ducy et al. 2000). On the other hand, parathyroid hormone-related protein (PTHrP) promotes osteoblastogenesis (Zuo et al. 2012).

Bone morphogenetic proteins (BMPs) are an essential regulator of bone remodeling. They play an important role in processes such as differentiation of osteoblasts, activity of osteoclasts, and bone metastasis. BMPs are a part of the family of TGFβ proteins. Their various activities are carried out by both Smad dependent and independent pathways (Chen et al. 2004). In the context of bone remodeling, BMPs are involved in various activities such as elevation of activity of alkaline phosphatase activity, osteoblast mineralization, and differentiation (Mandal et al. 2010, 2016). On the other hand, TGFβ are only involved in positive regulation of osteoblast proliferation (Chen et al. 2012). The bone formation function of BMPs is carried out with the help of transcription factors like Runx2/Cbfa1, Osterix (Osx), NFATc1, and Msx2 (Mandal et al. 2010, 2016). Evidence from the literature points out that NFARc1 is an important regulator of bone remodeling as an increase in expression of NFATc1 in osteoblasts promotes both osteoblast differentiation and osteoclast activity (Koga et al. 2005). The analysis of available literature points out that activation of P13K/Akt signaling by BMP-2 promotes osterix, NFATC1 and NOX4 medited osteoblast differentiation (Mandal et al. 2010, 2011). This was validated by the observation that suppression of PI3K/Akt signaling stops BMP2-mediated expression of NFATc1 and osterix. This, in turn, downregulates osteoblast and osteoclast activity (Mandal et al. 2009, 2010).

Thus from literature surveys, an inference can be drawn that bone remodeling is regulated by systemic as well as local environmental factors. The loss of this tight regulation of osteoclast and osteoblast activity is an essential event in the process of bone metastasis.

Formation of Bone Metastases in Different Types of Cancer

Bone Metastases in Breast Cancer

Breakdown of bone due to bone metastasis is one of the prominent issues faced by breast cancer patients. The investigation of literature points out that formation of bone metastases is mediated by interactions between cancer cells and bone resident cells like osteoblasts, osteoclasts, and osteocytes (Coniglio 2018). A variety of factors such as PTHrP, prostaglandin E2 (PGE2), TNFα, interleukins (IL-8, IL-6, IL-1, IL-11), MCP-1, CSF-11, RANKL, Jagged 1, and BMPs are released by cancer cells (Yamaguchi et al. 2014; Sethi et al. 2011). These factors take part in variety of functions such as RANKL, Jagged 1, BMPs, and IL-11 upregulate osteoclast activity (Coniglio 2018). Similarly, factors such as PTHrP, PGE2, IL-6, and IL-1 influence the functioning of osteoblasts in such a manner that they secrete osteoclastogenic factors like RANKL and CSF-1, upregulating the activity of osteoclast (Sethi et al. 2011).

Finally, literature study shows that FOX2 promotes BMP4/Smad1 signaling leading to epithelial to osteomimicry transition (EOT) (Wang et al. 2019a). This observation was further validated in an investigation where it was found that BMP-2 could upregulate the osteblast-like ability of cancer cells (Sharma et al. 2020). BMP-2 also elevates the expression of CSF-1 in osteoblast cells, which in turn, promotes osteoblast-mediated osteoclast activity (Mandal et al. 2009). Thus, the abovementioned instances illustrate the fact that TGFβ and BMPs are important drivers of bone metastasis.

Bone Metastases in Lung Cancer

Bone metastases present in lung cancer patients are mostly osteolytic. From a literature survey it was found that in lung cancer, bone metastases are associated with elevation in the level of factors like collagen fragments and bone alkaline phosphatase, tumor-derived microRNAs, Dickkopf-related protein 1 (DKK1), and insulin-like growth factor-binding protein 3 (IGFBP3). The elevation in the level of these factors leads to poor outcome for lung cancer patients (Lang et al. 2018). Lung cancer-mediated destruction of bone is also carried out by expression of factors like parathyroid hormone-related protein (PTHrP), IL-11, and downstream mediators of TGFβ (Vicent et al. 2008). Finally, in the course of the literature survey a study was found which defined the relationship between serum concentration of DKK1 and formation of bone metastases. It was observed that non-small cell lung cancer patients with bone metastases had increased concentrations of serum DKK1. The concentration of DKK1 in these patients was much more elevated compared to those without bone metastases (Chu et al. 2014). Finally, the role of DKK1 in promoting the breakdown of bones was determined with the help of an investigation in which it was seen that DKK1 impairs the activity of osteoblast. This was accomplished by

DKK1 by inhibiting β-catenin and Runt-related transcription factor 2 (RUNX2) in small-cell lung cancer (Pang et al. 2017).

Poor outcome in case of lung cancer is also related to increased expression of disintegrins and metalloproteinases (ADAMs). The role of ADAMs in bone metastasis was shown by an investigation in which it was found that truncated forms of ADAM8 elevate the expression of IL-8 and IL-6. It also promotes osteolytic bone metastases in mouse models (Hernández et al. 2010).

Bone Metastases in Prostate Cancer

Prostate cancer cells have an unusual ability of initiate new bone formation. From literature survey, an inference can be drawn that activation of fibroblast growth factor and Wnt signaling is essential for this process. The activation of fibroblast growth factor is mediated by tumor-derived endothelin 1 (Wan et al. 2014). A survey of literature also points out that hematopoietic stem cells (HSCs) are removed from their niche by prostate cancer cells in order to initiate metastasis (Shiozawa et al. 2011).

The process of bone metastasis in prostate cancer can be explained by the osteomimicry hypothesis. In accordance to this hypothesis, it has been observed that endothelial cells obtained from mouse prostate cancer express hematopoietic stem cell and mesenchymal stem cell markers. These endothelial cells in turn differentiate to form cartilage and bone tissues. This process is accompanied by elevation in the levels of a chondrocytic marker, sry-box transcription factor 9 (SOX9) and osteoblast marker, and osteocalcin (Dudley et al. 2008). These endothelial cells also facilitate the creation of blood vessels which help in tumor intravasation. These prostate cancer-associated endothelial cells are also capable of undergoing mesenchymal-like transition (Lin et al. 2017).

Bone Disease Observed in Multiple Myeloma

Multiple myeloma-associated bone disease is a condition in which plasma cells in the bone marrow become malignant. Bone lesions are commonly observed, which are osteolytic in nature with characteristic bone loss or osteoporosis. Myeloid cells are localized in a niche on the endosteal bone surface (Lawson et al. 2015). These cells enter a long duration dormant condition. This state is imposed via the activation of a unique myeloid gene signature (Khoo et al. 2019). The major event that characterizes advancement of diseased condition is the breaking of dormancy by a portion of myeloma cells. These cells form rapidly growing myeloma colonies. These colonies in turn introduce modifications into the bone microenvironment (Croucher et al. 2016).

Myeloma cells elevate the expression levels of an important osteoclastogenic factor, RANKL, and suppress the expression of RNKL decoy receptor, osteoprotegerin (Pearse et al. 2001). Literature survey has pointed out that

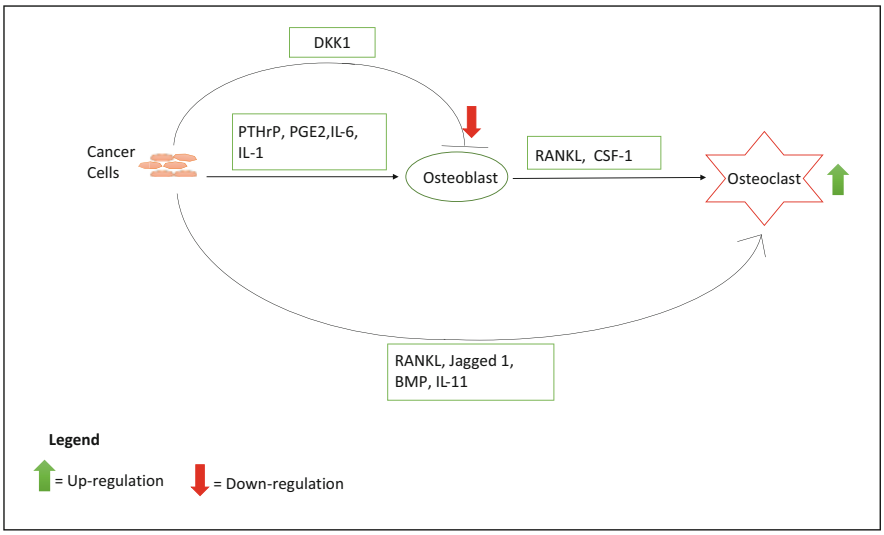

Fig. 2 A schematic representation of factors secreted by cancer cells that disturb the balance required for bone remodeling. These factors shift the balance in the direction of upregulation of osteoclast activity (breast cancer) and downregulation of osteoblast activity (lung cancer), resulting in osteolytic lesions

RANKL promotes osteoclastic bone resorption and formation of lytic bone lesions. In this context, in one study, an association between serum levels of RANKL and serum levels of osteoprotegerin in myeloma patients was investigated. It was observed that elevation of serum levels of RANKL is linked with suppression of levels of osteoprotegerin. This results in increased osteolysis (Terpos et al. 2003). The role of RANKL in breakdown of bone was further validated by another investigation in which its activity was impaired. It was found that suppression of RANKL activity resulted in preventing osteoclast formation. It also impaired the formation of osteolytic bone metastases and decreased the burden of myeloma (Croucher et al. 2001).

In order to develop further understanding of bone metastasis, further investigation needs to be done in an in vivo system. In the following sections, techniques of tumor generation in animals facilitating analysis of bone metastasis are discussed (Fig. 2).

Techniques to Induce Bone Metastasis in Model Organism

The need to develop techniques to induce metastatic cancers in animals arose from the observation that tumors that naturally develop in animals do not follow the sequence of events observed in case of human disease. For instance, mammary tumors that develop instantaneously in mice and rats do not undergo metastasis and display very little local invasion (Seely and Boorman 1999). This fueled research

into development of procedures that would lead to generation of tumors in animals that more closely resembled tumors observed in human.

Systemic Dissipation of Tumor Cells

One of the methods used to develop tumors in animals is via systemic dissipation of tumor cells. The advantage of this method is that it helps to analyze each step starting from the dispersal of cancer cells in circulation till the establishment of tumors at secondary sites (Lelekakis et al. 1999). One of the ways to carry out systemic dissipation of tumor cells is by intracardiac injection into the left ventricle (Fig. 3). This leads to systemic arterial circulatory dispersal of tumor cells, causing metastases at multiple locations. This procedure was utilized by Arguello and colleagues to generate a mouse model in which metastasis was consistently observed in the skeletal vertebrae and lone bone. For this purpose, they performed intracardiac injection of B16 (subG3.26) murine melanoma cells (Arguello et al. 1988).

Systemic distribution of tumor cells can also be carried out by tail vein injection, leading to hematogenous spread of cancer cells (Fig. 3). However, it is possible that the pulmonary system may filter and clear the cells (Wetterwald et al. 2002). Passineau and colleagues used this method to develop a synergistic mouse model to simulate the effects of dispersal of B cell lymphoma. They injected A20 murine lymphoma cells into tail vein leading to bony deposits on the femur, pelvis, and

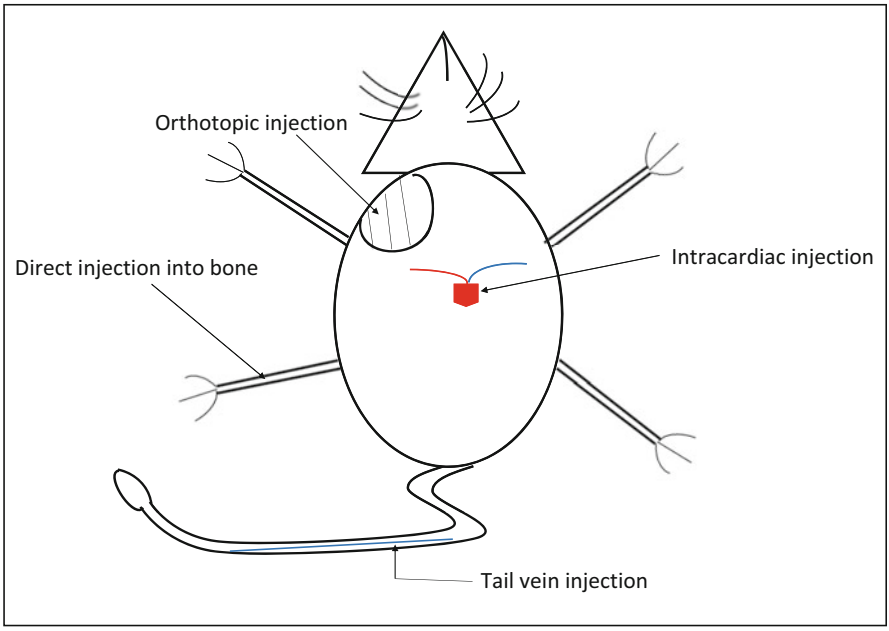

Fig. 3 Representation of the various techniques to generate tumors in animal models

vertebral column. Finally, this caused loss of bone and nerve compression (Passineau et al. 2005). The major disadvantages of this procedure are there is no means to ensure reproducibility of site and time of generation of metastatic deposition. There is also great variability in the number and sizes of metastases (Rosol et al. 2003). Excess tumor growth can also cause early death of experimental animals, hindering the study of prospective drug candidates.

Orthotopic Injection into Primary Tumor Site

Tumors can also be generated in animals by direct injection of tumor cells into primary tumor sites (Fig. 3). This procedure allows for complete investigation of the entire metastasis process starting from development of tumor at primary site. This method was used by Schubert and colleagues to establish a mouse model of breast cancer metastasis in which metastases were formed in the femur and lung. For this purpose, they injected MDA-MB-435 human breast cancer cells into mammary fat pad of nude mice (Schubert et al. 2011). This model enabled them to throw light on therapeutic effects of application of gonadotropin-releasing hormone (GnRH) analogs. These analogs were able to suppress formation and growth of metastases.

Direct Injection into Bone

In order to produce bone metastases, direct injection cancer cells into bone tissue of interest can also be done (Fig. 3). This helps in generating consistent as well as site-specific metastases. Direct injection of cancer cells into bone mass of interest does evade the entire process of metastasis, but it allows for extensive analysis of site-specific interactions between tumor and bone tissue. Choi and colleagues used this technique to inject VX2 carcinoma cells into the tibia of rabbits. This model was utilized for magnetic imaging (MRI) and histopathologic investigation of sequence of events taking place in the development of bone metastases (Choi et al. 2008).

Transgenic and Chemical Induction

Other methods of develop tumors include generation of tumors by using chemical agents and by transgenic manipulations. For instance, mammary neoplasia in rats can be developed by application of agents such as dimethylbenzanthracene, MNU, and N-ethyl-N-nitrosourea (ENU) (Ip 1996). Application of EMU promoted the development of adenocarcinoma in Sprague-Dawley rats. These rats were characterized by mild hypercalcemia. However, there were no occurrence of spontaneous bone metastases (Stoica et al. 1984). On the other hand, specific promoters can be used to generated tumors in mammary and prostate glands. For instance, whey acidic protein, C(3)1, and MMTV promoters can generate tumors in mammary gland. On the other hand, probasin, C(3)1, and PSA promoters can develop prostate-specific

tumors (Rosol et al. 2003). In spite of being consistent in the development of tumors in organs of interest, these methods display very low levels of occurrence of bone metastases. This phenomenon can be attributed to the rapid advancement of the primary tumor.

Animal Models of Bone Metastasis

Rodent Models of Bone Metastases

Of all the available animal models for investigation of bone metastases, rodent is the most widely used (Singh and Figg 2005). Rodents provide a variety of advantages. From a physiological point of view, there is a high level of genetic homology between human and rodent, and the anatomical location of organs is also identical. On the other hand, from a technical point of view, rodents are readily available, easy to handle and maintain, and comparatively cheap. They also provide us with a plethora of ways to maneuver cancer pathways via creation of knockout, transgenic, or over-expressing strains (Cossigny and Quan 2012). The versatility of the rodent model is validated by the fact that there are two immunodeficient strains available to create xenograft models. These two models are *Balb/c* athymic mice and severe combined immunodeficiency (SCID) mice. These strains of mice give us the means to investigate human cancer cell lines. Since SCID mice are more immunodeficient compared to nude mice, it has been observed that in case of some cancer cell lines, more rapid growth and greater occurrence of metastases is seen SCID mice (Taghian et al. 1993). In the course of literature survey, various examples were seen that validate the role of immunodeficient animal models in cancer. This is illustrated by a study carried out by an investigation carried out by Lefley and colleagues in 2019. For the purpose of their study, they used SCID mice bearing human bone implants. They also introduced xenografts of breast cancer patients into these mice. It was seen that for bone metastasis expression of IL-1B, IL-1R1, S100 calcium binding protein A4 (S100A4), cathepsin K (CTSK), secreted phosphoprotein 1 (SPP1), and RANK was affected in primary tumors. It was seen that downregulation of IL-1B was essential for preventing bone metastasis (Lefley et al. 2019) (Table 1).

In spite of the versatile applications of immune-deficient mice model, a major disadvantage of this process is that it excludes the role of immune system. However, this defect can be averted using syngeneic models. In syngeneic model, cancer cell lines and the host are of same species. In this context, Morony and colleagues created a model to mouse model of adenocarcinoma metastases to the bone. For this purpose, they used mouse colon adenocarcinoma (Colon-26) cells. These cells were injected into left ventricle of mice. This model was used to determine the efficacy of recombinant osteoprotegerin (OPG). OPG was able to remove lytic bone lesions in a dose-dependent manner (Morony et al. 2001) (Table 1). Finally, one more disadvantage associated with the use of rodent cancer model is the small size of

Table 1 Animal models used in bone metastasis

Model organism	Type of cancer	Role in cancer
SCID mice	Breast cancer	Xenografts from breast cancer patients were implanted in SCID mice bearing human bone implants. Suppression of IL-1B was essential for preventing bone metastasis (Lefley et al. 2019)
Syngeneic mice	Adenocarcinoma	Mouse colon adenocarcinoma cells were used to create syngeneic mice model to determine efficacy of OPG. Increasing the dosage of OPG was more effective in removal of lytic bone lesions (Morony et al. 2001)
Rabbit	VX2 carcinoma	Scaffold bearing n-HA was implanted into tumor-bearing rabbit with bone defect. The n-HA was anti-tumorigenic and also prevented osteolytic lesions (Zhang et al. 2019)
Cat	Pulmonary squamous cell carcinoma, pulmonary carcinoma	Development of spontaneous tumors resulted in painful digits due to carcinoma in digits (van der Linde-Sipman and van den Ingh 2000)
Dog	Prostate cancer	Orthotopic grafting of DPC-1 cells into dog prostate led to formation of mixed osteoblastic and osteolytic metastases (Anidjar et al. 2012)

bone. The miniscule size of rodent bone acts as a major roadblock in their use for development of therapeutic strategies for local drug delivery devices and invasive image-guided therapies (Tatsui et al. 2009).

Rabbit Model of Bone Metastases

Rabbits are also utilized as a model organism in cancer research. Rabbits provide a variety of advantages such as rapid growth, consistent generation of tumor by orthotropic allograft implantation, ease of propagation in skeletal muscle, capacity to graft donor tumors into many recipient rabbits, innate hypervascularity, and more detailed vascularity compared to rodents (Obeid 2018). Other than the abovementioned advantages, rabbits are more suitable to carry out surgical procedures and preclinical testing of skeletal metastases. However, the unavailability of immune-deficient rabbits restricts their use to species-specific cancer cell lines (Amundson et al. 2005). For utilization of rabbit in cancer research, VX2 carcinoma was obtained from a Shope papilloma virus-induced neoplasm in a domestic rabbit (Rosol et al. 2003). The role of rabbit as a model organism to carry out investigation on bone metastasis is illustrated by the work of Zhang and colleagues. In their work, they observed that hydroxyapatite nanoparticles (n-HA) upon implantation in tumor-bearing rabbit displayed anti-tumorigenic activity and also prevented osteolytic lesions (Zhang et al. 2019) (Table 1).

Cat and Dog as a Model of Bone Metastases

Other than rabbits and rodents, larger animals such as cats and dogs have also been used as model organisms in cancer research. The tumors that are generated in rodents are spontaneous in nature. Cats mostly develop invasive adenocarcinomas or ductal carcinomas. Bone metastases are rare occurrence in case of cats (Rosol et al. 2003). As early as 1998, an investigation was carried out to examine bone metastasis in 64 cats. All the animals in this study were found to experience painful digits. Of these, eight animals displayed primary squamous cell carcinoma. It was observed that these carcinomas occupied one digit or two digits of one leg. On the other hand, 56 animals displayed metastases of pulmonary carcinoma in digits. It was found that these carcinomas occupied multiple digits in different legs (van der Linde-Sipman and van den Ingh 2000) (Table 1).

As is the case with cats, tumors that develop in dogs are spontaneous in nature. Most of the times the tumors observed in dogs are hyperplasia, ductal carcinoma in situ, complex neoplasms with epithelial and myoepithelial components, and mixed neoplasms with cartilaginous and osseous differentiation of myoepithelial cells. However, formation of bone metastases is a rare occurrence in case of dogs (Rosol et al. 2003). Even still, Anidjar and colleagues used dogs as model organisms for the study of prostate cancer. For their investigation, they carried out orthotopic grafting of dog prostate cancer (DPC)-1 cells into prostate of 12 dogs. Out of 12 dogs, bone metastases were observed in case of two dogs. These metastases were of mixed osteoblastic and osteolytic nature (Anidjar et al. 2012). However, this observation is complicated by the fact that normal dog prostate tissue bears the capacity to form new bone in vivo (Rosol et al. 2003).

Conclusion

From the analysis of literature regarding the various animal models, an inference can be drawn that, in spite of their advantages, there is still room for further development. The ability to develop immunodeficient strains in larger animals will be a significant step in this direction. Moreover, the ability to carry out syngeneic grafting in larger animals will also be of great help.

Imaging Techniques to Investigate Bone Metastasis

Real-time imaging is an essential aspect of utilization of model organisms in cancer research. It helps us in detection, quantification, and allows us to observe the response to therapeutic agents (Rosol et al. 2003). The most frequently used imaging techniques for monitoring bone metastases include bioluminescent and fluorescent imaging, high-resolution radiography, micro-computed tomography, positron emission tomography, and single-photon emission tomography. Micro-magnetic resonance imaging (MRI) is also used; however, its use is less common (Simmons et al. 2015).

Bioluminescent Imaging of Bone Metastases

In order to carry out bioluminescent imaging, expression vector containing luciferase or other light-producing enzymes are used to transfect cancer cell lines. For the purpose of production of bioluminescence, cellular adenosine triphosphate (ATP) is needed. One advantage that can be attributed to this process is that this ensures detection of only live cancer cells, helping real-time visualization of neoplastic cells (Simmons et al. 2015). In order to initiate the activity of luciferase enzyme, the substrate of luciferase, luciferin is injected. Image is obtained with the help of a cryogenically cooled, intensified charge-coupled device (CCD camera) (Karam et al. 2003). There are many advantages of using bioluminescent imaging. It is sensitive and has good signal-to-noise ratio. The level of expression of luciferase determines the sensitivity. The presence of cells with elevated levels of luciferase expression which ensures that microtumors with total cell count lower than a few hundred cells are easily visualized. Imaging process and throughput is pretty quick, with instruments having the capacity to image as many as five mice in one go. Finally, time taken for complete imaging extends up to 60 s. One of the disadvantages of using bioluminescent imaging is that its use is restricted to only live animals and cells. Bioluminescent imaging of dead tissue can be done if it is carried out immediately after euthanasia (Simmons et al. 2015).

Fluorescent Imaging of Bone Metastases

It is identical to bioluminescent imaging in use; however, it has the advantage of a better spatial resolution. It also provides background fluorescence which accounts for decreased signal-to-noise ratio. The various sources of background resistance are bone, feed material on the skin or gastrointestinal tract, bedding on skin, and urine/feces on the hair or skin. For the purpose of imaging, near-infrared dyes are most suitable. The reason for using near-infrared dyes is that red light can easily pass through tissues and bones, and the background fluorescence is reduced to a greater extent. Finally, a light source and a filter ensure the separation of excited and emitted light, which are essential to carry out fluorescent imaging (Simmons et al. 2015).

High-Resolution Radiography of Bone Metastases

High-resolution radiography is suitable for skeletal imaging of osteolytic metastases in small animals. It has a resolution of up to 80 line pairs (1p)/mm (Simmons et al. 2015). The advantage of using radiography is that it is cheap and rapid. It can also be done both in vivo and ex vivo. However, there are a few limitations to the usage of radiography. It is unable to detect small bone metastases and metastases with very little break down of bone. It is also challenging to identify intramedullary bone formation using radiography (Simmons et al. 2015).

Micro-Computed Tomography of Bone Metastases

Micro-computed tomography (μCT) is an imaging procedure that enables us to get and analyze three-dimensional structure of bone at a micrometer resolution. This can be carried out in both in vivo and ex vivo (Mizutani and Suzuki 2012). There are many advantages of using μCT. It provides us with an increased value of spatial resolution. This property makes it ideal to carry out investigations in rodents. It has an in vivo resolution of 50–100 microns. On the other hand, it provides an ex vivo resolution of less than or equal to 20 microns (Koba et al. 2013). μCT also provides us information regarding bone mineral density, trabecular number and thickness, and bone surface area. Finally, with the help of finite element analysis, μCT data can also provide us with information regarding biomechanical data of bone, and also the effect of bone metastases on it (Simmons et al. 2015). However, there are a few limitations to the usage of μCT. The cost of μCT equipment is very high. Similarly, there is high price involved in its usage, software, and technician time as well. Its operation requires training. Finally, its processing speed is much slower compared to bioluminescent imaging.

Micro-Positron Emission Tomography and Micro-Single-Photon Emission Tomography

Micro-positron emission tomography (μPET) and micro-single-photon emission tomography (μSPECT) are two forms of nuclear imaging which enables us to get a three-dimensional image of the distribution of a radiopharmaceutical (Jang 2013). These techniques work by radiolabeling molecular markers. The resolution in these procedures lies between radiography and bioluminescent imaging (Simmons et al. 2015). The most frequently used marker in PET to identify tumors is ^{18}F-fluorodeoxyglucose (FDG) (Koba et al. 2013). Other than FDG, novel radiopharmaceuticals like very late antigen-4- and αβ3 integrin-targeted radiotracers are also used. These enable us to acquire images of intramedullary or osteoclastic bone metastases with greater sensitivity and selectivity (Soodgupta et al. 2013; Zheleznyak et al. 2012). There are certain limitations to the usage of PET compared to SPECT. In comparison to SPECT, PET is much more expensive and more widely available. Similarly, the tracers used in SPECT have a much longer half-life compared to tracers used in PET. The low half-life of PET tracers restricts its usage (Koba et al. 2013).

Therapeutic Interventions to Prevent Bone Metastasis

After lung and liver, bone is the site where metastases are most frequently observed. The process of formation of bone metastases is in turn driven by two of the most prevalent forms of cancer, i.e., breast and prostate cancer (Coleman 2001). These facts are the major driving force in the direction of development of the therapeutic agents that can prevent the activity of osteoclast activity as well as halt the spread of

Table 2 Therapeutic agents used in treatment of bone metastasis

Therapeutic agent	Mechanism of action	Adverse effects
Radiotherapy	Radiation treatment reduces tumor burden and provides relief from pain (Goblirsch et al. 2004)	Skin irritation and reaction, nausea or diarrhea, esophagitis, transient increase in bone pain (Johnstone and Lutz 2014)
Radiopharmaceuticals	Attach to areas of increased osteoblastic activity, radiation causes death in nearby cells, decreasing intraosseous mass (Ferreira et al. 2012)	Moderate hematologic toxicity, mild myelotoxicity (Ferreira et al. 2012)
Pamidronate	Inhibits binding of osteoclast precursor to hydroxyapatite crystals of bone matrix, suppresses action of mature osteoclast, initiate osteoclast apoptosis and prevents osteoblast-promoted osteoclast activation (Coukell and Markham 1998)	Hypocalcemia leading to secondary hyperparathyroidism, acute phase response, musculoskeletal pain, ocular events, and osteonecrosis of jaw (Papapetrou 2009)
Zoledronic acid	Causes suppression of FPPS activity and ApppI production and accumulation resulting in osteoclast apoptosis (Räikkönen et al. 2009)	Bone pain, insomnia, constipation, pyrexia (Chiang et al. 2013)
Ibandronate	Targets components of mevalonate pathway farnesyl diphosphate synthase, farnesyl diphosphate, and geranylgeranyl diphosphate (Cao et al. 2008)	Acute phase reaction (Diel 2010)
Denosumab	Obstructs the attachment of RANKL with RANK, repressing osteoclast activity and survival (Baron et al. 2011)	Osteonecrosis of the jaw observed in patients treated for bone metastases (Steger and Bartsch 2011)

FPPS farnesyl pyrophosphate synthase, *ApppI* ATP analog triphosphoric acid 1-adenosin-5-yl ester3-(3-methylbut-3-enyl) ester

bone metastases. In this context, a variety of treatment strategies were developed which will be explained in detail in the following sections as well as described in Table 2.

Radiotherapy in Treatment of Bone Metastases

Radiotherapy is recommended for patients that complain of pain and spinal cord compressions (Table 2). It is also advised for patients if there is chance of pathological fracture. For the purpose of treatment, external beam therapy can be applied. This treatment can be done via two ways, i.e., either fractionated routine or single fraction routine (Selvaggi and Scagliotti 2005). After conducting series of random

trials, it was found that the efficacy of single fraction routine was as good as that of fractionated routines (Nielsen 1999). The single fraction routine also provided the advantage that it was more suitable for patients as well as hospitals.

Radiopharmaceuticals in Treatment of Bone Metastases

Radiopharmaceuticals are also utilized for alleviation of bone pain (Table 2). Since there is a danger of exposure of healthy bones to radioisotopes, size of the tumor and characteristics of the radioisotope are also taken into consideration (Selvaggi and Scagliotti 2005). Of all the available Food and Drug Administration (FDA) approved radiopharmaceuticals, only ^{89}Sr ethylenediaminetetramethylene phosphonate (EDTMP) and ^{153}EDTMP display affinity towards bone (Maini et al. 2004). The effectiveness of ^{153}Sm in alleviation of bone pain was illustrated in a study in which it was used along with gamma and beta emitter with half-life of 2 days. It was found that 60–70% patients experienced pain relief after 1 month of treatment. This pain relief lasted for 2–4 months (Resche et al. 1997).

Bisphosphonates in Treatment of Bone Metastases

Bisphosphonates (BPs) prevent the activity of osteoclasts. The basic structure of bisphosphonate constitutes of a pyrophosphate with a phosphorus-carbon-phosphorus (P-C-P) central structure. The central structure enables the attachment to mineralized bone matrix. It also contains a variable $R^{/}$ chain that involved in providing potency, side effects, and mechanism of action (Fulfaro et al. 1998). From a functional point of view, all BPs are antiresorptive drugs. They work by initiating apoptosis of osteoclast. The analysis of available literature pointed out that bisphosphonates prefer to attach to hydroxyapatite crystals in bone matrix. They accumulate in high concentrations in the resorption lacunae. From here, they are taken in by osteoclasts, resulting in apoptosis (Rogers et al. 1999). Currently, the second and third generations of nitrogen containing BPs are in use. These include pamidronate, zoledronic acid, and ibandronate. The survey of literature points out that at the cellular level, these BPs act by targeting the mevalonate pathway. This is accomplished by impairing the activity of farnesyl diphosphate (FDP) synthase. This prevents the prenylation of small GTPase proteins, which results in apoptosis (Dionísio et al. 2019).

Pamidronate

It is most frequently used by patients who have metastatic breast cancer (Table 2). The established regimen for this disease constitutes of IV infusion of 90 mg every 3–4 weeks. This resulted in appreciable suppression of incidence. It also slowed down the progression of appearance of bone-related issues (Theriault et al. 1999; Hortobagyi et al. 1996). On the other hand, trials carried out in prostate cancer

revealed that it had no appreciable effect on reduction of pain in comparison to placebo (Small et al. 2003).

Zoledronic Acid

It was found to be effective in treatment of bone metastases in different types of cancers such as multiple myeloma, breast, kidney, and prostate cancer (Table 2) (Berenson 2005). In a phase III trial carried out along with pamidronate, it was found that over a period of 24 months, zoledronic acid was more efficient in halting the progress of SRE (Rosen et al. 2001). The toxic events experienced in this case constitute of bone pain, fever, fatigue, and nausea. None of the scenarios appear to affect the patient severely and can be taken care of. Other than these, renal toxicity was observed in 8% of patients (Selvaggi and Scagliotti 2005).

Ibandronate

It is administered via both IV and oral method (Table 2). The results from two phase III trials point out that 50 mg dosage was enough to appreciably decrease the duration of mean skeletal morbidity period. There was appreciable decrease in the number of scenarios that required radiotherapy and surgery. The observed toxic scenarios were not adverse in nature (Body et al. 2004).

Denosumab

Denosumab is a completely humanized monoclonal antibody that has shown the capacity to completely stop the activity of osteoclast in patients with solid tumor bone metastases and those with multiple myeloma (Table 2). It stops the activity of osteoclasts by inhibiting the action of RANKL (Baron et al. 2011). When randomized clinical trials to compare the efficacy of denosumab and zoledronate were carried out, it was found that when used in combination with standard cancer therapies, denosumab was more superior. It displayed greater efficacy in treatment of skeletal morbidity. In spite of these advantages, it was observed that the duration of progress of disease and overall survival in both the scenarios were similar (Stopeck et al. 2010).

Candidate Molecules with Potential to Act Anti-osteolytic

As it is very clearly visible from Table 2 that the long-term use of bisphosphonates leads to development of a variety of adverse effects in cancer patients. This has led search of novel candidate molecules bearing anti-osteolytic activity. In this context, the use of natural extracts, such as curcumin, benzyl isothiocyanate, and liensinine, for treatment of bone metastasis holds great promise (Mandal 2020). For instance, in an investigation carried out in a bone metastasis mouse model, it was found that curcuminoids are capable of downregulating TGFβ/Smad/PTHrP signaling (Wright et al. 2013).On the other hand, benzyl isothiocyanate and liensinine suppress the activity of RANKL (Kang et al. 2017; Pore et al. 2018).

In the course of investigation of the literature, it was found that groups of drugs can be "repurposed" for use in case of bone metastasis. This fact is validated by the use of statins in the treatment of bone metastasis. It is well known that the major function of statins is to suppress the activity of HMGCOA reductase to stop the synthesis of cholesterol. Observations like the increased level of cellular cholesterol in malignant and high metastatic breast cancer cells in comparison to benign and low-metastatic cancer cells point towards potential therapeutic effect of statins in preventing metastasis (Sharma et al. 2019). This possibility is validated by the observation that application of cholesterol-lowering simvastatin as well as cholesterol-depleting MBCD (methyl beta cyclodextrin) suppresses the expression of CSF-1 and RANKL in metastatic breast cancer cells. They also prevented breast cancer-mediated osteoclast activity by suppressing NFATc1/MMPs signaling (Chowdhury et al. 2017). Similar activity was also observed in case of pitavastatin (Wang et al. 2019b).

Another group of drugs with the potential to prevent bone metastasis is TGFβ inhibitors. In the course of survey through literature, it was found that a TGF β antibody developed by Mundy and colleagues was able to stop osteolytic bone metastasis. This was observed in an intracardiac injection model of breast cancer, via targeting of PTHrP signaling (Biswas et al. 2011). Another instance from the literature points out that the BMP inhibitor noggin inhibited BMP/Smad signaling, resulting in prevention of FOXF-2-mediated osteotropism of breast cancer cells (Wang et al. 2019a). Similarly, a combination of noggin and BMP receptor inhibitor (LDN-193189) prevented cancer cell migration and chemotaxis (Bellanger et al. 2017). Finally, ZL170, an oxindole extracted from cockroaches, acts as a suppressor of both TGFβ and BMP signaling. The analysis of literature shows that ZL170, when applied to triple negative breast cancer MDA-MB-231 cells, was capable of repressing cell migration, invasion, and EMT. Furthermore, investigation in animal model also showed that, its ability to prevent tumor growth and also osteolytic lesions (Di et al. 2019).

Conclusion

The analysis of literature points out that deregulation of the bone remodeling process lays the foundation of bone metastasis. This results in increase in activity of osteoclasts and decrease in osteoblast activity. Due to the vast number of factors involved in the development of bone metastasis, further investigation can only be done in a system where all of these factors can be analyzed together. This can be accomplished with the help of model organisms. The mouse model has been extensively used to investigate bone metastases. However due to its small size, it is not suitable to study all aspects of bone metastasis. This has led to research towards developing techniques to utilize bigger animals such as rabbits, cats, and dogs in the study of bone metastasis. But the unavailability of strains suitable for xenograft-based implantation of cancer tissue is the biggest hindrance in their use. Finally, the study of treatment strategies points out that many of them only target any one issue associated with bone metastases. However, this can be overcome by use of natural extracts which are anti-tumorigenic and also prevent bone metastasis. There

is a need to carry out further investigation into the effects of long-term use of natural extracts. This will help in overcoming the toxic effects of long-term use of bisphosphonates. Furthermore, bisphosphonates can also be used in combination with natural extracts which bring down their toxic effects. The use of animal models to test the effects of these combinations will help in their early utilization.

Limitations and Future Prospective

In the course of literature investigation, it can be inferred that bone metastasis occurs by deregulation of the process of bone remodeling. However, some important questions still remain unanswered. The sequence of events that lead to loss of dormancy and activation of cancer cells are yet to be determined. Development of techniques that allow us to manipulate spontaneous tumors in animals will help us in answering this question. Development of techniques to increase the application of cats and dogs in cancer research will bring us closer to human response. Animal models will help us to determine the long-term effects of usage of different combinations of drugs used. This would include determining the right time and the right route of application of drug.

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Available Preclinical Tools for Neuroblastoma

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Natarajan Aravindan and Sheeja Aravindan

Contents

Abstract

Neuroblastoma is the most predominant and deadly cancer in infants and in young children and contributes significantly to childhood cancer deaths. Despite the current intensive multimodal treatment strategies, the long-term survival of children who present with high-risk aggressive disease is unacceptable (<2%). After decades of intensive research, the genesis and the diversity in the disease process (spontaneous regression, favorable/therapy responsive, unfavorable high-risk disease) are thus far unrealized. Despite current intensive multimodal clinical

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therapy, children with high-risk disease display poor response and present with fatal progressive disease. Tumor heterogeneity and de novo acquisition of resistance endorse disease progression. Global efforts are in place to understand the tumorigenesis, disease process, complexity, evolution, and therapy response of NB and to identify new and improved therapy or therapeutic approaches to treat this deadly disease. Countless preclinical tools are developed and in the pipeline to underpin the biological events and to explore new and/or improved therapeutic options for neuroblastoma. Each model was favorably developed by considering unique experimental, objective, or disease (NB) variables or combinations, such as human NB-mimicking genetics, immune system, tumor features, ability to perform high-throughput and rapid experiments, preferential gene (s) manipulation, and real-time endpoint visualization. A better understanding of the available preclinical tools, their advantages and disadvantages, their reliability, and feasibility to be modified would allow us to exploit the system or develop new or improved clinically translatable models for neuroblastoma research. This chapter composes a list of preclinical neuroblastoma models and discusses their uniqueness, quality, and disadvantages. Since in vitro models generally require a second-step in vivo validation, this chapter is limited to the discussion of available in vivo tools for neuroblastoma research.

Keywords

Neuroblastoma · Mouse models of neuroblastoma · Zebrafish models · Spontaneous disease models · Xenografts · PDX

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in infants and heavily contributes to pediatric cancer deaths. With about 16,000 new cases (~800 in the USA) each year worldwide, NB occurs frequently in young children aged newborn to 4 years (median age of 2 years) and very rarely in children aged above 10 years. While most neuroblastomas are sporadic (not inherited), ~2% of cases present the familial, inherited form of NB. Although a number of risk factors, including neurocristopathies (e.g., Hirschsprung's disease, Klippel-Feil syndrome, Waardenburg's syndrome, Ondine's curse, Beckwith-Wiedemann syndrome, Cushing's syndrome), birth defects (e.g., congenital anomalies), fetal alcoholic syndrome, drugs taken by the mother (e.g., phenylhydantoin for seizure disorders), and mother's death before birth, are linked to sporadic NB, the definitive cause is undefined. NB is derived from a select subset of neural crest cells (NCCs) in the trunk region that show sympathoadrenal lineage (sympathoadrenal progenitors, SAPs) and lead to the formation of sympathetic ganglia and adrenal medulla. Mechanistically, SAPs undergo a Snail/Slug-dependent epithelial-to-mesenchymal transition (EMT) enabling their migration out of the neural tube, which makes them vulnerable to genomic alterations. Phox2b mutations in the neural/melanocyte

lineage-designated SAPs affect their differentiation into sympathetic ganglion, a predisposition for NB (Aravindan et al. 2019). Clinically, the disease progresses with hematogenous metastasis and frequent relapses (Santana et al. 2008; Simon et al. 2011). NB biology itself is characterized by somatically acquired genetic rearrangements leading to oncogene activation/addiction and tumor suppressor inactivation. Somatic amplification of proto-oncogene v-myc myelocytomatosis viral-related oncogene, neuroblastoma derived (MYCN), was observed in about 20–30% of the children with NB and serves as the reliable marker for progressive disease. Many other genetic rearrangements, including gain in chromosome 17q23 (in more than 50% of primary tumors) and loss in 1p36 (~30%), 11q23 (44%), and 14q23 (22%), have been linked to the clinical behavior of NB. Expression modifications in neurotrophins (and their receptors) associated with differentiation inhibition in neuroblasts could contribute to the malignant transformation.

The high level of complexity in the biology of the genesis, evolution, and treatment response of NB offers unprecedented uncertainties in the clinical management of this deadly disease. A better understanding of the spectrum of spontaneous regression, acceptable survival rates in patients with clinically favorable disease, and negligible <2% long-term survival in patients with high-risk disease displaying conditional acquisition of genetic/molecular rearrangements is needed. Three decades of intense research identified the requirement of a systematic, targeted multimodal management approach. In general, the current intensive multimodal clinical therapy (IMCT) includes the *induction phase*, an alternating regimens of high-dose chemotherapy (cyclophosphamide, cisplatin or carboplatin, vincristine, doxorubicin, etoposide, topotecan) and load reduction surgery; the *consolidation phase*, more intensive chemotherapy (carboplatin, etoposide, topotecan, busulfan and melphalan, thiotepa), along with radiotherapy (external beam RT, MIBG RT) and stem cell transplant (autologous bone marrow [BM] transplantation; peripheral blood stem cell reinfusion); and the *maintenance phase*, retinoid drug treatment (13-cis-retinoic acid, isotretinoin), immunotherapy (dinutuximab), and immune-activating cytokine (GM-CSF, IL-2) therapy. Despite this strategy, children with high-risk heterogeneous disease and poor hematological reserve have poor response. Progressive disease relapse after current clinical therapy is almost always fatal. Hence, an all-inclusive and in-depth understanding of the complexity of the disease (e.g., heterogeneity, spontaneous regression, disease progression); ongoing acquisitions of genetic/molecular rearrangements; unique cellular and intercellular events in disease evolution; cancer stemness and plasticity; and acquired therapy resistance is warranted to successfully treat this deadly disease.

A crucial step to achieving this understanding is to develop/design appropriate, cause-specific, clinically translatable preclinical tools that could be used to develop experimental designs that underpin the molecular/cellular events of NB genesis, growth, response, and evolution. Numerous preclinical tools have been developed, and others are in the pipeline for recognizing the biological events and to develop new/improved therapeutic options for NB (Fig. 1). Each model was favorably developed or improved by considering unique experimental, objective, or disease (NB) variables or combinations, such as human NB-mimicking genetics, immune

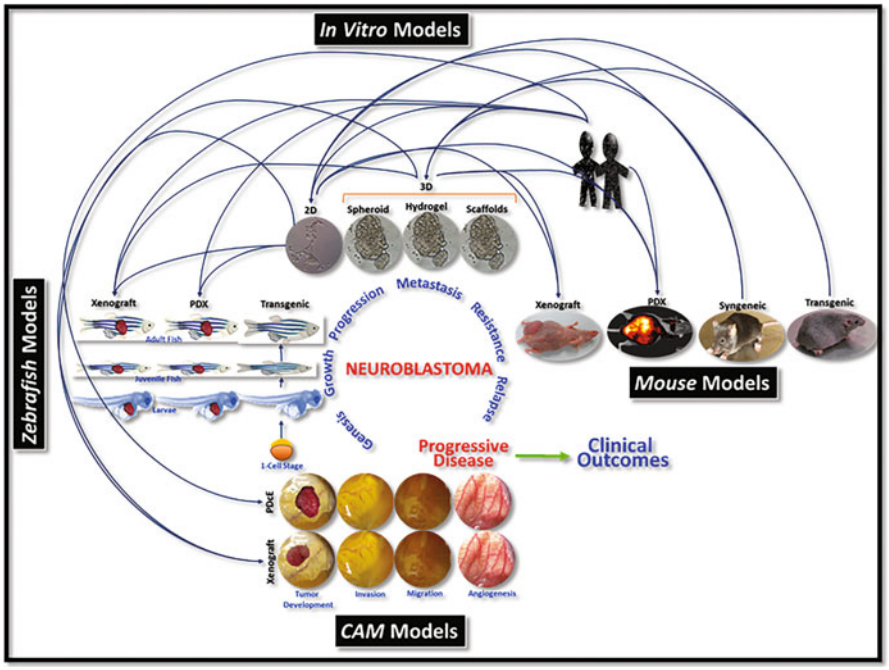

Fig. 1 Schema showing available preclinical tools for NB research and the brief overview on the flow through of workload in model development utilizing resources across platforms. The four major preclinical models that include *in vitro* cellular (2D and 3D), Chick chorioallantoic membrane (CAM), zebrafish, and mice are exclusively used in NB studies. Each model offers wide array of strategies, e.g., transgenic, PDX, and xenografts, for unique applications

system, tumor features, ability to perform high-throughput and rapid experiments, preferential gene(s) manipulation, and real-time endpoint visualization. A better understanding of the available preclinical tools, their advantages and disadvantages, their reliability, and their feasibility to be modified would allow us to exploit the system or to develop new or improved clinically translatable models for NB research. This chapter composes a list of preclinical neuroblastoma models and discusses their uniqueness, quality, crucial milestones reached, and shortcomings. Although in vitro NB models, especially the bed-to-bench reverse engineering studies, allowed researchers to gather critical information, these models often require a second-step in vivo validation. Hence, this chapter is limited to the discussion of the available in vivo preclinical tools for NB research.

Mouse Models of NB

Syngeneic Mouse Models of NB Syngeneic mouse models, otherwise termed allograft tumor models, are developed using tumor cells derived from the genetically

engineered mouse model (e.g., tyrosine hydroxylase [TH]-MYCN) that are introduced into mice of the same strain. Extensively characterized syngeneic mouse models of NB have existed for almost five decades (Finklestein et al. 1973). The transplantable (C1300 mouse NB cell line) model displayed spontaneous development of paraspinal region NBs that histologically (and in metabolizing catecholamines) resemble human NB, even mirroring occasional spontaneous regression. While these C1300 tumors are locally invasive and selectively spread to lymph nodes (LN), their sub-clone (TBJ) generates tumors and human NB-mimicking metastasis to the liver, adrenal glands, LN, and lung tissues. Another sub-clone of C1300, Neuro-2A, when injected into the retroperitoneal region, mirrors human disease as to localization, invasiveness, and disease dissemination to the bone marrow (BM). Developed NB in syngeneic A/J mice with Neuro-2a NB cells derived from the same syngeneic line was subjected to in situ transduction of adenoviral vector encoding both subunits of the murine IL-12 heterodimer (Davidoff et al. 1999). With this model, the authors recognized the immunomodulatory efficacy of IL-12 by showing its benefit in tumor regression, protective tumor immunity, and cytotoxic T lymphocyte (CTL) activity. The hybrid NXS2 cells (fusion of C1300 with murine dorsal root ganglion cells) with high dopamine secretion and high expression of GD2 consistently produced spontaneous metastasis in the liver, lung, and BM. Similarly, TH-MYCN tumors offered generation of hemizygous-, homozygous-dependent diverse cell types with various levels of MYCN expression and with different tumorigenicity potentials that were used to develop syngeneic tumors (Cheng et al. 2007). TH-MYCN mouse-derived cell lines preserved several human NB genetic features. Transplantation of these cells into the background strain (with matched, unaltered immune systems and stroma) designated this as the best clinical NB-representing syngeneic model with which to study immunotherapy.

The major limitations of the syngeneic mouse models are that they do not recapitulate human disease due to the lack of human NB-specific genetic alterations (e.g., gain of 17q23-qter; loss of 1p36, 11q23, and 14q23-qter) and inconsistency in tumor engraftment. Still, the syngeneic mouse model is one of the most useful models for understanding therapy efficiencies and, more importantly, for studying immune-mediated gene therapy. These models mirror human NB in low expression of class I antigens with the possibility of increasing expression with cytokines and the substantiated stimulation of cellular/humoral response. Syngeneic mouse models are advantageous in that the genetic mutations are confined to the transplanted tumor cells; the abundant availability of cells allows high-throughput screening. Further, it is feasible to use these models for in vitro genetic modification for signaling/functional studies, and use of the animals permits the inclusion of imaging markers for visualization. Since the tumors developed with syngeneic models are thoroughly vascularized, they perfectly suit the investigation of angiogenic components in NB development and antiangiogenic interventions.

Transgenic Mouse Models of Spontaneous NB The era of using the transgenic (Tg) mouse for NB research began in early 1990 with the establishment of a Tg line

with polyoma virus middle T-antigen delivery linked to thymidine kinase promoter (Aguzzi et al. 1990). The Tg line with selective expression in the neurons of the central nervous system (CNS) and peripheral nervous system (PNS) led to the rapid (within 3 months) generation of multiple NBs with striking site, histological, and molecular analogy to human NB (Aguzzi et al. 1990), identifying a novel tool for understanding NB pathogenesis. Interestingly, Skalnik and colleagues developed Tg mice with the simian virus 40 T-antigen gene (SV40-Tag) linked to that 91-kDa cytochrome b heavy chain (gp91-phox) gene that resulted in the development of NB selectively in the prostate gland in all males carrying the transgene (Skalnik et al. 1991). This model histologically and biochemically mimicked human NB, allowed researchers to identify a tissue-specific neuroectodermal cell origin, and hence provided a system in which to study NB.

Likewise, mice with SV40-Tag linked to olfactory marker protein (OMP) gene promoter develop NB in the nasal cavity that invades maxillary and cranial structures through the palate and cribriform plate of the ethmoid bone (Largent et al. 1993). Servenius and colleagues generated four independent Tg lines for SV40-Tag linked to OMP gene promoter. All resulted in the development of human-mimicking (histological, ultrastructural) MYCN non-amplified (MYCN-na) metastatic NB in four months (Servenius et al. 1994). This Tg system offers a unique platform to study the biology and evolution of MYCN-na metastatic disease that comprises nearly 70% of all high-risk disease patients. Further, work with this model indicated the possible function of OMP in the regulation of sympathetic neuron differentiation (Servenius et al. 1994). Similarly, tetracycline-inducible (tetracycline-responsive elements, TRE) Tg mice carrying the SV40-Tag with cytomegalovirus promoter displayed MYCN-expressing adrenal NBs with comparable (histology, neuron-specific enolase [ENO2] and chromogranin A expression, and elevated dopamine) human analogy (Iwakura et al. 2008). The inducible TRE-SV40 Tag Tg model of NB could be a useful tool for developing and/or characterizing therapeutic strategies and understanding disease evolution. In an attempt to understand the extent of RAS pathways in modifying neuroblast fate, Sweetser and colleagues developed HRAS-activated Tg mice using the dopamine-beta-hydroxylase (DβH) promoter (Sweetser et al. 1997). These HRAS Tg mice developed about 30–40% of NB in the adrenal medulla with TH, Ret and RAS immunoreactivity, and measurable metastases to the LN, liver, and lungs. This model provides a platform to understand the molecular basis for the select subset of human NB that expresses high RAS with varying degrees of MYCN expression.

One of the most accepted, characterized, and widely used Tg mouse models for NB is the TH-MYCN mouse, in which the active promoter in migrating NCCs early in the development, tyrosine hydroxylase (TH), drives the human MYCN expression (Weiss et al. 1997). These mice displayed unique NCC-derived tissue-specific expression of MYCN (e.g., adrenal gland) and produced thoracic paraspinous NB masses (expression NB-specific ENO2, synaptophysin [SYP]) with varying degrees of neuronal differentiation. TH-MYCN Tg mice exhibited nearly four copies of the MYCN gene and displayed gross (liver, lung, ovary) and microscopic (liver, lung,

kidney, brain, lymphatics, ovary, testes, muscle) metastasis (Weiss et al. 1997). NB studies on this Tg model utilizing comparative genome hybridization (CGH) and latent period for tumor formation recognized that additional genetic lesions beyond MYCN overexpression are required, as those mirror human disease. More importantly, with microarray-based CGH analysis, the same group demonstrated the conservation of many human genetic changes in this model. Further independent studies characterizing this model affirmed the differences in tumor incidence, growth physiognomies, and treatment window between the hemi- and homozygous mice (Rasmuson et al. 2012).

The TH-MYCN model aided as an unparalleled tool in multitude of basic biology and therapeutic studies and served as an apt platform for multimodal imaging of the characteristics of tumor evolution and/or response. TH-MYCN tumors (i) arise spontaneously; (ii) have a high penetrance within NCC-derived sympathetic paraspinal, celiac, and periadrenal sympathetic ganglia; (iii) have syntenic genetic changes of human MYCN-amplified (MYCN-a) disease; (iv) preserve native tumor-stromal interactions and vasculature; (v) recapitulate human MYCN-a disease progression; and (vi) display MYCN-dependent oncogene addiction (Chesler and Weiss 2011). More recently, investigators made use of this clinically mirroring TH-MYCN Tg model to develop more advanced and selective scenario setups to address specific clinically relevant questions in NB research. For instance, Yogev and colleagues engineered a chemoresistant Th-MYCN^{CPM32} model by developing cyclophosphamide (CP)-resistant tumors with BM metastasis in TH-MYCN mice (Yogev et al. 2019). Uniquely, this platform offers NBs with an altered immune microenvironment and enriched gene expression that is relatively syntenic to progressive disease post-IMCT in humans.

In addition to offering a tool to determine the mechanisms of therapy resistance and tumor evolution, the Th-MYCN^{CPM32} model also underscored JAK-STAT3 signaling in progressive disease (Yogev et al. 2019). TH-MYCN mice bred with TH-Cre caspase-8 KO mice displayed pro-metastatic molecular rearrangements, alterations in ECM structure, and heightened BM metastasis when compared with TH-MYCN mice (Teitz et al. 2013). With the feasibility to imitate clinical therapy-dependent primary tumor load reduction, and with ongoing metastasis, this combination is a perfect fit to investigate and/or to develop therapies for progressive NB. Similarly, the tamoxifen-inducible TH-MYCN/Trp53(KI/KI) mouse was generated to underscore the function and mechanism of Trp53 in survival and radioresistance (Yogev et al. 2016).

Expanding further on the MYCN-amplified disease model, Althoff and colleagues developed Cre-conditional-MYCN Tg mice (LSL-MYCN; DβH-iCre) utilizing DβH-expressing cells that developed (>75% incidence) strain background-independent NB (Althoff et al. 2015). The tumors closely recapitulated human NB in tumor localization, histology, marker expression, and genomic makeup. Work with this model recognized the expression of MYCN-dependent miRs (miR-17-92 cluster), MYCN-associated genes, and oncogene addiction to MYCN and identified syntenic chromosomal aberrations to human NB (Althoff et al. 2015).

The ALK Tg approach has produced another high-value Tg mouse model for NB research. Single-base missense mutations in the regulatory regions of ALK kinase domain prompt ligand-independent signaling by interrupting the kinase auto-inhibition. Three mutations (R1275, F1174, F1245) contribute to ~85% of all ALK mutations in NB, where R1275Q is common in familial (45%) and sporadic (~35%) NB, while F1174 (30%) and F1245 (12%) mutants are exclusive to sporadic NB. F1174L, a cytosine-to-adenine alteration in exon 23, directs phenylalanine-to-leucine substitution in kinase domain codon at 1174 that results in increased autophosphorylation. In general, ALK mutations are associated with MYCN amplification and poor prognosis and could serve as a better platform from which to understand ALK-positive NB. Consistently, Tg mice developed with human ALK F1174L cDNA ligated downstream of TH promoter (Th-ALK-F1174L) selectively overexpress ALK F1174L in sympathetic precursor committed NCCs (Berry et al. 2012). This model allows additional MYCN overexpression to neuroectodermal cells. Although the TH-ALK-F1174L mice do not form spontaneous NB, co-expression of ALK and MYCN (when crossed with TH-MYCN mice) results not only in an early onset of disease (compared with TH-MYCN), but the mice also presented with disease of higher penetrance and boosted lethality. In addition, this ALK mutation model uniquely harbors molecular events, including activation of the PI3K/AKT/mTOR and MAPK pathways and suppression of MYCN pro-apoptotic effects (Berry et al. 2012).

Heukamp and colleagues generated Cre (DβHi-Cre; TH-IRES-Cre)-conditional NCC-specific expression of ALK-F1174L Tg mice that displayed NB formation that mirrored human NB histologically, with their immune reactivity to NB-specific markers (TH, Dβh, ENO2, SYP, Ncam1, Phox2b), and with comparable genomic aberrations (Heukamp et al. 2012). Interestingly, two knock-in (KI) Tg models, ALK-R1279Q and ALK-F1178L, failed to show any development of spontaneous NB but displayed synergism in the development of Ret-inhibitor-sensitive NB in vivo when crossed with TH-MYCN mice (Cazes et al. 2014). Other Tg approaches for the development of spontaneous NB include the NCC-specific overexpression of Lin28b. LSL-Lin28b mice crossed with Dβh-iCre mice resulted in mice with CAG-driven Lin28b expression selectively in Dβh-expressing sympathoadrenergic lineage cells of the neural crest (Molenaar et al. 2012). These mice developed adrenal, thoracal, and superior cervical ganglion tumors that aligned histologically and molecularly (DβH, Th, Phox2b) with human NB. Work with this model identified that LIN28B directly regulates MYCN and that therapeutic targeting of MYCN may be useful in the cure of LIN28B-driven NB. More importantly, the lack of embryonic neuroblast proliferation modifications or Let-7 functions in the *LSL-Lin28B*^{DBHiCre} model affirmed the requirement or involvement of additional genetic abnormalities (Hennchen et al. 2015).

Human Xenograft Models: Xenotransplantation of Human NB cells in immune-deficient mice is widely used in NB research. Studies have demonstrated the usefulness of this model for gaining a rapid understanding of key events of disease initiation, biology of progression, or the therapy response. NB xenograft mouse

models are generally developed by subcutaneous, intraperitoneal, or intravenous transplantation of genetically homogeneous cancer cell lines in athymic (nu/nu) or severe combined immunodeficiency (SCID) mice. Xenotransplantation results in the formation of ectopic tumor masses that recapitulate the genetic complexity of human NB, but lack human stroma interaction, and in most cases inadequately reflect the disease and clinical outcomes in humans. NB research has extensively utilized this tool, and numerous human NB cell lines (e.g., SH-SY5Y, SK-N-AS, IMR-32, SK-N-BE(2), LAN5) have been successfully xenografted into the mice. With access to a wide array of genetically diverse NB cell lines, diverse and unique models can be developed to suit research objectives. Innumerable studies of NB signaling, mechanisms, and functional and therapeutic (chemotherapy, radiotherapy, siRNA, inhibitors) screening utilized xenograft models. For instance, it has been shown that molecular iodine supplementation in metronomic CP treatment in NB xenografts of SK-N-BE(2) potentiates chemotherapy by targeting proliferation (survivin), malignancy (MYCN, TrkB), and vasculature remodeling and increasing differentiation (PPARγ, TrkA) and apoptosis, which collectively substantiate inhibition of tumor growth while reducing chemo-drug side effects, including weight loss and hemorrhagic cystitis (Alvarez-Leon et al. 2021). Xenografts generated with subcutaneous/orthotopic implantation of NB cells alone or in combination with human BM-mesenchymal stem cells (BM-MSCs) and cancer-associated fibroblasts (CAFs) were treated with JAK2/STAT3 and MEK/ERK1/2 inhibitors, and researchers examined the role of BM-MSCs/CAFs in therapy response (Borriello et al. 2017).

Xenograft models are easier to develop, to a certain extent mimic the heterogeneity of human NB, and allow high-throughput studies. Conversely, xenografts are generated in an unrealistic T cell-lacking tumor microenvironment (TME) that limits its use for immunotherapy studies. The other major limitation in this model is that developed xenografts generally do not metastasize. Although metastasis biology studies have extensively used this model using tail vein or intracardiac injection (pseudo-metastasis model), the lack of the initial stages of the metastatic process and the pattern of clinical metastatic spread is an undeniable shortcoming. Despite these limitations, xenograft models remain the prime model for proof-of-concept or new strategy studies (Table 1). In addition to the flank region xenograft development, a number of studies utilized orthotopic transplantation of in vitro cultured cells, generally called orthotopic models. For instance, Brignole and colleagues transplanted wild-type and luciferase-transfected IMR-32 or SH-SY5Y cells in the adrenal glands of nude mice to investigate the therapeutic target relevance of nucleolin (NCL) for NB (Brignole et al. 2021). Due to the wide array of studies utilizing xenograft and/or orthotopic mouse models, a brief list of its applications is provided in Table 1.

Patient-Derived Orthotopic Xenograft (PDX) Mouse Models for NB As discussed above, the mouse xenograft model with the subcutaneous transplantation of human NB cells suffers some serious limitations. In this regard, generation of PDXs by the implantation of human NB explants with patient-derived cellular features offers a

Table 1 Sample list of studies/applications that utilized mouse models of NB. □ = xenograft, □ = orthotopic, □ = PDX, □ = syngeneic, □ = transgenic

Molecular Target	Manipulation	Mice	Cell-Line	Endpoint
Serine-Threonine Kinase Receptor Associated Protein (STRAP)	Knockout	Athymic nude	SK-N-AS	Tumor regression
Interleukin 15 (IL-15)	N803 + Dinutuximab + ex vivo NK cells	NSG	SK-N-FI-Luc	Tumor regression, survival
Neuronal Leucine-rich repeat 1 (NLRP1)	mAb	SCID	SH-SY5Y	Tumor regression
Carbonic anhydrase isoform 1X (CAIX)	Acetazolamide + Cisplatin + Fenidiline	NOD-SCID	SK-N-BE(2)	Tumor regression, survival
Glypican 2 (GPC2)	mAb CT3-derived CAR-T cells	NSG	IMR-5-Luc, NBEB-Luc	Complete tumor regression
PCF11 cleavage and polyadenylation factor it	PCF11 depletion	Athymic nude	BE(2)C	Tumor regression
pERK	*i*Yap, CA3 + *i*MEK, Trametinib	Athymic nude (KSN/Slc)	SK-N-AS	Tumor regression, survival
Norepinephrine transporter (NET)	BG-P1600-TAT + Thyrointegrin αvb3	SCID	SK-N-AS, SK-N-FI	Inhibited tumor growth
Growth Harmobe Receptor (GHR)	Knockdown – sHRNA	BALB/c nude	SH-SY5Y	Potentiates etopside
Minichromosome maintenance complex component 6 (MCM6)	Knockdown – sHRNA	BALB/c nude	SK-N-BE(2)	Inhibit NB growth
Butyrylcholinesterase (BChE)	Knockout CRISPR-Cas9	NCr-nu/nu	BE(2)-C	Reduced tumor growth
Gamma-aminobutyric acid B-type receptor 1 (GABBR1)	Adipose-derived stem cell-derived extracellular vesicles	BALB/c mice	SKNBE-2	Reduced tumor growth
Survivin, MYCN, TrkB	Molecular Iodine + Cyclophosphamide	Foxn1 nu/nu	SK-N-BE(2)	Tumor regression
Somatostatin receptors (SSTR)	^{177}Lu-octreotate	BALB/c nude	CLB-BAR, CLB-GE, IMR32	Therapy for metastatic NB
Alternative lengthening of telomeres (ALT)	ATM knockdown (shRNA) or ATM inhibitor AZD0156	Athymic nude	CHLA-90m, SK-N-FIm, COG-N-515m, Felix-m	Event free survival
xportin-1 (XPO1)	XPO1 inhibitor verdinexor	Nude mice	SK-N-BE(2)	Inhibit tumor growth
MEK pathway	Trametinib + GD2-specific chimeric antigen receptor (CAR)-T cells	SCID Beige	AS-High-FFLuc, SY5Y-FFLuc	Enhances the antitumor efficacy of GD2-CAR-T cells
Glucose transporter 1 (GLUT1)	Stably transfected with sh-LINC00839-1	BALB/c athymic	SK-N-SH	Reduces NB Cell Growth
Retinoic acid receptor-related orphan receptor alpha (RORα)	agonist SR1078	athymic Ncr nude	NGP	Inhibited MYCN-amplified NB growth
Ubiquitin-specific protease USP36	*Depleteion with sHRNA*	Bagg Albino nude	IMR-32	Reduced tumor volume
Hypoxia-sensitive moiety	nitroimidazole	Nude mice	SK-*N*-BE(2)	Imaging tumors
LncRNA differentiation antagonizing nonprotein coding RNA (DANCR)	sHRNA for DANCR	BALB/c-nude	SK-N-SH cells	Inhibits tumor growth
LINC01410	sh-LINC01410	BALB/c nude	SK-N-BE(2) c	NB regression radiosensitization
Chromatin stability	Curaxin CBL0137 +*i*HDAC, panobinostat	BALB/c-Foxn1nu/Arc	BE(2)-C	Tumor regression in HR-NB
T cell-based immunotherapy	EATs using IgG-[L]-scFv-platformed BsAb, anti-CD3 (huOKT3) scFv	BALB-*Rag2*−/−IL-2R-*γc*-KO (BRG) mice	IMR32Luc	long-term remission, prolonging survival
Cell-based immunotherapy	Labeling CD45, pan-leukocyte; BSA-I, dendritic cell; CD11b, NK cell; CD68, macrophage markers.	SCID Mice	LAN-1, IMR32	Immune Competent Cells Infiltration in to tumors
Cell-based immunotherapy	Hu3F8-BsAb armed ATCs	BRG	IMR-32-Luc	T cell infiltration
Glypican 2 (GPC2)	mAb CT3-derived CAR-T cells	NSG	imr5	Complete tumor regression
NEDD8	NEDD8 Inhibitor Pevonedistat	Nude	SH-SY5Y, S-K-NAS	Decreased tumor growth
Nucleolin (NCL)	Doxorubicin-loaded nanocarriers with the NCL-recognizing F3 peptide (T-DXR)	Foxn1*nu* (*nu/nu*)	IMR-32, SH-SY5Y	Delay of tumor growth
ALK, TRK, JAK2/STAT and Src/FAK	Repotrectinib ± chemotherapy	Diabetic/ NSGH	MSKNBL30595, -82180	Anti-tumor activity and EFS
Multi kinase	Rigosertib	Athymic nude	LU-NB-3	Tumor regression, survival
T cell-based immunotherapy	EATs using IgG-[L]-scFv-platformed BsAb, anti-CD3 (huOKT3) scFv	BALB-*Rag2*−/−IL-2R-*γc*-KO (BRG) mice	NBL50a, Piro20Lung	long-term remission, prolonging survival
Ornithine decarboxylase (ODC)	Eflornithine +Probenecid	Athymic nude	COG-N-623	Potentiates Eflornithine
Alternative lengthening of telomeres (ALT)	ATM knockdown (shRNA) or ATM inhibitor AZD0156	Athymic nude	COG-N-625x, -519x, -564x, -452x, -623x	Event free survival
BCL-2	Venetoclax	NSG	COG-N-561x, -415x	Controlled tumor growth
Chromatin stability	Curaxin CBL0137 + *i*HDAC panobinostat	BALB/c-Foxn1nu/Arc	COG-N-424x	Tumor regression in HR-NB
Cell-based immunotherapy	Hu3F8-BsAb armed ATCs	BRG	Piro20-lung	suppressed growth of NB
Chromatin stability	Curaxin CBL0137 + *i*HDAC, panobinostat	wild-type 129/SvJ	TH-MYCN	Tumor regression in HR-NB
Chromatin stability	Curaxin CBL0137 + *i*HDAC, panobinostat	TH-MYCN	TH-MYCN	Tumor regression in HR-NB

better platform. Advantages of the NB-PDX model include (i) intact tumor explants with stroma; (ii) no in vitro culture-associated genetic/molecular modifications; (iii) retention of human NB features like tumor histopathology, TME architecture, and mutational and proteomic profiles; (iv) formation of vascularized tumors in mice that substantiate development of spontaneous metastasis; and (v) the presence of hallmarks of the TME, vascular infiltration, CAFs, and tumor-associated macrophages (Braekeveldt et al. 2016).

With the feasibility of subcutaneous (or orthotopic) implantation into the mice or experimental teratomas, use of the PDX model is progressing for understanding NB mechanisms of progression, metastasis and therapy resistance, and for preclinical drug screening. Utilizing a PDX model, Dorneburg and colleagues identified the benefit of γ-secretase inhibitor I (GSI-I) on survival after MYCN-na disease (Dorneburg et al. 2016). Due to limited sporadic access to fresh viable NB tumors, researchers have demonstrated the feasibility of establishing NB-PDXs from viably cryopreserved NB samples (Braekeveldt et al. 2015). The same group later indicated

the feasibility of serial passaging and molecular analysis of NB-PDXs and showed that PDX could consistently and strongly maintain intrinsic genetic, transcriptional, and phenotypic stability for more than 2 years (Braekeveldt et al. 2018). Crucially, findings from this PDX study warn about complications in the translation of PDX outcomes to the clinics, particularly with the realization of spatial intratumor heterogeneity.

Recently, Nguyen and colleagues used the orthotopic NB-PDX model and unveiled the benefit of a soluble IL-15/IL-15Rα complex in potentiating the anti-GD2 antibody-mediated NB regression by lowering the numbers of immature tumor-infiltrating NK cells (Nguyen et al. 2019). Investigating the generation of reproducible NB-PDXs within the clinically useful time frame, Kamili and colleagues showed that NB-PDXs could be reliably established from diverse (solid tumor, BM, pleural fluid, residual cells from cytogenetic analysis) samples and further recognized that orthotopic implantation develops rapid PDX when compared with subcutaneous or intramuscular engraftment (Kamili et al. 2020). Utilizing an NB-PDX model coupled with a selective targeting hydrophobic pocket in the HIF-2α PAS-B domain with a potent antagonist (PT2385), additional functions of HIF2α in NB other than ARNT-dependent transcriptional activity have been recognized (Persson et al. 2020). Similarly, Sime and colleagues used the NB-PDX model to define the efficacy of a novel lead drug candidate (small molecule, epi-enprioline) for vincristine-resistant NB (Sime et al. 2020). The function of kinesin spindle protein (KSP) in NB progression and the benefit of targeting KSP with ARRY-520 (filanesib) in regressing MYCN-a disease and improving survival were documented utilizing subcutaneous and orthotopic (adrenal glands) NB-PDX (using LU-NB-1, LU-NB-2, LU-NB-3 PDX cells) mouse models (Hansson et al. 2020). In addition, the efficacy and synergistic benefit of targeting bromodomain-containing protein 4 (Brd4) and its binding partner cyclin-dependent kinase with Brd4 inhibitor (AZD5153) and CDK inhibitor (dinaciclib) for augmenting tumor lymphocyte infiltration, necrosis, and tumor regression were investigated using MYCN-a, TERT-overexpressing orthotopic (adrenal) NB-PDX (ST16 cells) mouse model (Wood et al. 2021).

Limitations of the NB-PDX model include (i) the lack of an intact immune system; (ii) high costs for development; and (iii) its use that warrants expertise and creates a significant labor load. Since immunosuppressed (athymic nude, lacking T cells; NOD-SCID, lacking T and B cells; NSG, lacking T, B, and NK cells) mice are currently used for PDX generation, immunotherapy studies are not feasible in this setting. Moreover, the functional interaction between murine stroma and human tumor cells is unfounded. It is nearly impossible to avoid intratumoral heterogeneity and yet cover all response-driving mutations. Other crucial considerations for the generation of PDX models include the requirement for establishing linear cohorts of PDX (e.g., progressive disease, relapsed disease, metastatic sites, postmortem); tumor cell cross-contamination or EBV-infected B-lymphoblast contamination; biological/metastatic research benefitting orthotopic implantation (vs. subcutaneous); limitations in engraftment and tools (Matrigel/co-injection with stroma) to enhance engraftment rate; and the choice of mouse strain.

Humanized (Immune) Mouse Model One of the major setbacks of the DX model is that they lack an intact immune system, and due to the functional significance of the immune system→tumor interactions in therapy response, recent efforts are focused on developing humanized mouse models. Multiple strategies for humanizing the mouse immune system have been employed, including (i) the transplantation of human peripheral blood mononuclear cells (PBMCs) generally referred as the "immuno-avatar" mouse model; (ii) the transplantation of $CD34^+$ or $CD133^+$ human hematopoietic stem cells (hSCs) and progenitor cells (hPCs), referred as the hemato-lymphoid humanized mouse model; and stromal tissue injection alongside tumor tissue into immunocompromised mice prior to the xenograft or PDX development. Since the injection of $CD34^+$ or $CD133^+$ into the BM of irradiated immunocompromised mice allows T and B cell (and macrophage) generation, it is feasible to use matched patients' resources. Nguyen and colleagues generated a next-generation humanized $GD2^+$ HLA-deficient orthotopic NB-PDX model coupled with $CD34^+$ HPCs transplantation and showed that developed NK cells in these mice permitted human NB engraftment but could be redirected to inflict antibody-dependent cell-mediated cytotoxicity (Nguyen et al. 2021). They showed that these mice lack activated NK cells mirroring the human NB, indicating that this could be a tumor-mediated suppression.

Although this humanized PDX model presents challenges due to technical complexity, this model could serve as a reliable platform for immunotherapy studies. When compared with the proof-of-principle studies conducted with syngeneic, transgenic, xenograft, or PDX models, a humanized mouse model is the only alternative that offers a human-appropriate model for investigating endpoints like immune checkpoint blockers, adoptive cell therapy, oncolytic viruses, cytokine therapy, and combinational immunotherapies. Despite the benefits (human immune system→tumor [xenograft, PDX] interactions) of this model, the development itself is highly sophisticated, extremely expensive, time-consuming, and unlikely to be useful for any high-throughput screening, and remnant murine immune system interaction is likely.

Zebrafish Models for NB

One fast-growing alternative to the preclinical mouse model of NB is the zebrafish model. The increase in the popularity of this tool for NB research is because of the high fertility rate, low cost, feasibility for genetic remodeling and/or patient-derived cancer cell xenotransplantation (PDX), and the simplicity of in vivo imaging. Gene annotation from the sequenced zebrafish genome showed 71.4% orthologs to human genes with >9500 protein coding orthologs (Howe et al. 2013). Although there is a five-decade history of the use of zebrafish for cancer research (Stanton 1965), the evolution of a clinically translatable zebrafish genetic cancer model commenced with the development of transgenic animals for T cell leukemia in 2003 (Langenau et al. 2003). This opened the doors for an influx of zebrafish models for diverse

tumor types, including NB (Hason and Bartunek 2019). Zebrafish models of NB present an advantage over other models as one can longitudinally observe the early onset of tumorigenesis (the unique requisite for NB as opposed to adult tumors) without euthanizing the animals. Since the zebrafish are translucent and develop from externally fertilized eggs, the early detection of tumor onset is feasible in live animals.

Zebrafish NB Model for Understanding Disease Evolution The development of dβh promoter-controlled, GFP-expressing MYCN stable transgenic zebrafish [Tg (dβh:EGFP-MYCN)] was the first step in the use of zebrafish models for NB (Zhu et al. 2012). This transgenic approach showed initiation of small, undifferentiated tumor formation in the anterior abdomen (mirroring adrenal gland) with strong expression of NB-specific markers. Beyond opening the doors for the use of zebrafish for NB research with a known transgenic strategy, this study also developed additional transgenic animals with ALK gene harboring the F1174L mutation (dβh:ALKF1174L) and demonstrated the feasibility of performing double transgenic approaches in zebrafish for a better understanding of NB progression (Zhu et al. 2012). Developing zebrafish-NB Tgs with human genes (and not with zebrafish genes) evades crucial hurdles like transgene(s) targeting and defining the efficacy of human protein-targeting drugs. The dβh promoter was highly preferred for building transgene constructs in zebrafish due to the limitations in using TH promoter.

The pioneering study discussed above led to the effective use of zebrafish NB models in innumerable independent investigations of NB disease biology that changed the thought process regarding disease evolution. Tao and colleagues recognized that the gain of function of chromatin modifier chromatin assembly factor 1 subunit p150 (CHAF1A) blocked neuronal differentiation and promoted malignancy (Tao et al. 2021). CHAF1A activates the polyamine metabolism, and targeting induced polyamine synthesis with DFMO an ornithine decarboxylase inhibitor promotes NB differentiation and potentiates retinoic acid (RA) antitumor activity. Anderson and colleagues showed that high dihydrolipoamide S-succinyltransferase (DLST) predicts progressive NB and poor therapy response. In a MYCN model of zebrafish NB, increases in DLST prompted tumor progression, while targeting DLST with clinical pipeline drug IACS-010759 suppressed NADH production, impaired OXPHOS, and regressed NB growth (Anderson et al. 2021). Likewise, co-injection of dβh-EGFP (or dβh-mCherry) DNA with dβh promoter-driven transgene led to their co-integration and co-expression and allowed real-time tracking in embryos within one day after fertilization. Such controlled approaches led independent studies to develop many Tg lines for ALKwt, PTPN11, GAB2, LIN28B WT, LIN28B_MU, LMO1, and DEF, which cooperate with MYCN and contribute to NB pathogenesis.

Utilizing this strategy Tao and colleagues developed an improved TgMYCN_TT model (dβh-MYCN, cDNA+30UTR, and dβh-EGFP) with inclusion of the 30 UTR of the MYCN gene, containing microRNA recognition sites (Tao et al. 2017). This strategy allows the posttranscriptional regulation of MYCN expression that could

substantiate increased penetrance of NB. Further, studies have used the MYCN zebrafish Tg model to couple with switch target genome editing strategies (e.g., clustered regularly interspaced short palindromic repeats, CRISPR; transcription activator-like effector nucleases, TALENs) to address specific questions in NB research. CRISPR-based arid1aa and arid1ab KO in a MYCN zebrafish NB model designated arid1a as the NB suppressor gene, where loss of arid1aa and arid1ab augmented MYCN-driven disease penetration (Shi et al. 2020). Likewise, gas7 KO fish with TALEN prompted extensive metastatic disease in a MYCN transgenic line (Dong et al. 2021). As the use of zebrafish models for NB research is in the early stages and constantly evolving, many additional strategies are in the pipeline. For instance, studies have indicated that developing a Cre-LoxP conditional model could be highly beneficial when NB research warrants preferential activation/inhibition of gene of interest in specific subset of cells. In addition, inducible Cre recombinase models and the use of CRISPR/Cas9 techniques in zebrafish will serve as an ideal tool for NB research [88].

Zebrafish NB Models for Drug Response Zebrafish NB models have been extensively used in investigating acquired drug resistance and the mechanisms and/or identifying genetic determinants for developing new and improved targeted therapies against progressive disease. Dubiella and colleagues developed Sulfopin, an inhibitor targeting the Cys113 site of oncogene activator Pin1, and showed survival benefit in a zebrafish model of MYCN-driven NB by targeting c-MYC target genes (Dubiella et al. 2021). Further, epidermal growth factor receptor (EGFR) kinase inhibitor lapatinib can prolong and enhance the cytotoxicity of YM155, an anticancer drug, by inhibiting the multidrug-resistance efflux transporter ABCB1 (Radic-Sarikas et al. 2017). This led to the synergistic inhibition of the growth of MYCN-overexpressing NB in vivo. Similarly, using Tg fish with overexpression of both MYCN and proliferation-associated 2G4 (PA2G4), Koach et al. showed that small molecule (WS6) can competitively bind to PA2G4 to prevent its interaction with MYCN, leading to destabilization and reduced expression of MYCN that led to NB regression (Koach et al. 2019). A study utilizing a double KI Tg zebrafish model expressing MYCN and GAB2 recognized the chemosensitizing potential of MEK and histone chaperone FACT inhibitors (Zhang et al. 2017). A unique MYCN KI-NF1 KO model was developed and used to demonstrate the synergistic antitumor activity of a trametinib-isotretinoin combination (He et al. 2016). Together, these zebrafish models and others are an excellent model with which to investigate the efficacy of new drugs and novel combinations for improved therapeutic strategies and to recognize the mechanisms/genetic determinants that contribute to acquired therapy resistance.

In addition to the zebrafish Tg models, tumor transplants and xenograft strategies have been widely used in NB research. With the feasibility of using both adults and/or embryos, zebrafish models are a suitable platform for high-throughput drug screening. Since zebrafish embryos offer permeability for small molecules, in these models it is easy to investigate/identify small molecules that could inhibit NCC

induction (in NCC-transplanted embryos), an unparalleled approach that benefits the NB experimental therapeutics field. Utilizing this strategy, Seda and colleagues screened hundreds of FDA-approved drugs in NCC-transplanted zebrafish embryos and indicated the benefit of repurposing an immunosuppressive drug used for rheumatoid arthritis to inhibit NCC induction (Seda et al. 2019). In parallel, using adult zebrafish xenografted with human NB cells served as a platform for several studies defining the antitumor potential of select lead drug candidates. One such study described the therapeutic benefit of dubermatinib (TP-0903) by targeting AXL receptor tyrosine kinase (RTK) and countering acquired chemoresistance, immune evasion, and the metastatic potential of NB cells (Aveic et al. 2018). More importantly, the PDX zebrafish model is a current possibility, constantly evolving and immensely facilitating the understanding of NB disease evolution and aiding in developing novel therapeutic strategies. For example, the NB-PDX zebrafish model identified the function of CNR2 and MAPK8 in high-risk NB evolution and defined the tolerance and anti-NB potential of two lead drug candidates in this setting (Almstedt et al. 2020). The development of the first humanized (mirroring patients' cytokine-enriched microenvironment) zebrafish PDX model (Rajan et al. 2020) is a huge leap forward in the use of zebrafish as a model for cancer research and could be useful for NB drug response research.

Benefits and Shortcomings of Zebrafish NB Models There are a number of advantages of using zebrafish as a model for NB research. The primary advantage is its translucent appearance in the embryonic and adult stages, which favors early detection of tumor onset. Since NB originates from migrated NCCs (Aravindan et al. 2019), it is not feasible, and/or it is technically and financially demanding to identify the initial events of NB genesis in mouse models. Conversely, zebrafish models offer grouped (*n* of a cohort can be in hundreds), multiplexed (many molecular targets) high-content real-time fluorescent visualization in weeks after fertilization without requiring euthanasia (Zhu et al. 2012). The translucent appearance also allows longitudinal measurements of tumor progression and dissemination. For instance, fluorescent-labelled NB cells expressing oncogenes or with loss of tumor suppressors allowed real-time linear measurement of early dissemination of tumor to distant sites (Zhu et al. 2017).

Further, zebrafish models allow the real-time investigation of the molecular or cellular events that drive NB progression. Increased extracellular matrix (ECM) stiffness that contributes to metastasis could be uniquely profiled in real time in zebrafish (Zhu et al. 2017). Due to the requirement of a large population and whole organisms to signify (statistically) the safety and benefit of novel drug candidates and/or combinations, zebrafish would allow the preclinical evaluation of the antitumor efficacy of new/repurposed drugs for NB. Zebrafish NB models would also provide robust benefits for genome editing and targeted genetic rearrangements. Unlike time-consuming in vitro fertilization and embryo implantation in mouse models, zebrafish offers external fertilization, allowing access to a large collection of fertilized embryos for single-cell stage genetic editing that rapidly generates

innumerable transgenic lines in a short time. In addition, zebrafish permit the feasibility of performing high-content PDX transplantation and drug screening. Xenotransplantation of tumor cells with diverse inherent/modified genetic characteristics or PDX in large cohorts allows linear and parallel comparisons on the mechanisms of disease, acquired cellular function in disease progression, or drug efficiency in NB. Thus, zebrafish models uniquely offer tracking of NB initiation/progression; an immune system that facilitates early embryonic NB development or that defies immune rejection of transplantation; and availability of unparalleled magnitudes of embryos. The major limitation is that the zebrafish culture conditions (28 °C) are not ideal for the transplantation of NB cells and PDX (Nolan et al. 2020). Although modified fish/embryo acclimation could be suited to transplantation requirements, this approach warrants technically demanding unique expertise and infrastructure. In addition, the dissimilarities in zebrafish and human physiology make zebrafish less appropriate for any clinical translation of the research outcomes. However, zebrafish models could be relatively useful for NB study, as the NCC lineage (from which the NB originate) is highly conserved between zebrafish and mammals (Zhu and Thomas Look 2016).

Future Prospective With the recent technological advances in the cancer biology and developmental therapy fields, zebrafish NB models would serve as a perfect tool for deriving meaningful statistical preclinical conclusions. For instance, the feasibility of a single-cell strategy in zebrafish could effectively couple with high-tech, high-content platforms (e.g., single-cell RNA sequencing, metabolic profiling) for unveiling genetic/metabolic/cellular blueprints along the lineage lines of NB pathogenesis. In addition, with the complementing upcoming technologies, the zebrafish platform would facilitate the understanding of: (i) the evolution of heterogeneous disease; (ii) intercellular communications and interactions within tumor cells and between tumor cells and TME; and (iii) the signal transduction flow-through and/or mechanisms of disease progression and acquired therapy resistance. With the evolving new classes of drug candidates, zebrafish will offer an unparalleled platform with which to comprehensively (i) classify large cohorts of drug candidates; (ii) measure the linear response to drug treatments; and (iii) evaluate the response at the single-cell level. Further, with the recent advances and understanding of NB immunotherapy, zebrafish could be the perfect tool for the development of improved immunotherapy-based approaches. With the unparalleled in vivo imaging capabilities of zebrafish, the recruitment of T cells to the NB could be precisely validated and will lead to the development of strategies to enhance T cell recruitment to NB and prompt the conversion of phenotypically cold tumors into inflamed tumors.

Chick Chorioallantoic Membrane (CAM) Model of NB

Although the CAM model is used less often than are the mouse models, researchers have used this model to investigate NB tumor development (differentiation, proliferation) and metastatic state (invasion, migration). Studies showed that seeding

labelled or unlabeled (e.g., GFP) human NB cells (e.g., BE(2)C, IMR32) in the Matrigel or as suspension onto the CAM followed by enhancing the tumor formation by trauma or with stimulants (e.g., trypsin EDTA) results in tumor development (Herrmann et al. 2015). In addition, researchers recognized that hypoxic preconditioning alters tumor cell phenotype and effect metastasis into the chick organs. For drug screening investigations, tumors are exposed to drugs topically delivered to the CAM or through allantoic cavity injection. The development of this model led to studies that unveiled crucial cellular events and function of genetic determinants (e.g., FOXO3, stanniocalcin-2, neurotrophin-receptor Trk-B) in NB evolution and therapy response (Ribatti and Tamma 2018). The use of the CAM-xenograft model for drug delivery research in NB settings is postulated by tracking RA-inflicted changes in NB cell morphology, proliferation, gene expression, and differentiation (Swadi et al. 2018). More importantly, this model has been extensively exploited to investigate angiogenesis in NB. Using the CAM model, studies showed that human NB cells stimulated angiogenesis in vivo and localized in a perivascular/intravascular position. NB cells were frequently arranged in clusters and occupied a perivascular position, as demonstrated immunohistochemically with NB markers (Ribatti et al. 1998). Moving forward, the same group identified the feasibility of implanting NB biopsies on the CAM and identified an antiangiogenic compound in this setting (Ribatti et al. 2001). Interestingly, a higher efficiency of angiogenesis measures (in vivo antiangiogenic potential of IFNγ) was realized with the NB-CAM model than with the NB xenograft model (Ribatti et al. 2006). Consistently, many studies showed the benefit of the NB-CAM model (conditioned medium from NB cells, NB xenografts, or primary NB biopsy specimens) in screening the antiangiogenic potential of diverse drug candidates (e.g., bortezomib, fenretinide, liposomal doxorubicin, CPT-11, EZN-2208). Further, Mangieri and colleagues seeded NB cells in Matrigel onto the CAM initially to a confined region (with the use of silicon ring) to underscore the angiogenic and invasive responses (Mangieri et al. 2009).

Studies are evolving to identify the mechanism(s) and genetic determinants (e.g., IGFBP-2, ANG-2) of intrinsic and acquired angiogenic response in NB. Overall, the advantages of NB-CAM model include that it (i) is cost-effective; (ii) is less demanding than mouse and zebrafish models; (iii) is an efficient alternative for reducing and replacing animals; (iv) allows real-time endpoint visualization; and (v) offers high-throughput capability. However, the limitations include: (i) the lack of an immune system that mirrors human NB; (ii) it is non-feasible for long-term linear therapeutic investigations; (iii) zebrafish have a different drug metabolism from humans; (iv) limitations in distinguishing existing and new vessels.

Concluding Remarks

The search for an NB cure is fast approaching due to the evolving progress in identifying novel and improved therapeutic strategies that compose the current standard frontline high-risk therapy. The current clinical management is maximally

intensive and employs a multimodal approach, including multi-agent induction chemotherapy, consolidation therapy with tandem high-dose chemotherapy with autologous peripheral blood stem cell rescue and radiotherapy, and post-consolidation therapy with anti-GD2 immunotherapy. Despite the completion of IMCT, children with NB suffer disease- and treatment-induced toxicities. The ability to detect/predict NB initiation; develop low-toxicity, tumor- and/or genetic determinant-targeted therapies; and design personalized treatment strategies would highly benefit the survival and quality of life of children with NB. Although studies are slowly beginning to unveil the underpinnings of NB evolution, there remains a paucity of knowledge on the mechanisms of tumor initiation, clonal selection and plasticity, evolution from favorable to high-risk disease, and acquired therapy resistance. The preclinical mouse, zebrafish, and CAM models discussed in this chapter are valuable tools with which to reach these milestones. However, the lack of NB in nonhuman vertebrates suggests that human NB predisposition could be related selectively to human evolutionary genomes. Although a number of preclinical models are developed and could be useful in reaching different milestones, experimentally it is not feasible to recreate this deadly disease in animal models, at least for the initiation of NB formation. Conceptually, the development of adrenal chromaffin cells or sympathetic neurons from human-induced pluripotent stem cells derived from normal cells of NB patients that may harbor intrinsic genetic risk factors in vitro would serve as the perfect model with which to investigate NB initiation. With all of these appropriate preclinical tools and with more in the pipeline, NB is becoming a curable tumor.

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Animal Models in Cancer Research

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Abstract

Cancer remains one of the most debilitating diseases in the world as it ranks the second leading cause of mortality. Researchers are relying on animal models to bridge the gap between bench-to-bedside investigations and clinical trials as they race to find a solution for this complex disease. Because of their similarities to humans, rodents are the most utilized preclinical test animals. Cancer research can use a variety of mouse models, from spontaneous and autochthonous to transplantation systems. A growing number of murine cancer models are based on cell line-derived xenografts, patient-derived xenografts, and humanized animals, due to their capacity to closely imitate tumor initiation, progression metastasis, and a realistic tumor microenvironment. As a result of their propensity to form tumors in immune-competent animals, genetically modified mouse models are also commonly used. Researchers have also used other lower animals such as Zebrafish and *Drosophila* to study tumorigenesis. This is because of their inexpensive cost, high fertility, shorter breeding period, and low upkeep; they are also relevant in whole animal drug screening. Larger animals like cats, canine, and porcine models offer a more crucial link between mice and men and have also been used in cancer investigations. This chapter explains in detail the types of animal models used in cancer research, emphasizing both their merits and shortcomings.

Keywords

Cancer · Mouse model · Xenograft · CRISPR-CAS · Tumor microenvironment · Zebrafish · *Drosophila* · Tumorigenesis

Introduction

Animals have been used in scientific studies for hundreds of years. Animal models of research have provided investigators with an understanding of complex diseases such as diabetes, neurological disorders, obesity, and cancer; their use has also helped tremendously in drug development. Animals are frequently employed in testing because they are biologically comparable to humans, are susceptible to similar health issues, and can be housed in a controlled environment.

"Cancer" is a comprehensive term that encompasses a group of diseases with over 250 different forms (Hassanpour and Dehghani 2017). It is one of the most rampant chronic diseases of the twentieth century, and its prevalence is still increasing in recent times (Roy and Saikia 2016). Cancer continues to be one of the most enfeebling diseases and the world's second leading cause of death (Hassanpour and Dehghani 2017). Despite conventional and new approaches to treat cancer, about 19 million new cancer cases and over 9 million cancer deaths were recorded globally in 2020 (Sung et al. 2021); nonetheless, the survival rates of cancer patients have improved owing to the accessibility of screening modalities and targeted therapies (Pacharinsak and Beitz 2008).

To minimize the overall prevalence of cancer, it is necessary to develop more efficient diagnostic methods and innovative treatment strategies (Li et al. 2021). Experimentation with animals is used to bridge the gap between in vitro assays and clinical trials. Animal models are valuable tools for studying the biology of human cancers. They provide better understanding of the etiology of various cancer types, the function of genes and gene mutations in tumorigenesis, as well as the identification and screening of curative therapies (Li et al. 2021). As technology advances and our understanding of cancer deepens, new animal models are being developed to more accurately reflect a particular cancer type (Yee et al. 2015).

Several small animals are being used for cancer research. Rodents, particularly mice and rats, are the commonly used animal models, accounting for more than 90% of all animals utilized in studies. Rabbits, dogs, cats, pigs, and fish are some of the other cancer models used in research, although their use is not as widely popular.

Murine Models in Cancer Research

Mouse is a widely recognized mammal that is employed in biomedical research all around the world. Its peculiarity stems from its tiny size, ease of handling, and ability to simulate human physiology (Waterston et al. 2002). Furthermore, the mouse genome was one of the first animal genomes to be entirely sequenced, revealing a high percentage of comparison between human and mice (Waterston et al. 2002). This similarity allows for a rich supply of genetic variation, genome modification, and a valuable tool for modeling human disease (Justice et al. 2011). We will describe four commonly used mouse models in detail.

Transplantation Murine Models

Murine models have been used in translational cancer research for decades (Day et al. 2015). It involves the transplantation of mouse tumor cells into another immunocompetent mouse (Fig. 1) with the same genetic background as the cell line or tissue (Chavan 2013). Tumors can be injected intraperitoneally or subcutaneously into the host (heterotropic transplant) or to more precise injection locations (orthotopic transplant) (Chavan 2013). Heterotropic transplants stimulate cellular proliferation and the production of ascites (Chavan 2013; Talmadge et al. 2007) and were used as a simple model for anticancer drug screening (Day et al. 2015; Talmadge et al. 2007). However, they were ineffective for cytotoxic screening against solid tumors and could not imitate tumor interactions or metastasis (Talmadge et al. 2007; Workman et al. 2010). As a result of this setback, orthotopic transplantation models were developed, which entail the transplantation of mouse tumor cells into specific anatomic locations or primary sites of the host in order to precisely mimic the cancer of interest, metastasis, and host-microenvironment interaction (Chavan 2013; Workman et al. 2010).

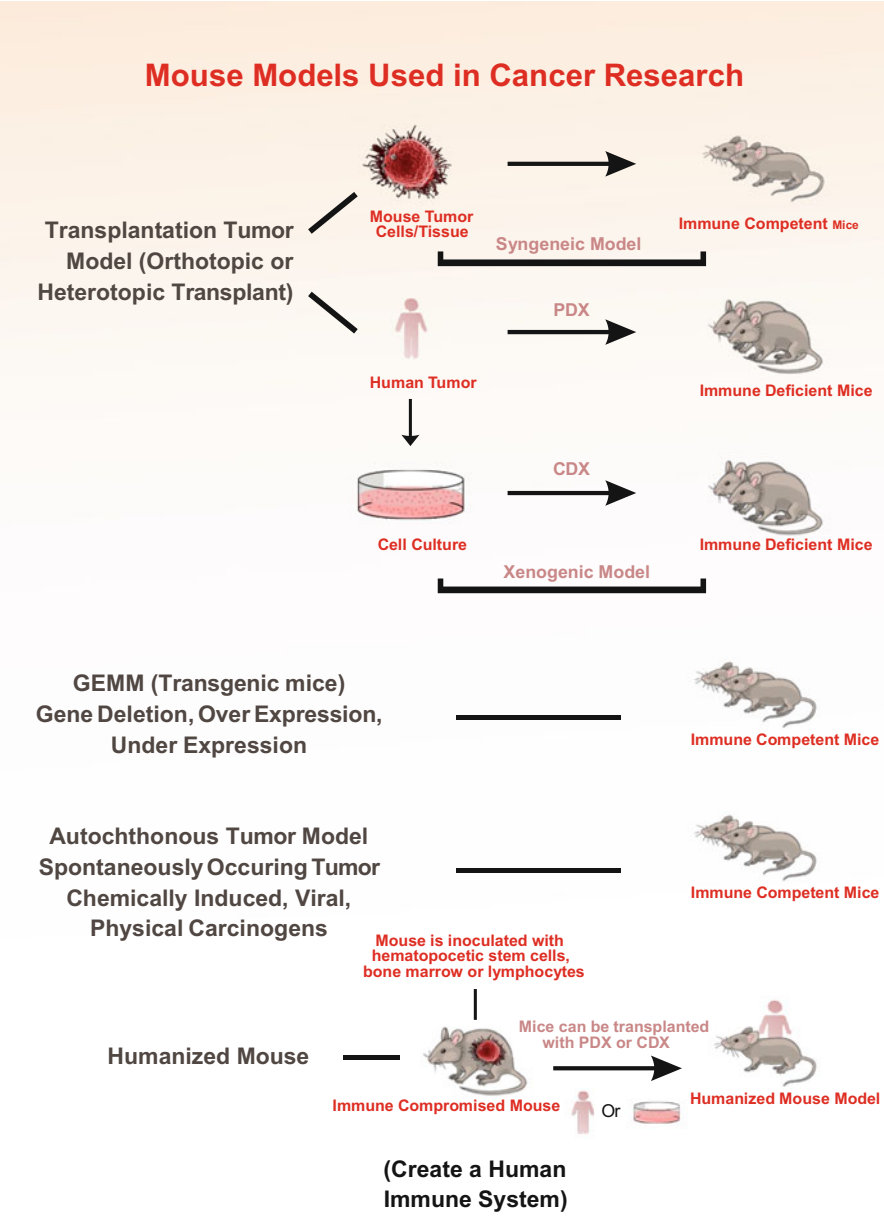

Fig. 1 An illustration of mouse models used in cancer research. PDX: patient-derived xenograft, CDX: cell line-derived xenograft, GEMM: genetically engineered mouse model

As scientific knowledge of cancer progressed, using mouse tumor cells to research cancer was no longer sufficient to understand the biology of cancer, and it became vital to develop mouse models that accurately matched human cancers, leading to the development of xenograft models. These murine models were first utilized by the National Cancer Institute (NCI) to investigate therapeutic efficacy (Chavan 2013; Jung 2014). It involves implantation of tumor cells or tissues into an immune-compromised host. Athymic mice or severe immunocompromised mice were used as host since they did not produce an immune response to reject the inoculated human cells (Chavan 2013). The xenograft model is a significant improvement in translational research in mice and has been widely used in cancer investigation to study tumor progression and treatment action (Chavan 2013; Jung 2014).

Human cancer cell lines can be implanted into an immune deficient host (cell line derived xenograft CDX) as ectopic transplants through subcutaneous injection or intraperitoneally, with the implantation site differing from the origin of the cultured cells (Jung 2014). These ectopic xenografts were widely used because it was straightforward to replicate, consistent, and easy to use (Li et al. 2021; Chavan 2013; Jung 2014). However, it has its limitation in that it cannot imitate human cancer's biological behavior, heterogeneity, malignant invasion, or metastasis (Li et al. 2021; Jung 2014). Orthotopic xenograft, on the other hand, is a more advanced technique that involves injecting human cancer cells into the same anatomic site as the donor cell's origin. In an orthotopic system, human lung cancer cells, for example, would be injected into the thoracic cavity of the host mice. Several studies have reported that orthotopic xenografts could simulate the development of cancer in humans and predict medication response, tumor heterogeneity, tumor metastasis, and invasion (Chavan 2013; Jung 2014; Richmond and Su 2008). Orthotopic xenografts were a substantial advancement in the use of murine cancer models, but they too have limitations. Orthotopic xenografts are more expensive; the method requires specialized technique and specificity, the need for live imaging systems to observe tumor spread in the live animal, and the use of immunocompromised hosts that fails to simulate a realistic tumor microenvironment (Jung 2014; Richmond and Su 2008).

Another significant improvement in the xenograft model of cancer is the use of patient-derived tumor xenograft (PDX, PDTX) also known as primary tumor graft model. This method involves the transfer of cancer patient tissue directly into an immunocompromised mouse either ectopically or orthotopically to emulate the natural course of cancer (Fig. 1). This method helps preserve tumor histology, tumor genetics, stromal component, and tumor microenvironment and is tailored towards personalized drug discovery (Li et al. 2021; Day et al. 2015; Jung 2014; Ruggeri et al. 2014). Patient-derived xenografts have a lot of potential in cancer research since they can closely imitate the activity of the tumor of origin while also providing a preclinical setting for resolving some of the most significant barriers to

cancer investigations (Day et al. 2015). However, the use of the PDX system is limited because use of an immune deficient mice may not replicate the same microenvironment as humans. It is expensive (Li et al. 2021; Jung 2014) and time-consuming (Day et al. 2015) and has ethical and clinical concerns (Jung 2014).

Autochthonous Murine Models

Cancer can arise spontaneously in some murine species, although it can also be driven through viral infection or toxins. These animals are known as autochthonous tumor models (Fig. 1) because they can grow tumors from inception within the organisms (Talmadge et al. 2007; Gargiulo 2018). Some autochthonous malignancies occur spontaneously in inbred mouse strains (Chavan 2013; Workman et al. 2010). A noteworthy example is the Donyru (DA/Han) rat, where more than 60% of female animals succumb to endometrial adenocarcinoma (Chavan 2013). Similarly, Martinsen found that inbred cotton rats produce spontaneous Enterochromaffin-like or ECL-derived gastrointestinal malignancies (Martinsen et al. 2003). Radiation-induced tumor formation in inbred hairless SKH-1 mice also falls within the same category (Chavan 2013). Autochthonous tumors have also been linked to chemical carcinogens such as dimethylhydrazine, azoxymethane, diethylnitrosamine, and dimethylbenzanthracene in several murine models (Workman et al. 2010).

According to Talmadge and colleagues, the advantages of using autochthonous tumor models include the ability to exhibit orthotopic tumor growth, tumor histology free of transplant-induced alterations (Talmadge et al. 2007), and ability to replicate metastasis (Workman et al. 2010). Autochthonous systems for cancer research were part of the original models utilized by researchers, but this method is defective due to its inability to simultaneously generate large numbers of homogenous tumors for screening purposes.

Genetically Engineered Murine Models

To better understand the molecular basis of tumor development and response to therapy, researchers developed genetically modified murine models (GEMM) that can be orchestrated to possess mutations that can lead to carcinogenesis (Fig. 1). GEMMs develop tumors naturally in an immune-proficient microenvironment by manipulating the mouse genome in the form of gene function loss, overexpression, under expression, or mutation (Walrath et al. 2010). These transgenic models are genetically engineered immune-competent hosts that have had their genomes altered through the random integration of gene sequences. An embryo or fertilized egg is often inoculated with an exogenous gene, which is then expressed by the progeny and passed on to their generations (Chavan 2013). Most transgenic hosts are created to demonstrate loss of function or gain of function mutations (Lampreht Tratar et al. 2018). Also, there are now other variations of the transgenic model, including non-germline genetically engineered mouse models (nGEMM), which carry DNA

changes only in somatic cells rather than germline cells (Lampreht Tratar et al. 2018). To study the biological function of targeted genes, transgenic mice can be genetically engineered to have the gene of interest deleted or silenced, resulting in loss of gene function or knockout murine models (Lampreht Tratar et al. 2018). When it comes to translational cancer research, knockout mice experiments are essential for elucidating the cause-and-effect relationships during cancer development. They also serve as a valuable tool for evaluating the potential validity of specific genes in targeted therapy (Day et al. 2015; Lampreht Tratar et al. 2018). Researchers can either use a constitutive knockout system, where the loss of function mutation is directed towards specific gene deletion in the complete body of the mouse, or an inducible knockout model, where the loss of function mutation can be controlled spatially to a specific location. Constitutive knockout models can be used to validate a novel oncogene by monitoring changes in phenotypes including appearance, behavior, and other observable traits; however, this method is limited because germline loss of function generally leads to either embryonic lethality or severe developmental defects in the animals (Lampreht Tratar et al. 2018). Recombinases, such as Cre-lox or FLP-FRT that is site-specific, or hormone-sensitive domains, such as the tamoxifen-inducible estrogen receptor, can all be employed to create inducible knockout models (Day et al. 2015; Lampreht Tratar et al. 2018). Oftentimes, the Cre-lox or FLP-FRT system is usually aligned in the same direction as the gene of interest, and the expression of Cre or FLP proteins invariably promotes homologous recombination of the complex, eventually leading to the deletion of the gene of interest. The conditional knockout model is favored because multiple inducible systems can be coupled within the same animal (Day et al. 2015); likewise, the model can more accurately replicate spontaneous carcinogenesis that is similar to tumor formation in humans (Walrath et al. 2010).

Aside from knockout animal models, researchers can also investigate how overexpression of a gene (knock-in) can promote tumorigenesis due to gain of function. The knock in design can be constitutive, in which a transgene is randomly inserted into the genome to overexpress the gene of interest (Lampreht Tratar et al. 2018). Knock-ins have also been constructed using site-specific loci such as Rosa26 in conjunction with Cre-regulated gene expression (Rappaport and Johnson 2014). It is also possible to create conditional knock-in models utilizing tissue-specific promoters such as loxP or FRT sites lined with a termination or (STOP) sequences (Lampreht Tratar et al. 2018; Rappaport and Johnson 2014).

The GEMM systems have been extensively used in cancer research to study and comprehend the molecular basics of tumor onset, development, and therapeutics. According to Day and associates, GEMM is the most comprehensive model for studying cancer progression (Day et al. 2015). Other advantages of GEMM include the capacity to imitate mutations seen in human tumors, sufficient portrayal of cancer growth from the starting phase, presence of an intrinsic stroma, and the use of an immunologically competent mouse that portrays a realistic microenvironment (Day et al. 2015; Richmond and Su 2008). The GEMM, like other cancer models, has some flaws, such as tumor formation being slow and unpredictable in different animals utilized and the ability to test only a limited number of genes at a particular

time, which is usually not reflective of the heterogeneity seen in human cells (Richmond and Su 2008; Ruggeri et al. 2014).

Researchers have developed a way to bypass the drawbacks of regular GEMM by using non-germline genetically modified mice. "Chimeric mice" that spontaneously form tumors in a tissue-specific pattern or through direct injection of cells have been used to create these models. It is necessary to implant genetically altered embryonic stem cells into pre-implantation embryos to create chimeric mice. These mice end up with a mixture of predisposed cells that are derived from ESCs and from wild-type host cells. Eventually, tumors form in the context of normal tissue simulating human carcinogenesis (Heyer et al. 2010). Non-germline GEMs are thought to be more efficient because they can circumvent breeding and save costs and, in some cases, improve flexibility, homogeneity, and timeliness (Heyer et al. 2010).

Humanized Murine Models

As the use of immunotherapy in cancer treatment progressed, the use of immunocompromised mice, which cannot directly predict human immune responses to cancer therapy, prompted the discovery of humanized mice. Several comprehensive papers on the concept of humanized mice have been published (De La Rochere et al. 2018; Shultz et al. 2007; Shultz et al. 2012; Tian et al. 2020; Yin et al. 2020). Human hematopoietic cells, lymphocytes, stem cells, and bone marrow have been transplanted into mouse hosts in humanized models to recreate the human immune system (Li et al. 2021; Shultz et al. 2007; Shultz et al. 2012). On this foundation, tumors can be implanted into a humanized murine host (Fig. 1) and can be used to research cancer immunotherapy treatments (Li et al. 2021; Shultz et al. 2012; Yin et al. 2020).The use of humanized mice in cancer therapy has provided a better understanding of how the immune system may influence tumor progression and treatment; similarly, the establishment of cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) tumors in humanized mouse models (Fig. 1) is a significant step forward in understanding cancer etiology, pathogenesis, and drug discovery (Yin et al. 2020). Nonetheless, humanized mouse models also suffer from several shortcomings. The difference in immune systems between mice and humans is significant, as some transplanted stem cells may fail to differentiate or function (Tian et al. 2020). Another setback is the emergence of graft-versus-host disease, as well as the presence of residual murine immune responses, which may affect immune cell reconstitution (De La Rochere et al. 2018; Tian et al. 2020; Yin et al. 2020).

Murine models (mouse and rat) remain the most often employed animals in cancer research and preclinical evaluation of therapeutics because of their size, affordability, biological similarity, and ease of handling. Several murine models for cancer studies exist each with its own advantages and limitations. The traditional methods used were autochthonous and primitive transplantation systems; however,

as science evolved, cell line-derived xenografts, patient-derived xenografts, genetically reformed models, and humanized mice have become mainstay in the use of mouse models for cancer studies.

Zebra Fish as a Model for Cancer Investigations

Studies have recognized the Zebrafish as a model for studying human diseases since the early nineteenth century (Lieschke and Currie 2007). Originally, the fish models were used to investigate embryology and development. As advancement in the area proceeded, Lieschke and Currie reported that genetic techniques that can modify the Zebrafish genome in order to study cloning, mutagenesis, and transgenesis were discovered in the late nineteenth century (Lieschke and Currie 2007). Scientists recognized the potential for using this invertebrate as a significant tool for modeling human diseases at this point (Amatruda and Patton 2008).

Zebrafish (*Danio rerio*) has been used as a model animal of choice because their embryos are transparent and can be observed in real time as they develop, making it possible to observe biological processes. Furthermore, housing is relatively inexpensive, and animals undergo external fertilization, which results in a high fertility rate, a short generation time, and rapid embryonic development. Additionally, the genome of Zebrafish has been fully sequenced and can be easily manipulated to contain mutations; multiple strains of Zebrafish are also available, making it an excellent choice for biomedical research (Lieschke and Currie 2007; Amatruda and Patton 2008; Hason and Bartůněk 2019; Kobar et al. 2021; Teame et al. 2019).

Zebra Fish in Cancer Research

Fish have long been used in cancer research. It was reported that the first fish models used to study tumorigenesis were *Xiphophorus*, medaka, and trout, which were able to generate carcinogen-induced tumors (Amatruda and Patton 2008). An increased focus on Zebrafish has emerged in recent years because genome sequencing of the Zebrafish revealed a similarity between human disease-causing genes and those found in the animal (Kirchberger et al. 2017). Similarly, reports have shown that cancer gene sequences are conserved between the two species, with tumors replicated in Zebrafish resembling human cancers histologically and genetically (Kirchberger et al. 2017).

Cancer can develop spontaneously in Zebrafish, or in addition to exposure to mutagens and cancer-causing substances (Fig. 2) (Lieschke and Currie 2007). In the nineteenth century, Zebrafish were exposed to the carcinogen diethylnitrosamine (N-nitrosodiethylamine, DEN), which caused hepatic neoplasms (Raby et al. 2020). N-nitrosodimethylamine (NDMA), Dibenzo(a,l)pyrene (DBP), and N-methyl-N′-nitro-N- nitrosoguanidine (MNNG) have all been reported to cause liver, testis,

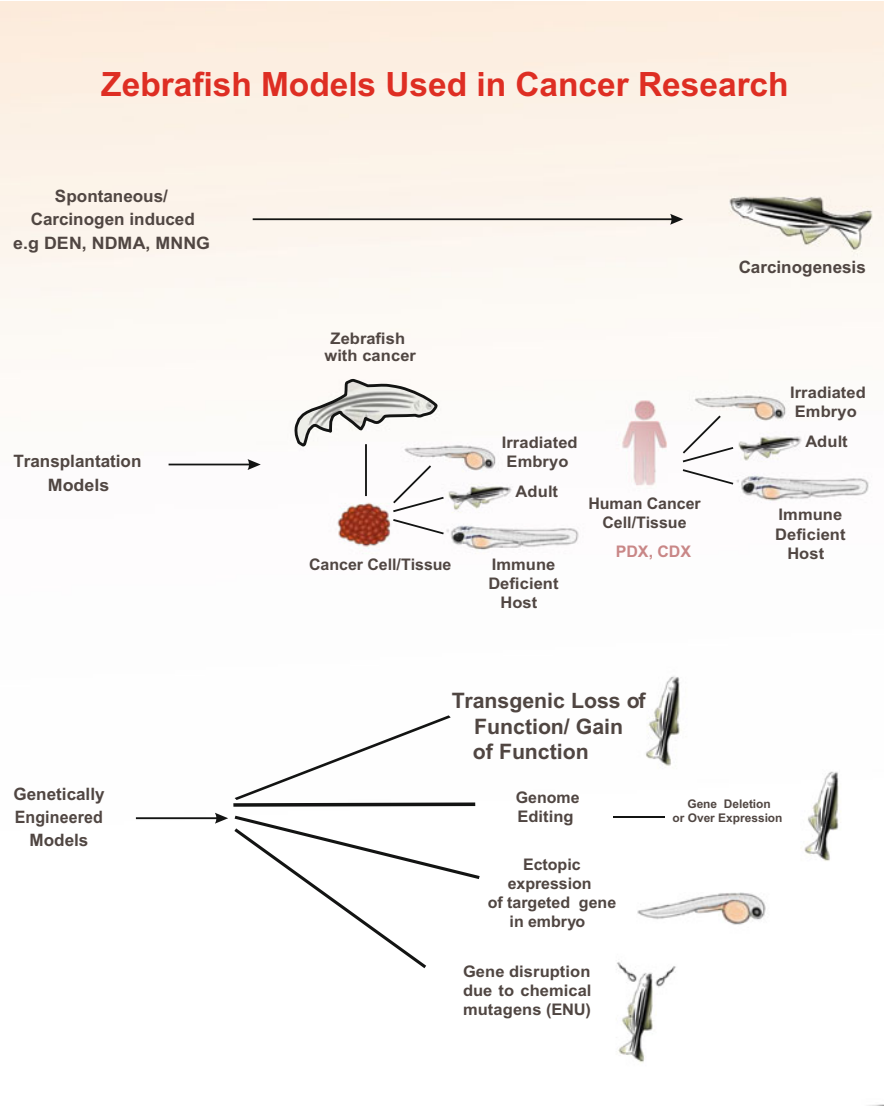

Fig. 2 An illustration of Zebrafish models used in cancer research. DEN: N-nitrosodiethylamine, NDMA: N-nitrosodimethylamine, MNNG: N-methyl-N′-nitro-N- nitrosoguanidine, PDX: patient-derived xenograft, CDX: cell line-derived xenograft, ENU: N-ethyl-N-nitrosourea

and muscle tumors in zebra fish models, respectively (Raby et al. 2020). To study cancer initiation and development, the use of spontaneous cancer formation has disadvantages owing to its late carcinogenesis, low tumor incidence, and heterogeneity of the tumors formed.

Transplantation Models

Cancer cells from the same organism (allografts) or a different organism (xenografts) can be inserted intraperitoneally or through intracardiac injection into the zebra fish embryo (Fig. 2) and can be monitored for tumor formation from day 2 to day 7 post-fertilization. During this time, the embryo does not reject implanted cells since the animal's adaptive immune system is still dormant (Hason and Bartůněk 2019). The advantage of using Zebrafish embryos for implants is the opportunity to visually monitor tumor growth and development (Hason and Bartůněk 2019).

Some researchers use sublethal irradiation of recipient embryos to prevent rejection of injected cancer cells; similarly, adult fish are frequently mildly irradiated to suppress the immune system and prevent rejection of transplanted cells. Single doses of 20–25 Gy are considered sublethal because they are tolerated by more than 80% of the fish (Taylor and Zon 2009). Aside from irradiation, dexamethasone treatment can also be used to reduce the immune response of Zebrafish. In this case, larval fish from 1 week to 1 month of age can be treated in 25 to 250 g/mL of dexamethasone a few days before transplant (Taylor and Zon 2009).

There have been recent advancements in Zebrafish research that have resulted in the generation of adult zebra fish that can keep their translucency throughout adulthood, a notable example of this is the "Casper" strain (White et al. 2008). Tumor cells can be inoculated intraperitoneally into sublethally irradiated Casper fish, and tumor progression can be observed. Colored or GFP-labeled cancer cells can also be used to track tumor growth after transplantation (Taylor and Zon 2009).

Another method of transplantation is to employ genetically immunocompromised fish as recipients (Hason and Bartůněk 2019). In Zebrafish, just a few of these immune-deficient variants are accessible. The rag2 (E450fs) mutant Zebrafish, which have few functioning T and B cells but are active and fertile, was one of the first systems produced. These mutant fish have a reduced amount of functional T and B cells, but are nonetheless viable and reproductive, and have been used to engraft numerous cancer cells (Tang et al. 2014).

Human cancer cells have recently been implanted as xenografts into host zebra fish (Fig. 2) in order to investigate tumor formation, invasion, metastasis, and effect on angiogenesis (Taylor and Zon 2009). Human melanoma cell lines were injected into the blastula stage of Zebrafish embryos to develop the first established human tumor implants in Zebrafish (Veinotte et al. 2014). Over time, researchers have created and perfected the scenarios for transplanting human cancer cell lines into Zebrafish and studied the grafted recipients for cell behavior and sensitivity to medications (Veinotte et al. 2014). Also, patient-derived tumor xenografts have been successfully used in the Zebrafish. Venoitte and co-workers reported that patient-derived gastrointestinal tumor cells demonstrated cell proliferation, migration through the blood, and metastasis within 24 hours of transplantation (Veinotte et al. 2014). Likewise, PDX from human glioblastoma and multiple myeloma (MM) in the bone marrow was implanted into the blastocyst or adult fish to mimic human carcinogenesis (Hason and Bartůněk 2019).

Transplantation models of Zebrafish have been used to examine leukemia, solid tumors, and other forms of cancer in vivo. For instance, Zebrafish leukemic cells were transplanted into irradiated adult Zebrafish in the research of mMyc-induced T-cell leukemia in Zebrafish (Langenau et al. 2003). Solid tumors, like melanomas, have also been observed to retain their invasiveness following repeated transplantation into other host fish (reviewed in (Taylor and Zon 2009)). Other benefits of using these models include the ability to observe tumor progression in real time due to the translucent nature of the fish; fluorescently tagged or pigmented cells can easily be imaged as the tumor develops and moves around in vivo; this model can also require less development time when compared to rodents.

Genetic Model of Cancer in Zebra Fish

Genome-specific mutations, deletions, overexpression, or underexpression of genes can also be used to construct genetically modified zebra fish to mimic cancer progression in vivo.

Gene Disruption Using Chemical Mutagens

N-ethyl-N-nitrosourea (ENU) has been utilized as a strong point mutagen in *Drosophila*, mouse, and Zebrafish (Fig. 2) (Raby et al. 2020). Investigators "Wolfgang Driever" and "Christiane Nüsslein-Volhard" conducted two large-scale ENU-based mutagenesis screenings in Zebrafish in the 1990s, which resulted in the discovery of hundreds of mutants with developmental abnormalities (Raby et al. 2020). Other findings have reported that ENU and MNNG (N-methylnitrosoguanidine) can cause a variety of tumor types, including adenoma and rhabdomyosarcoma, in early Zebrafish screens (Hason and Bartůněk 2019). In one of the initial cancer models, mutant p53 from Zebrafish was identified. In these mutant p53 animals, malignant peripheral nerve sheath tumor (PNST), an unusual form of sarcoma, develops. In humans, TP53 is the most commonly mutant tumor suppressor gene; hence, p53−/− fish were thought to exhibit characteristics similar to those seen in TP53-deficient individuals (Hason and Bartůněk 2019).

Transgenic Zebrafish

In the last few years, many novel strategies for gene editing and transgenic insertion into the Zebrafish genome have emerged. The goal of these procedures is to create a loss-of-function mutation or to overexpress orthologous genes involved in carcinogenesis (Fig. 2). Transgenic models are created using promoter-oncogene constructs and inducible or bipartite expression methods such as Gal4/UAS, Cre/loxP, or LexA/lexAOP (Kirchberger et al. 2017). One advantage of the inducible paradigm is that before sexual maturity, the oncogene-related lethality of the systems is prevented

(Kirchberger et al. 2017). For example, when directing $KRAS^{G12V}$ mutation solely to the liver with the Cre ERT2 system, it was feasible to create an effective cancer model in Zebrafish. A wide spectrum of liver cancers develop in the fish, from benign adenoma to malignant hepatocellular carcinoma (Kirchberger et al. 2017). Similarly, overexpression of the mMyc oncogene in lymphoid tissue caused lymphoma in fish generating similar phenotype to acute lymphoblastic leukemia (T-ALL) (Hason and Bartůněk 2019).

Ectopic Expression of Targeted Gene in the Embryo

In Zebrafish research, early embryonic injection of the protein of interest encoded by manufactured cap-mRNA is a popular technique. In this approach, all newly generated embryonic cells are injected with an equivalent amount of mRNA for the desired protein. However, because gene expression is not cell type specific, this method is limited. Despite its limitations though, it provides a quick and valuable method for functional gene investigation of either gain or loss of function variants by the targeted gene in the entire embryo. Because the injected mRNA is only stable during the early stages of development, this approach can only be used to study early embryonic processes (Wiebke and Sassen 2015).

Genome Editing in Zebra Fish

Using this technique, site-specific endonucleases are utilized to cause double-strand DNA breaks at specific genomic target sites. It is possible to turn off a gene by inducing double-strand DNA breaks that initiate DNA repair systems in the cell to insert or delete random nucleotides at the cut locations (Raby et al. 2020). This method can also be used to introduce precise genomic alterations in the presence of a designated homologous DNA template (Wiebke and Sassen 2015). Large deletions can also be generated with programmed site-specific endonucleases that induce several double-stranded DNA breaks on the same genomic DNA molecule (Raby et al. 2020). Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 nucleases are the three most common types of programmable site-specific endonucleases utilized for genome editing in Zebrafish models (Raby et al. 2020; Wiebke and Sassen 2015).

Zebrafish Models in Drug Discovery

The whole organism screening has been recognized as a promising option to testing thousands of pharmacological compounds; in this regard, Zebrafish offers a dependable, low-cost, and quick method for screening vast drug libraries and assessing their immediate therapeutic value (Letrado et al. 2018). Depending on the research goal, a wide range of assessment characteristics such as reaction rate, potency, pharmacokinetic

properties, and therapeutic efficacy can be applied to these screenings. Zebrafish larvae are also transparent, making it easy to see morphological effects of the therapy in the early stages of development (Letrado et al. 2018). A classic example is the discovery of two novel Notch inhibitory compounds after a genetic screen compared the effects of more than 200 chemicals on larval development to the classic Notch inhibitor DAPT; results also showed that the novel compounds inhibited the proliferation of human oral cancer cell lines (Kirchberger et al. 2017).

Limitations to the Use of Zebrafish

Zebrafish are an excellent model for biomedical research due to their transparent embryos, high fecundity, and low maintenance costs. However, its use is limited by the fact that fish and humans have different anatomical features; there is also the inability to replicate all aspects of human tumors; the immune systems between humans and fish are also different, and the dissimilar rate of metabolism of different drugs, which may lead to cytotoxicity in Zebrafish but not in humans.

Fruit Fly as an Animal Model for Cancer Research

Even though flies are simpler invertebrates with a completely different architecture from humans, their diminutive size has not hindered them from revealing answers to complex human diseases. For decades, the fruit fly "*Drosophila*" has been used in scientific research and is renowned for its substantial contribution to genetics. Due to its short lifespan, ease of maintenance, low cost, and genetically accessible genome, *Drosophila* has become increasingly popular in scientific research (Gonzalez 2013; Yadav et al. 2016). In addition, molecular signaling pathways in *Drosophila* are highly conserved, allowing it to be used in complex functional experiments and genome-wide research (Yadav et al. 2016; Mirzoyan et al. 2019).

According to several reports, *Drosophila* is increasingly being used as a model for cancer research (Yadav et al. 2016; Mirzoyan et al. 2019). This is because the organism has been acknowledged with having a genome that is 60 percent identical to that of humans (Yadav et al. 2016; Mirzoyan et al. 2019) and for being crucial in the discovery of cancer-linked genes (Tipping and Perrimon 2014). In addition, manipulating cancer-related genes in *Drosophila* can simulate key features of cancer, such as avoidance of apoptosis, persistent proliferation, invasion, prolonged survival, and genetic mutations (Tipping and Perrimon 2014).

Cancer in *Drosophila*

The short lifespan of *Drosophila* prompted the first argument about its use in cancer research. Scientists advised that an organism with a lifespan of 6–8 weeks could not imitate human malignancies (Gonzalez 2013). Yet, Salomon and Jackson reported

that wild-type laboratory strains of *Drosophila melanogaster* are more likely to develop testicular and intestinal tumors as they age (Salomon and Jackson 2008). To examine cancer in *Drosophila*, researchers have recently used cultured cells, organs (eyes, wings, imaginal discs), entire larvae, and whole flies (Tipping and Perrimon 2014; Yadav et al. 2016).

The benefits of employing *Drosophila* to study carcinogenesis include the capacity to perform genetic screens that can probe the entire genome for tumor suppressor functions; some models may also imitate the loss and gain of function mutations that cause human malignancies (Gonzalez 2013). This design has helped develop cancer models that can overexpress or underexpress certain genetic components. A good example is the *Drosophila* model of glioblastoma. Gliomas are the most common type of malignant brain tumor, characterized by rapidly proliferating glial cells. They are also aggressively invasive and treatment resistant (Louis et al. 2016). Witte and colleagues used the Gal4-UAS system to generate *Drosophila* glioma models by overexpressing human tyrosine kinase receptor homologs under the control of the glia-specific promoter. This resulted in uncontrolled proliferation of larval glial cells, as well as tumor-like growth on the optic stalk, which eventually led to glial cell invasion of the optic nerve (Witte et al. 2009). This model can be used to better understand the molecular basis of gliomas and potential therapeutic strategies.

Drosophila has also been recognized for its use in high-throughput anticancer drug screening. Cells, larvae, or adult flies are cultured with a targeted medication and oncogenes tagged with a luciferase or green fluorescence protein (GFP) reporter. Observing the organism's morphology, luciferase activity, GFP expression, or other biochemical assays can then be used to assess the medication's influence on the organism's development (Gonzalez 2013; Yadav et al. 2016). Its short lifespan and ease of reproduction also make *Drosophila* ideal for use in whole-organism drug screening, which can effectively analyze absorption of drugs, dispersion, toxicology, and metabolism. Using whole organisms also enables for drug testing in a multicellular setting and can mimic the disease's complexity (Yadav et al. 2016). A *Drosophila* model of multiple endocrine neoplasia was treated with inhibitors of RET oncogenic activity; the model was also utilized to uncover the pathways responsible for the efficacy and dose-limiting toxicity of other active drugs (Gonzalez 2013).

New medications for integrative cancer treatment are also being discovered through screening in live flies. Combining two or more complimentary treatments may result in lower effective dosages and fewer side effects. To this effect, *Drosophila* can be used to conduct large-scale, impartial screenings that could lead to the discovery of wholly new and more efficient molecular entities (Gonzalez 2013). In addition, Yadav and colleagues proposed that *Drosophila* could significantly contribute to drug repurposing by examining the efficacy of non-cancer drugs for cancer activities, owing to the fact that several diseases share similar molecular pathways and drug targets (Yadav et al. 2016). This will enable expedited screening of existing medications that may be useful in the treatment of cancer.

One significant disadvantage of using *Drosophila* is the large difference in morphology and physiology between flies and humans. Likewise, drug dosages,

formulations, and method of administration in *Drosophila* differ greatly from those in humans. Similarly, because murine models are more closely related to humans, using *Drosophila* as a model for therapeutic testing will require validation by rodent models. Furthermore, these organisms can demonstrate efficacy that is not always replicated in higher vertebrates. However, before using higher animals such as mice, this model organism can be used for early screening of therapeutics and molecular targets.

Cancer Research with Higher Animals

Cancer in higher animals like cats, dogs, and pigs has been documented. It has been reported that cancer is the leading cause of death in older dogs, accounting for about 25% of deaths (Overgaard et al. 2018). Canine cancer models are ideal in preclinical cancer research because canine cancer occurs spontaneously and exhibits similar characteristics to many human tumor (Overgaard et al. 2018). Cancer is also common in domestic cats, though it is probably less common than in canines, cats with cancer, like dogs, have clear advantages over laboratory models of human cancers. In addition to being immune competent, cats and dogs are exposed to the same environmental risk factors that humans do, which reflects the complex interplay between genetics, environmental risk factors, immune system, and tumor microenvironment. Cats and dogs also have a higher degree of homology for specific genes with humans (Cannon 2015). Similarly, oncologists are also turning to porcine models for cancer research due to the high degree of homology between pig and human anatomy and physiology. In addition, the pig's long lifespan allows researchers to track and characterize disease development and progression over a period of time that is comparable to that of humans (Overgaard et al. 2018). When it comes to emulating the human system, higher-level animal research models of cancer appear to be promising, but they come with a slew of drawbacks. Breeding of larger animals is labor-intensive, time-consuming, costly, and monitored by several ethical regulations. There are also a limited number of commercially available reagents for their research (Overgaard et al. 2018).

Conclusion

Humans are impressively complicated species that differ greatly in physiology, heredity, and immunology. Since humans cannot be used as a study model, animals are continually offering new ideas for cancer research because they are such an excellent tool for studying the pathophysiology of human diseases. No one universal animal model for preclinical testing or studying the complicated pathways of tumor/immune cell interactions appears feasible, because cancer is not a single disease, and different tumor forms require diverse treatment strategies. Determining what type of model to utilize in cancer research will be heavily influenced by the study's goals. Drug screening or repurposing experiments can be easily assessed in simpler models

like fruit fly or zebra fish embryos, while more complicated studies can rely on rodents and higher animals. Oncology research may benefit from larger animal models such as cats, dogs, and pigs, but these animals cannot replace the vast diversity of mouse models, which continue to provide valuable information. It will become increasingly important as our understanding of cancer grows to build more personalized animal models for drug discovery.

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Animal Models of Gynecological Cancers

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Abstract

Gynecological cancers develop in the reproductive organs of a woman. The common gynecological cancers are cervical, ovarian, uterine, vaginal, and vulvar cancers. Most of the gynecological cancers including the ovarian cancers undergo metastasis to distant organs, acquire resistance to cancer therapy, and relapse. There are no specific diagnostic biomarkers available to detect the early stages of gynecological cancers. The challenges to diagnose and treat gynecological cancers stem from the fact that these cancers are heterogeneous diseases and, therefore, need physiologically relevant animal model systems in order to develop strategies to discover targeted therapeutics. In this book chapter, the authors elaborate on mouse models that serve as helpful tools to understand the biology of gynecological cancers as well as the identification of biomarkers and novel therapeutics.

Keywords

Ovarian cancer · Endometrial cancer · Animal model · Gynecological cancer · Cancer

Introduction

Cancer is a major global health problem with millions of people being diagnosed with cancer throughout the world, and nearly 50% of patients die from this disease (Ma and Yu 2006). Cancer is one of the primary causes of global mortality as it led to nearly ten million deaths in 2020. Gynecological cancer is a type of cancer that originates in one of the female reproductive organs, which include the ovaries, cervix, uterus, vagina, and vulva. In 2018, the American Cancer Society (ACS) reported over 110,000 newly diagnosed cases of gynecological cancers, resulting in over 32,000 deaths alone just in the United States. Each gynecological cancer is unique in its origin, symptoms, prognosis, and risk factors. Women with increased age are at a higher risk of developing gynecological cancers. In general, early detection of gynecological cancers can lead to an increased chance of survival. To improve the current treatment and increase the survival and quality of life of gynecological cancer patients, we need to understand the pathobiology of gynecological cancers. The extensive molecular characterization of gynecological cancers will facilitate the identification of new biomarkers of early growth, progression, and treatment outcome. Importantly, the gynecological cancers can be established in

animal models and could be used to determine the role of key genes/proteins by overexpression or knockdown studies, which can lead to the development of novel targeted therapies (Ramachandran et al. 2012; Ramadoss et al. 2017). In this book chapter, we discuss about the major types of gynecological cancers and the specific animal models that facilitate deeper understanding of the tumor biology of gynecological cancers.

Ovarian Cancer

Ovarian cancer (OC) ranks fifth among cancers in women, with more than 22,530 women diagnosed each year. In every 78 women, 1 will develop ovarian cancer in their lifetime, and about 1 in 108 women will die from ovarian cancer. The risk of developing ovarian cancer increases with age, and the lifetime risk is about 1.6%. Genetic predisposition is a major risk factor in the development of ovarian cancer (McLemore et al. 2009). Thus, women with a family history of ovarian cancer, especially first-degree relatives, have a 5% risk of developing ovarian cancer. Mutations in breast cancer gene 1 (BRCA1) or BRCA2 in women present a risk between 25% and 60% (McLemore et al. 2009; Pruthi et al. 2010).

Ovarian cancer subtypes are classified as endometrioid, mucinous, serous, and clear cell carcinoma (Bast et al. 2009). The most common form of ovarian cancer diagnosed in women aged 40 years or older is epithelial ovarian cancer (EOC). The incidences of other types of ovarian cancer include serous (about 50%), endometrioid (about 25%), mucinous (6–16%), and clear-cell (5–11%) ovarian carcinoma.

The high mortality rates associated with ovarian cancer are partially due to misdiagnosis or lack of diagnosis at an early stage, due to nonspecific or misinterpreted symptoms. About 70% of patients are diagnosed at an advanced stage with approximately 66% of late-stage patients eventually relapse and develop resistance to standard therapy. Importantly, the cancer stem cells (CSCs) are important contributors to tumor development and therapeutic resistance (Phi et al. 2018), and thus lead to tumor aggressiveness (Chengizkhan et al. 2020). Several pathways including Notch, Wnt/β-catenin, TGF-β, Sonic Hedgehog, PTEN, FGF, IGF1, and BMI1 are implicated in regulating the proliferation, survival, self-renewal, cell fate determination, as well as maintenance of CSCs in ovarian cancer (McAuliffe et al. 2012).

Animal Models to Study Different Types of Ovarian Cancer

Immunodeficient xenograft mouse models: The xenograft mouse model represents an immunocompromised mouse harboring a human tumor that is generated through orthotopic or heterotopic implantation of human tumor tissue, cell line, or primary cell culture. Strong immune rejection is the profound barrier for the engraftment of human cancer cells in immunocompetent mice (Yang and Sykes 2007). Therefore, immunodeficient mice were developed to overcome this rejection of human cancer cells facilitated by the mouse adaptive (B- and T-cells) as well as

innate system cells like macrophages and natural killer cells through perturbation of the genes critical for immune function (Tian et al. 2020). The discovery of congenitally athymic nude mice was a monumental breakthrough in the investigation of human cancers using immunodeficient mice in the 1960s. The nude mice are naturally homozygous for the *Foxn1*^{nu} or nude mutation. *Foxn1* gene encodes a transcription factor that is required for both hair follicle and thymic development. Hence, nude mice lack a thymus (where $CD4^+$ and $CD8^+$ T-cells differentiate and mature) and hair (nude). Due to T-cell deficiency, nude mice cannot support the majority of immune responses, including cell-mediated immune responses, antibody formation, hypersensitivity responses that are delayed, destruction of malignant T-cells, as well as graft rejection, thereby making the nude mouse immunodeficient. Essentially, nude mice can accept every type of human tumor. This attribute, however, is limited by the existence of both an intact humoral adaptive immune system and an innate immune system, which hinders the successful engraftment with some primary human tumor cells (Olson et al. 2018).

The next major development in the field was the identification of spontaneous mutations in C.B17 mice termed "severe combined immunodeficient (SCID)" (*Prkdc*^{scid}, protein kinase, DNA-activated, catalytic polypeptide) (Shultz et al. 2014). This mutation impedes the recombination of antigen receptor genes, thereby resulting in the arrest of B and T lineage-committed cells in their early development (Bosma and Carroll 1991). Though SCID mice are more receptive hosts to the engraftment of human cells and tissues as compared to athymic nude mice, they have an intact innate immune system consisting of NK cell action that limits the engrafting and growth of human tumors. Further research generated non-obese diabetic (NOD)/SCID strain mice that have intrinsic deficiencies in innate immunity, leading to lower activity of NK cells and decreased activation of macrophages, abnormal functioning of dendritic cells, and no hemolytic complement (Shultz et al. 2014). A major leap forward in developing higher-order immunodeficient mice was achieved when *NOD-Prkdc*^{scid} was combined with desired mutation in the interleukin-2 (IL-2)-receptor common gamma chain gene (*IL2rγ*^{null}), resulting in NOD-*Prkdc*^{scid} *IL2rg*^{null} (NSG) mice with the absence of adaptive immunity and deficiency in innate immunity and therefore being more susceptible to the engraftment of human tumors (Olson et al. 2018).

Cell line-derived xenograft (CDX) model involves the injection of established human cancer cells subcutaneously or orthotopically into immunodeficient mice. This model is used in cancer drug discovery and research (Day et al. 2015). Another widely used models include the intraperitoneal or intrabursal injection of tumor cells in immunodeficient mice (Shaw et al. 2004; Cordero et al. 2010; Magnotti and Marasco 2018). Intraperitoneal model closely represents the late-stage metastatic ovarian cancer, while the intrabursal model represents localized disease as tumors are mostly confined to the ovary. In contrast to mice in which the ovary is covered by the bursal membrane, humans do not have bursal membrane which allows the easy and rapid metastatic spread of tumor cells (Lengyel 2010). Because of this

anatomical difference, the intrabursal ovarian cancer models may not truly represent the metastasis in human ovarian cancer.

CDX models are useful to some extent, however, these models don't recapitulate the actual patient scenario as the biology and heterogeneity of human cancer cell lines differ greatly from the original tumor tissue. Furthermore, CDXs have failed to predict patient response to targeted therapies as observed by a very low approval rate of about 5% by United States Food and Drug Administration (FDA) (Day et al. 2015).

Syngeneic Mouse Models of Ovarian Cancer

Studies that require an intact immune system rely on immunocompetent animal models. Syngeneic mouse models can be used to examine the antitumor immune response in the tumor microenvironment including immune cell infiltration in ovarian cancers. The tumors can be initiated using established ovarian cancer cell lines that originate in the same mouse strain with similar genetic background, thereby, minimizing the immune rejection (Fig. 1). The syngeneic mouse models with orthotopic injection of tumor cells in the ovarian bursa/intraperitoneal cavity can model histopathological characteristics of ovarian cancer that can allow the examination of mechanisms underlying tumor immune evasion and ovarian cancer metastasis (Nunez-Cruz et al. 2010). The orthotopically implanted tumors into the organs from where the cancer originated can model early disease progression in the physiologically relevant tumor microenvironment (Quinn et al. 2010).

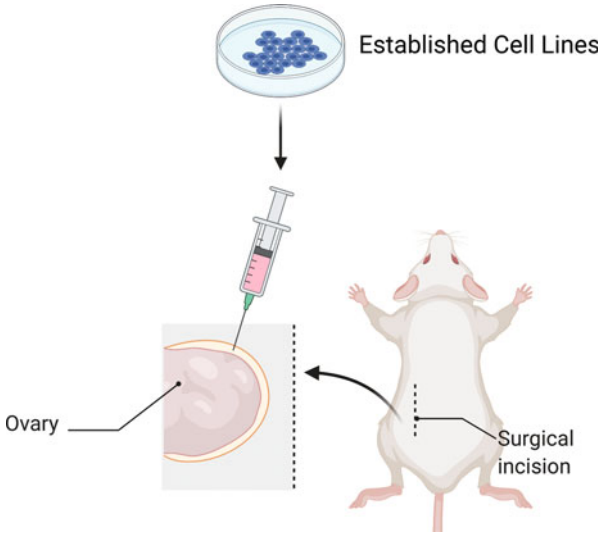

Fig. 1 Syngeneic orthotopic mouse model: Established ovarian cancer cell lines are prepared for injection. A dorsolateral incision is made, the ovary is located, and cells are injected

Applications of Syngeneic Mouse Models

Syngeneic mouse models are valuable preclinical tools used in identifying the response to immunotherapy. The ovarian syngeneic preclinical mouse models have led to several clinical trials investigating novel immunotherapy treatment strategies. Examples of such trials include testing of chemotherapy drug cisplatin in combination with rintatolimod, a toll-like receptor 3 (TLR3) agonist, and pembrolizumab, a programmed cell death protein 1 (PD-1) immune checkpoint inhibitor in patients with recurrent ovarian cancer (https://clinicaltrials.gov/ct2/show/NCT03734692).

Immune checkpoint inhibitors are used to treat various cancers including melanoma, where patients display durable response to immunotherapy. This new paradigm shift in cancer treatment led to numerous clinical trials using immune checkpoint inhibitors and ultimately approval of pembrolizumab (monoclonal antibody for PD-1) for patients who harbor the advanced stage of disease with a high mutational burden and high microsatellite instability (Matulonis et al. 2019). Although this is a huge milestone for treatment for patients with advanced ovarian cancer, many patients remain refractory to immunotherapy. Notably, current research efforts are geared toward the identification of novel factors that drive resistance to immunotherapy and investigation of the underlying mechanisms using these preclinical models.

Predictive biomarkers have also been investigated in syngeneic models to assess the efficacy of immune checkpoint inhibitors. Established syngeneic mouse models using validated mouse ovarian cancer cell lines are used to identify the biomarkers, such as CXCL9, as a driver of effective immune checkpoint blockade of PD-L1 in preclinical ovarian cancer (Seitz et al. 2022). The convenient and easy-to-use syngeneic models have facilitated a deeper understanding of the mechanism underlying the efficacy of immunotherapy and help to identify the proteins/factors that can be targeted to increase the response to immunotherapy in ovarian cancer patients.

Challenges and Limitations of Syngeneic Mouse Models

The main advantage of syngeneic mouse models is their feature of full murine immunity which allows to study the cross talk between the tumor, its microenvironment, and the surrounding immune cells. This model provides an opportunity to study various factors including secreted factors from immune cells, tumor intrinsic factors, and immune cell infiltration, among many other possible interactions, and explore the novel interventions that can overcome the challenges associated with the treatment of advanced ovarian cancer. However, this model also has major limitations that can often lead to the findings that are difficult to translate. This limitation is primarily due to the differences between mouse and human tumors. While they may share many similar features, human cancers are more complex and contribute significantly to the difficulty of interpreting the results derived from this model. Similarly, mouse cell lines do not recapitulate the heterogeneity present in human tumors, further limiting the physiological relevance of this model (House et al. 2014).

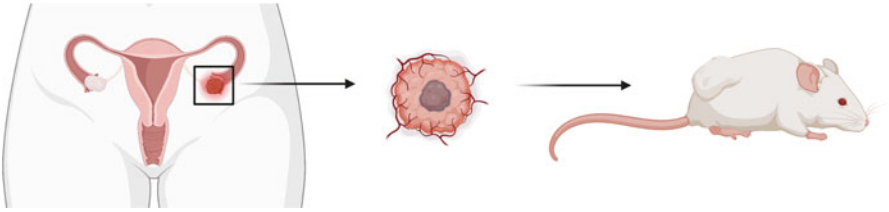

Fig. 2 Patient-derived xenograft (PDX) mouse model: Cancer tissue from patient surgery or biopsy is obtained and processed. Cancer tissue is engrafted in orthotopic or nonorthotopic sites in immunocompromised mice

Patient-Derived Xenograft Models of Ovarian Cancer

Patient-derived xenograft (PDX) models are widely used animal models that are developed by implanting human tumors derived from patients directly into an immunocompromised mouse (Fig. 2). PDX models are useful in representing the tumor of origin by retaining the complexity and heterogeneity that are deficient in other preclinical models. The development of PDX models overcomes some of these major limitations. The extensive handling of cell lines *in vitro* can result in drastic changes in their genome. PDX models, on the contrary, can be maintained *in vivo,* avoiding the *in vitro* culture steps and reducing the changes to the genome while retaining the histopathological features of the primary human tumors (Domcke et al. 2013). However, cultured cancer cells display altered genetic information including gain- and loss-of-function mutations that can lead to changes in the growth and invasion abilities (Gillet et al. 2011). PDX models have become one of the preferred methods for drug efficacy studies, for biomarker identification, and for other preclinical research on ovarian cancer to overcome the limitations of using conventional cell lines.

Applications of Patient-Derived Xenograft Models

There are several applications of PDX models that are employed to understand the different stages of ovarian cancer, and this depends on the study design including the location of the tumor implantation. This model provides the opportunity to study the ovarian cancer using subcutaneous or orthotopic engraftment of the tissue or a dissemination model to study the metastasis. The preclinical drug development for ovarian cancer relies on models that can recapitulate human disease and drug response by retaining the molecular and histological characteristics. PDX models can predict the clinical outcomes of new therapeutic approaches, identify the biomarkers, and shed lights on the biology of ovarian cancer to generate a strategy for personalized medicine (Hidalgo et al. 2014). Previous studies have shown a highly positive correlation between patient response and drug interventions and comparable experimental drug intervention in PDXs (Butler et al. 2017).

An earlier study using 20 distinct human ovarian tumors implanted into SCID mice demonstrated that >65% of the tumors reach a size large enough for passage into other SCID mice suggesting a moderate to high penetrance. Of those, several mice further developed metastasis and ascites representing the natural clinical progression of ovarian cancer (Xu et al. 1999). This study illustrates the flexibility of PDX model to conduct large-scale studies and to recapitulate human tumor progression. PDXs are powerful models for preclinical testing of new therapeutic strategies that can connect the findings from scientific studies to clinical translation. Several clinical trials have implemented the use of PDX models already, including combination therapy trials for patients with platinum-resistant ovarian cancer (https://clinicaltrials.gov/ct2/show/NCT02312245), thus highlighting the significance of utility of this animal model.

Challenges and Limitations of Patient-Derived Xenograft Models

PDX models are adaptable and can be used in parallel with other models to generate valuable preclinical data. While this model is promising, there are challenges and limitations to this model. Primarily, this model requires more time as tumor engraftment can be labor-intensive, and it takes several weeks for this model, to be prepared to passage into more animals. Additionally, obtaining patient samples can often be challenging for researchers and requires the generation of a tumor bank for this model to be more accessible. Cost can also become a challenge for researchers to maintain this model due to the requirement of expensive genetically engineered mice and the facility costs to continue studies over a long period of time. One major limitation of PDX models is the lack of immunity, which impedes the study of the immunotherapy and the immune response. For sufficient tumor engraftment, ovarian cancer PDX models require an immunodeficient host, and the absence of an immune system prevents the examination of the role of the immune system. Other limitations include the loss of human stroma and vessels, which is replaced by the mouse stroma and vessels 15–25 weeks after tumor engraftment. Therefore, this model may not be suitable to study the interventions targeting the human stromal components or vasculature (Hylander et al. 2013).

Specialized Mouse Models of Ovarian Cancer

Genetically engineered mouse models: Over the last decade, the development of genetic engineering techniques has resulted in an extraordinary growth in our understanding of the genetic basis of cancer (Frese and Tuveson 2007). Collectively, these pioneered the establishment of mouse models with the ability to incorporate specific genomic alterations to induce tumor development in a tissue-specific manner. Genetically engineered mouse models (GEMMs) are systems in which genetic changes are made in mice that can promote the development of a particular disease.

These models primarily employ tissue-specific promoters to enhance expression of an oncogene related to tumor formation or tissue-specific expression of recombinase enzymes to facilitate the deletion of tumor suppressor genes (Olson et al. 2018). The primary application and strength of GEMMs are the study of tumor growth and progression, as well as the identification of the role of specific genetic alterations in cellular transformation. In ovarian cancer studies, tumors are created by silencing appropriate tumor suppressor genes or activating the oncogenic genes using knock-out or knock-in approaches (Fig. 3). The use of this approach can examine driver mutations involved in tumorigenesis by the genetic manipulation of specific genes that are observed in human patients. The invaluable feature of GEMMs is the power to study the tumor initiation, progression, and metastasis of ovarian cancer while assessing the physiological relevance of specific genetic mutations found in human diseases (House et al. 2014).

GEMMs can be categorised as endogenous or transgenic. Endogenous GEMMs represent mutant mice with a loss of tumor suppressor genes (TSG) and over-expression of dominant-negative TSGs, as well as oncogenes. The transgenic mice models are developed through pronuclear injection of cDNA constructs consisting of promoter regions fabricated to inhibit tissue tropism. Transgenic GEMMs recapitulate identical genetic composition of amplified or translocated proto-oncogenes. To generate transgenic GEMMs, a direct injection of fertilized oocytes or through the gene targeting and lentiviral transduction in embryonic stem cells is often employed (Frese and Tuveson 2007). The advantages of transgenic GEMMs include the capability to control the target gene expression in a reversible manner.

Altered Genes	Cancer Histology	Reference
p53, c-Myc, Kras, Akt	Ovarian Carcinoma	Orsclic S. et al., 2002
p53, Rb1	Epithelial Ovarian Cancer	Flesken-Nikitin A. et al., 2003
p53, Rb, Brca1 or Brca2	Serous Epithelial Ovarian Cancer	Szabova L. et al., 2012
Pten, Apc	Ovarian Endometrioid Adenocarcinoma	Wu R. et al., 2007

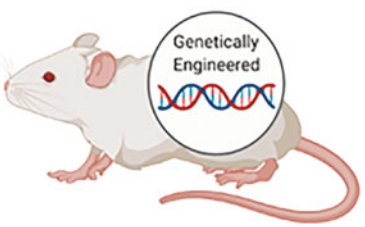

Fig. 3 Genetically engineered mouse model (GEMM): Specific genes are knocked out in the cell of interest to model the tumor development of ovarian cancer. The table summarizes the most utilized altered genes to create mouse models that develop into different ovarian cancer histologies

Applications of Genetically Engineered Mouse Models

A major advantage of GEMMs is the ability to examine the role of specific genes in the malignant cellular transformation in cancer. The study of prevention of disease can further be investigated using this model by observing the effects of interventions in models with particular gene alterations. The use of this model has led to a deeper understanding of cancer initiation, angiogenesis, invasion, metastasis, and the immune system overall in the context of cancer. In particular, GEMMs can provide the opportunity to study the spontaneous metastasis during tumor progression, which recapitulates human patient scenario (Hasan et al. 2015). GEMMs allow for specific gene alteration in a tissue-specific manner that can be regulated at critical times in the development or adulthood to mimic the human diseases. There are two methods of transforming cells in GEMMs: RCAS/TVA gene delivery system, which is performed *ex vivo*, and the Cre-LoxP system, which delivers the Cre recombinase to the orthotopic site. Both of these approaches allow for transformation specifically in the ovarian surface epithelia cells and are temporally controlled (Sale 2009). To study high-grade serous ovarian cancer (HGSOC), an investigation that utilized GEMMs found PAX8 as a driver of mouse HGSOC, and these PAX8-driven murine tumors were shown to have a strong correlation with human tumors. In this GEM model, the genetic alterations of *Brca, TP53,* and *Pten* resulted in intraepithelial precursor lesion. This study also outlined that HGSOC can initiate from the fallopian tube secretory epithelial cells (Perets et al. 2013).

GEMMs are suitable models to investigate the response of immunotherapy and can aid in the discovery of novel treatment strategies to treat patients with ovarian cancer. The study of immunotherapy in GEMMs requires a distinct approach compared to syngeneic models or other animal models; nonetheless, the use of GEMMs in immunological studies is increasing. GEMMs can model tumor intrinsic and extrinsic features that can initiate *de novo* tumor formation and the natural advancement of the cancer including metastasis, making these models essential for preclinical research and can be used in parallel with clinical trials, termed "co-clinical trial." The successful use of GEMMs has led to the validation of drug targets and cancer-causing genes, as well as for the assessment of the efficacy of therapeutics and evaluation of the mechanisms that contribute to treatment resistance.

Challenges and Limitations of Genetically Engineered Mouse Models

Several advantages of GEMMs have led to a more comprehensive understanding of the precise role of essential genes involved in tumor development and have enhanced our understanding in tumorigenesis. However, certain caveats exist with this model. The major limitation of using GEMMs is the time to develop this model. There is a latency period for *de novo* tumor formation to occur, and the mice can develop cancer at different stages with different levels of penetrance of mutations varying from 50% to 100%. This can result in complex and prolonged studies that can be

costly. The generation of GEMMs usually takes over a year to develop, and once developed, faster tumor growth and high tumor burden can force the termination of the study. This can impede the study of metastatic disease since animals are required to be sacrificed at an early tumor developmental stage. One more major limitation of this model is the absence of a complex genomic landscape that is prominent in human disease. Although GEMMs are labor- and resource-intensive and require time and careful consideration, they deliver valuable information that cannot be obtained from other animal models (Mullany and Richards 2012).

Humanized Mouse Models of Ovarian Cancer

PDX mouse models have been the most successful among the established models of gynecological cancers; however, they are primarily useful in the preclinical testing of chemotherapeutics drugs. Due to the site-specific differences, studies investigating the efficacy of immunotherapeutic drugs involving syngeneic animal models have largely been unsuccessful. Therein exists a void in the translatability of the immune system from mice to human, which has recently been addressed by the emergence of humanized mouse models. Humanized mouse models have been developed with the aim of incorporating an intact human immune system in mouse models to generate and study the immune responses following immunotherapeutic intervention (Choi et al. 2018). This model represents a paradigm shift in understanding the human immune response in the context of gynecological cancers and imparts high translational utility for novel immunotherapies for these cancers.

In order to develop this model, investigators engraft a functional human immune system into mouse strains that are murine immunodeficient. The three major humanized mice models are Hu-PBL-SCID, Hu-SRC-SCID, and BLT (bone marrow, liver, thymus) models. Hu-PBL-SCID mice models are established by engrafting human peripheral blood leukocytes (PBL) through intraperitoneal or intravenous injection into adult immunodeficient mice. Immunodeficiency can be achieved by irradiating the mice at sublethal doses. This method is relatively less time-consuming, and the development process is fairly simple. The Hu-SRC-SCID model is established by injecting the human $CD34^{+}$ hematopoietic stem cells (HSCs) into adult immunodeficient mice that are derived from various sources including HSCs from the peripheral blood mobilized with granulocyte colony-stimulating factor (G-CSF), blood from the umbilical cord, bone marrow, or fetal liver HSCs. The third major humanized mice model, the BLT model, is developed through the implantation of thymus and liver tissues derived from the same human fetus and then engrafted into immunodeficient mice. These mice are usually conditioned using renal capsule followed by the injection of autologous $CD34^{+}$ fetal liver HSCs intravenously. This model presents the most physiologically equivalent engraftment of a human immune system, which can be further strengthened when used in NSG mouse models (Yin et al. 2020; Tian et al. 2020).

A number of studies have employed advanced humanized mouse models in evaluating the efficacy of various immunotherapies. A combined dual-blockade

therapy of programmed cell death protein 1 (PD-1) and the immune checkpoint T-lymphocyte-associated protein 4 (CTLA-4) with autologous tumor-associated leukocytes has been shown to effectively reduce the ovarian cancer progression in PDXs of the humanized mice. Furthermore, it was also reported in this model that the combination of anti-CTLA-4 and anti-PD-1 increased the tumor-recognizing $CD8^+$ T cells that infiltrated the tumor microenvironment, showing an acquired memory phenotype in the T cells, and led to the protection of tumor growth upon tumor rechallenge in the animals (Odunsi et al. 2020).

Endometrial Cancer

Endometrial cancer (EC) is the sixth leading cause of cancer-related deaths among women in the United States, with an estimated ~76,000 deaths annually among women globally (Tang et al. 2021; Urick and Bell 2019). It usually originates in the cells that line the endometrium of the uterus and is also sometimes referred to as uterine cancer. Endometrial cancer accounts for about 5% of all diagnosed cancers and affects mainly postmenopausal women. An estimated 5% of EC occurs due to inherited cancer genetic predisposition syndromes, commonly Lynch syndrome, while the majority of EC diagnoses are considered sporadic. Notably, recent studies demonstrated that the number of deaths attributed to endometrial cancers is increasing. There are persistent racial disparities in the survival of patients with endometrial cancers, and this is illustrated by the variation between 60% and 80% 5-year survival rates depending on the different region of global population (Mukerji et al. 2018). Endometrial cancer consists of four distinct histological subtypes, which include endometrioid endometrial cancer (EEC, type I), serous endometrial cancer (SEC, type II), clear-cell endometrial cancer (CCEC, type III), and mixed endometrial cancer and uterine carcinosarcoma (type IV) (Urick and Bell 2019). Type I EC tumors represent around 70% of the diagnosed cases and are considered the most frequent subtype; they are considered low grade and are linked to estrogen stimulation. Type II EC tumors, in contrast, are less common, more aggressive, and commonly considered high grade, metastatic, and independent to estrogen stimulation and exhibit a higher risk of relapse after treatment. Around 10% of diagnosed endometrial cancers are type II tumors, accounting for 40% of deaths, and are associated with a poor prognosis (Sorosky 2012; Urick and Bell 2019).

Animal Models to Study Different Types of Endometrial Cancer

In accordance with other cancers, animal models of endometrial cancers provide a strong impetus for translational research, in vivo disease modeling, and therapeutic testing. Several animal models for endometrial cancers have been proposed and are presently utilized by research scientists and are highlighted in the sections below.

Rodent Models of Endometrial Cancer

Rodent models have been widely investigated in endometrial cancers, especially after the seminal discovery by Deerberg et al. (1981), where it was observed that there was an incidence rate of uterine tumors around 40% in female Wistar rats. Furthermore, Nagaoka et al. (1990) also demonstrated a 35.1% incidence rate of endometrial adenocarcinoma in Donryu rats, around 60% of which eventually develop tumor lesions in the endometrium. Moreover, findings by Tanoguchi et al. (1999) suggested similar signatures in tumors arising in Donryu rats to mutated KRAS as compared to endometrial cancers in humans. BDII/Han rats are also known for their high spontaneous tumor development of greater than 60% in their lifetime and are highly characterized at both genomic and molecular levels. Samuelson et al. (2009) showed how tumors in these rat models are comparable to type I human endometrial cancer and thus serve as excellent models to recapitulate the genomic and molecular features identified in the human endometrial cancers.

Chemical-induced rodent models of endometrial cancer are also of significant interest to researchers as tumors induced through chemical treatment serve as translational models that can be utilized to study the effects of chemoprevention. To study the effects of danazol on endometrial tumorigenesis, Niwa et al. (2000) utilized female ICR mice. The only major limitation of this type of model is the inimical effects on the metabolism and nonspecific toxicity upon chemical treatment.

Transgenic Mouse Models of Endometrial Cancer

Transgenic mouse models are the most widely used animal models to investigate the biological mechanisms related to endometrial cancer development. Phosphatase and tensin (PTEN) is one of the most altered genes in endometrial cancers, and knocking down its expression resulted in one of the first transgenic endometrial cancer models. To generate hyperplasia, knocking out of any one of the two alleles ($PTEN^{+/-}$) is adequate to form carcinoma in 20% of all cases. Knocking out both alleles ($PTEN^{-/-}$) is embryonically lethal for the mouse; however, to address this issue, conditional knockout systems including tamoxifen-inducible transgenic systems and AAV-mediated Cre-Lox are often utilized. The investigations with these systems have eluded that PTEN inactivation leads to rapid induction of endometrial carcinoma. This model also set the stage to investigate other genes associated with endometrial cancer development. A study conducted by Contreras et al. (2010) depicted that inactivating LKB1 further drives the progression of endometrial cancer development. Another study (Cheng et al. 2014) succeeded in establishing a transgenic mouse model with the combined deletion of LKB1 and PTEN that depicted reduced survival and modeled an advanced state of the disease.

TP53 mutations are often present in both type I and type II human endometrial cancer. The investigations by Daikoku and colleagues (2011) demonstrated that a combined deletion of $PTEN^{-/-}$ and $TP53^{-/-}$ led to an aggressive phenotype as well as reduced survival when compared to deletion of just $PTEN^{-/-}$ alone. While type I

endometrial cancer is the most commonly studied endometrial cancer, type II endometrial cancer is generally more aggressive and has a higher mortality in patients. Interestingly, studies (Akbay et al. 2013) demonstrated that the combined gene deletion of POT1A (along with TP53) resulted in an endometrial cancer resembling a type II phenotype at 9 months of age in these mice and further led to metastasis in all of the mice with the genetic deletions of POT1A and TP53 at 15 months of age.

Mitogen-inducible gene 6 (MIG-6) is a gene that is regulated by stress stimuli and mitogens, and it is known to negatively regulate EGFR signaling (Kim et al. 2017). Thus, another endometrial cancer model was developed using a transgenic mouse model with MIG-6 knockout in the uterus, and this model was utilized to uncover the estrogen-dependent tumor suppressive function. All these aforementioned transgenic mouse models have been widely investigated to assess the therapeutic response to various agents including Akt-mTOR inhibitors, PARP inhibitors, as well as palbociclib (CDK4/6 inhibitor). While these well-characterized models serve as excellent resources to further disseminate the tumor biology of endometrial cancers, they are insufficient to recapitulate the native heterogeneity of endometrial cancer and thus are not physiologically accurate (Van Nyen et al. 2018).

Recently, an orthotopic immunocompetent mouse tumor model of metastatic endometrial cancer was established from endometrial cancer that was developed in a GEMM. In this model, Fedorko et al. (2020) generated an immortal cell line MECPK (mouse endometrial cancer PTEN deleted K-ras activated) from a 4-week-old $Pgr^{cre/+}Pten^{f/f}K\text{-}ras^{G12D}$ GEMM that developed endometrial cancer. After tumor cell engraftment, the mice developed local and metastatic endometrial tumors particularly lung metastasis. This immunocompetent orthotopic tumor model may offer advantages of exposure of tumor cells to the physiologically appropriate microenvironment in the uterus and establishment of tumor in mice with an intact immune system.

Patient-Derived Xenografts (PDXs) of Endometrial Cancer

PDX models for endometrial cancer are generated by directly engrafting the tumor piece subcutaneously into immunocompromised mice (Jia et al. 2021). However, few studies have generated endometrial cancer orthotopic PDX models by intrauterine injection of tumor-derived cells (Fonnes et al. 2020). PDX models commonly used for uterine cancer research are generated by 2012 subcutaneous implantations (Shin et al. 2022). The generation of first PDX model where resected tumor tissue from an endometrial tumor was implanted orthotopically through transvaginal injection into nude mice (Cabrera et al. 2012). In another study, an orthotopic PDX model was developed by dissociating the primary tumor biopsy into cell suspension and then injecting them to uterine horn of NSG mice (Haldorsen et al. 2015). Over the last decade, seminal studies by Depreeuw and colleagues (2017) as well as Unno et al. (2014) have characterized over 24 endometrial cancer PDX models representing primary, metastatic, and recurrent endometrial cancer as

well as from patients undergoing surgery. Importantly, the development of PDX from patient-derived tumor organoids has gained a lot of attraction. Pauli et al. (2017) reported the generation of PDXs from 18 different endometrial cancer types. These PDXs had 86.4% engraftment rate and successfully formed endometrial cancer in multiple mice models. These PDXs offer an exciting avenue for personalized medicine and can lead to maximizing treatment efficacy in endometrial cancer patients.

Conclusion

Several gynecological cancer-related animal models are available. These animal models are useful tools to improve our knowledge on gynecological cancers. In particular, PDX-based models of gynecological cancers are expected to add significant value in animal model-based gynecological cancer research. The next few decades will likely witness further advancement in the modeling of various diseases with the ultimate goal of progressing patient care and increasing treatment outcomes for patients with gynecological cancers.

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In Vivo, Ex Vivo, and In Vitro Model Systems for Liver Cancer Research

18

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Contents

Abstract

Cancer claims the lives of millions of people worldwide. Liver cancer represents one such form of cancer that has a high incidence with a poor survival rate when diagnosed late and increased mortality. Hence, pursuing research to understand

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the disease pathology and progression is indispensable to curtail mortality. In the past century, methods for studying liver cancer have advanced greatly, giving significant insight into the hallmarks of cancer. For many years, several model systems have been carried out to imitate the characteristics of liver cancer cells in humans; for instance, to understand virus-mediated cancer occurrence in living things and to study liver cancer in cultured cells. In this chapter, several in vivo, ex vivo, and in vitro models are elaborately described such as transgenic and knockout mice, the type of cancer cell lines, 3D spheroid cultures, gene-editing techniques, and the idea of cancer stem cells pertaining to liver cancer. In vivo, ex vivo, and in vitro model systems are important for cancer research because they help to understand liver cancer biology and development of therapeutic measures for better liver cancer treatment.

Keywords

Hepatocellular carcinoma · Non-invasive · Magnetic resonance imaging · Angiogenesis · Viral hepatitis

Introduction

The most common type of primary liver cancer (PLC) is hepatocellular carcinoma (HCC). It accounts for nearly 90% of all PLCs, including intrahepatic, ductular, and perihilar cholangiocarcinoma (CCA). Hepatoblastoma is the most common type of PLC in children (Marin et al. 2019). According to IARC Program, infectious agents constitute a significant grouping of agents as "carcinogenic to humans" (Group 1) (Baan et al. 2009). Among the numerous probable etiological conditions linked to HCC, HBV, and HCV viruses, O. *viverrini* and C. *sinensis* are Group 1 infectious pathogens that can cause cancer in the liver (Wrighton et al. 2019). The relative risk of developing PLC in patients with HCV infection is 2.5 (De Martel et al. 2012; El-Serag and Kanwal 2014; Lemon and McGivern 2012).

Diagnosis and Staging of HCC

The primary goal of HCC tumor staging is to assess the disease's prognosis. The appropriate therapy option for the patient also contributes to developing clinical studies and research. The degree of hepatic dysfunction, tumor stage, and overall patient state influence prognosis and treatment. Most HCC patients have concomitant liver cirrhosis, which is frequently as essential as or even more critical than the tumor stage.

Imaging Techniques

Imaging is crucial in the diagnosis of HCC. Imaging technological advancements over the last two decades have contributed to better diagnosis of hepatic lesions with a greater variety of opportunities. However, detecting smaller tumors remains

challenging, especially in cirrhotic patients with poor parenchymal morphology. Differentiating HCC from benign cirrhosis lesions or subsequent cancers remains difficult. Since noninvasive approaches are available, they are much preferred. Computed tomography (CT), Magnetic Resonance Imaging (MRI), and Contrast-Enhanced Ultrasound (CEUS) are the currently available noninvasive technologies that have displaced mainly biopsy and classical angiography in the diagnosis of HCC. Variations in attenuation on CT, return on the signal on CEUS, and signal intensities on MRI between the HCC and the surrounding liver tissue are all used to diagnose (Bialecki and Di Bisceglie 2005).

Detection of HCC

Patients who have abnormal results, such as a liver nodule on abdominal ultrasonography (USG) or elevated alpha-feto protein (AFP) values (>20 ng/ml), are at a higher risk of HCC and should be further examined as soon as possible. Usually, USG-detected liver lesions 1 cm in diameter are not HCC and are harder to predict. As a result, cross-sectional imaging is unnecessary, and a 3-month follow-up with repeat USG is sufficient. Enhanced technologies of CT (Quadruple phase) or MRI (Dynamic contrast) can detect lesions larger than 1 cm. Due to the tumor's altered blood supply relative to the liver background, HCC lesions show brighter than the surrounding liver in the delayed phases of a CT scan or MRI (Llovet et al. 2021; Choi et al. 2014). However, outcome measures are increasingly suggesting biopsies to define the molecular features of HCC. In classic HCC, the arterial phase increases, followed by a portal washout and a delayed phase with a false capsule around the nodule. Dynamic MDCT with the high spatial and temporal resolution is also available and can effectively reconstruct 3D or even 4D pictures of liver tumor evaluation. Perfusion CT (PCT) allows for a thorough assessment of hepatic hemodynamics on tumor-related angiogenesis. After administering an iodinated contrast agent, acquire serial pictures by PCT. This technique quantifies tissue perfusion parameters and determines segmental liver function (Hennedige and Venkatesh 2012).

Biopsy and Histopathology

Whereas most HCCs show characteristic findings in imaging, 10% of tumors have an unusual appearance. The International Consensus Group for HCC has recommended the glandular pattern, fatty alterations, stromal invasion, intratumoral portal tracts, and increased cell density as essential biopsy-observed hallmarks of HCC. Because of the risk of undiagnosed lesions and the difficulty of distinguishing well-differentiated HCC from dysplastic nodules, the sensitivity of a biopsy is 70% in tumors when it reaches 2 cm in size. Patients who have tested negative for biopsy should be diagnosed with serial contrast-enhanced imaging. A biopsy-based diagnosis is needed if a false-positive result is observed in the patient. In addition, the tumor growth is still retained with an unusual appearance for HCC (Forner et al. 2008).

Staging of HCC

The prognosis for HCC is influenced by the stage of the tumor, with curative approaches (Forner et al. 2008; Llovet et al. 2021). The Barcelona Clinic Liver Cancer (BCLC) staging system connects HCC prognosis with the optimal treatment strategy based on robust clinical evidence. HCC staging is dependent on imaging to assess pathological changes: the number, size, and location of tumors, as well as the presence or absence of vascular invasion. Patients with BCLC 0 and A are considered for therapies (resection, ablation, and liver transplantation) and have a better 5-year overall survival rate of 50–75%. With multinodular HCC, patients with BCLC B have impaired liver function. There is no evidence of vascular invasion or extrahepatic metastases. For these patients, transarterial chemoembolization is the only treatment option. The disease progresses in BCLC C stage patients. With vascular invasion or extrahepatic dissemination, they show cancer-related symptoms (Ayuso et al. 2018).

Serum Tumor Markers for Detection of HCC

The serum tumor marker test is a refined blood test that can measure the tumor marker released into the bloodstream. An elevated amount of tumor marker is a sign of cancer. According to international health guidelines, undergoing a serum tumor marker test for HCC patients is recommended (Jungner et al. 2017). It facilitates the early identification of HCC in HBV and severe cirrhosis cases (Trinchet 2011). In this context, the rise in detecting serum markers like AFP, AFP-L3, and DCP marked the sensitive improvement in HCC surveillance and diagnosis. The increased level of AFP indicates a lower survival rate and higher recurrence rate. Some HCC patients show an elevated range of AFP up to 1000 ng/ml (Lee et al. 2014), suggesting tumor reprogramming and invasion of the microvasculature. DCP is a biomarker for HCC detection. It is generally associated with larger tumors, poor differentiation, and invasion of the microvasculature. The serum level of DCP is higher after tumor hypoxia. The most commonly used cut-off value for DCP as a prognostic biomarker is >40 mAU/ml (Okuda et al. 2002; Toyoda et al. 2017).

Emerging research suggests the following proteins as serum tumor biomarkers for HCC diagnosis. They include Osteopontin (OPN) (Abdel-Hafiz et al. 2018); Serum angiopoietin 2 (ANG-2) (Ao et al. 2021); Glypican 3; Golgi protein 73 (GP-73); VEGF; Insulin growth factor 1 (IGF-1); and Hepatic growth factor (HGF). Detection of Serum OPN and augmented AFP serum levels improve the test's sensitivity and suggest a potential vascular invasion, dedifferentiated HCC, and needy prognosis. Nevertheless, its precision in the early detection of HCC is not validated and is further warranted.

Serum dickkopf-1 (DKK-1), a secretory antagonist of the Wnt signaling pathway, is highly expressed in HCC tissue and absent in nontumor liver tissue. It is a novel HCC serum biomarker, with selective and sensitive diagnostic detection of HCC, even in early stages and in patients with normal AFP levels (Shen et al. 2012).

Another highly expressed HCC protein, Glypican 3, is identified as a prognostic marker in recent meta-analyses. Glypican 3 expression is elevated in patients with HCC in its aggressive histological tumor features, resulting in a relatively poor survival rate and notable heterogeneity (Liu et al. 2018). The HGF is another molecular biomarker identified in the early and progressive stages of HCC. It encourages tumor growth and spread. THE MET proto-oncogene encodes the HGF tyrosine kinase receptor, activating downstream pathways in response to tumor proliferation, invasion, and antiapoptotic signals (Giordano and Columbano 2014). Although numerous biomarkers are available to detect HCC, there are no ideal biomarkers for HCC diagnosis of liver cancer at very early stages. Further research is still in the nascent stage. Identifying selective and sensitive biomarkers alone or in combination for diagnosis can further help identify each tumor stage at an early phase. Emerging research is underway in this regard. Such a strategy could be highly beneficial in determining appropriate therapy modalities and increasing patient survival rates.

In Vitro Model Systems of HCC

This section represents the various experimental model systems available to investigate liver and HCC (Table 1).

Primary Human Hepatocytes

Among the various in vitro models in HCV research, adult primary human hepatocytes are the most relevant cell-based liver model system (Pichard-Garcia et al. 2010). HCV isolated from human serum potentially infects these cells in vitro. Viral genome replication is permissible in primary adult human hepatocytes; however, the replication level usually is less (Buck 2008). In producing more realistic, lipid-associated HCV particles, primary cultures of adult human hepatocytes come closer to the animal model system-based analysis (Podevin et al. 2010). Importantly, this model system elicited an early innate antiviral response to HCV infection, indicating that interferon inhibition was effective (Molina et al. 2007). Although being desirable features of this model, the interferon response and innate immune response limit the spread of HCV infection (Andrus et al. 2011). These cells have limited access, are challenging to handle, and only support poor HCV replication; thus, they are not the common choice of use. Despite these drawbacks, this model has much potential for researching HCV-induced liver disease (Rau et al. 2013).

According to a recent report, the HCV virus may cause an epithelial-mesenchymal transition in primary human hepatocytes (Bose et al. 2012). This research discovered that exposing hepatocyte cultures to HCV causes them to change into fibroblast-like cells, which explains the fibrotic response seen in clinical HCV patients. Research also suggests that HCV infection of hepatocytes can lead to

Table 1 Experimental model systems of HCC

Virus/Viral proteins	Type of mutation	Phenotype (+/− and −/−)	Metastasis	References
Hepatitis B virus large envelope protein	BgIII-A fragment of HBV encoding large envelope protein under the control of albumin promoter and enhancer	Focal necrosis, inflammation, and subsequent HCC in 72% males	No metastases; rare local invasion	Schwartz et al. (2012)
Hepatitis B virus X protein	EcoRI–BglII fragment of HBV, including the X gene under its own promoter and enhancer	HCC in 84% after 13–24 months in mice with high HBx expression	Lung metastasis	Koike et al. (1994)
Hepatitis C virus	HCV core-E1–E2 transgenic under albumin promoter and HCV core transgenic under HBV X promoter	No DEN: no HCC in either strain by 21 months. +DEN: 100% HCC at 32 weeks; HCV core-E1–E2 with the largest tumors ($p = 0.008$)	DEN injected weekly – 6 weeks	Toshkov et al. (1994)
Hepatitis C virus	HCV core under HBV X promoter; HCV E1–E2 under HBV X promoter	Core transgenics: 32% HCCs in male mice at 16–23 months; E1–E2 transgenics: no HCC. No evidence hepatitis	None reported	Kamegaya et al. (2005)
Hepatitis C virus	HCV core-E1–E2 transgenic under albumin promoter and the entire HCV transgenic under albumin promoter	HCC in core-E1–E2 transgenic and entire HCV transgenic after 13 months	None reported	Lerat et al. (2002)

transformation and cancer phenotype generation (Fukuhara et al. 2012). This parenchymal liver cell shift from epithelial to mesenchymal phenotype occurs due to the modulation of critical signaling pathways or key oncogenic factors.

Liver-Derived Cells

The HuH-7 cell line and its closely related variations represent commonly utilized in vitro HCV research models. HCV replication is very effective in these cells, presumably due to the liver-specific microRNA's high basal expression level, miR-122. The binding of miR-122 to its HCV genome target regions does not hinder the viral RNA's function but positively promotes its reproduction (Narbus et al. 2011). Endogenous expression levels of miR-122 change significantly between hepatic and nonhepatic cells (Kambara et al. 2012). MiR-122 expression in HuH-7 cells increased more than two orders of magnitude than HepG2 or Hep3B cells. Nonetheless, MiR-122 expression in nonliver derived cells was significantly lower

(NCI-H2030, SK-OV-3, Caki-2, MC-IXC, 293T, HEC-1-B, RERF-LC-A1, 769-P, A-427, SW-780, SW-620, and SK-PN-DW). Hence it is believed that MiR-122 expression allowed for efficient HCV RNA replication and infectious virion production in HepG2 cells (Pascale et al. 2019).

Nevertheless, missing HCV receptor cells also encourage viral access and the complete HCV life cycle (Bukong et al. 2013). HuH-7 cells are a benchmark model system for HCV replication-based studies. However, HuH-7 cells express mutant p53 gene that is transcriptionally inactive, rendering them inefficient for studies on HCV-associated carcinogenesis. Several studies have also shown that Hep3B cells, without selection or adaptation, may support HCV replication and viral particle production at levels comparable to HuH-7-derived cells. Furthermore, interferon-stimulated gene expression is differentially upregulated in Hep3B cells following viral infection (Sarasin-Filipowicz et al. 2009).

In Hep3B cells, earlier stages of HCV infection are effective. In HuH-7 cell cultures, however, subsequent phases of the viral life cycle such as steady-state replication, de novo virus generation, and dissemination are impeded (Sarasin-Filipowicz et al. 2009). HCV-induced innate signaling, comparable to that found in primary cultures of adult human hepatocytes, was blamed for the reduced response. HuH-7 and other classic cell-based models may not completely mirror the events during a genuine HCV infection in vivo because they utilize cell-culture-adapted viruses (Ndongo-Thiam et al. 2011). HepaRG cells, which can differentiate into hepatocytes and biliary epithelial cells, have recently been demonstrated to be sensitive to in vitro infection with human serum-derived HCV and capable of producing infectious lipoprotein-associated enveloped HCV particles for lengthy periods (Lau et al. 2013). HepaRG cells have the potential to be used as a surrogate infection system for testing viral entry inhibitors. HCV infection models in vitro are beneficial in translational clinical studies. While interferon alfa (IFN-a) treatment for HCV infection reduces HCV infection in many people by altering cellular response pathways of innate immunity, the response to such therapy is not uniform (Lau et al. 2013). HuH-7 cells were exposed to low doses of IFN-a-2a, and interferon-stimulated gene expression rose along with HCV RNA levels, showing that persistent IFN exposure can create some level of interferon tolerance. Studies from mouse livers also suggest the same (Chew et al. 2019).

Stem Cell-Derived Liver-Like Cells

The introduction of stem cell-derived liver-like cells has lately enriched the field of in vitro research in HCV. According to emerging research, embryonic (Gastaminza et al. 2008) and induced pluripotent (Funakoshi et al. 2011) stem cells (iPSCs) differentiate into liver cells that are phenotypically comparable to human fetal liver. The human iPS-derived hepatocyte-like cell-based model (Si-Tayeb et al. 2010) allows researchers to investigate genetic abnormalities that influence HCV infection. Induced human liver-like cells produced known HCV host factors involved in HCV entry, supported the whole life cycle of genotype 2a HCV reporter virus, and reduced

viral production significantly when incubated with antiviral medicines. HCV infection induced an antiviral inflammatory response in hepatocytes similar to that found in chronic liver disease, and cell culture supernatants from this model were able to infect HuH-7 cells. Cell culture supernatants also contained TNF-a and IL-28B/IL-29 (Coll et al. 2018). As a result, we might conclude that iPSCs produced from patients with a genetic background that influences HCV infection could be used to investigate the role of innate immunity in the infection, perhaps improving our understanding of interferon-based therapy.

In Vivo Experimental Models of HCC

In vivo models are a well-developed system used to study the molecular mechanism of disease development, prognosis, chemoresistance, and targeted drug therapy. The result of disease complexity and the impact of phenotypic and functional heterogeneity in tumor progression led to the development of experimental models of HCC, which are comparable to the human disease condition. Mammalian models are generally used to study liver cancer. They include rats, mice, rabbits, woodchucks, and porcine subjects (Fig. 1). Nonetheless, limited studies on zebrafish, chick embryos, and drosophila as model systems exist to investigate liver cancer.

Rat Experimental Models

Around 1937, in the first experiment successfully conducted by Riojun Kinosita, a Japanese researcher used 4-dimethylamino benzene to develop chemically induced liver cancer in rats (*Rattus norvegicus*) (Macek Jilkova et al. 2019) models. Since then, chemically induced liver cancer in vivo models have been used to study the physiological changes, immune modifications, disease development, and progression in the tissue relevant to liver cancer. Lately, numerous chemical compounds have been identified as carcinogens based on exposure, varying from short to long term. Depending upon the toxicity, metabolic derivative, exposure period, and carcinogen concentration, further can be categorized into a nongenotoxic or genotoxic hepatic carcinogen. Under nongenotoxic carcinogen induction, two main carcinogens are proven and studied extensively to induce liver cancer. They are carbon tetrachloride (CCl4) and thioacetamide (TAA). Nonetheless, CCL4-mediated induction rat models also exist, which cause cellular structure disruption, promoting cell proliferation status, liberating excess tissue ROS, and partaking in cirrhosis and furthering HCC. Numerous studies have also shown that CCl4 is preferred to be administered with Diethynitroamine (DEN) to mediate liver cancer in rat and mice model systems (Santos et al. 2017; Zhang et al. 2019).

DEN is known to be teratogenic, mutagenic, and procarcinogenic in genotoxic cancer-inducing models. Magee and Barnes reported its variant dimethylnitrosamine

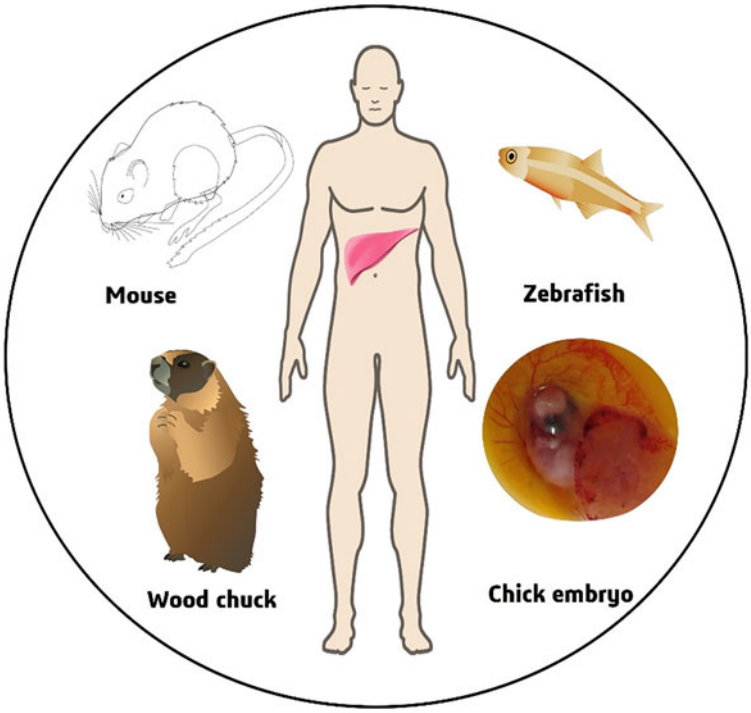

Fig. 1 In vivo model system of HCC

(DMN) as a carcinogen in rat models in 1956 (Santos et al. 2017). Multiple studies are conducted with DEN to understand liver tumor initiation, promotion, progression, and metastasis. As an individual agent, DEN directly binds with DNA via forming a covalent bond, creating a complex thereby initiating mutation at guanosine residues. The DNA damage is associated with oxidative stress, where the metabolic pathways of liver cancer-induced rat models are similar to humans. This induced damage by DEN causes hepatic cell proliferation, impairment in the liver, fibrosis or cirrhosis, recruitment of neutrophils, and platelets in the immune cell-mediated niche for enhanced destructive ROS injury (Kurma et al. 2021; Macek Jilkova et al. 2019; Zhang et al. 2019). Knowledge and evidence-based studies have revealed numerous phytochemicals prospects as chemopreventive agents against HCC. One such polyphenol – Curcumin, generated from the root of *Curcuma longa*, has been widely explored in the traditional and rational system of medicine for its hepatoprotective property. In mouse and rat models, curcumin inhibited DEN-induced overexpression of oncogenic HRAS, localized dysplasia, and HCC. Metformin is often used as an antidiabetic drug and suppresses hepatic progenitor cell activation and advanced glycation end products in chronic DEN-induction rat models, potentially preventing hepatocarcinogenesis (Shankaraiah et al. 2019).

Mouse Experimental Models

Among all the rodent models, the mouse (*Mus musculus*) is considered the best experimental system for studying HCC due to its life span, size, breeding ability, and, most importantly, sharing similar genetic and physiological functions with humans. Carcinogen-induced, Syngenic, Xenograft, GEM (Genetically induced models), and Viral models (Liu et al. 2020a; Macek Jilkova et al. 2019; Santos et al. 2017) represent excellent HCC mice model systems. Likewise, the same compounds are used independently or combined in rat models to develop tumors. Thioacetamide, otherwise known as TAA, is initially used to cause fibrosis in rats and mice models. The administration of TAA to mice through i.p. (intraperitoneal) injection is approximately 200 mg/kg body weight, and it causes hepatic centrilobular necrosis. Individually chronic administration of TAA leads to cholangiocarcinoma. On the contrary, TAA, in combination with DEN, is significantly responsible for the induction of HCC (Zhang et al. 2019).

Syngeneic and Xenograft Experimental Models

Human tissue culture techniques and animal models have become immensely expanded these days. Due to tumor heterogeneity of the cell line, it is often challenging to study the evolution of the tumor. In such cases, understanding molecular-related transformation processes like (epithelial-mesenchymal transition) EMT potential, cellular response to external factors, and signal-mediated pathways promotes an opportunity to study cancer in vivo. As an impact of tumor cell line heterogeneity, using multiple cell lines of HCC is encouraged for the drug-screening test to prevent false positives. For this kind of study, immune-compromised or immune-competent cells can be used (Santos et al. 2017). Syngenic model systems allow the growth of tumor cells in vitro derived from inbred mice, followed by their implantation in the same inbred species of mice. These models enable the study of the molecular mechanisms, pathology of HCC, the surrounding lymph system, vasculature, and the tumor niche. HCC syngeneic models allow studying the anti-tumor immune response elicited by novel drug combination therapy strategies. Xenograft model systems of HCC include subcutaneous implantation of tumor cells from one species into the tissue of another species. Such a strategy involves challenging an immunocompromised mouse with human tumor-derived cell lines. These models are rapid and relatively easy for preclinical studies to analyze the drug's toxicity. A significant drawback of the xenograft model in the tumor is the need to grow in immune-competent mice, which do not reflect a human's definite functional system.

Another way of preserving the actual disease state observed in HCC patients (Blidisel et al. 2021; Liu et al. 2020a; Shankaraiah et al. 2019) includes the patient-derived xenograft (PDX) model. This approach involves maintaining the genomic,

transcriptomic, and histopathological characteristics of the actual HCC condition. Therefore, PDX models remain the best strategy for studying precision medicine-based personalized therapy. A benefit of PDX is that it allows the investigation of human tumor tissue-derived cells as an autograft or an allograft in another organism, which is more reflective of the features and mutations found in human cancer (Macek Jilkova et al. 2019).

Genetically Modified Experimental Models

The first-ever report in 1984 stated the generation of transgenic mice which carry the cloned oncogene, which confirmed the hypothesis that expression of an oncogene in a mammalian model could develop tumor growth (Santos et al. 2017). This progress extended the study of molecular characteristics of malignancy in experimental models. The benefit of genetically modified (GM) models has significantly elevated tumor biology research. GM models generated via activation or overexpression of an oncogene by specific tissue promoters, e.g., KRAS or inactivating tumor suppressor gene using CRISPR technology (Blidisel et al. 2021; Liu et al. 2020a), are used for experimental purposes. An extensive set of GM mice models for HCC studies include oncogene overexpressed models, such as B-catenin, Myc, or growth factors (EGF). GM mice models generated from the inactivation of tumor suppressor genes (PTEN, TP53) also help understand HCC.

Multiple studies have shown that mutations or inactivations in a specific gene alone cannot cause liver cancer. Accumulation of mutations or overexpression of oncogenes, or loss of tumor suppressor genes, leads to the generation of HCC. Exceptions exist, and downregulation of Glycine N-methyltransferase (GNMT) at epigenetic levels establishes HCC. GNMT knockout mice instantly promote HCC and help understand it as a metabolic syndrome, the molecular mechanism behind the metabolic dysfunction through degradation of PREX2, a PTEN inhibitor in the PI3K/AKT pathway. In GNMT knockout mice, tumor nodules generated in the liver allow for identifying biomarkers in HCC (Li et al. 2017; Zhang et al. 2019). MST1/2, a family of class II germinal center kinases, also function as tumor suppressors. Knockout mice of these genes generated spontaneous HCC, indicating the role of Hippo-Lats-Yorkie signaling in oncogenesis (Shankaraiah et al. 2019). According to World Health Organisation (WHO), in worldwide distribution, chronic hepatitis B (HBV) and chronic hepatitis C virus (HCV) are considered highly prevalent and are associated with HCC. Hepatitis B virus core antigen X protein (HBx) is a multifunctional HBV protein. HBx-regulatory gene inserted into mice's germline resulted in the development of HCC in transgenic mice. This viral gene insertion established multifocal regions of disrupted hepatocytes, accompanied by the emergence of adenomas followed by carcinomas. HBx decreases HCC patient survival during metabolic stress by triggering inflammation, regulating fatty acid oxidation, and inducing oxidative stress in liver cells (Sukowati et al. 2019; Xu et al. 2016; Zhang et al. 2019).

Zebrafish Liver Cancer Experimental Model

Over a decade, zebrafish (*Danio rerio*), a vertebrate model, has emerged as a successful platform for cytotoxicity investigation and drug discovery. Zebrafish models maintain many benefits in favoring human studies, including fast growth, high fecundity, smaller size, cost-effectiveness, and similar pathophysiology to humans (Lin et al. 2019). Three strategies facilitate the promotion of HCC in zebrafish. Administering carcinogens such as methyl nitrosourea mediates HCC. Furthermore, overexpression of an oncogene and xenotransplantation approaches also enable studying HCC in zebrafish (Blidisel et al. 2021). The first-ever liver cancer transgenic zebrafish model has expressed the overactive mutant KRAS under the regulator of fabp10, a liver-specific promoter. Zebrafish transgenic model contributed a favorable circumstance to produce an in vivo liver cancer model which is histopathologically similar to mammalian. It also confirmed the incidence of hyperplasia and adenoma in liver cancer. The introduction of the fusion proteins like hepatitis B protein x (HBx) and mCherry in the transgenic zebrafish model resulted in the induction of liver cancer with or without the association of TP53 (Nakayama and Gong 2020). Mifepristone-treated zebrafish developed tumors in a dose- and time-dependent manner. After a week of exogenous administration of HBx fusion proteins, transgenic zebrafish larvae turned hyperplastic. Furthermore, it proceeded to HCC condition after additional 4 weeks of treatment with mifepristone at increasing concentrations (Lee et al. 2021; Nakayama and Gong 2020). The Raf-MEK-ERK expression increased in transgenic zebrafish larvae treated with mifepristone, and their intervention with suitable inhibitors led to their suppression and decreased oncogenesis (Lee et al. 2021).

Another transgenic approach in zebrafish wherein a brief chemical pulse activates oncogene expression by integrating the mifepristone-inducible KRAS model with the Cre/loxP system, which induces irreversible genetic alteration. The liver-specific promoter fabp10a controls transcription of KRAS; however, in the novel transgenic strain, loxP sequences flanking a mCherry-STOP cassette sequence prevent it. In the presence of mifepristone, Cre/loxP system excises the STOP cassette sequence facilitating the stable expression of KRAS (Lee et al. 2021; Lu et al. 2015; Wrighton et al. 2019). The zebrafish model expedited the drug screening of therapeutics. Intersegmental vessel formation (ISV) is an angiogenic process during embryonic growth in zebrafish. When exposed to the drug for 24 h postfertilization, the embryos revealed ISV inhibition, suggesting angiogenesis-targeted drug ability (Lin et al. 2019). Multiple such toxicity assays also allow ascertaining cytotoxicity, neurosensory organ toxicity, and cardiotoxicity potential of the drug in vertebrate model systems like zebrafish.

Woodchuck Experimental Model

The woodchuck (*Marmota monax*) is a clinically relevant in vivo model system for studying Hepatitis B virus-related illnesses like HCC. It is a multistage process.

According to a study of intratumoral transcriptional profiles in woodchucks and HBV-infected people, HCC metastasis outside of the liver is almost nonexistent in woodchucks, except for a few rare incidences of pulmonary metastasis. After injection of the woodchuck hepatitis virus (WHV), they develop chronic hepatitis that progresses to liver cancer in adults. In terms of viral life cycles, infection and replication mechanisms, nucleotide sequence, genomic structure, and virion shape, there are many analogies between WHV and human HBV. The development of liver cancer takes about 24–32 months after infection with WHV. However, Woodchucks infected with the virus do not develop cirrhosis. Hepatic neoplasms in woodchucks are often well-differentiated and result in trabecular HCC. WHV-induced hepatic carcinoma shares molecular similarities with other subtypes of human HCC. WHV-induced HCC shares a highly comparable disease course to HBV-induced HCC and preserves the pathophysiology due to the local tumor niche. The preclinical woodchuck model of virally induced HCC is an ideal paradigm for studying the efficiency of therapeutics to combat HCC (Blidisel et al. 2021; Chauhan et al. 2017; Liu et al. 2020b).

Chick Embryo Model

The Chick embryo (*Gallus gallus domesticus*) model is a well-established model for angiogenesis study. Limited studies report their role as a model system for studying liver disease conditions. CAM (chorioallantoic membrane) present in chick embryos is a proven xenograft approach for studying various cancers (Komatsu et al. 2019). Also, it is cost-effective, reproducible, and less time-consuming. For HCC developing an Ovo xenograft model, studies are rare. Considering this method could provide an opportunity to understand the underlying association between HCC and angiogenesis. These models are also capable of developing and propagating the viral proteins. In that case, studying HBV or HCV and its association with HCC is also feasible (Harper et al. 2021; Li et al. 2015).

Ex Vivo Models in Liver Cancer Research

Liver cancer ranks fifth position in the global cancer ranking. Liver cancer is highly malignant and equally fatal when diagnosed at late stages (Anwanwan et al. 2020). One of the causes of the growth of the tumor is its invasion of other cells and tissues and reoccurrence because of the inevitable presence of highly resistant cancer stem cells (Sun et al. 2016). A recent study has stated that the septic liver can alter the gut microbiome and the mechanism of metabolism that can lead to dysbiosis (Ma et al. 2018).

The personalized treatment options for primary liver cancer management include surgical resection and adequate preservation of residual liver depending on the tumor size. By enhancing patient outcomes, oncosurgical methods such as portal vein

embolization and parenchymal-sparing resections have raised the number of patients eligible for curative liver resection (Orcutt and Anaya 2018).

The ex vivo platform (Table 2) is an excellent platform to culture liver cancer in several kinds of matrices like 2D, 3D. This system has more advantages than in vitro and in vivo methods. It is feasible to understand the underlying cell-cell interaction in the tumor niche without hassle. Transarterial chemoembolization (TACE) is a turned-up cornerstone treatment against unresectable liver cancer. The organ is de-cellularized along with the preserved ECM (Extra Cellular Matrix) and vasculatures to employ this strategy. This decellularization maintains a translucent appearance, which can aid in the easy investigation of chemoembolization. This exemplary de-cellularized ex vivo model makes it easier to efficiently understand the occlusion and the drug mechanism in the TACE treatment, which is much more demanding to carry out in vivo (Gao et al. 2021).

It is not easy to simulate the exact human host physiology while using animal models for research. However, experimental human 3D model systems can closely replicate the steps involved in any infection, such as *Entamoeba histolytica*. It can provide insights into mechanisms like barrier crossing, tissue migration, and pro-inflammatory mediator release to understand human disease conditions better (Petropolis et al. 2014).

Table 2 The existing ex vivo methods in liver cancer research and their advantages

	Ex vivo models	Advantages	References
HCC research	2D models	To understand the majority of genetic and epigenetic alterations present in the tumor of origin	Orcutt and Anaya (2018)
	3D cocultures	Allows cell-cell interaction, which plays a critical role in cancer invasion	Gül et al. (2012)
	3D spheroids	It helps in retaining the cell morphology, phenotype, microenvironment, cell-cell interactions, and cell-ECM communication	Song et al. (2015)
	3D organoids	Preserve the identity of the modeled organ. Enables long-term culture, cryopreservation, and genetical manipulation	Griffith et al. (2014)
	3D scaffold-based models	Provides a physical matrix on which cells can aggregate, divide, and migrate	Griffith et al. (2014)
	Bioprinted and 3D printed models	Innovative platforms that enable the deposition of bioinks containing multiple types of living cells, signaling molecules, de-cellularized extracellular matrix constituents, nutrients, growth factors, and cell-laden biomaterials using a computer-aided design (CAD) to engineer 3D constructs with tissue-like architecture	Clark et al. (2016)
	Organ-on-a chip	The construction of a biomimetic organ platform on a multichannel microfluidic chip, recreating the structural and functional features of human physiology	Clark (2021)

Organ on a chip is a highly advanced 3D tool for research. Legacy LiverChip® is an ex vivo hepatic microphysiological system where the 3D microperfusion culture format can mimic the entire state of promotion and progression of cancer. This system also enables further proteomic and genomic data analysis (Clark 2021). The fetal liver on a chip method is another organ on a chip tool to investigate ex vivo. Initially, sacrificing the E12.5–E14.5 pregnant dam is performed by allowing inhalation of CO_2 and cervical dislocation. Initially, remove the uterus and transfer it to a culture plate with phosphate-buffered saline (PBS). Then the fetal liver is removed from the rostral portion with sterile equipment to generate the ex vivo biomimetic system. The ex vivo microfluidic fetal liver system allows tracking of hematopoietic stem cell (HSC) homing to and interaction with the hepatic environment underflow and matrix elasticity conditions characteristic of embryonic development. Tweaking this model facilitates investigating critical microenvironmental and biophysical inputs that support HSC homing and growth (Mohammadalipour et al. 2021).

The 3D organotypic culture of the liver can abolish the limitations of the 2D ex vivo models. This 3D culture can potentially reestablish the structural and signaling relationship between tissues necessary for organ function. Rational recreation of the 3D system facilitates a successful understanding of the drug mechanism and their metabolism (Gül et al. 2012). In addition, challenging the 3D organotypic cultures with appropriate carcinogens to mimic the tumor condition and metastatic phenotypes has been partially successful among the currently available approaches (Griffith et al. 2014). The bioartificial liver platforms presently available for research include the PEARL perfusion liver system, Liver Chip microphysiologic system, and Self-assembly liver system (Clark et al. 2016; LeCluyse et al. 2012). The PEARL perfusion liver system is a revolutionary platform enabling long-term primary hepatocyte culture with the flow. Each microfluidic array contains 32 independent perfusion units designed to mimic the natural liver architecture, resulting in more clinically relevant results validated with freshly isolated and cryopreserved hepatic cells from human and rat origin. The PEARL perfusion platform lets to perform multiple hepatocyte experiments at a fraction of the time and cost of alternate methods while obtaining data not currently possible in vitro. The 96-well format of the PEARL perfusion platform enables easy usage of high-quality microscopy, fluorescence, luminescence, and biochemical studies of the hepatocyte culture (Nelson et al. 2015).

Cryopreservation of human hepatocytes has prolonged cell survival and maintained intact tissue integrity (Tsamandouras et al. 2017; Sarkar et al. 2015). When the 3D cultures are developed, it is vital to maintain the tissue integrity, thereby facilitating better functioning of the cells. 3D cultures generated from liver cell aggregates or spheroids also enable understanding of the hepatic differentiation process. Augmented liver cell differentiation occurs when iPS cells and stromal cells are seeded into polydimethylsiloxane (Song et al. 2015). Another 3D culture method is commercially available RAFT™ for culturing iPS aggregates for over 40 days (Gieseck et al. 2014).

HBV is hepatotropic which infects only human hepatocytes. It is also known as hepatocarcinogen. Due to limited in vitro and in vivo modalities, the HBV infection

in HCC is still in a nascent stage. Emerging research focuses on developing a better ex vivo technique to understand the unclear mechanism (Torresi et al. 2019).

It is crucial to precisely understand all the molecular signaling pathways and mechanisms in any diseased liver condition. There exist limitations to the results obtained using in vitro and in vivo methods. A large sample size of the animals required in the case of in vivo methods is a dampening for the study. And when it comes to in vitro, the microenvironment may not be exactly mimicked. Hence, the ex vivo approach is the preferred method for overcoming such limitations (Pearen et al. 2020). In a recent study, the BSA (albumin) is encapsulated with Au nanoparticles to target liver cells. BSA bound to Au nanoparticles was inoculated intra-arterially onto the specimen to determine the specific delivery of the nano-bioconjugate into the malignant liver tissue using the capillary bed. The results obtained from the selective ex vivo photothermal nano-therapy of solid liver tumors mediated by albumin-conjugated gold nanoparticles were exemplary (Buzoianu et al. 2017).

Conclusion

Evidence that genetic mutations alone do not always result in tumors unless triggered by a proinflammatory agent. The former statement highlights the need for new models in which HCCs develop spontaneously in a fibrotic environment to best mimic the human disease process. Furthermore, recent integrated functional genomic investigations have revealed crucial human HCCs subgroups based on the activation of novel molecular pathways such as “Hippo”. Comparing gene expression in mouse models with human HCC may 1 day allow us to construct mouse models that accurately represent the various subgroups, making them suitable models for preclinical research.

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Animal Models for Prostate Cancer Research: A Mechanistic Outlook on the Challenges and Recent Progress

19

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Contents

Abstract

Molecular pathogenesis of prostate cancer (PCa) still remains poorly understood in human. Difficulties in understanding of prostate oncogenesis and lack of efficient noninvasive therapeutic measures are primary obstacles in PCa management. Among animal models of human prostate cancer, rat, mice, and canine models are mostly used. However, genetic differences of these model animals and anatomical as well as histological differences of their prostate with respect to human make the PCa research quite tricky. Also, stages of disease development from benign to malignant and the progression of malignant tumors to more

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aggressive forms in these animal models often vary substantially from those seen in human. Therefore, animal models of prostate cancer must be selected carefully with respect to the specific research purpose. In this context, present study aimed to summarize a thorough idea of purpose and efficacy of different animal models suitable for different aspects of prostate cancer research from the published literature. The information may serve as a reference in further research for better understanding of the disease pathogenesis and developing novel as well as effective therapeutic approaches.

Keywords

Prostate cancer · Challenges · Animal models · Oncogenesis · Castration resistance · Metastasis · Pathways

Introduction

Prostate cancer (PCa) is a malignancy of aged male, associated with higher mortality rate primarily because of insufficient knowledge on disease pathogenesis and lack of effective management (Dasgupta et al. 2012). Conventional treatment modalities of prostate cancer include surgery, chemo-radiation, and androgen deprivation that have limited efficiency for more aggressive and advanced disease stages (Litwin and Tan 2017). To understand molecular events associated with disease pathogenesis and progression, research works involving animal models of human prostate cancer are conducted, and for this purpose, rat, mice, and dog are mostly used (Russell and Voeks 2003). However, unlike other cancers, prostate cancer research comes with a set of problems, most notable of which are the heterogeneity in its oncogenesis and lack of an all-purpose animal model (Lamb and Zhang 2005). This leads to a fragmented research approach in PCa, where the utility of a given animal model gets restricted to a very limited number of study purposes. On the aforesaid background, present review work aimed to shed light on the current scenario of animal model-based PCa research and discuss how different existing animal models can be used in different aspects of the study to increase the efficiency of the research outcome (Hayashi et al. 1991).

Animal Models of Prostate Cancer; the Challenges

The aim of using animal models in PCa research is to replicate the steps of disease development in animal for understanding details of the disease pathogenesis and develop novel targeted therapeutics. Rodents are the conventional pick for PCa research due to their small size, ease of handling, and maintenance. Additionally, rodent genome can also be manipulated efficiently for experimental purposes. Apart from rodents, Canines, mainly dogs also serve as animal model for PCa research, although their maintenance cost is high and genetic modification is very difficult.

Table 1 Comparison of prostate characteristics between human male and animal models

Characteristics	Human	Mouse	Rat	Dog
Prostate structure				
Anatomy	Single gland five lobes	Four distinct lobes	Four distinct lobes	Bilobed
Functional differentiation	Heterogeneous	Partly heterogeneous	Partly heterogeneous	Homogenous
PCa development and progression				
Incidence of spontaneous PCa	Occurs naturally	Occurs rarely	Occurs in a few strains	Occurs naturally
Resistance to castration	In advanced stages	In advanced stages	In advanced stages	In early stages
Bone metastasis	Frequent	Rare	Rare	Frequent

The usage of canine animal model is restricted for studying mainly bone metastasis, an aggressive phenotype frequently seen in human PCa (Simmons et al. 2014b). However, using animal models for PCa research itself has problems, which makes the management of animal model-based study quite convoluted.

Human prostate differs from that of rodent and canine anatomically and histologically in several aspects (Table 1). Rodents have four anatomically different prostate lobes while canines have two that are homogenous with respect to cellular differentiation. On the contrary, human prostate gland can be subdivided into five lobes (anterior, posterior, right lateral, left lateral, and median) and functionally differentiated into three distinct zones (peripheral, transitional, and central). Rodents have compound ductules in prostate and in dog ducts are branched into alveolar glands, whereas characteristic acinus is present in human prostate (Lamb and Zhang 2005). Discontinuous basal layer of prostate in dogs and rats is a norm while in human, the criteria can rather be an indicator of PCa (Hayashi et al. 1991; Hameed and Humphrey 2005).

In dogs, both benign and malignant prostatic lesions develop spontaneously and more likely in aged animals similar to human (Waters et al. 1997). Whereas, although a few rat strains can develop spontaneous PCa (Nascimento-Gonçalves et al. 2018), the phenomenon seems to be a rare one in mice. In rodent, malignant tumor of prostate progresses to adenocarcinoma, sarcoma, and neuroendocrine carcinoma while it mostly progresses to adenocarcinoma in human (Grabowska et al. 2014). Similar to humans, rodent PCa may acquire androgen independence in advanced stages. On the other hand, canine PCa show pathological and molecular criteria similar to androgen-independent subtype from very early stage of tumor development (LeRoy and Northrup 2009). Rodent PCa does not naturally metastasize to bones, but the phenomenon is frequent in canine PCa and is very similar to that of human (Cornell et al. 2000).

Testing the efficacy of novel therapeutics for PCa on animal models is also challenging. Rodents are smaller in size, and their life span is shorter than humans; hence in preclinical trial with rodents, the data on effective dosage, toxicity measurements, and retentions of a given therapeutic is expected to differ in humans and

therefore needs extrapolation during clinical trial. Canines might be a better research model on this background, although here, the latent period of PCa is relatively longer than human (Ryman-Tubb et al. 2022).

Purposefulness of Animals Models in Prostate Cancer Research

Several approaches are followed to create different types of animal models suitable for achieving different specific purposes of human PCa research.

Animal Models to Understand the Stages of PCa Development

To understand the pathogenesis of human PCa, it is essential to study all developmental stages of the disease namely benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), and prostate adenocarcinoma as well as its more aggressive subtypes separately. In human, availability of tissue samples from all the developmental stages seems to be limited indicating the emergence of animal model in PCa research.

Benign Prostatic Hyperplasia

Benign prostatic hyperplasia (BPH) is the enlargement of prostate gland due to increase in both cell number and size. Studying animal models of BPH will provide information about the key changes of mainly prostatic epithelial cells leading to their uncontrolled proliferation and hyperplastic growth of the organ as a whole. Existing animal models generated through genetic manipulation for studying exclusively BPH are listed below.

p27 Knockout mice model: This model was generated by targeted knockout of p27 through vector-mediated standard protocol at the embryonic stage of C57BL/6 mice. Histological findings showed incidence of prostatic hyperplasia in acinar epithelial cells of 14-month-old mice of this model, and this is also comparable to human BPH (Cordon-Cardo et al. 1998).

ERβ knockout mice model: Mice model lacking oestrogen receptor β (ERβ) was generated by knocking out the *ESR2* gene through inserting a neomycin resistance genetic element into exon 3 of the coding region in embryonic stem cells. Aged male mice showed hyperplastic growth in prostate and bladder. In prostate of ERβ (-/-) mice, increased AR level was seen and the tissue contained multiple hyperplastic foci (Krege et al. 1998; Abdulkadir and Kim 2005).

Mice model of prolactin deregulation: In this model, a construct of rat prolactin (PRL) gene was cloned under the metallothionein-1 (Mt-1) promoter of MtbGH 2016 plasmid and was microinjected into C57BL/6JxCBA-f2 mice embryos to overexpress prolactin. Although prolactin overexpression is neither prostate specific nor aggressive, nonetheless it causes enlargement of prostate gland in the model animal comparable to the BPH lesions of human (Wennbo et al. 1997).

Mice model of Int2 and kgf deregulation: In this model, mouse mammary tumor virus (MMTV)-regulatory sequence from pMMTV-neu-NT plasmid was inserted between the SV 40 promoter and murine Fibroblast growth factor 3 (int2) gene cloned in a pKC3–9 plasmid. Following the same approach, keratinocyte growth factor (kgf) was inserted under the MMTV regulator sequence. In either case, the recombinant plasmid (pKC3-9) was microinjected into female pronuclei of FVB mouse strain following implantation in the foster mother. In the transgenic animals, overexpression int2 and kgf resulted in the development of benign prostatic hyperplasia (Muller et al. 1990; Kitsberg and Leder 1996).

Prostatic Intraepithelial Neoplasia

Prostatic intraepithelial neoplasia (PIN) is the first sign of prostate malignancy, and here the neoplastic growth is restricted to epithelial cells within the ducts or acini of preexisting benign prostatic lesion. An understanding of molecular events associated with the development of PIN will be helpful in identifying the driver steps of PCa oncogenesis.

TGFBR2knock out model: In this model, the gene-encoding TGFβ receptor 2 (TGFBR2) is inactivated in mice fibroblasts by crossing $Tgfbr2^{floxE2/floxE2}$mice with mice having fibroblast-specific protein 1 (FSP1) gene under a cre-recombinase system. The resulting transgenic mice were nonresponsive to TGFβ stimulation and neoplastic lesions comparable to the PIN developed in their anterior and dorsolateral prostate lobe at the age of 5–7 weeks. This model may be useful for studying PIN and understanding the role of TGFβ as a tumor suppressor in PCa development (Bhowmick et al. 2004).

Rb knockout model: Mice homozygous for lox introduced retinoblastoma (Rb) gene were crossed with heterozygous mice for cre-recombinase gene cloned under the rat probasin (PB) promoter (−426/+28 region). The filial generation was then inter-crossed to obtain the concerned model in which Rb gene was deleted selectively in prostate gland under the influence of PB promoter resulting in development of PIN lesions. In this model, development of prostate adenocarcinoma was not found probably because of redundant function of other Rb family members such as p130 that might reverse the effect of Rb deletion (Maddison et al. 2004; Abdulkadir and Kim 2005).

ERG/ETV1 deregulated model: In this model, a TMPRSS2-ERG fusion construct was produced by inserting noncoding exon-1 of transmembrane protease serine 2 (*TMPRSS2*) gene adjacent to the exon-2 of ETS-related gene (ERG). The resulted shortened ERG product was then put under the control of a modified PB (ARR_2PB) promoter through recombination events. Finally, the construct was microinjected into fertilized egg pronuclei and following their implantation into FVB surrogate mice, consecutive crossings were performed to establish the model. Total 12–14-weeks-old mice acquired PIN in their ventral lobe of prostate (Tomlins et al. 2008).

Akt deregulated model: The coding sequence of Akt1 along with a myristoylation sequence and hemagglutinin epitope sequence from pCDNA3 plasmid was cloned into pBSK plasmid under rat PB promoter. This construct was

linearized and microinjected into fertilized oocytes of FVB mice. The resulted transgenic model showed constitutive expression of Akt in prostate and developed PIN lesions. This model is also known as murine prostate AKT (MPAKT) model (Abdulkadir and Kim 2005; Cunningham and You 2015).

High-Grade Prostatic Intraepithelial Neoplasia

High grade PIN (HGPIN) is considered as the precursor stage of prostate adenocarcinoma and characterized by uncontrolled proliferation along with cytological abnormalities of glandular epithelial cells restricted to prostatic duct and acini. Studying HGPIN pathogenesis seems to be essential to understand the molecular events associated with gradual progression of PIN lesions toward the invasive adenocarcinoma.

NKX3.1$^{-/-}$; PTEN$^{+/-}$ knockout model: An inducible (conditional) double knockout model for NKX3.1$^{-/-}$ and PTEN$^{+/-}$ was generated by crossing PSA^{Cre}NKX3.1 with PSA^{Cre} PTEN conditional knockout mice. The resulted double knockout animals developed HGPIN lesions at a higher frequency which was shown to be 60% by 26 weeks and about 100% by 52 weeks in comparison to PTEN$^{+/-}$ knock-out models where lesion incidence was only 25% by 52 weeks (Valkenburg and Williams 2011).

Conditional PTEN knockout model: PTEN was conditionally knocked out in mouse prostate by crossing PTEN$^{loxP/loxP}$ mice with MMTV-cre mice. The transgenic model developed HGPIN (2 weeks) lesions with atypic papillary proliferation of epithelial cells (Backman et al. 2004).

Prostate Adenocarcinoma

Prostate adenocarcinoma is the final stage of prostate oncogenesis characterized by the malignant tumor. Studying prostate adenocarcinoma will be helpful to understand the key molecular events associated with invasion, migration, and other aggressive phenotypes of malignant cells.

Animal model by carcinogen/hormone treatment: Rats are commonly used to generate carcinogen-/hormone-treated animal models of prostate cancer. Subcutaneous application of carcinogen 3,2'-dimethyl-4-aminobiphenyl (DMAB) in F344 rat and intraperitoneal/ intravenous injection of N-methyl N-nitrosourea (MNU) in Sprague Dawley and Lobund-wistar rats alone or in combination with testosterone in silastic transplant was found to induce prostate adenocarcinoma. In WU rats, DMAB treatment followed by cyproterone acetate and testosterone propionate consecutively can induce mild adenocarcinoma in prostate lobes of dorsolateral and anterior regions. In various inbred rat strains, only hormonal treatment with testosterone orestradiol-17β can also induce prostate adenocarcinoma and thus providing an ideal model for studying the role of hormonal (gonadal) dysregulation in carcinogenesis of prostate (Bosland 1992; Nascimento-Gonçalves et al. 2018).

Transgenic animal model: Mice are generally used to generate this transgenic model in which ERG/Bmi gene is overexpressed or Gata3 gene is deleted under the background of conditional PTEN knockout resulting development of prostate adenocarcinoma. For overexpression of ERG gene, mice model carrying TMPRSS-

ERG fusion construct is used and crossed with $PTEN^{+/-}$mice; the resulted transgenic animal developed aggressive prostate adenocarcinoma within 26 weeks (Carver et al. 2009). Bmi1 is a component of polycomb-repressive complex 1 (PRC1) frequently upregulated in prostate cancer of human (Zhu et al. 2018). By crossing Bmi^{LSL} mouse having a Lox-STOP-Lox (LSL) sequence with PB-Cre4 strain, overexpression of Bmi1 allele is effectuated in the prostate gland of resultant transgenic model. Upregulation of Bmi1 under background of $PTEN^{+/-}$ haploinsufficiency resulted in development of invasive prostate adenocarcinoma (Nacerddine et al. 2012). GATA3 is a transcription factor frequently inactivated in human prostate cancer and the phenomenon under condition of PTEN deficiency favors tumor progression. Transgenic animal model carrying double PTEN; GATA3-mutant [$PTEN^{-/-}$; $GATA3^{-/-}$] was generated by crossing PB-Cre4; $PTEN^{flox}$ mice with $GATA3^{flox}$ one, and the model was shown to develop invasive prostate adenocarcinoma within shorter time in comparison to $PTEN^{-/-}$ one (Nguyen et al. 2013) (Fig. 1).

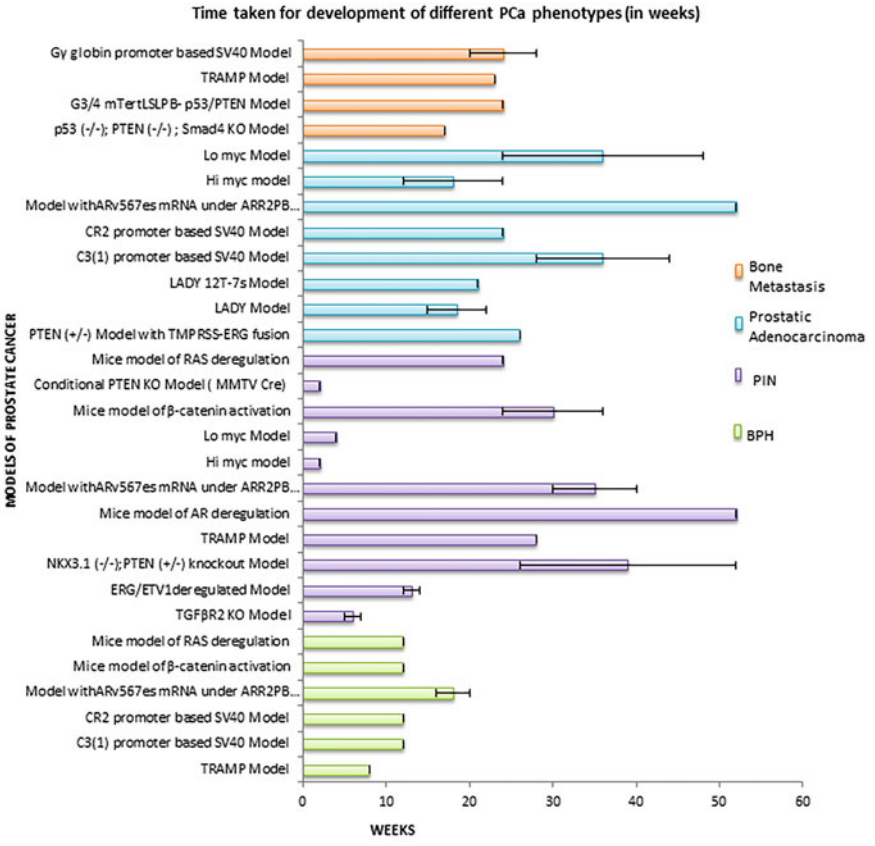

Fig. 1 Time taken for mice models to develop different phenotypes of PCa

Animal Models for Studying Aggressive Phenotypes of Prostate Cancer

In several instances, PCa shows aggressive phenotypes like androgen-independent progression and metastasis to distant places. Prostate cancer develops androgen independence after getting exposed to androgen deprivation therapy and then considered under the castration resistance category which is more aggressive in nature and associated with poor patient outcome. Among incidence of distant metastasis, bone is considered as the most affected organ in prostate cancer of human and the phenomenon was found to affect the disease outcome. Therefore, these aggressive disease phenotypes must be studied in detail to design effective therapeutic measures with an aim for better disease management.

Development of Castration-Resistance of Prostate Cancer

LNCaP/Neo xenograft model: LNCaP cell line derived from prostate adenocarcinoma of a 50-year-old Caucasian male metastasized to the left supraclavicular lymph node was infected with pLNSXNeo retrovirus overexpressing Neu oncogene. Resulting LNCaP/Neu cells were injected subcutaneously in castrated SCID mice (male) resulting in development of prostate tumor even in absence of androgens, which thus resembled the properties of CRPC in human. Additionally, the latent period for the development of this xenograft tumor was shortened and the level of PSA was also increased. This model can be useful for understanding the role of Her2/Neu-mediated signaling in the pathogenesis of CRPC (Craft et al. 1999).

Metastasis

Transgenic Models (Mice)

p53; Rb double knock out model: PB-cre4 male mice (C57BL/6xDBA2 background) were crossed with $p53^{loxP/loxP}Rb^{loxP/loxP}$ female mice (FVB/N;129 background), and the transgenic mice model of desired genotype was generated in the F3 generation. In the transgenic animals, PCa metastasized to lymph nodes, muscle, and blood vessels as well as to several other organs and tissues. This model provides information about potential sites where PCa can metastasize; however, the model is not appropriate for studying skeletal metastasis, a phenomenon more frequently found in humans (Zhou et al. 2006).

Smad4 knockout model on the background of $p53^{-/-}$; $PTEN^{-/-}$ genotype: Smad4 is a tumor-suppressor gene of TGFβ pathway showing frequent promoter methylation and concordant reduced expression in prostate cancer (Aitchison et al. 2008). The model was generated by crossing a $p53^{-/-}$; $PTEN^{-/-}$ mice with a Smad4-conditional knockout strain to test the effect of Smad4 deletion in prostate on the background of $p53^{-/-}$; $PTEN^{-/-}$ genotype. Total 12.5% of the resulted animals showed bone metastasis of prostate tumor with a very short period of survival (17 weeks) (Ding et al. 2012).

G3/4 $mTert^{LSL}$ PB- p53/PTEN: TERT gene encodes telomerase, aberrant activation of which was reported in malignancy of different organs including prostate

(Graham and Meeker 2017). In transgenic animal model, aberrant activation of TERT gene was achieved in prostate and the model was generated by crossing p53/PTEN conditional knockout mice under rat PB-promoter with $mTert^{LSL}$; Cre mice continued up to 3rd/fourth generation. Among 25% of the model animals at the age of 24 weeks, PCa metastasized to lumbar spines, although the type of metastatic phenomena with respect to the effect on target sites (osteoblastic or osteolytic) was not determined (Ding et al. 2012).

Xenograft model: In this model, prostate cancer cell lines are transplanted to the host animal and allowed to grow into solid tumor. However, xenograft tumors as derived from a single malignant cell type are more or less homogenous in nature and therefore may not be representative to the primary tumor with respect to tumor heterogeneity and associated microenvironment.

Xenograft Models of Rat Origin

MATLyLu cell line: This PCa cell line, known as Metastatic Anaplastic Tumor Metastasizing to Lymph node and Lungs (MATLyLu), was derived from a poorly differentiated androgen-insensitive prostate carcinoma of a 22-month-old inbred Copenhagen male rat. After introducing this cell line into Copenhagen rats by intravenous (tail) and intracardiac injection, osteoclastic bone metastasis of this xenograft tumor was noticed (Simmons et al. 2014b).

PA-III cell line: The PA-III androgen-insensitive cell line was derived from spontaneous prostate carcinoma of a Lobund-Wistar rat. When this cell line is implanted over the calvaria/scapula of rodents after periosteal disruption, mixed osteoblastic/osteolytic metastatic lesions are formed in bones (Koutsilieris 1992).

Xenograft Models Canine Origin

DPC-1 cell line: This cell line was developed from poorly differentiated prostate adenocarcinoma of an 11-year-old Doberman Pinscher. DPC-1 is used to develop xenograft tumor following its orthotropic seeding in the prostate tissue of immunosuppressed dogs, and the incidence of mixed osteoblastic/osteolytic metastasis is frequently resulted in the pelvic bones (Simmons et al. 2014b; Chevalier et al. 2015).

Leo cell line: This cell line established from primary prostate carcinoma of a 5-year-old mixed breed dog can metastasize to the brain, spinal cord, and long bones (characterized by loss of cortical and trabecular bone) in nude mice after intracardiac injection. This is an almost exclusive model for studying brain metastasis of PCa (Thudi et al. 2011a; Simmons et al. 2014b).

Probasco cell line: The Probasco cell was developed from the primary prostate carcinoma of a 10.5-year-old mixed-breed dog that underwent castration and then received palliative radiation therapy and metronomic chemotherapy (piroxicam, cyclophosphamide, toceranib phosphate, and chlorambucil) afterward as treatment measures. After intracardiac injection in nude mice, these cells primarily metastasize to the appendicular skeleton leading to the development of metastatic osteoblastic tumors. Probasco cells transfected with parathyroid hormone-related protein were

found to result increase growth of the metastatic tumor with higher extent of osteolysis (Simmons et al. 2014a, b).

Multipurpose Animal Models to Study Prostate Cancer

Transgenic adenocarcinoma of mouse prostate (TRAMP) model: The Simian Virus 40 T/t antigen (SV40 Tag) from pSV plasmid was cloned to pBSK plasmid under the minimal rat PB promoter (-426/+28 region) and the recombinant plasmid was microinjected into pronuclei of fertilized mice oocytes. Among the transgenic mice, the strain showed higher SV40 Tag expression in dorsal and ventral lobes of prostate was designated as TRAMP. Mice from this model developed epithelial hyperplasia at eighth week of age that progressed to PIN at 28th week, and all of the lesions progressed to lymphatic metastasis among which 66% displayed pulmonary metastasis. Skeletal metastasis has also been found at around 23rd week of age. Castration of 12-week-old TRAMP mice resulted in development of androgen independence of the PCa showing more aggressive hyperplasia as well as metastatic potential than that of noncastrated mice (Abdulkadir and Kim 2005; Grabowska et al. 2014). This model is also useful in preclinical trials of several targeted and chemopreventive therapeutics (Abdulkadir and Kim 2005). TRAMP-derived tumor cells can also be introduced in immunocompromised and syngeneic mice for serving specific purposes, i.e., subcutaneous injection of TRAMP-C2 castration-resistant PCa cells in RAG mice is useful for assessing antitumor activity of TGF-β-insensitive $CD8^{+}$T cells (Zhang et al. 2006).

LADY models: LADY models are generally constructed by ligating SV 40 small tag deletion mutant under long PB promoter (-11,500/+28 region) followed by microinjection of the construct into the pronuclei of fertilized mice oocyte. The resulted transgenic mice developed prostate adenocarcinoma in 15–22 weeks with no instances of metastasis. The LADY 12T-7s (slow progressing PCa) model was crossed with PB-hepsin mice to express SV40 T antigen and the hepsin transgene specifically in the prostate of the next generation animals. As a result, by the age of 21st week, male mice developed invasive adenocarcinoma followed by distant metastasis to liver, lymph nodes, and bones. The 12T-7f/MT-DNIIR is another LADY model important for studying metastasis. LADY model is also used to study therapeutic potential of antioxidants like vitamin E, lycopene, and selenium for PCa management (Abdulkadir and Kim 2005; Grabowska et al. 2014).

Other SV40 models: Several mice models of PCa have been developed by overexpressing SV40 T/t antigen (Tag/tag) under promoters other than PB to study the stepwise process of development and aggressive phenotypes of the disease.

In C3(1) promoter-regulated model, the steps of disease development from low-grade PIN to invasive adenocarcinoma are well-characterized and consistent. Male C3(1)-Tag mice can develop BPH by 3 months and prostate adenocarcinoma by 7–11 months which metastasized to lungs occasionally (Yoshidome et al. 1998).

Intestinal epithelial cells (Paneth cells) produce antimicrobial peptides known as cryptdins. In absence of androgen, transgenic male mice expressing SV40 T antigen

in neuroendocrine cells of prostate under cryptdin-2 (CR2) promoter developed PIN by the age of 12th week. The lesion proceeded to locally invasive PCa by the age of 24 weeks followed by its further progression to the distant metastasis (Garabedian et al. 1998).

Transgenic mice overexpressing SV40 Tag and tag under embryonic Gγ globin promoter showed high incidence of neuroendocrine and epithelial prostate tumors with very short latency. The tumors developed androgen-independence at fourth to sixth week and later metastasized to kidneys, adrenal glands, and lymph nodes, also occasionally micrometastasized to thymus, lung, and bone at about fifth to seventh months (Perez-Stable et al. 1997).

Mouse Prostate Reconstitution (MPR) model: For development of prostate reconstitution model, fetal urogenital sinus tissue from a p53 knock out background was microdissected followed by enzymatic dissociation into mesenchyme and epithelium; next mesenchymal or epithelial or both cell types were subjected to retroviral transduction of RAS and MYC oncogenes, and subsequently grafted to renal capsule of immuno-compromised mice. This model showed 100% incidence of prostate cancer in both p53 homozygous and heterozygous backgrounds with very high frequency of distant metastatic deposit in lung, lymph node, bone, and liver (Buttyan 1997; Simmons et al. 2014b).

Mice model of AR deregulation: Androgen receptor (AR) is a marker as well as a primary determinant for the development of differentiated luminal epithelium in prostate. Osr1 promoter-mediated overexpression of human AR in mouse prostate leads to the development of PIN lesion in 50% and adenocarcinoma in 5% of animals (mice) by 52 weeks (Zhu et al. 2011). In another study, transgenic male mice overexpressing murine AR under rat PB promoter developed variety of prostatic lesions including BPH, PIN, and microinvasive HGPIN. Additionally, castrated transgenic mice overexpressing ARv567es mRNA (a transcript variant of AR lacking exons 5,6,7) under ARR_2PB promoter developed BPH in 16–20 weeks that progressed to PIN in 30–40 weeks and adenocarcinoma in 52 weeks (Liu et al. 2013). This animal model, as showed development of malignant prostate tumor in absence of endogenous androgen, can be considered as a novel model for studying CRPC.

Mice model of Myc deregulation: In this approach, myc-Pal (prostate antigen1) constructs were cloned under two types of PB promoter, and the constructs were then inserted into fertilized eggs of FVB mice followed by implantation into surrogate mother for establishment of transgenic animal models namely Hi-myc and lo-myc (Ellwood-Yen et al. 2003). In Hi myc model, where the *MYC* gene was put under ARR_2PB promoter, PIN developed at 2 weeks and progressed to adenocarcinoma at about 3–6 months. In the lo-myc mice model where the *MYC* gene was put under minimal rat PB promoter, PIN developed at 4 weeks of age and adenocarcinoma at about 6–12 months. In the Hi-Myc model, it has been found that increasing expression of myc and decreasing expression of NKX3.1 mark the transition from PIN to invasive adenocarcinoma. Additionally in this model, constitutive activation of the NF-κB pathway renders the adenocarcinoma castration resistant (Ellwood-Yen et al. 2003). Furthermore, transgenic mice overexpressing Z-Myc under PTEN and p53

knockout background [Z-Myc; PB-Cre4; PTEN$^{(-/+)/(-/-)}$; p53$^{(-/+)/(-/-)}$] was also generated and the animals developed very aggressive adenocarcinoma with very high incidence of lymph node metastasis (Valkenburg and Williams 2011; Grabowska et al. 2014).

Mice model of RAS deregulation: Rat sarcoma virus (RAS) is an important oncogene which is significantly activated/ overexpressed in various types of cancer. Although, in transgenic mice models where H-RAS-mutant G12V is expressed under rat PB promoter, malignancy of prostatic cells only progressed to PIN lesions (Valkenburg and Williams 2011). Another model has been established by crossing PB-cre mice with K-RAS$^{+/V12}$ mice which developed atypic hyperplasia at 3 months which progressed to low-grade PIN in 6 months. These models indicate that aberrant activation/expression of RAS may be important for occurrence of primary noninvasive lesions, but it does not seem to be sufficient for progression of the disease to the higher grades of PCa (Pearson et al. 2009).

Mice model of β-catenin activation: In this model, transgenic animal was developed by crossing between PB-cre4 mice with Catnb$^{flox(ex3)}$ mice, expressing truncated β-catenin (exon3 deleted) insensitive to proteasomal degradation specifically in the prostate epithelial cells. The transgenic animals developed BPH in 12 weeks that progressed to PIN at 6 months and finally to HGPIN at about 9 months. Following 2 weeks of castration, the prostate gland in the transgenic mice continued to grow indicating acquisition of androgen independence (Yu et al. 2009) (Tables 2 and 3).

Key Signaling Pathways Associated with Molecular Pathogenesis of Prostate Cancer

For studying the molecular pathogenesis of prostate cancer, transgenic animal models are frequently used. In transgenic models, organ-specific knockout or ectopic expression of selected tumor suppressor gene(s) or oncogene(s) was achieved to deregulate the associated signaling pathways that can be identified and characterized to explore the molecular pathogenesis associated with different disease parameters. Signaling pathways, deregulation of which was mostly studied in animal model of prostate carcinogenesis, are summarized from the available literature. The pathways found to be deregulated in animal models of PCa are portrayed in Fig. 2.

PI3K/Akt/mTOR Pathway

This pathway was found to exert an oncogenic effect in the pathogenesis of prostate cancer. Phosphatidylinositol 3 kinase (PI3K) is a membrane-associated enzyme which phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) that activates Akt. Akt activates mTORC1 which phosphorylates its downstream targets like 4EBP1 and P70S6 Kinase (S6K) that in turn induce synthesis of proteins responsible for cell proliferation. PTEN is a well-

Table 2 Uses of animal models in PCa research

Purpose	Mouse	Rat	Dog
Carcinogenesis and oncogenesis	p27 knockout (Cordon-Cardo et al. 1998) TRAMP (Abdulkadir and Kim 2005; Grabowska et al. 2014) *TGFBR2* knockout (Bhowmick et al. 2004) MPAKT (Abdulkadir and Kim 2005) AR upregulation (Zhu et al. 2011) Myc upregulation (Ellwood-Yen et al. 2003) Rb knockout (Maddison et al. 2004) NKX3.1; PTEN knockout (Valkenburg and Williams 2011) RAS upregulation (Pearson et al. 2009) β-catenin activation (Yu et al. 2009)	Lobund-Wistar (Bosland 1992; Nascimento-Gonçalves et al. 2018) Sprague Dawley (Bosland 1992; Nascimento-Gonçalves et al. 2018) F344 (Bosland 1992; Nascimento-Gonçalves et al. 2018) WU (Bosland 1992; Nascimento-Gonçalves et al. 2018)	
CRPC	SCID (Craft et al. 1999) AR upregulation (Zhu et al. 2011; Liu et al. 2013) Myc upregulation (Ellwood-Yen et al. 2003) SV40/Gγ globin (Perez-Stable et al. 1997)		
Bone metastasis	TRAMP (Abdulkadir and Kim 2005; Simmons et al. 2014b) p53; Rb knockout (Zhou et al. 2006) Smad4; PTEN; p53knock out (Ding et al. 2012) MPR (Buttyan 1997; Abdulkadir and Kim 2005)	MATLyLu (Simmons et al. 2014b) PA-III (Koutsilieris 1992)	DPC-1 (Chevalier et al. 2015) Leo (Thudi et al. 2011a) Probasco (Simmons et al. 2014a)
Therapeutics	TRAMP (Abdulkadir and Kim 2005) LADY (Abdulkadir and Kim 2005; Grabowska et al. 2014) RAG (Zhang et al. 2006)		

Table 3 Modulated genes in animal models of PCa

Promoter	Induced gene	Model for BPH	Model for PIN	Model for adenocarcinoma	Model for metastasis	Model for CRPC	Reference
Metallothionein-1 (Mt-1)	PRL	✓	–	–	–	–	(Wennbo et al. 1997)
Probasin	Murine AR	✓	✓	–	–	–	(Zhu et al. 2011)
	RAS	✓	✓	–	–	–	(Valkenburg and Williams 2011)
ARR_2PB	MYC	✓	✓	–	–	–	(Ellwood-Yen et al. 2003)
	ERG	✓	✓	–	–	–	(Tomlins et al. 2008)
C3(1)	SV40 T-antigen	✓	✓	✓	–	–	(Yoshidome et al. 1998)
MMTV	Int2	✓	✓	–	–	–	(Muller et al. 1990)
	Kgf	✓	✓	–	–	–	(Kitsberg and Leder 1996)
Cryptdin-2	SV40 T-antigen	✓	✓	✓	✓	–	(Garabedian et al. 1998)
Gγ globin	SV40 T-antigen	✓	✓	✓	✓	✓	(Perez-Stable et al. 1997)
Long PB	SV40 large T-antigen	✓	✓	✓	✓	–	(Grabowska et al. 2014)

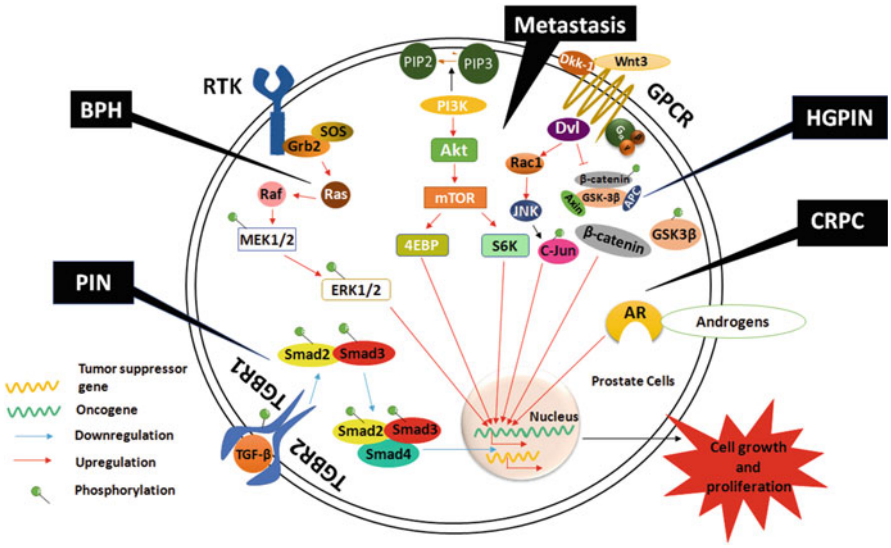

Fig. 2 Signaling pathways associated with animal models of PCa

known tumor suppressor which acts antagonistically to PI3K/Akt/mTOR pathway by de-phosphorylating PIP3 into PIP2. Conditional PTEN knockout in the prostate of aged mice displayed significant activation of PI3K/AKT/mTOR/S6K and PI3K/AKT/mTOR/4EBP1 axes leading to the development of neoplastic growth in the organ concern (Ma et al. 2005). This confirms that PI3K/Akt/mTOR pathway is crucial for cell growth and proliferation during prostate carcinogenesis. Additionally, in MPAKT mice model, activation of mTOR and S6K through ectopic Akt expression also led to the development of PIN, and treatment with the mTOR inhibitor RAD001 was found to cause reversal of the neoplastic phenotype through increase of apoptosis in epithelial cells (Majumder et al. 2003).

RTK/ERK Pathway

This pathway is involved in transducing signals mostly from extracellular growth factors and hence associated with cell growth and proliferation accordingly. Deregulation of RTK/ERK pathway involving aberrant activation of several components is frequently reported in malignancy of several organs including prostate. In RTK/ERK pathway, activation of RTKs (EGFR, FGFR, etc.) brings son of sevenless (SOS) protein (Guanine Exchange Factor or GEF) to RAS via adaptor protein Grb2 leading to its (RAS) activation through exchange of bound GDP by GTP. Activated RAS then binds to and activates Raf which initiates a cascade of phosphorylation reaction in the downstream leading to activation of mitogen-activated protein kinase (MEK) 1/2 first and then extracellular signal-related kinase (ERK)1/2. ERK1/2

phosphorylates and thereby activates transcription factors of several candidate genes actively associated with cell growth, proliferation, and migration (Chen et al. 2017). Transgenic mice with only RAS activation progressed to only PIN in male mice (Valkenburg and Williams 2011). Deregulation of RTK/ERK pathway through K-RAS upregulation in the background of PTEN knockout is frequently used to develop transgenic mice model for studying BPH, PIN lesions, and aggressive phenotypes of PCa (Mulholland et al. 2012).

TGFβ Pathway

Transforming growth factor-beta (TGF-β) pathway involves in the regulation of diverse cellular functions like proliferation, differentiation, motility, and apoptosis. Upon binding of the ligand (TGF-β), TGF-β receptor 2 (TGFBR2) recruits TGF-β receptor 1 (TGFBR1) which gets subsequently phosphorylated and in turn phosphorylates Smad2/3 that form a complex with Smad4; the Smad complex is then translocated into the nucleus leading to transcriptional upregulation of downstream target genes-regulating cell proliferation. Smad 6/7 act as inhibitors in this pathway through interfering the TGFBR-mediated activation of Smad2/3. Tumor-suppressive role TGF-β pathway in carcinogenesis of prostate has been documented in several studies (Tu et al. 2003). Downregulation of TGF-β pathway is achieved through partial knockout of *TGFBR2* intransgenic mice models leading to the development of PIN lesions and stromal inflammation (Bhowmick et al. 2004).

Wnt Pathway

Wnt signaling includes both canonical (Wnt/β-catenin pathway) and noncanonical pathways, regulating vital cellular functions like proliferation, differentiation, migration, apoptotic cell death, stem cell renewal, and so on (Many and Brown 2014). In canonical Wnt signaling, in absence of Wnt, β-catenin is phosphorylated in cytosol by a protein complex consisting adenomatous polyposis coli (APC), casein kinase 1α (CK1α), and glycogen synthase kinase 3β (GSK3β) leading to its proteasomal degradation. Upon binding of Wnt ligand to its receptor Frizzled (G-protein-coupled receptors; GPCR), GSK3β-mediated phosphorylation and subsequent degradation of β-catenin are inhibited. Now β-catenin after getting stabilized translocates into the nucleus and acts in cooperation with TCF/LEF to induce the transcription of several target genes (Pai et al. 2017). Among noncanonical Wnt signaling pathways that are operated independent of β-catenin and TCF/LEF, the planar cell polarity (PCP) is one of the best characterized candidates. In PCP pathway, Frizzled receptor after binding to Wnt activates RAC1 (a small GTPase) that in turn activates JNK as the downstream effector. JNK phosphorylates and thereby stabilizes c-JUN which then translocates into the nucleus leading to transcriptional upregulation of target genes (Kagey and He 2017). Aberrant activation of both canonical and noncanonical Wnt signaling has been reported in prostate cancer indicating importance of the pathways

in disease pathogenesis (Schneider and Logan 2018). In the development of animal model for HGPIN and CRPC in transgenic mice, de-regulation of canonical Wnt signaling was noted and found to be achieved through constitutive activation of β-catenin (Yu et al. 2009). Aberrant activation of canonical Wnt signaling was also noticed in TRAMP mice models (Shukla et al. 2007). When Dickkopf-1 (Dkk-1)-transfected Ace-1 (canine adenocarcinoma cell of prostate) was xenografted in athymic mice, upregulation of JNK signaling through noncanonical Wnt pathway was found leading to the development of solid tumor followed by the incidence of metastasis to bones (Thudi et al. 2011b).

AR Signaling Pathway

The development of prostate is hormonally regulated, and therefore in this process, androgen and associated signaling pathway seem to play an important role. In AR signaling, binding of androgens (testosterone, dihydrotestosterone, etc.) to androgen receptor (AR) in cytosol causes its dimerization followed by translocates into the nucleus. Active AR then binds to the androgen response elements (ARE) located in promoter regions of AR-regulated genes to induce their transcriptional expression. In prostate tumorigenesis, de-regulation of AR signaling pathway has been found to be achieved through AR overexpression mediated by Akt, TGF-β, and so on. Aberrant activation of AR signaling independent of androgen has also been reported in the disease progression more aggressive form. Thus, AR pathway seemed to contribute not only to the development of normal prostate but also to the development and progression of its malignant form (Lonergan and Tindall 2011). In transgenic mice models, aberrant activation of AR signaling is achieved through ectopic expression of AR leading to the development of malignant prostate tumor that showed stepwise progression to more aggressive form capable of growing in absence of androgen. Consequentially, all the pathways described above can modulate the each other (Gao et al. 2017) as well as AR signaling in PCa.

Conclusion and Future Perspectives

The pathogenesis and associated risk factors of PCa are not well characterized. PCa is a disease of aged male. Along with age, food habit and tobacco smoking are also considered as important epidemiological factors, and incidence of oxidative stress as well as inflammation has been well documented as associated cellular phenomena. However, to explore the disease pathogenesis in detail at molecular level, animal models of prostate cancer including rat, mice, and canine are commonly used. In the present chapter, a summarized view of commonly used animal models of prostate cancer categorized according to the study purposes was presented. Carcinogen models may help us to understand how chronic carcinogenic exposure particularly to the aged male in daily life can play a role in emergence of PCa. Transgenic and immunodeficient models are primly helpful to understand the details of molecular

pathogenesis associated with the PCa development and its progression to advanced stages. Castration-resistant rodent models and some canine models help in dissecting the driver molecular events leading to the development of androgen independence of PCa. Canine models are almost inevitable in studying bone metastasis; some bone metastasis-specific transgenic rodent models are also used in this purpose. Xenograft models of PCa may serve the purpose of preclinical studies for novel therapeutic measures. However, in developing effective therapeutics, it is important to keep in mind that dose-dependency and toxicity assays are subject to animal sizes and metabolic rates. So, the therapeutics approaches must be standardized for human body before incorporation. In conclusion, animal models of prostate cancer are of diverse types, each having specification to address research issues and limitations of their own. However, the genetic dissimilarities of the model animals from human may affect the bench side to bedside implementation of the research output, and to overcome this, the scientist may use more humanized animals for this purpose in future.

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Animal Models for Angiogenesis on Cancer Research

20

Sweta Makwana and Chandi C. Mandal

Contents

Abstract

The process of formation of blood vessels follows an intricate cascade of mechanisms, having an array of positive and negative regulators. Angiogenesis is essential in various day-to-day physiological processes such as tissue growth, embryonic development, and wound healing; however, when the normal balance is tipped off, it contributes to various pathological conditions such as diabetic retinopathies, rheumatoid arthritis, cancer, and obesity. Evidence suggests that growth, proliferation, the persistence of tumors, and cancer metastasis are all in a manner dependent on angiogenesis. It is crucial for us to elucidate the mechanistic events taking place in the process of angiogenesis and then develop an efficient therapeutic strategy for cancer. A good antiangiogenic agent would be one that not only prevents the formation of new blood vessels but also disrupts the preexisting microvessel which surrounds the tumor and feed the cancerous mass. In this search for antiangiogenic agents as anticancer therapy, it is necessary

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to investigate and assess how these agents hinder the process of angiogenesis. To test the antiangiogenic agents, assays are required which can activate crucial steps in angiogenesis and also act as a tool to monitor the efficacy of the drug. Multiple antiangiogenic drugs have been developed to date such as endostatin, angiostatin, canstatin (endogenous peptide inhibitor), marimastat, and thalidomide (synthetic inhibitors). These inhibitors either show kinase activity against VEGFR (vascular endothelial growth factor receptor) or inhibit metalloproteinases. Since the development of an antiangiogenic agent primarily depends on the type of assays used for the preclinical study, estimation of the relevance, benefits, and pitfalls of the angiogenesis assays in use is necessary. One single assay will not allow us to understand the influence of the tissue microenvironment, structural activities, and dosage effect; therefore, it is also essential to test a therapeutic agent by more than one assay. In this chapter, a discussion on the methodologies of various assays and their benefits, pitfalls, and economical, technical, and ethical perspective is carried out.

Keywords

Angiogenesis · Corneal micropocket assay · Sponge implant assay · Danio rerio · Animal model · Cancer

Introduction

Tumor angiogenesis is a crucial step involved in tumor progression and metastasis. Tumoral angiogenesis is essentially a series of multistep processes leading to the formation of new blood vessels which go on to feed the cancerous mass. Angiogenic factor secreted by the tumor cells, adjacent tissue/cells, and infiltrating immune cells interacts with the receptors on the endothelial cells. The interaction between the ligand and receptor results in endothelial cell growth, proliferation, invasion, and migration, and ultimately capillary tube formation. However, under normal circumstances, proangiogenic substances are counterbalanced by antiangiogenic substances, thus maintaining homeostasis (Risau 1997). When this balance between pro- and antiangiogenic factors is corrupted, formation of new blood vessels takes place. To combat neovascularization in diseased state condition, development of antiangiogenic agents is essential. Assays which help in quantification and visualization of angiogenesis are critical for assessing the therapeutic agents. These assays should be able to activate certain steps in angiogenesis and validate data that has been observed in in vitro studies. The basic pattern that these assays follow is to either observe for entirely new blood vessels in avascular tissue or be able to distinguish between preexisting microvessel and newly formed microvessel. The assays that are being employed currently all have their specific pros and cons. Corneal micro-pocket assay requires a highly skilled technician to perform it; however, it has its own benefits. Chorioallantoic

membrane (CAM) assay allows us to carry out large-scale screening of the therapeutic agent. Angiogenesis assay can be grouped into three groups: (i) one which requires polymer matrix implants for the vascularization to develop onto, e.g., sponge implant assay; (ii) one which requires microcirculatory preparations, e.g., CAM assay; and (iii) one which requires excision of some animal tissue, e.g., corneal micro-pocket assay (Hasan et al. 2004).

In the last few years, the assays have been modified and refined in such a manner that enables us to carry qualitative as well as quantitative analysis of tumor angiogenesis, thus improving our ability to analyze a potential antiangiogenic therapeutic target.

Tumor Angiogenesis/Angiogenic Switch

Judah Folkman, in the year 1971, first proposed the hypothesis that tumor progression and growth is driven by development of new microvessels from preexisting vasculature (Folkman 1971). Folkman and his associates also isolated proangiogenic factor called "Tumor angiogenesis factor." His hypothesis stated that it is the endothelial cells that might switch from a state of resting to a rapidly growing phase, and that the switch is made chemical signal secreted by the tumor cells (Folkman et al. 1971). A plethora of diverse biological processes contributes to the formation of tumor vasculature starting from tissue type, anatomical location, and tumor type. Several signaling pathways and secretory factors such as chemokines and growth factors orchestrate tumor angiogenesis, one of the hallmark features of cancer. A major challenge as well as target in therapeutic regimen for cancer is tumor angiogenesis. It allows a continuous supply of growth factors, nutrients, oxygen to the cancer mass along with facilitating dissemination of the tumor cells to a distant site. The communication taking place between the tumor microenvironment and tumor cells is quite dynamic in nature, which is facilitated by the delicate balance of pro- and antiangiogenic signals. The phenotype and characteristics of the tumor vasculature is vastly different from that of preexisting blood vessels. Analysis from in vivo microscopic studies indicate that the tumor mass has a disorganized, bidirectional blood supply with highly variable blood flow. The first step of angiogenesis involves degradation of the local basement membrane which is present around the capillaries. Followed by the invasion of the stroma underlying the epithelial cells (ECs), this occurs in the direction from which the stimulus for angiogenesis is received. Once the stimulus is received, the EC start to proliferate and organize themselves into 3D structures which would further go on to connect with similar structures, leading to the formation of new blood vessels. Tumor invasion is promoted by the ECs since they secrete matrix degrading proteinases and various growth factors. As the ECs keep on growing and expanding, it provides more opportunity to the tumor cells to enter blood circulation and metastasize to distant location. It has been observed that the growth of a solid tumor occurs in two phases: one is avascular, which is followed by a

vascular phase. Such a kind of development is facilitated by the acquisition of proangiogenic ability that makes the ECs undergo neoplastic transformation. The angiogenic switch is what drives tumor angiogenesis, which in turn is dependent on positive and negative regulatory growth factor and neoplastic factors released by ECs. These molecules are responsible for hindering and downregulating cells' inherent regulation of angiogenesis inhibitors (Ahmad et al. 2002). In a normal cell, angiogenic stimulus is met with the dominant influence of antiangiogenic factors which help maintain vascular quiescence. The process of angiogenesis can occur by two methods, sprouting and non-sprouting.

1. Sprouting: New microvessels branch out from preexisting blood vessels.
2. Non-sprouting/intussusceptive microvascular growth (IMG): Preexisting blood vessel formed by the rapid growth of endothelial cells fuse, enlarge, and split within the wall of the vessel. They create capillary mesh like structure.

Sprouting and non-sprouting angiogenesis can go on simultaneously in organs such as heart and lungs, whereas non-sprouting angiogenesis is predominantly found in case of brain metastasis. The angiogenic switch is started by upregulation of positive regulatory of neovascularization such as fibroblast growth factor-2 (FGF-2), transforming growth factor-β (TGF-β), placental growth factor (PlGF), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), pleiotrophins, platelet-derived growth factor (PDGF), and many more (Ferrara et al. 2003). These factors can be exported or secreted in the tumor microenvironment; the angiogenic switch is a simple balance between the net positive and negative regulators of angiogenesis. Among them, the key player is VEGF which has a crucial role in the development, pathology, and physiology of angiogenesis. VEGF has four isoforms depending upon the splicing pattern of the mRNA transcript; it is a homodimeric glycoprotein which binds to heparin. The isoforms of VEGF are named depending upon the number of amino acids that are present in the protein, like VEGF-165 and VEGF-205. VEGF-B is involved in vasculogenesis, whereas VEGF-C is involved in lymphangiogenesis. Another endothelial cell-specific molecule having role in angiogenesis is angiopoietin, and it is involved in tumor malignancy, vascular extravasation, inflammation, and angiogenesis. Apart from these proangiogenic factors and receptors, hypoxic condition also influences tumor angiogenesis. Chronic hypoxic condition is developed within the tumor mass due to the distant diffusion occurring in the tumor vessels. Hypoxic microenvironment is linked with resistance to treatment and poor prognosis. In areas where there is the presence of hypoxic conditions and poor vasculature, tumor-associated macrophages increase the level of proangiogenic factors like FGF2, TNF-α, TGF-β, IL-8, PDGF, urokinase, MMPs, and VEGF (Kumar et al. 1998). So, indirectly hypoxic condition regulates tumor vasculature mainly via HIF-1. Since the regulation of tumor angiogenesis occurs by multifaceted cascades, observation of all those avenues is possible to develop a sensitive, efficient antiangiogenic therapeutic drug for cancer treatment (Fig. 1).

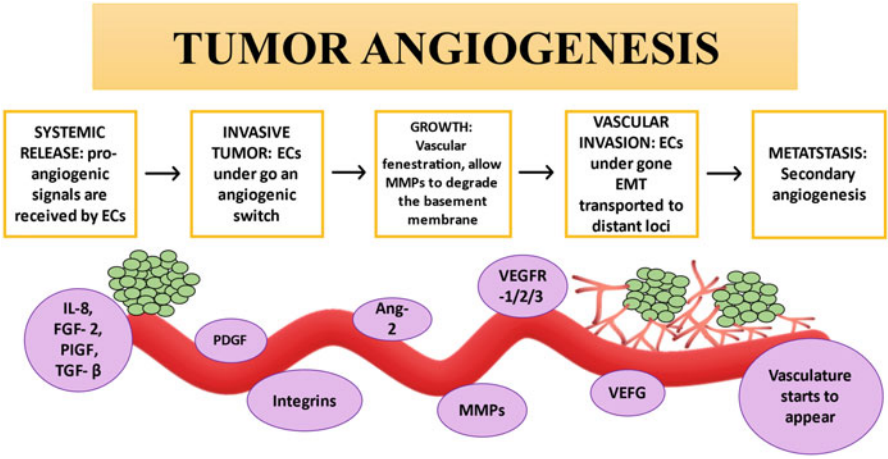

Fig. 1 Diagrammatic representation of the process of angiogenesis

Animal Models Used in Angiogenesis Study

Rodent and Mice as a Model

Corneal Micro-pocket Assay

Corneal micro-pocket assay and chick CAM assay were both introduced in the year 1974 by Folkman and his associate (Folkman 1974). The avascular nature of a cornea appears to be one of the key advantages of this technique. Vascular sprouting that develops in an avascular tissue is called as neovascularization. Using this assay, neovascularization caused by angiogenic inducers such as tumor cells can be observed. The avascularity of the cornea is contributed by various factors such as production of antiangiogenic factors, immune deviation associated with the anterior chamber, epithelial cells in the cornea having an angiostatic nature, and absence of proangiogenic factor (Ambati et al. 2006). Integrity of the corneal epithelial cells plays an important role in imparting the avascularity (Azar 2006). The basic procedure of the assay is to create a point of insertion where the angiogenic inducer can be introduced. This point of insertion is the corneal micro-pocket and hence the name of the assay. Anatomically, the cornea is divided into five distinct layers, the first being the epithelial layer on the surface. Next is the Bowman's membrane, which is an acellular structure made of collagen, stroma that occupies around 80–95% area of the cornea and is transparent due to the presence of collagen fibrils. In between the stroma and monolayer of polygonal-shaped internal endothelial cells lies the non-cellular Descemet's membrane. Homeostatic conditions in a cornea are maintained by the collagen matrix of the stroma since it contains fibroblast-like cells and keratocytes, which secrete certain essential substances. For studying in vivo angiogenesis, it is a reliable method since the cornea can replicate the cellular and

molecular cascade essential for angiogenic sprouting in an adult. Angiogenic response in the absence of corneal inflammation and edema was demonstrated by implanting hydron pellets having VEGF or FGF-2 and sucralfate into the mouse cornea (Kenyon et al. 1996). The assay is carried out by anesthetizing the mouse using methoxyflurane; once that is done, the micro-pockets are created to the depth of 1 mm of the limbus in both the eyes. Hydron, which is the casting solution, is prepared at 37 °C by dissolving the polymer in absolute alcohol (Langer and Folkman 1976). Hydron-coated pellets containing the test substance are implanted into the micro-pocket. Sucralfate is added when the test sample contains peptide; it is meant to stabilize the peptide molecule as well as ensure slow-paced release of hydron (Chen et al. 1995). Using slit lamp biomicroscopy, the microvessel formation is scored at fixed time intervals (5 and 7 days), and the vessel length is also measured to monitor the vascular response. Corneal neovascularization can also be visualized by intravenously injecting FITC dextran, i.e., fluorochrome-labeled high-molecular-weight dextran (Kenyon et al. 1996). The impact that a drug has on corneal angiogenesis can be determined by administering microinjection into the thickness of the cornea or by administering the drug in the form of ointment or ocular drops (Presta et al. 1999). The systemic impact of a drug on corneal angiogenesis can also be studied using this assay. Inhibitors of angiogenesis which are produced by human tumors were identified in immunodeficient mice using a modified version of this assay (Chen et al. 1995) (Fig. 2).

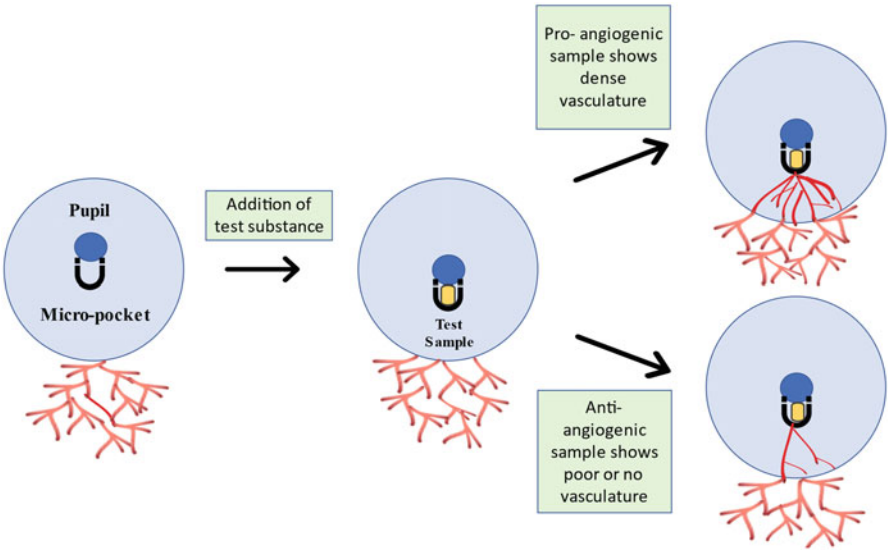

Fig. 2 Diagrammatic representation of corneal micro-pocket assay. A point of insertion (micro-pocket) is created in the cornea. After the addition of test sample, vasculature starts to appear after an appropriate amount of incubation in the avascular tissue

Rodent Mesentery Angiogenesis Assay

Norrby et al. developed the rodent mesentery angiogenesis assay in the year 1986 (Norrby et al. 1986), and since then, it has been modified by various researchers (Norrby and Østergaard 1996). Observing microcirculation is best done using an intravital microscope in the rodent mesentery angiogenesis assay. For physiological measurements, it is considered an ideal system due to the transparency of the small gut mesentery tissue and even the ease with which it can be exteriorized from the abdomen (Zweifach 1973). Adult male rats have around 40–50 mesenteric windows that are surrounded by fatty tissue and show the presence of portal artery-vein pair. Mesenteric tissue is around 5–10 μm thick and is surrounded on both sides with a layer of mesothelial cells on the basal membrane; between the tissue space are macrophages, mast cells, fibroblasts, lymphocytes, and eosinophiles. Microscopical examination of these windows on an objective slide shows the central part being larger avascular whereas the microvessel develops towards the edges in an asymmetrical manner. The mesentery is the thinnest tissue in the body and can contain elastic fibers, elastin, and collagen. The assay is performed in adult male Sprague-Dawley (SD) rats. Along the elastin fibers, pericytes and capillary sprouts migrate into the rat mesenteric window during angiogenesis. The anatomical position of sprouting and angiogenic stimulus which has been applied in the network influences this phenomenon (Ponce and Price 2003). When the microvascular bed of the rat mesenteric window is stained using immunostains, mid-capillaries, pre-capillaries, post-capillaries, venules, and metarterioles can be easily observed (Nehls and Drenckhahn 1991). Since the mesenteric window is covered on both sides by a highly permeable mesothelial layer, the test substance that is injected intraperitoneally reaches the target tissue rapidly. Due to this reason, angiogenesis induced because of wound healing does not take place (Rasio 1987). Mast cell-mediated microvessel formation was demonstrated using an agent that selectively activated the mesenteric mast cells (Norrby et al. 1989). In a similar experimental setting, molecules such as growth factors bFGF (Norrby and Østergaard 1996), VEGF (Norrby 1996a), and inflammatory cytokines TNF-α (Norrby 1996b), IL-1-α, and IL-8 (Norrby 1996c) have been tested. Mesenteric windows in adult mice, and adult and fetal rats were used to demonstrate that TNF-α, IL-1-α, IL-8, and bFGF and VEGF induce angiogenesis by sprouting (Nehls et al. 1992; Wang et al. 2004).

Dorsal Air Sac Model

To monitor the vascularization developing in tumor grafts, Selye and his colleagues developed the dorsal air-sac model in the year 1953 (Selye 1953). This technique involves lifting up the skin on the dorsal side of the mouse so as to expose the white fascia area and injecting air into it. Next, a Millipore ring is covered from both sides by a filter, leading to the formation of a chamber into which the test tumor sample can be injected (Yonekura et al. 1999). The Millipore ring containing the test sample is inserted into the air sac of an anesthetized mouse. After 48 h of tumor implantation, vasodilation and clear vascular channels can be observed. This is followed by removal of the test sample containing the Millipore chamber, and rings of the same

dimensions are placed on the site which was directly exposed to the chamber. A dissecting microscope is used to count the newly formed microvessels lying under the area of the ring. Gamma counter can be used to measure the volume of blood, by estimating the number of erythrocytes which are labelled with ^{51}Cr (Funahashi et al. 1999). Another method to distinguish between preexisting and newly formed blood vessels is by injecting Evan's blue dye into the mice; preexisting vessels will retain the dye, whereas in newly formed blood vessels, it will leak out. The amount of dye accumulated is assessed, giving us a semiquantitative estimate of the extent angiogenesis (Yamakawa et al. 2004). Angiogenic activity of TAF (tumor angiogenesis factor) was determined using 3 H-labeled thymidine in the rat air-sac model, and the examination of tissue was done using electron microscopy and autoradiography (Funahashi et al. 1999).

Disk Angiogenesis System

The disk angiogenesis system technique was introduced by Fajardo et al. in the year 1998 to study angiogenesis in vivo. It is a reliable technique to observe wound healing or angiogenesis caused due to tumor tissue and other angiogenic substances. This technique involves an 11 mm synthetic disk made up of polyvinyl alcohol foam to be subcutaneously implanted into a mouse model via a distal skin incision (Fajardo et al. 1988). On both sides except the edge area, the disk is covered with Millipore filters, the test samples such as antagonist, tumor cells, or other angiogenic factors are loaded on the center of the disk. The disk might also contain either agarose or ethylene-vinyl acetate copolymer that would facilitate the slow and steady release of the test sample. These disks are generally implanted in the thorax or abdomen region of the mice since it is well-tolerated and convenient. The incubation period for the disk can vary on whether or not any stimulator is present. When a disk contains proangiogenic factors, then the disk is examined after 7–12 days, whereas in the absence of a stimulator, the disk is examined after 12–20 days of implantation. During this period, the disk can be seen being encapsulated by granules along with having new vasculature being formed around it in a centripetal manner. Visual analysis of the newly formed blood vessel can be done using histochemical techniques and observing the sections of the disk under a microscope, where you can see the vascular growth along with mast cells, fibroblasts, lymphocytes, and other connective tissue components, while the quantitative analysis of the vessel can be done by determining the centripetal vessel growth and intravascular volume. The centripetal vessel growth is the radial distance between the edge of the disk and the central portion of the blood vessel. Fibroblasts are constantly present around the vasculature, thus giving rise to inflammation-mediated angiogenesis, which is the major drawback of this technique (Hasan et al. 2004; Fajardo et al. 1988). Vessels can be visualized using intravascular dyes, e.g., India ink, microspheres, or inert substances under an electron microscope or combined with a light microscope.

Sponge Implant Assay

Sponge implant assay was introduced by Andrade and his colleagues in the year 1987 (Andrade and Fan 1987). Subcutaneous pockets are made on the dorsal side of

the mice. In the subcutaneous pocket, sterile sponge disk is inserted in which angiogenic test substance or inhibitors can be injected. These sponge matrices have absorbable gel foam, and can be made up of polyurethane, polyether, polyester, cellulose acetate, gelatine and polyvinyl alcohol either alone or in combinations. Within a period of 5 days post implantation of the sponge it is invaded by micro-vessel which have just been formed from the pre-existing micro-vessels. The quantification of the neovascularization in the sponge can be checked after 1–2 weeks via histological or immunohistochemical techniques. Since the implants originally being avascular it is easy to measure the amount of blood flowing into the sponges once the vascularization starts. The blood flow is measured using ^{133}Xe clearance technique, an indirect method to observe the vascular changes taking place. The rate at which ^{133}Xe loss occurs is proportional to extent of neovascularization. Drabkin method can also be used to monitor vascularization by measuring the amount of haemoglobin (Drabkin and Austin 1935). This model was used to demonstrate how thymidine phosphorylase influences platelet derived endothelial cell growth factor (PDECGF) in imparting angiogenic effect (Miyadera et al. 1995), and also the anti-tumorigenic and anti-angiogenic role of suramin by blocking fibroblast growth factor activity (Pesenti et al. 1992). Antiangiogenic activity of EGF and cartilage-derived growth factor was studied using polyvinyl alcohol sponges, and analysis was done by measuring the collagen, protein and DNA content in the sponges(Buckley et al. 1985)

Chamber Assay

Study of chronic angiogenesis in vivo has improved since the development of various types of transparent chamber assay including cranial window chamber, dorsal skinfold chamber, and rabbit ear chamber. The procedure of this assay includes removal of a part of the skull, i.e., cranial window chamber or certain piece of skin, e.g., skinfold and ear chamber from an anesthetized animal. On the exposed surface, the test substance, i.e., pro/angiogenic factor or tumor cells, is placed and covered with glass which has to be properly secured in its place. After the animal has recovered from the injury, continuous monitoring of various parameters can be carried out. Using the chamber assay model, in a living animal, analyses of blood flow, pH, angiogenesis, and gene expression are carried out. It is possible to demonstrate the impact the tumor microenvironment on neovascularization. The 3D imagining vessel growth can be done in one mouse using chamber assay, within a period of 2–3 weeks. Due to this reason, each measurement point does not require separate animal, thus minimizing the number of animals used. In cranial window chamber assay, transplant ability is higher and angiogenic response is also induced at the rather rapid pace. The drawback of this assay is that it requires high technical expertise and all of them are invasive. Out of all chamber assays, the rabbit chamber is the most expensive for daily use; apart from that, after the surgery, the recovery of the animal takes around 4–6 weeks. Sample testing can only proceed after full recovery has occurred. While imaging a new vessel in the cranial window chamber, administering fluorescent marker injection is necessary, whereas in the case of a

skinfold chamber, due to the thickness of the skin, visual observation is very poor. Xenografts in immunodeficient rats were used to study tumor angiogenesis in the dorsal skinfold chamber (Dellian et al. 1996). Vessel density, diameter, and blood flow are determined by transillumination techniques (Menger et al. 2002). Vascular permeability, platelet adhesion, and erythrocyte flow are determined using epi-illumination (Menger and Lehr 1993).

Chick

Chick Chorioallantoic Membrane (CAM) Assay

Another robust technique to study tumor angiogenesis and the potential of a substance to be pro- or antiangiogenic is the CAM assay. Developed by Folkman and his associates, earlier this technique was used to study embryonic development and embryonic tissue grafts (Folkman 1974; Ausprunk et al. 1975). Chick chorioallantoic membrane till the 19th day of incubation is meant for gaseous exchange and acts as the first respiratory system in the embryo. Allantois (extraembryonic membrane) of the splanchnic mesoderm fuses with the chorion of the somatic mesoderm on the 4th day of incubation to form the chorioallantois. The vascularization of CAM can be divided into three distinct phases. The initial phase of development from day 5 to day 7 includes sprouting of the capillary network leading to the formation of primary capillary plexus. From day 8 to day 12 is the intermediate phase where you can see tissue profiles with intussusceptive microvascular growth, that further go on to form intercapillary meshes. At this stage, no sprouts can be observed as they have been replaced by intussusceptive microvascular growth. In the late stage, around days 12–13, the entire shell is layered by the chorioallantois, completing the expansion (Schlatter et al. 1997). The procedure of the assay is to incubate fertilized chick eggs at 37 °C; since chick eggs are easily contaminated by fungi, it is necessary to check if they are free of pathogens. After 3 days of incubation, 2–3 ml of albumen is aspirated from the pointed side of the egg. This is done to make the CAM detached from the shell easily, and with the help of scissors, a small cut is made into the shell. Initially, tumor angiogenesis was studied by implanting tumor cell samples on the surface of CAM. Within 2–5 days of grafting the tumor sample, vessels originating from CAM are visible in the tumor xenograft which is followed by a phase of rapid increase in the number of microvessels. When glioblastoma cells were grafted onto CAM, within a period of 2 days, formation of an avascular tumor was promoted by VEGF. One drawback of this assay is that it is difficult to monitor the test area prior to the complete period of incubation. However, electron microscopic analysis can be performed, even synthesis and content of protein, DNA, collagen can be determined. Autoradiographic evidence suggests that all types of cells receive proliferation stimulus in CAM. Study of matrix and vascular proteome was done using CAM assay, in vivo biotinylation along with bioinformatic tools and high-resolution mass spectrometry (Soulet et al. 2013).

The Chick Embryo Aortic Arch Assay

Modification of mouse aortic ring angiogenesis assay is the chick embryo aortic arch assay, primarily developed to test thalidomide. This assay was introduced by Robert and his colleagues (Nicosia et al. 1990). But a rather clear understanding of this assay is provided by Muthukkaruppan et al. (Muthukkaruppan et al. 2000). One of the main advantages of this assay is that test substance can be administered during a specific time while the culture period is going on, this enables us to test effect on angiogenesis in a serum-free culture medium or growth factor-depleted medium (Auerbach et al. 1974). Chick eggs that are incubated for 12–14 days at 37 °C, the shell is first cracked open, and the contents are emptied into a petri dish containing PBS. Onto another petri dish using scissors and forceps, the embryo is placed, removing its surrounding material. The embryo has to be placed in a ventral position followed by rapid removal of its head. With the help of a tweezer, the tissue above the breastbone is lifted up, and the heart and aortic arch are exposed by trimming the tissue above the thoracic cavity. The heart and aortic arch are now accessible and need to transferred into another petri dish containing PBS and streptomycin/penicillin (Akhtar et al. 2002). Observing the isolated aortic arch under a dissecting microscope allows the user to observe a pair and threesome of the arches, and any excess tissue should be removed at this point. Further, the best fit arches for the assay are transferred into a dish containing antibiotics and saline. The obtained aortic rings are placed on 24-well culture plates. These plates are prefilled with 1–2 ml of Matrigel; immediately after the aortic arch is placed in the 24-well culture plates, fresh 10 ml cold Matrigel needs to be added. Before the Matrigel starts to harden, aortic arch needs to be repositioned in such a way that the opening of the aortic arch ring is facing upwards. The culture is incubated at 37 °C with 5% CO_2 after growth medium has been added. If the culture has to be maintained for a longer duration, the medium has to be changed every 3–4 days. Time lapse photography can be used to observe the angiogenesis, cells can be harvested for biochemical analysis, and they can be stained with appropriate antibodies to carry out histological analysis (Isaacs et al. 2002; Zhang et al. 2011) (Fig. 3).

Zebrafish

Danio rerio (zebrafish) is a freshwater fish found in the tropics. Along the longitudinal axis of their body, they have five horizontal pigmented stripes, hence the name zebrafish. The size of an adult zebrafish reaches up to 3–4 cm long, whereas the generation time is 3 months. They can be easily cultured in large numbers due to the small generation time and size. The common ancestors between humans and zebrafish separated around 400 million years ago, but still, there is an astounding amount of similarity at the physiological, anatomical, and molecular levels. The first use of zebrafish as an whole animal model to analyze molecules showing pro- or antiangiogenic potential was demonstrated in the year 1999 (Serbedzija et al. 1999). There is strong similarity in the vascular development occurring in vertebrates and

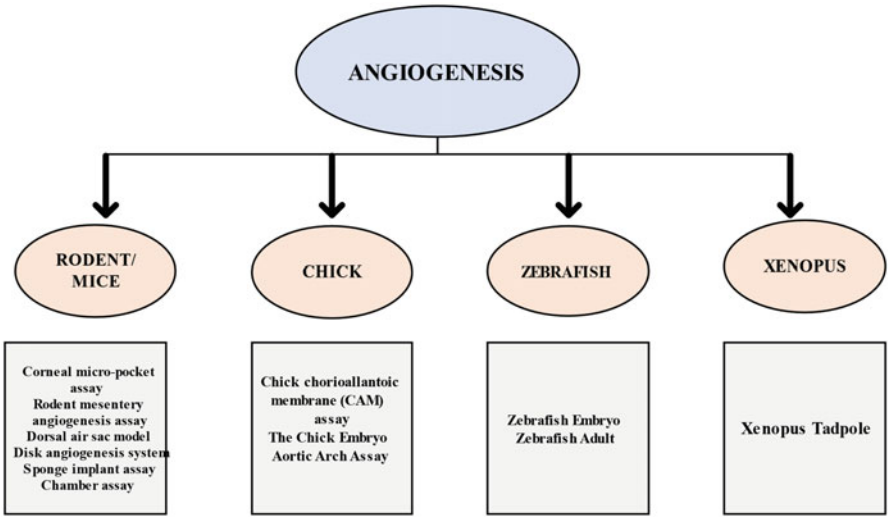

Fig. 3 Various angiogenesis model organisms and their assays

that of zebrafish, and it even has a complex circulatory machinery like that of the mammals (Weinstein 2002). The vascular structure of a zebrafish embryo includes a posterior cardinal vein and dorsal aorta. From these vessels, other secondary vessels branch out, which consist of longitudinal parachordal vessel, two longitudinal anastomotic vessels that connect the intersegmental vessels (ISVs) and the dorso-ventrally aligned ISVs (Eriksson and Löfberg 2000). In a zebrafish embryo, the test substance is injected into the yolk sac, and when the test sample is lipophilic in nature, it can easily diffuse into the embryo. Visual inspection of the neo-vasculature is possible due to the transparent nature of the zebrafish embryo. The inhibition of VEGFR by a kinase inhibitor PTK787, leading to reduction in angiogenesis in a dose-dependent manner, was demonstrated in zebrafish embryos (Chan et al. 2002). Using zebrafish assay, it is possible to differentiate between antiangiogenic substances which act by nonselective cytotoxicity and the one's inhibiting VEGFR (Chimote et al. 2014). Pathological angiogenesis was studied by Cao et al.; using an adult zebrafish, they created a hypoxia-induced retinal angiogenesis model. From the study it was concluded that hypoxic conditions promoted vascularization in the retina and VEGF is a major player in this process. Regenerative angiogenesis is also studied in zebrafish by partial fin amputation (White et al. 1994). Another method to observe newly formed microvessel with time lapse is done using transgenic zebrafish embryos. These embryos have endothelial cells that can express green fluorescent protein which are under the regulation of promoter specific for endothelial cells. This assay is a modified version of tumor xenograft/zebrafish embryo assay, and an in vivo confocal microscope or epifluorescence microscope can be used to observe new vasculature in the live embryo (Nicoli 2007). In the zebrafish model, applying antisense technologies is not possible; however,

Morpholinos (MO), which are oligonucleotide substitutes, help in knocking down genes either by splicing of pre-mRNA or interfering with translation of mRNA. MO are successful in blocking mRNA translation because they show specificity towards target mRNA molecule; these mRNA analogs are highly stable in the cytosol. This knockdown technology can help us get a better hold on understanding the molecular cascade involved in the process of vascularization using the zebrafish model. The molecular events of angiogenesis can be investigated with the help of antisense and mutagen MO oligonucleotide via genetic engineering (Currie and Ingham 1996).

Xenopus Tadpole

Xenopus laevis is a clawed-toed frog found in South Africa; since its initial use demonstrated in pregnancy testing (1950s), it has been popularized as an ideal amphibian model organism. It is a very potent model organism to study the process of lymphangiogenesis and angiogenesis (Gurdon and Hopwood 2003). It shows greater vascular similarity to higher vertebrates as compared to zebrafish. Study of lymphangiogenesis is done in Xenopus as it develops a lymphatic system, which is not possible with the zebrafish model. After fertilization, within a few days, visualization of proper functional vasculature is possible through microscopic imaging. The advantages of using Xenopus are that it is resistant to diseases, maintaining it in the laboratory is easy, its life span is 10 years, and it can also ovulate thousands of eggs four times a year by injecting human gonadotropins (Hardwick and Philpott 2015). Recently, a semisolid tumor model has been developed in which, before subcutaneous engrafting, the tumor cells are embedded in a collagen matrix, and this was done using LG6 and LG15 tadpoles. Intravital imaging can be used to visualize the tumor vasculature by infusing intracardiac labelled dextran and fluorescently labelling tumor cells. Using the semisolid graft visualization of infiltrating melanophores, collagen rearrangement and real-time analysis of the interaction between the stroma and the tumor is possible due to the transparent nature of Xenopus tadpoles. During the study of TP53 (tumor suppressor p53) in a normal embryo, the initial tumor phenotype in Xenopus was discovered. Oncogenic role of TP 53 was well established in mouse model and tissue experiments through observations of genetic instability and aberrant cell cycle control. Therefore, Xenopus was used to understand the role of TP53 in tumorigenesis and embryogenesis. The Xenopus homolog of TP53 is very similar to the human TP53 protein, making it an ideal model to study tumorigenesis (Wallingford et al. 1997) (Table 1).

Drugs for Angiogenesis

In the context of tumor growth and distant metastasis, a pivotal role is played by neoplastic angiogenesis. One of the key players of neoplastic angiogenesis is vascular endothelial growth factor (VEGF), and therefore, it is one of the main drug targets for angiogenesis. Other antiangiogenic agents include multi-kinase

Table 1 Advantages and disadvantages of the angiogenesis assays

Models/assays	Advantages	Disadvantages	References
Corneal micro-pocket assay	1. Monitoring the formation of a new vessel is easy 2. The model organism that can be used are mice, rats, and rabbits 3. Monitoring can be done for a longer duration 4. Minimal chances of cross-reaction 5. Angiogenesis type: sprouting	1. Since the cornea is avascular, atypical angiogenesis is formed 2. Cornea as a site for tumor initiation is not highly relevant 3. Ethical issues: technique requires causing trauma to the model organism 4. Highly skilled and experienced operator required 5. The test sample can cause nonspecific inflammation 6. The assay can be hindered by exposure to oxygen 7. Overall an expensive technique	Folkman (1974), Auerbach et al. (1974), Gimbrone Jr et al. (1974)
Rodent mesentery angiogenesis assay	1. Dose-dependent study possible 2. Molecular activity can be studied 3. Visualization using intravital microscope is possible	1. Real-time observation not possible 2. High technical expertise required 3. Amount of test agent required to be administered is high in rats	Norrby and Østergaard (1996), Norrby (1996a), Norrby and Jakobsson (1990), Norrby et al. (2001), Norrby (1998)
Disk angiogenesis system	1. Can be used to study angiogenesis as well as wound heal 2. Newly formed vasculature is easily identified and observed 3. Simple technique 4. Reproducibility and statistical accuracy: for each dose point or time point, multiple disks can be used 5. Easy quantification of result 6. Inexpensive	1. Encapsulated by granulation tissue 2. Not possible to monitor the concomitant kinetic changes occurring in vivo 3. Subcutaneous tissue is not a suitable site for tumor initiation	Fajardo et al. (1988)
Sponge implant assay	1. Does not require a high level of expertise 2. Cost-effective	1. Nonspecific inflammatory response 2. Each animal needs to	Andrade and Fan (1987)

(continued)

Table 1 (continued)

Models/assays	Advantages	Disadvantages	References
	3. Continuous assessment possible 4. Reproducible 5. Can replicate hypoxic tumor microenvironment	be kept separately 3. Quantification of angiogenesis can differ depending upon the type of sponge matrices been used	
Chamber assay	1. 3D vessel growth can be observed 2. Time lapse and real-time analysis possible 3. Ideal for quantitative analysis	1. Expensive 2. Nonspecific inflammatory response 3. Technically demanding and invasive	Dellian et al. (1996)
Chick chorioallantoic membrane (CAM) assay	1. Reproducibility 2. Large-scale screening is possible 3. Can be used to study both pro- and antiangiogenic substances 4. Appropriate for mammalian xenograft studies 5. Low cost	1. Nonspecific inflammation 2. Angiogenesis by intussusceptive microvascular growth and sprouting 3. Sensitive to environmental factors: oxygen stress, pH, making it tedious to work with 4. Can show false-positive angiogenesis 5. Assessment of drugs that need metabolic activation not possible	Folkman (1974), Ausprunk et al. 1975, Ausprunk and Knighton (1974)
The chick aortic arch assay	1. Rapid: 1–3 days required to perform the assay 2. No use of laboratory animal 3. Cost-effective	1. One major disadvantage being that adult vessels involved in angiogenesis are in dormant or quiescent stage whereas embryo vasculature consists of actively dividing cells	Nicosia et al. (1990), Muthukkaruppan et al. (2000)
Danio rerio (zebrafish)	1. Microscopic analysis is possible due to optical transparency. 2. Early-stage tumor metastasis 3. Can be mimicked in a zebrafish xenograft model 4. Large-scale screening allowing proper statistical	1. Nonmammalian 2. Sustaining breeding conditions is expensive	Serbedzija et al. (1999)

(continued)

Table 1 (continued)

Models/assays	Advantages	Disadvantages	References
	analysis 5. Time-saving		
Xenopus laevis (tadpole)	1. Easy maintenance 2. Long life span 3. Intravital visualization possible	1. Nonmammalian	Hardwick and Philpott (2015), Wallingford et al. (1997)

inhibitors which show activity against receptors such as epidermal growth factor receptor (EGFR), platelet-derived growth factor-receptor (PDGFR) along with VEGFR. An inhibitor of VEGF, bevacizumab, which is a humanized monoclonal antibody, has shown to improve the effect of chemotherapy on tumor mass. Combination therapy of irinotecan or 5-fluorouracil (cytotoxic chemotherapy drug) along with bevacizumab administered as frontline therapy for metastatic colorectal cancer exhibited improvement in response by 35% to 45% as compared to only chemotherapy. Apart from VEGF inhibitors, there are small molecule tyrosine kinase inhibitors. Multi-kinase inhibitors are small molecules which are administered orally and target various receptors essential for angiogenesis, such as PDGFR and VEGFR. Pericytes, which provide structural support to endothelial cells, express PDGFR. Tyrosine kinase receptors play a crucial role in pathophysiology of clear-cell carcinoma. Sunitinib and sorafenib both are inhibitors of tyrosine kinase (O'Farrell et al. 2003). Sorafenib is a biaryl urea Raf kinase inhibitor. Raf kinase which is a part of RAF/MEK/ERK pathway in the activated state promotes cell growth and proliferation. Inhibition of tumor angiogenesis was observed in xenograft model when administered with sorafenib, which was measured using anti-CD31 immunostaining. It is also able to directly inhibit PDGFR-ß, VEGFR-2, and VEGFR-3 (Lyons et al. 2001). A new type of antiangiogenic drug administration has been developed called as metronomic chemotherapy. Generally, chemotherapy drugs work by disrupting microtubules of proliferating cells, damaging the DNA, etc. Recent studies suggest that administering cytotoxic agents at a very low dose leads to an increase in the antiangiogenic activity of the drug. This cytotoxic agent has to be administered at a low dose that is around a tenth to a third of the maximum tolerated dose. In metronomic chemotherapy, the advantage is that, as compared to conventional therapy option, it has less chances of emergence of drug-resistant cancer cells and lower toxicity (Muthukkaruppan et al. 2000) (Table 2).

Limitations

In the current scenario with all the angiogenesis assays which are at our disposal, there are certain aspects that need to be elucidated and dwelled deeper into for optimizing our studies related to angiogenesis and to further use it for clinical purposes. A few of

Table 2 List of drugs targeting angiogenesis in cancer therapeutics

SR NO.	Name	Mode of action	Purpose	References
1.	Axitinib	Kinase inhibitor Inhibits vascular endothelial growth factor receptor (VEGFR1, VEGFR2, VEGFR3)	Renal cell carcinoma. Administered as first treatment along pembrolizumab and avelumab	Rini et al. (2011)
2.	Bevacizumab	Human monoclonal antibody (IgG1) inhibits binding of VEGF to its cell surface receptors	Cervical cancer, colorectal cancer, glioblastoma, hepatocellular carcinoma, non-squamous renal cell carcinoma, non-small cell lung cancer, and ovarian epithelial cancer	Kazazi-Hyseni et al. (2010)
3.	Cabozantinib	Tyrosine kinase inhibitor Inhibits specific receptor tyrosine kinases such as TIE-2, VEGFR-1, VEGFR-2, VEGFR-3, TRKB, KIT, RET, AXL, FLT-3, and MET	Thyroid cancer, hepatocellular carcinoma, renal cell carcinoma	Cochin et al. (2017)
4.	Everolimus	Mammalian target of rapamycin (mTOR) kinase inhibitor Binds to FKBP-2 forming an inhibitory complex with mTOR complex 1	Breast cancer, pancreatic cancer, renal cell carcinoma, gastrointestinal cancer, subependymal giant cell astrocytoma	Houghton (2010)
5.	Lenalidomide	4-amino-glutamyl (lenalidomide) analogue of thalidomide Immunomodulator drug shows potent anti-inflammatory, antiangiogenic properties Causes tyrosine phosphorylation of CD28 on T-cells	Anemia, follicular lymphoma, multiple myeloma, mantle cell lymphoma	Kotla et al. (2009)
6.	Pazopanib	Synthetic indazolylpyrimidine Antineoplastic activity: multi-kinase inhibitor	Renal cell carcinoma, soft tissue sarcoma	Kasper and Hohenberger (2011)
7.	Ramucirumab	Human monoclonal antibody (IgG1) Inhibitor of VEGFR-2, prevents its binding to ligands VEGF-A, VEGF-C, and VEGF-D,	Colorectal cancer, stomach adenocarcinoma, non-small cell lung cancer, hepatocellular carcinoma	Refolo et al. (2020)

(continued)

Table 2 (continued)

SR NO.	Name	Mode of action	Purpose	References
		thus blocking VEGF-mediated receptor phosphorylation		
8.	Regorafenib	Multi-kinase inhibitor Inhibits activity of FGFR1, FGFR-2, DDR-2, KIT, RAF-1, VEGFR1, VEGFR2, VEGFR3, PDGFR-α, and PDGFR-β	Colorectal cancer, hepatocellular carcinoma, gastrointestinal stromal tumor	Frenette (2017)
9.	Sorafenib	Multi-kinase inhibitor Inhibits c-KIT, VEGF-receptor 2 and 3, RAF-kinase, and PDGFR	Renal cell carcinoma, hepatocellular carcinoma, thyroid cancer	Frenette (2017)
10.	Sunitinib	Tyrosine kinase inhibitor Interferes with cellular signaling by targeting RTK's like PDGF-R, RET, VEGF-R, CSF-1R, flt3, and KIT (CD117)	Pancreatic cancer, gastrointestinal stromal tumor, and renal cell carcinoma	Mena and Pulido (2010)
11.	Vandetanib	Antineoplastic kinase inhibitor Selective inhibitor of RET (rearranged during transfection), VEGFR (vascular endothelial growth factor receptor) and EGFR (epidermal growth factor receptor), and tyrosine kinases	Medullary thyroid cancer	Lee et al. (2017)
12	Ziv-aflibercept	Recombinant protein Consist of Fc region of IgG fused with binding domain of two VEGF Used when metastatic colorectal cancer has become resistant to oxaliplatin	Metastatic Colorectal cancer	Patel and Sun (2014)

Reference: National Cancer Institute

the key points being: (i) difference related to age, (ii) difference in the strain and species used for experimental purpose, and (iii) gender of the animal (Hasan et al. 2004).

The inherent differences and variability from one animal to another need to be addressed. It would not be advisable to use just one assay to determine the angiogenic activity of the test sample, since each and every assay does not provide the whole picture in terms of quantitative and qualitative analysis. In CAM assay, due to

Table 3 Assays used in combination for screening novel angiogenesis inhibitor

SR NO.	Novel inhibitor of angiogenesis	Mode of action	Assays used in combination	References
1.	CP-547,632 (isothiazole)	Inhibitor of basic fibroblast growth factor (FGF) kinases and vascular endothelial growth factor receptors	Corneal micro-pocket assay and Sponge implant assay	Beebe et al. (2003)
2.	Anlotinib	Inhibitor of vascular endothelial growth factor receptor-2 (VEGFR2)	Epithelial tube formation assay and rat aortic ring assay	Xie et al. (2018)
3.	FK228 (*Extracted from Chromobacterium violaceum*)	Inhibitor of histone deacetylase	Chorioallantoic membrane (CAM) assay and mouse Matrigel plug assay	Kwon et al. (2002)
4.	Vasostatin	Inhibitor of angiogenesis induced by basic fibroblast growth factor (FGF)	Chorioallantoic membrane (CAM) assay and corneal micro-pocket assay	Xiao et al. (2002)

the respiratory function of the tissue being used, it allows easy gaseous exchange through it. Whereas in corneal micro-pocket assay, there is exposure to high concentration of oxygen. Therefore, these two assays will not be the best option to study tumor angiogenesis since the tissue air interface is more and the tumor microenvironment is predominantly hypoxic. Use of a combination of two or three assays to study a putative pro/antiangiogenic compound is the best-suited option. Combining a microcirculatory preparation like corneal micro-pocket assay or CAM assay along with a matrix implant assay would provide a better understanding in regard to the angiogenic agent (Beebe et al. 2003). A single angiogenesis assay model cannot elucidate the whole mechanism of angiogenesis since there are many variables like tissue or organ site, whether the tissue is adult tissue or embryonic tissue, difference in species, how the angiogenic agent is being administered, and the type of microenvironment (Table 3).

Conclusion and Future Direction

In the last decade, the extent of advances made in our knowledge of angiogenesis mechanism has made it possible to get an insight into not only the functional but also genetic aspect of neovascularization. This is due to the availability of reliable, sensitive, and simple assays of angiogenesis. The criteria for a good angiogenesis assay include it being easy to perform, cost-effective, sensitive, reproducible, and reliable. Apart from all this, the final analysis and visualization is also a very important component and can act as a guiding factor while choosing the assay to be used. A good assay should allow us to differentiate between preexisting microvessels and newly formed microvessels, like in the case of zebrafish, due to the

transparency of the embryo, observing angiogenic activity is quite easy (Serbedzija et al. 1999). However, there is still a lot of progress yet to be made in terms of trauma caused to the animal during the procedure of the assay. Assays need to be developed which allow them to carry out continuous monitoring for a longer time and are less invasive towards the animal. As and when the delicate balance between pro- and antiangiogenic factors is tipped off, the process of angiogenesis then has severe repercussions and an impact on the health of an individual. Understanding the cell signaling mediated via various receptor tyrosine kinases like PDGF and VEGF has led to the development of therapeutic agents against angiogenesis; however, the regulation and cross talk of these cues in humans has not been understood fully (Kumar et al. 1998). In future, it is necessary to put emphasis on testing various concentration of the antiangiogenic substances by keeping the clinical viewpoint in mind, whereas physiological concentrations should be used for proangiogenic substances. In a recent study it was determined that these models are unable to provide sufficient prognostic importance in the case of human cancer, which needs to be looked into. Another issue that requires to be looked into is the vast difference in the dosage and scheduling of the antiangiogenic therapy between mouse models and humans. The rapid surge in angiogenesis research and the need for better therapeutic drug targets the demand for assay which can predict the activity of the drug in a clinical setting will increase (Norrby 2006).

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Genetically Engineered and Spontaneous Animal Model: Utilization in Preclinical Cancer Therapy Development

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Contents

Abstract

Comparative transcriptomics demonstrate that various animal cancer models share molecular signatures with human cancers. Preclinical evaluation with animal model is thus a crucial step in successful clinical translation of novel drugs and techniques developed for cancer therapy. The main focus of animal model studies is therefore on genetically engineered and spontaneous animal models of disease. In particular, small animals like zebrafish are more amenable to cellular and molecular testing and are chosen for generating cancer model. Spontaneous mutations arising in zebrafishes mirror the pathological conditions in human and are capable of generating humanized zebrafish models. This chapter offers an understanding of zebrafish models of cancer, focusing on the spontaneous models and the genetic approaches, particularly CRISPR/Cas in developing the zebrafish an excellent model for cancer research.

Keywords

Zebrafish · CRISPR/Cas · Spontaneous · Transgenics · Preclinical

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Introduction

The emergence of experimental animal models in preclinical study has revolutionized the field of oncology research. The most reliable and fitting animal model framework is imperative in the comprehensive understanding of cancer biology as these models represent the heterogeneous and variable cancer system (Schachtschneider et al. 2017). The main focus of animal model studies of late has been on genetically engineered and spontaneous animal models of disease. Small animals in particular are more amenable to cellular and molecular testing along with monitoring the progression of cancer, while large animals are restrictive to these advantages (Zimta et al. 2019; Raby et al. 2020). In cancer research, zebrafish serves as a robust model. This is due to the fact that cancer-associated mechanisms are well conserved evolutionarily between zebrafish and humans (Hason and Bartůněk 2019). The availability of various genetic engineering tools facilitates the manipulation of zebrafish to match the human tumor condition by inducing mutations at germline as well as somatic level and those which are inheritable (Raby et al. 2020). Zebrafishes are also known for spontaneous development of various types of tumor and offer more translatable results (Feitsma and Cuppen 2008).

This chapter thus focuses on the technological advances in the development of genetically engineered and spontaneous zebrafish animal models for preclinical cancer therapeutics.

Genetically Induced Cancer Models

Various methods/techniques for altering genes and their functions have been developed and used in recent years, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the RNA-guided Clustered Regularly Interspaced Short Palindromic Repeats CRISPR – Cas nuclease system (Ran et al. 2013). Because of its ability to edit the genomes of numerous organisms, CRISPR/Cas9 technology has transformed and emerged as one of the most reliable gene-editing tools. Its applicability has been very beneficial for gene therapies, developmental studies, mutation studies (Sharma et al. 2021), and the generation of various animal models and human pluripotent stem cell models for preclinical drug studies (Gonzales and Yeh 2014). However, this area's potential has yet to be completely realized. Further advancements are likely to bring major new advances that can be applied to a wide range of important biomedical issues, including progress toward more efficient drug discovery, the development of novel diagnostics and biosensors by combining genome editing tools and various molecular technologies, genome-wide screens to discover gene functions, and more.

CRISPR-Cas9 gene editing has been tested in a variety of model species, including the zebrafish (Danio rerio). The zebrafish is a tiny freshwater fish that has long been used as a developmental biology model for studying the genetics of vertebrate embryonic development. Furthermore, the zebrafish's characteristics,

such as its tiny size, quick life cycle, optical transparency, rapid external development, high fecundity, and low-cost care, make it a particularly useful and potent study model. The model has been utilized for studying and simulating human illnesses, as it has 70% genetic similarity with humans, and it is also commonly used for drug testing (Cornet et al. 2018; Rubbini et al. 2020). Similarly, in addition to wildtype strains, a vast number of transgenic zebrafish lines have been produced and utilized in vivo to research many human illnesses in various organs and cells (Raby et al. 2020). The zebrafish has established itself as one of the best vertebrate models for cancer research including the manipulation of cancer-related genes and further investigation of their consequences (Gallo and Bellipanni 2017).

According to various studies using aqueous carcinogen to induce cancer in zebrafish, these fish may develop a variety of benign and malignant tumors, most of which are histologically similar to human tumors, suggesting that genetic processes causing pathogenic alterations associated with malignancy are conserved. It was also discovered that zebrafish had several orthologs of oncogenes and tumor suppressor genes discovered in mouse models and humans (Berghmans et al. 2005a). This chapter will provide an introduction of zebrafish models of cancer, with a focus on how genetic tools, particularly CRISPR/Cas, have helped to make the zebrafish a viable cancer model.

TALENs (transcription activator-like effector nucleases) are another essential new technique for genome engineering. TALENs were originally utilized to inactivate somatic genes in zebrafish embryos to examine the formation of the vasculature. A transcriptional activator-like (TAL) effector DNA-binding domain linked to a DNA-cleaving domain produces TALEN, a chimeric nuclease that binds to any DNA sequence of interest. TALEN pairs have also been used to create a wide variety of mutations in zebrafish with up to 98.5% efficiency by injecting encoding mRNAs (Sertori et al. 2016). In a number of model species, this technique allows for targeted gene disruption. In comparison to zinc-finger nucleases, this approach is easy to develop and build (Vitale et al. 2014). TALENs are more consistent than ZFNs, with higher efficiency and more target specificity, resulting in less off-target effects (Sertori et al. 2016).

The activation of single or multiple oncogenes or the inactivation of tumor suppressor genes has typically been utilized to construct genetically engineered animal tumor models; however, the production of live animal models has proven problematic due to the fact that cancer phenotypes are more commonly induced by somatic mutation instead of germline genetic inactivation. TALENs have been reported to generate alterations in endogenous zebrafish genes, along with locus-specific DNA breaks in somatic and germline tissues, according to recent study. Shim et al. claim to have used TALEN-mediated somatic gene silencing of the cdkn2a/b or rb1 tumor suppressors to create somatically modified tumor models in zebrafish. The TALEN mRNA injection into fertilized eggs from a tp53e7/e7 mutant background accelerated the development of Malignant Peripheral Nerve Sheath Tumors by silencing the cdkn2a/b gene. TALEN-mediated rb1 somatic inactivation resulted in the growth of brain tumors in zebrafish. We performed RNA sequencing, as well as histological and immunohistochemical analysis, to

show that brain tumors generated by rb1 somatic inactivation share molecular characteristics with medulloblastoma-like neuroectodermal malignancies (Shim et al. 2017).

CRISPR-/Cas-Mediated Zebrafish Cancer Models

The CRISPR/Cas9 method has made significant progress in the previous decade and is now frequently employed in zebrafish illness models. Cas9 is an endonuclease that recognizes certain DNA sequences in an RNA-dependent way. The guide RNA (gRNA) is designed to interact with the Cas9 enzyme while also binding to and targeting certain regions of genomic DNA (Hason and Bartůněk 2019). Because of the advances made in the deployment of advanced genetic and reverse genetic technologies, a great range of genetically modified zebrafish cancer models has emerged.

The reverse genetic techniques attempt to either induce a loss-of-function (knock down) phenotype or transfer genes changed in human cancer patients into zebrafish (knock in). This might also imply developing a zebrafish model with a mutation in a human orthologous gene that results in a cancer-related phenotype (Sassen and Köster 2015). Several studies in zebrafish have been successful in this area, using programmable site-specific endonucleases to produce mutant models containing mutations in tumor suppressor genes (e.g., nf1, rb1); to engineer novel genetic modifications in tumor suppressor genes (e.g., tp53del/del); to exemplify the tumor suppressor function of novel candidate genes (e.g., irx1, spred1, arid1a); to investigate the interaction of different carcinogenesis onset mutations (e.g., tp53 and nf1) with atrx or suz12; and to define the role of tumor-forming genes (e.g., twist3) (Raby et al. 2020). Melanoma is a tumor of melanocytes with common mutations discovered in $BRAF^{V600E}$, $NRAS^{Q61L}$, or $NRAS^{Q61R}$, HRAS and KRAS for cutaneous melanoma; KIT^{V559A} for mucosal and acral melanoma; and $GNAQ^{Q209L}$ or $GNA11^{Q209L}$ for uveous melanomas (Idilli et al. 2017). Zebrafish and mammals have the same melanocyte development, making them good models of pigmentation and melanoma (McConnell et al. 2021).

In addition, a recent zebrafish melanoma model has proven the supporting role of the tp53-/- mutation in combination with the activating mutation in the serine/threonine kinase BRAF. As a result, the transgenic animal expresses a mutant version of $BRAF^{V600E}$, which is widely detected in human melanoma (Hason and Bartůněk 2019). Langenau et al. observed that transgenic zebrafish expressing mouse c-myc under the influence of the zebrafish Rag2 promoter produced clonally generated T cell acute lymphoblastic leukemia. This transgenic animal could be used to conduct pharmacological and genetic screens to find mutations that prevent or promote c-myc-induced tumorigenesis (Langenau et al. 2003).

A zebrafish pancreatic cancer model developed by Park and Leach substantially reflects the course of human pancreatic intraepithelial neoplasia. They created a new technique that combines CRE/Lox technology with the GAL4/UAS system to produce oncogenic KRAS in the ptf1a domain for a short period of time. This

zebrafish model has been used to evaluate various treatments for pancreatic tumors, and it might eventually lead to the creation of experimental and preclinical systems that could improve patient outcomes (Park and Leach 2018). Following that, when oncogenes such as xmrk, KRAS, and Myc were overexpressed in zebrafish, the zebrafish developed hepatoma in both the larval and adult stages. The zebrafish has also been used to simulate neuroblastoma and Rhabdomyosarcoma by boosting oncogenic KRAS $^{(G12D)}$ expression and overexpressing MYCN and fgf8 (Zhao et al. 2015).

In addition, Casey and Stewart's study from 2020 found that the LIM-only domain gene (LMO1) is highly linked to high-grade, metastatic Neuroblastoma (NB) cancers. It has been established that coexpression of LMO1 and MYCN genes causes only little changes in tumor penetrance but dramatically increases the formation of NB metastases. Furthermore, overexpression of LMO1 also increases human NB cell motility and upregulates extracellular matrix remodeling genes. These discoveries have paved the way for the rapid identification of other genetic events and/or medicines that regulate NB metastasis in vivo (Casey and Stewart 2020). It is now feasible to simulate practically all forms of cancer using zebrafish, making it an excellent model system for evaluating the antitumor characteristics of various anticancer medications. Several big chemical screens have been conducted using zebrafish as a model throughout the last decade (Fig. 1).

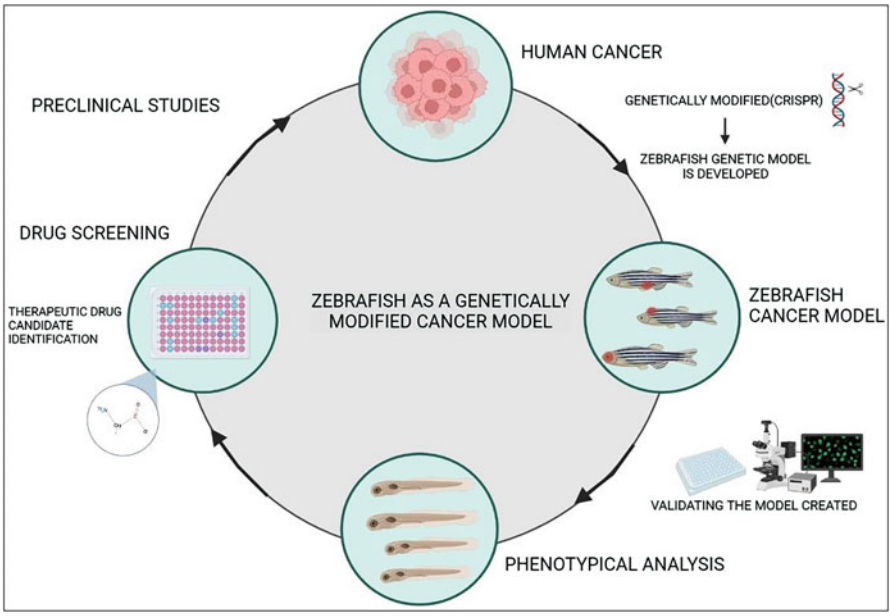

Fig. 1 A representation of the use of genetically modified zebrafish in cancer research. (Created using Biorender)

Transgenic Zebrafish as Cancer Model

Transgenic zebrafishes carrying the mammalian oncogene are an excellent model to study the mechanism and process of cancer development including metastasis. Zebrafish/tumor xenograft models have been basically used for studying angiogenesis, invasion, and metastasis. One of the major advantages of zebrafish is that the embryos are transparent, allowing the observation of labeled tumor cells and the evaluation of response to candidate molecules in a high-throughput format in vivo (Lu et al. 2011). Transgenics are generated by injecting a foreign DNA (desired transgenic cDNA) at one cell stage of zebrafish embryo. These embryos with the construct develop into adult stage (F0) to form transgenic mosaics. The F0 animals when propagated produce F1 offsprings that are partially transgenic due to the expression of transgene by offsprings which had taken up the cDNA construct integration at germline level alone. As the transgene expression is dominant, transgene positive F1 animals are heterozygous. For homozygous transgenic F2 generation, the F1 heterozygous needs to be in crossed (Berghmans et al. 2005b).

When a GFP tagged murine Myc oncogene is injected into the zebrafish developing lymphocyte using *rag*2 promoter, the transgenics developed T-cell acute lymphoblastic leukemia at the age of 1 and 5 months. The leukemic cells that GFP tagged are seen to be metastasizing to surrounding tissues from thymus and found to invade the visceral organs' skeletal muscles. Thus, a successful expression of oncogene in zebrafish initiates cancer formation (Langenau et al. 2004). Cancer formation induced by oncogene expression is mediated by a number of tissue-specific promoters other than *rag*2 in transgenic zebrafish (Table 1).

Furthermore *tol*2, a transposable element, is shown to create potential transgenics by efficient insertion in zebrafish genome (Clark et al. 2011). *Tol*2 is used in

Table 1 Tissue-specific promoters used in transgenic zebrafish generation

Tissue-specific promoter	Type of cancer	References
spi1 (myeloid-specific promoter)	Acute myeloid leukemia	Lu et al. 2016
mitfa (melanocyte-inducing transcription factor a)	Melanoma	Patton et al. 2005
mylz2 (myosin light chain) and *Cdh15* (M-cadherin)	Rhabdomyosarcoma	Storer et al. 2013
Ptf1a (Pancreas-associated transcription factor 1a)	Exocrine pancreatic carcinomas	Park et al. 2008
fabp10a (fatty acid-binding protein 10a)	Hepatocellular carcinoma	Lu et al. 2013
dbh (dopamine hydroxylase)	Brain tumors	Zhu et al. 2012
pomc (proopiomelanocortin)	Pituitary adenoma	Zimmerman et al. 2018
tg (thyroglobulin)	Papillary thyroid carcinoma	Anelli et al. 2017
ftck (Fugu lymphocyte-specific protein tyrosine kinase)	Testicular germ cell cancer	Gill et al. 2010

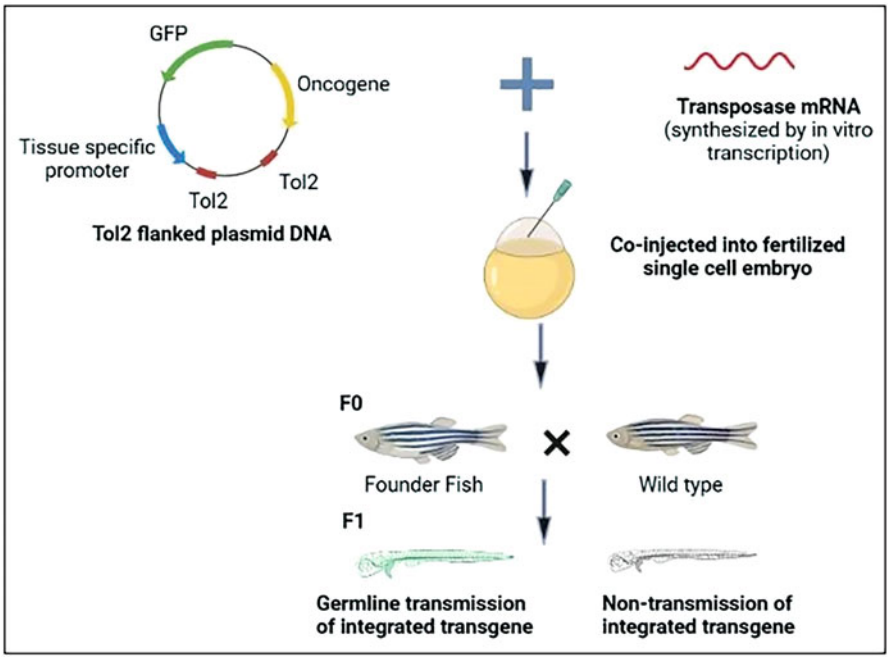

Fig. 2 Tol2-based generation of transgenic zebrafish. (Created using Biorender)

transgenesis upon modification of transposase enzyme by separation of the short terminal repeats (cis transposable elements) from it. The transposase enzyme upon transcription in vitro is injected along with the circular plasmid that carries the transgenic cassette (Fig. 2). The transgenic system thus created permits the foreign DNA up to 11kbp to be inserted in the genome and produces around 50% of injected zebrafish expressing the transgene with germline transmission. *Tol*2-based transgenics are thus widely used in cancer research (Suster et al. 2009).

Additionally, certain fluorescent markers when coexpressed permit the real-time monitoring of progression of tumor and characterization of transcriptome and phenotype of isolated tumor cells. Due to the abovementioned properties, transgenic zebrafish models are emerging as powerful cancer models. The expression of various oncogenes in transgenic zebrafish makes it a suitable model to mimic human cancer environment and thus utilize in preclinical therapy development.

Advantages of Genetically Engineered Models over Xenografts

Though xenograft models are inexpensive, rapid, and easy to use, they are not representative of the histology and genetics of the tumors in human. However, genetically engineered models (GEM) are more accurate in this aspect by

recapitulating the genetic and phenotypical features of human cancers. Xenograft models when used to screen the potential of drugs fail to show correlation with clinical efficacy. On the other hand, GEMs address a number of therapeutic challenges in cancer as they permit the interaction of tumor microenvironment and tumor cells (Holland 2004).

GEMs also have certain drawbacks as they have low penetrance, require extended length of time to develop neoplasia by GEMs, and possess indefinite and unpredictable nature in which they develop tumor and hence lead to delayed tumor formation. To overcome these limitations, various imaging techniques have been introduced like magnetic resonance imaging (MRI) (for organ) and micro-computed tomography (CT) (for bone) are used for anatomical imaging (Koutcher et al. 2002). The changes at molecular and cellular level can be studied accurately using in vivo bioluminescence imaging (Lyons et al. 2003).

Spontaneous Models

Spontaneous mutations arising in zebrafishes mirror the pathological conditions in human and are capable of generating humanized zebrafish models. Zebrafishes which are known to develop any tumor spontaneously have certain target tissues. These spontaneous neoplasia tissues include testis, gut, thyroid, liver, peripheral nerve, connective tissue, and ultimobranchial gland (Kent et al. 2002). Certain mutations in zebrafishes are displayed in commonly known tumor suppressor genes found in mammals too (Berghmans et al. 2005; Haramis et al. 2006; Faucherre et al. 2008).

pten which is found in human is the second most frequently mutated tumor suppressor gene and displays gene duplication in zebrafish. Homozygous mutations in both *ptena* and *ptenb* alleles failed to show developmental phenotype, whereas mutant animals which lack both *ptena* and *ptenb* display increased proliferation and cell survival and hence die at 5 days post fertilization (dpf). Adult fish mutants with deficient *ptenb* spontaneously develop ocular tumor at an incidence rate of 33% at 18 months, while those adult mutants lacking *ptena* do not develop any tumor (Faucherre et al. 2008).

Adenomatous polyposis (*APC*), a gene found in humans, upon mutation activated the Wnt signaling pathway and causes inherited and sporadic colorectal cancer (Giles et al. 2003). In zebrafish, this *apc* gene undergoes nonsense mutation and results in fatality of the animal in homozygous condition, whereas in heterozygous condition, >30% of fishes develop tumor in liver and intestine spontaneously 15 months onward. When these *apc* heterozygotes are treated with 7,12-dimethylbenz[a] anthracene, it results in tumors of liver, pancreas, and intestine with a three-fourfold increase in frequency than wild type (Haramis et al. 2006).

These spontaneous zebrafish models mimic the human cancer profiles. The genetic background of such animal models correlates well with mechanisms of human pathologies and hence helps in the development of several disease-specific animal models in preclinical cancer therapy.

Large Animal Models in Spontaneous Cancer Research

Large animal models with the ability to develop spontaneous cancer are of great significance in the preclinical cancer therapy development. Canines and cats are reliable models with great translational values due to their ability to develop various types of cancers spontaneously. These animals also share high similarity with humans in treatment response and biological characteristics (Gardner et al. 2015).

Sequencing of dog genome revealed that in humans and dogs, common tumor suppressor and oncogene are responsible for cancer initiation and progression (Sahabi et al. 2018; Shao et al. 2018). Melanomas which are one of the deadliest cancers are also found in dogs. They resemble that of humans in molecular alterations and histopathological features. Homologous genes for *NRAS, BRAF,* and *V-RAS* are found in both human and canines which are responsible for melanomas. Apart from dogs, other animal models like pig, horse, and cat also serve as efficient models for preclinical cancer research (Hernández et al. 2018; Van Der Weyden et al. 2015; Prouteau and Andre 2019).

Hence cancer research on large animals developing spontaneous cancer will yield translatable data for preclinical research. However, these animals are less explored due to constraints like ethical reasons, cost, and time.

Conclusion and Future Perspectives

Animal models have always provided several advantages over conventional in vitro cell models. Over the years, application of sophisticated genetic engineering tools in the manipulation of animal models has guided the establishment of remarkable tumor models in the field of cancer research. Generation of genetic modifications in the animal model that efficiently recapitulates the genetic alterations in cancer patients is critical in testing the novel hypotheses for cancer therapeutics before clinical implementation. The ability to associate the genetic alterations thus helps in developing efficient preclinical models for successful clinical translation. Genetically engineered zebrafishes establish a platform for the identification of tumor suppressors through high-throughput chemical screening. Further the complexity of the cancer mechanism can be understood in a profound manner by involving epigenetic modulators. Having said the advantages, however, a great deal of difficulties lie in generating a large homogenous cohort of spontaneous animals and comprehensive animal models. Therefore, better technical advances are vital for the profound understanding of the molecular pathogenesis of cancer and to unravel the entry points of preclinical cancer therapeutics.

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DEN-Induced Hepatocellular Carcinoma in Animal Model

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Abstract

Cancer is a major cause of death both in developed and developing countries. Among the various types of cancers, primary liver cancer represents about 4% of all cancers worldwide. Hepatocellular carcinoma (HCC) is a histological type of liver cancer; the fact being that the aspects related to the development of hepatocellular carcinoma and its metastasis are not yet known, and here animal models play an important role in diagnosis, prognosis, and treatment strategies of the disease. Animal models also provide an opportunity to explore new treatment strategies. N-nitroso compounds mainly N-diethylnitrosamine (DEN) is a hepatic carcinogen, which is well known to cause liver necrosis. The current review is based on research and review of works on animal models treated with DEN-induced hepatocarcinogenesis. Despite ongoing debate, animal models could provide valuable information about biotransformation of toxicants and how they worsen the damaging effects on DNA and cell proteins that result in the development of cancer. Today, the emergence of various therapies that target the immune system and the tumor microenvironment emphasizes the importance of the host, conditions of chronic inflammation, and fibrosis. Thus, the use of animal models for anti-HCC drug screening will find our best ability to successfully discover new drugs to combat HCC.

Keywords

Liver cancer · HCC · DEN · Herbal drugs

Introduction

One of the leading causes of cancer-related fatalities worldwide is hepatocellular carcinoma (HCC), a prevalent form of liver illness. People with chronic liver diseases, such as cirrhosis caused by hepatitis B or C infection, severe drinking and diabetes, obesity, and non-alcoholic fatty liver disease are more likely to develop HCC (Balogh et al. 2016; Manimekalai et al. 2016; Rajesh et al. 2016; Hemalatha et al. 2020; Flora Priyadarshini et al. 2020; Rajesh and Sivakumari 2020; Angalammal et al. 2021; Padmavathy et al. 2021). The incidence of HCC is higher in men than in women (2.4:1), with higher incidence in East and South Asia, Central and West Africa, Melanesia, and Micronesia/Polynesia (Ferlay et al. 2010). According to Altekruse et al. (2009), the rate of HCC among Native Americans and Alaskan Indians has increased from 1.6/100,000 to 4.6/100,000 people followed by Blacks, Whites, and Hispanics (Altekruse et al. 2009).

To study the efficacy of drugs against HCC, chemicals are used to induce HCC both in vitro in cell lines and in vivo in animal models. N-nitroso compounds are well-known hepatic carcinogens that cause liver necrosis, especially N-diethylnitrosamine (DEN) (Tricker et al. 1991). N-nitrosamines are known to cause varieties of tumors in several animal models, and are also known to cause

health hazards in humans too (Piot and Sirica 1980; Simonsen and Uirji 1984). These chemicals and their precursors can be present in the environment, in specific workplaces, in foods like meat and dairy products, in tobacco, pharmaceutical and cosmetics items as well as endogenously generated in human body from dietary components (Shank 1975; Bartch and Montesano 1984). Because of this, DEN promotes oxidative stress and cell damage due to increased reactive oxygen species (ROS) generation (Bartsch et al. 1989). By creating free radicals, the cytochrome P450-dependent monoxide system's enzymes increase oxidative stress by producing hydrogen peroxide (H_2O_2) and superoxide anions (Farber and Gerson 1984). The most harmful products of cellular metabolism are reactive oxygen species (ROS), which have a direct impact on cell development, proliferation, and its survival in cancer development. As liver is the primary metabolic biotransformation site for DEN, oxidative stress produced by liver injury may be generated by ROS generation in the liver (Gey 1993). Lipid peroxidation (LPO) is a measure of cell damage caused by ROS (Spiteller 1996). The liver, on the other hand, has a powerful antioxidant system that prevents ROS from causing damage to essential bio-molecules like lipids, proteins, and deoxyribonucleic acid when they are exposed to oxidative stress.

Several research works have been reported the hepatotoxic and carcinogenic effects of DEN (Schmahl et al. 1960; Druckrey et al. 1967; Dhanasekaran et al. 2009; Janani et al. 2009, 2010; Khan et al. 2017; Nithya 2021). In 1963, a study found that giving DEN to rats caused N7 atomic ethylation in nucleic acid guanines in the liver (Magee and Lee 1963), which was a key step toward understanding the chemical mechanisms behind the carcinogenic impact of DEN. A pathway that depends on cytochrome P450 enzymes like CYP2E1 connects the biotransformation of DEN and DMN (dimethylnitrosamine) to alkylating metabolites that result in the production of a DNA adduct (Yang et al. 1990; Verna et al. 1996). CYP2E1 is essential for the bio-activation of nitrosamines, according to studies done on CYP2E1 null mice (Kang et al. 2007). These mice had considerably fewer and smaller tumors, according to observations. After DEN treatment, these mice had a significant drop in tumor size and repetition compared to wild animals. Because of nitrosamine's carcinogenic qualities, it's becoming increasingly popular to utilize these chemicals, particularly DEN, to induce liver tumorigenesis in mice as a test model for human hepatocarcinogenesis (Kang et al. 2007).

Plants and their derivatives have long been recognized as efficient and versatile chemopreventive treating agents for various malignancies. Medicinal plants have been utilized for treating and preventing several diseases, as well as for the promotion of good health, since antiquity. Anticancer therapy has progressed significantly, as a result of medicinal plant-derived drug research, which has resulted in considerable advancements in anticancer therapies. India is known as the "Medicinal Garden of the World" because of the vast quantity of medicinal plants nature has bestowed upon us. In the armory of modern medicine, the drugs manufactured from phytocompounds or medicinal plants have been investigated for their efficacy against specific diseases, so that they would be valuable therapeutic agent in modern medicine.

Effects of Herbal Medicine Against DEN-Induced HCC

Curcumin

Chuang et al. (2000a) looked into the effect of curcumin on DEN-HCC mice model. Curcumin was demonstrated to be a potential inhibitor of DEN-induced hepatocarcinogenesis in C3H/HeN mice. p21 (ras) levels, nuclear antigen (PCNA) expression, and CDC2 protein levels increased significantly in DEN-treated mice's hepatic tissues, but curcumin decreased the levels of all these biological indicators (Chuang et al. 2000a). Curcumin might also significantly inhibit liver inflammation induced by DEN and hyperplasia in rat HCC model, according to Chuang et al. (2000b). The oncogenic p21 (ras), p53 proteins, PCNA, cyclin E, factor NF-, and p34 (cdc2) proteins were likewise suppressed by curcumin, but not Cdk2, c-Jun, and c-Fos, as revealed by immunoblotting studies (Chuang et al. 2000b). Curcumin's antioxidant, anti-inflammatory, and apoptotic potential in HCC models in vitro as well as in vivo, as well as its role in multiple molecular signaling mechanisms, have all been well documented (Table 1). The potential challenges, viz., the bioavailability, drug delivery, pharmacokinetics of curcumin in HCC, and the lacunae in its clinical studies have also been reviewed (Darvesh et al. 2012).

Terminalia arjuna

In male Wistar albino rats, the antioxidant potential of *Terminalia arjuna* bark ethanolic extract (EETA) against DEN-induced liver cancer was investigated by Sivalokanathan et al. (2006). Induction of liver cancer by DEN (200 mg/kg) was followed after 2 weeks by Phenobarbital (PB) for cancer promotion up to 14 weeks. Then, the HCC bearing rats were fed with EETA extract (400 mg/kg) and the samples of serum, liver and kidneys were collected for biochemical analysis. LPO levels such as H_2O_2, ascorbate, and $FeSO_4$ were estimated in serum, liver, and kidney of control and DEN treated rats. Likewise, non-enzymatic antioxidants like Vitamin C (Vit-C) and Vitamin E and enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Vit-E) were also assessed. With DEN therapy, LPO levels increased significantly, but enzymatic and non-enzymatic antioxidant levels declined. In DEN-treated rats, EETA administration at a dose of 400 mg/kg dramatically improved these changed enzyme levels. As a result, EETA's protective impact was linked to DEN-induced LPO inhibition and antioxidant enzyme levels being maintained (Sivalokanathan et al. 2006).

Annona squamosa

In DEN-induced Swiss albino mice, Raj et al. (2009) investigated the hepatoprotective effects of custard apple (*Annona squamosa*). Total protein, GOT, GPT, ACP, ALP, AFP, Total bilirubin and Direct bilirubin in serum and liver along

Table 1 Effects of herbal products against DEN-induced HCC

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
Curcumin (*Curcuma longa*)	C3H/HeN mice	5 weeks	48 weeks	Curcumin reversed the increased levels of p21 (ras), PCNA and CDC2 proteins to normal values in hepatic tissues.	Chuang et al. (2000a)
Curcumin (*Curcuma longa*)	Male Wistar rat	4 weeks	42 days	Curcumin strongly inhibited DEN-mediated the increased expression of oncogenic p21(ras) and p53 proteins in liver tissues of rats. Curcumin selectively reduced the expression of proliferating cell nuclear antigen (PCNA), cyclin E and p34 (cdc2), but not Cdk2 or cyclin D1. Curcumin also inhibited the DEN-induced increase of transcriptional factor NF-kappa B. However, Curcumin failed to affect DEN-induced c-Jun and c-Fos expression	Chuang et al. (2000b)
Terminalia arjuna	Male Wistar albino rat	4 weeks	28 days	EETA significantly decreased LPO levels and maintained enzymic and non-enzymic antioxidants, thus exhibiting its protective effect	Sivalokanathan et al. (2006)
Annona squamosa	Swiss albino mice	30–40 g	30 days	The levels of GOT, GPT, ALP, Total and Direct Bilirubin (both in serum and tissue), ACP, AFP (only in serum) decreased in DEN-induced plus *Annona squamosa* extract groups. Total proteins increased in DEN-induced plus *Annona squamosa* extract groups. Histopathology also confirmed the hepatoprotective effect of *Annona squamosa*	Raj et al. (2009)
Tinospora cordifolia	Male Wistar albino rats	120–150 g	20 weeks	Treatment of ECD in both preventive and curative DEN-induced animals increased the level of antioxidants (SOD, CAT) and detoxification	Dhanasekaran et al. (2009)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
				enzymes (GSH, GPx), and decreased serum transaminase level and hepatic marker enzymes (SGOT, SGPT, LDH) to near normal. Histopathological and nodular incidence also confirmed that ECD remarkably reduced tumor incidence and reversed damaged hepatocytes to normal	
Bacoside A (*Bacopa monniera*)	Male albino rats	160–180 g	7 days	The liver weight, lipid peroxidation (LPO), and activity of serum marker enzymes (aspartate transaminases, alanine transaminases, lactate dehydrogenase, alkaline phosphatase, and gamma-glutamyl transpeptidase) were near normal in bacoside A-pretreated rats. Activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutatione-S-transferase, and reduced glutathione) in liver also significantly elevated in bacoside A-pretreated rats. It is concluded that pretreatment of bacoside A prevents the elevation of LPO and activity of serum marker enzymes and maintains the antioxidant system and thus protects the rats from DEN-induced hepatotoxicity	Janani et al. (2009, 2010)
Saffron	Rat	4 weeks	22 weeks	Saffron significantly reduced the DEN-induced increase in the number and the incidence of hepatic dyschromatic nodules. Saffron also decreased the number and the area of placental glutathione S-transferase-positive foci in livers of DEN-treated rats. Furthermore, saffron counteracted DEN-induced oxidative stress in rats	Amin et al. (2011)

				as assessed by restoration of superoxide dismutase, catalase, and glutathione-S-transferase levels and diminishing of myeloperoxidase activity, malondialdehyde and protein carbonyl formation in liver. The results of immunohistochemical staining of rat liver showed that saffron inhibited the DEN-mediated elevations in numbers of cells positive for Ki-67, cyclooxygenase 2, inducible nitric oxide synthase, nuclear factor-kappa B p-65, and phosphorylated tumor necrosis factor receptor. Saffron also blocked the depletion in the number of cells positive for TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) and M30 CytoDeath in liver tissues of DEN-treated rats. In vitro experiments carried out using HepG2 cells also confirmed these findings and showed inhibition of nuclear factor-kappa B activation, increased cleavage of caspase-3, as well as DNA damage and cell cycle arrest upon saffron treatment	
Luteolin	Male Wistar albino rats	130–150 g	16 weeks	Administration of LVME as 200 mg/kg b.w. (to NDEA treated rats) significantly (i) reduced the preneoplastic lesions alleviated lipid peroxidation through scavenging free radicals, (ii) enhanced antioxidant status and reverted liver/disease marker enzymes (ALP, ALT, AST) plausibly by modulating xenobiotics metabolizing enzymes (XMEs) and by exhibiting antiproliferative and cytoprotective effects	Balamurugan and Karthikeyan (2012)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
Leucas aspera	Wistar albino rat	150–180 g	6 weeks	A significant lowering of the activity of ALP indicated the inhibition of pre-cancerous transformation in the liver on hydro-ethanolic and aqueous extract treatment in DEN + CCL4 animals, indicating the chemo-preventive efficacy of both the extracts in decreasing cell proliferation and hepatic nodulogenesis	Gupta et al. (2015)
Graptopetalum paraguayense	Male Wistar albino rats	150–180 g	84 days	*Graptopetalum paraguayense* enhanced PTEN expression and decreased AKT phosphorylation at Ser473 in a concentration-dependent manner in HCC cells. Moreover combination of GP or HH-F3 and sorafenib synergistically inhibited the proliferation of Huh7 cells. The treatment of a rat model with diethylnitrosamine (DEN)-induced liver cancer with extracts of GP and HH-F3 decreased hepatic collagen contents and inhibited tumor growth	Hsu et al. (2015)
Oldenlandia diffusa	Male Sprague-Dawley rats	180–200 g	60 days	The survival in *Oldenlandia diffusa* treated groups was shown to have a greater therapeutic effect than the control group. 28 days after drug treatment. *Oldenlandia diffusa* treated groups resulted in a significant reduction in tumor number, size, ^{18}F-FDG uptake, and serum levels such as alanine transaminase, aspartate transaminase, and alkaline phosphate compared to the control group. Also, proliferated cells in tumor sites by *Oldenlandia diffusa* were reduced compared to the control group. Furthermore, several rats in *Oldenlandia diffusa* treated group	

				survived over 60 days and liver morphology of these rats showed the difference between tumor mass and normal tissue	
Celastrol (*Tripterygium wilfordii*)	Male Sprague-Dawley rats	130–150 g	20 weeks	Celastrol significantly decreased the mortality rate, the number of tumor nodules and the index of liver in the Celastrol groups compared with DEN-treated group. Moreover, Celastrol obviously improved the hepatic pathological lesions and decreased the elevated levels of ALT, AST, ALP and AFP. Meanwhile, Celastrol suppressed the expression of the protein MDM2, activated the intrinsic mitochondrial apoptosis pathway induced by p53, inhibited anti-apoptotic Bcl-2 and Bcl-xl, induced the pro-apoptotic Bax, cytochrome C, PARP and caspases	Chang et al. (2016)
Tetilla dactyloidea	Male Sprague-Dawley rats	4 weeks	14 weeks	Oral administration of crude methanolic extract of *Tetilla dactyloidea* at a dose of 400 mg/kg body weight to DEN treated rats restored the nodule incidence, body weight, liver marker enzymes, enzymatic and non-enzymatic antioxidant, Phase I metabolizing and liver macromolecular damaging enzymes and immuno-histopathological changes were to near normal levels compared to control. The biochemical results were consistent with histopathological observations suggesting marked hepatoprotective effect of Crude Methanolic Extract of *Tetilla dactyloidea* in a dose-dependent manner	Gowri Shankar et al. (2017)
Phoenix dactylifera	Aalbino male rats	5–6 weeks (or) 100–120 g	10 weeks	Histological features of HCC in rats treated with extract of ajwa dates (ADE) showed partial to complete reversal of normal liver architecture.	Khan et al. (2017)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
				Antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), glutatione peroxidase (GPx) and catalase (CAT) increased, while the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels and lipid peroxidation significantly decreased than that of DEN treated groups. Pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1β, GM-CSF increased in the serum of DEN treated rats, while the anti-tumor cytokines (IL-2, IL-12) increased in ADE treated groups. In addition, Alpha-Feto Protein (AFP) and IL-6 gene expression levels were up-regulated in DEN treated groups, while they were significantly down-regulated in ADE treated groups	
Garcinia mangostana	Wistar rats	–	16 weeks	Significant increase in serum AFP, CEA, hepatic hydroxylproline, and total tissue protein levels in HCC group versus the negative control group. In contrast, the groups with HCC subjected to either high or low dose of GME elicited significant reduction of AFP, CEA, hepatic hydroxylproline, and increase in total protein in serum compared to the untreated HCC rats. Interestingly, treatment with *Garcinia mangostana* extract elicited marked improvement in the liver histological feature and down-regulation of tumor necrosis factor-alpha levels in HCC groups. *Garcinia*	Priya et al. (2018)

				mangostana extract possess chemopreventive benefits by reducing the tumor promoting growth factor levels in HCC-induced group	
Wedelia calendulacea	Male Swiss albino Wistar rats	150–200 g	22 weeks	19-α-Hydroxyurs-12(13)-ene-28 oic acid-3-O-β-D-glucopyranoside (HEG) in *Wedelia calendulacea*, confirmed the reduction of growth and deoxyribonucleic acid synthesis of both cell lines. DEN successfully induced HCC in all group, which was significantly altered by the HEG in a dose-dependent manner. The decreased level of pro-inflammatory cytokines and altered membrane-bound enzyme activity were also observed. HEG inhibited the phase I, II and antioxidant enzymes at the effective dose-dependent manner, which is considered as the precursor of the HCC. The alteration of phase I, II and antioxidant enzymes confirmed the inhibition of inflammatory reaction and oxidative stress, which directly or indirectly inhibited the NF-kB expression. Collectively, we can conclude that the HEG inhibited the growth of hepatocellular carcinoma via attenuating the NF-kB pathway	Verma et al. (2018)
Cynanchum auriculatum	Male Sprague-Dawley rats	150–180 g	20 weeks	Baishouwu extract pre-treatment successfully attenuated liver injury induced by DEN, as shown by decreased levels of serum biochemical indicators (AST, ALT, ALP, TP, and T-BIL). Administration of Baishouwu extract inhibited the fibrosis-related index in serum and live tissue, respectively from inflammation stage to HCC stage after DEN treatment. It significantly reduced	Ding et al. (2019)

(continued)

Table 1 (continued)

Herbal products	Animal model	Selected for the study ages/weight	Treatment duration	Observations	References
				the incidence and multiplicity of DEN-induced HCC development in a dose-dependent manner. Macroscopic and microscopic features suggested that pre-treatment with Baishouwu extract for 20 weeks was effective in inhibiting DEN-induced inflammation, liver fibrosis, and HCC. Furthermore, TLR4 overexpression induced by DEN was decreased by Baishouwu extract, leading to the markedly down-regulated levels of MyD88, TRAF6, NF-κB p65, TGF-β1 and α-SMA in hepatitis, cirrhosis, and hepatocarcinoma	
Echinacoside	Male C57BL/6 J mice	6–8 weeks	8 weeks	Echinacoside (ECH) attenuated diethylnitrosamine (DEN)-induced HCC in mice, and exerted anti-proliferative and pro-apoptotic functions on HepG2 cell line. ECH exposure in HepG2 cells dose-dependently reduced phosphorylation of AKT (p-AKT) and enhanced the expression of p21 (a cell cycle inhibitor) and Bax (a proapoptotic protein). Furthermore, ECH significantly suppressed insulin-like growth factor-1-induced p-AKT and cell proliferation. These data indicated that phosphoinositide 3-kinase (PI3K)/AKT signalling was involved in the anti-HCC activity of ECH. Gene set enrichment analysis results revealed a positive correlation between the PI3K pathway and triggering receptors expressed on myeloid cells 2 (TREM2) expression in HCC tissues. ECH	Ye et al. (2019)

				exposure significantly decreased TREM2 protein levels in HepG2 cells and DEN-induced HCC. Furthermore, ECH-mediated proliferation inhibition and AKT signalling inactivation were notably attenuated by TREM2 over-expression	
Ginger	Male Wistar albino rats	180–210 g	22 weeks	Ginger restored the activities of superoxide dismutase, catalase, GST and glutathione. Immunohistochemical bleaching in rat livers showed that ginger prevented the increase in cell-positive numbers for Ki-67, cyclooxygenase-2 and nuclear factor kappa B p65. Ginger also inhibited the number of positive cells in DEN/2-AAF-treated rats for TUNEL, M30 and caspase-3 liver tissues	Hamza et al. (2021)
Cow ark with *Allium sativum*	Wistar rats	–	28 days	Cow ark has the potential to accelerate Reactive Oxygen Species production, and allow to increasing membrane permeability (MP) and efficient discharge of Cytochrome c in HCC cancer cells while, no remarkable change was recorded in control hepatocytes	Nithya (2021)

with histological investigations of the liver were carried out. GOT, GPT, ALP, Total and Direct Bilirubin (both in blood and tissue), ACP, and AFP (only in serum) levels increased in DEN-treated groups, while all values reduced in the DEN and *Annona squamosa*-treated groups. Total protein levels were lower in DEN-treated mice and higher in DEN and *Annona squamosa*-treated mice. *Annona squamosa*'s hepatoprotective activity was further validated by histopathological examinations (Raj et al. 2009).

Tinospora cordifolia

With a diterpenoid (5R, 10R)-4R, 8R-dihydroxy-2S, 3R: 15, 16-diepoxycleroda-13 (16), 17, 12S: 18,1S-dilactone (ECD), eluted from *Tinospora cordifolia*, Dhanasekaran et al. (2009) assessed the chemopreventive inhibitory efficacy against DEN that produced HCC in mice. Antioxidant activity (SOD, CAT) and detoxifying enzymes (GSH, GPx) decreased in DEN-treated animals, but hepatic signaling activity increased (SGOT, SGPT, LDH). The treatment of ECD resulted in an increase in antioxidants and detoxification enzymes, as well as a drop in blood transaminases and hepatic indicators to normal levels in both treatment groups. ECD effectively reduced tumor incidence according to histopathological and nodular incidence (Dhanasekaran et al. 2009).

Tinospora cordifolia ECD also helped to avoid additional damage. In a solid tumor model, it proved efficient in inhibiting tumor growth. This work indicated ECD's chemopreventive potential in DEN-induced hepatocarcinogenesis, which was attributable to ECD's antioxidant and detoxifying mechanisms. Reduced serum transaminase secretion preserved membrane function, which is also attributable to ECD's protective impact. ECD's chemopreventive activities were also validated by biochemical and histological tests. ECD performs a dual effect by suppressing carcinogen metabolic activity and increasing carcinogen detoxification, according to Dhanasekaran et al. (2009).

Bacoside A

Janani et al. (2009) reported that bacoside A (BA), a substance derived from Bacopa monniera Linn., protects rats from DEN-induced liver damage. The activity of serum marker enzymes, viz., AST, ALT, LDH, ALP, and GGT rose significantly, as did liver weight, lipid peroxidation (LPO), and liver weight in rats treated with DEN, while the markers enzymes were on par with the levels of the above parameters in rats treated with BA. Antioxidant enzyme activity, viz., SOD, CAT, GSH-Px, GR, GSTs and reduced GSH in the liver was diminished in rats treated with DEN. The findings imply that pre-treating BA decreases LPO and serum marker enzyme activity while maintaining antioxidant activity, protecting rats from DEN-induced hepatotoxicity (Janani et al. 2009).

Likewise, the impact of BA on the activity and expression of MMP-2 and MMP-9 during HCC was examined by Janani et al. (2010). Co-treatment with BA considerably reduced the activity of MMP-2 and MMP-9, which increased during HCC, according to the results of a gelatin zymography investigation. Immunoblot analysis demonstrated a decrease in the expression of MMP-2 and MMP-9 in BA co-treated rats, than that of DEN-induced HCC rats. By suppressing MMP-2 and MMP-9 activity as well as expression, BA inhibits the metastasis of DEN-induced HCC (Janani et al. 2010).

Saffron

Saffron was found to be a strong medication against HCC in another investigation by Amin et al. (2011). The number and incidence of hepatic dyschromatic nodules caused by DEN were considerably reduced by saffron. In the livers of rats treated with DEN, saffron decreased the intensity and distribution of placental GST positive foci. It also protected rats against the oxidative stress caused by DEN by restoring the levels of SOD, CAT, and GST. Similarly, MPO activity, MDA activity, and COb formation were inhibited in liver. According to immunohistochemical labelling of rat liver, Saffron lowers the amount of cells positive for Ki-67, COG 2, inducible NOS, NF-kB, p-65, and phosphorylated TNF receptors in rats treated with DEN. In the liver tissues of mice treated with DEN, saffron decreased the number of cells positive for TUNEL and M30 Cyto-Death (Amin et al. 2011).

Luteolin

Balamurugan and Karthikeyan (2012) investigated the effectiveness of luteolin in Wister albino rats with DEN-induced HCC. The researchers looked at non-enzymatic antioxidant enzymes including AST, ALP, LDH, and c-GT, as well as enzymatic antioxidants like SOD, CAT, GSH, and GPx, along with histopathological alterations. In the DEN-treated groups, tissue-damaging enzymes were higher, while enzymatic antioxidants were lower. The DEN-treated rats developed severe lesions and cirrhosis. The levels of tissue-damaging enzymes and enzymatic antioxidants recovered in DEN-treated rats after treatment with luteolin, which almost entirely healed the damaged lesions in the liver induced by DEN. In albino rats, luteolin functions as a potential anti-HCC agent (Balamurugan and Karthikeyan 2012).

Leucas aspera

In Wister rats, Gupta et al. (2015) investigated the chemoprotective efficacy of *Leucas aspera* against DEN-induced and CCL4-stimulated hepato-carcinogenesis.

Except control, all other groups got a single dose of CCl4 (2 ml/kg i.p.) 2 weeks after the commencement of the test protocol to enhance liver cell proliferation and regeneration. The extent of protection was measured once the treatment period was completed by analyzing blood antioxidant indicators. To validate the effect of toxicants on the liver and to assess the chemoprotective potential of *Leucas aspera* extracts, biochemical parameters of the liver were measured. In addition to an increase in GGT levels, which indicated hepatic carcinogenesis, DEN administration in animals resulted in an increase in ALP activity, which could be attributable to changes in enzyme production, as in other examples of hepatotoxicity. The extracts normalized serum GGT levels and lowered serum AST and ALT levels, indicating a hepatoprotective action and suppression of carcinogenesis. In rats treated with aqueous and hydro-ethanolic extracts of DEN + CCL4, a significant reduction in ALP activity indicated suppression of pre-cancerous alterations in the liver. As a result, both extracts of *Leucas aspera* were found to be efficient in suppressing cell proliferation and hepatic nodulogenesis (Gupta et al. 2015).

Graptopetalum paraguayense

In HCC cells, the release of *Graptopetalum paraguayense* (GP) suppressed the expression of many oncoproteins, including AURKA, AURKB, and FLJ10540, according to Hsu et al. (2015). When the fractions eluted from the extracts were tested for their effects on onco-protein exposure in HCC cells, it was discovered that the HH-F3 fraction enriched with active components had cytotoxic effects and inhibited onco-protein expression. Studies on apoptosis showed that HH-F3 caused HCC cells to undergo apoptosis by increasing the energy loss from the mitochondrial membrane and the production of active oxygen species. In a concentration-dependent manner, HH-F3 improved PTEN expression and reduced AKT phosphorylation at Ser473 in HCC cells. The combination of GP or HH-F3 and sorafenib also suppressed Huh7 cell proliferation. Treatment with GP and HH-F3 reduced hepatic collagen levels and prevented tumor growth in DEN-treated mice. The results clearly depicted the protection of liver by GP and HH-F3 extracts liver, and that they could be used to treat HCC (Hsu et al. 2015).

Oldenlandia diffusa

Demonstrated *Oldenlandia diffusa*'s therapeutic potential in vitro and in vivo. *Oldenlandia diffusa* enhanced apoptosis and anti-proliferation activities while reducing the ability of HCC cells to migrate. In in vivo experiments, *Oldenlandia diffusa*, when given twice daily for 28 days following confirmation of the HCC model utilizing two images – [^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) imaging, showed a higher survival rate in *Oldenlandia diffusa* treated group than in the control group. Tumor counts, size, tumor cell proliferation, ^{18}F-FDG uptake, and serum enzyme levels such as ALT, AST, and ALP were all considerably less in the *Oldenlandia*

diffusa treated group than control group after 28 days of therapy. Furthermore, several *Oldenlandia diffusa*-treated rats lived for more than 60 days, and their liver morphology revealed variations between tumor mass and normal tissue compared to control rats.

Celastrol

Chang et al. (2016) investigated the anti-tumorogenic activity of Celastrol, an active component in *Tripterygium wilfordii*, in DEN-induced HCC in Sprague-Dawley rats. For 16 weeks, DEN (10 mg/kg) was given intragastrically 6 days a week. Hematoxylin-Eosin (HE) staining was used to determine the number of nodules developed and hepatic pathological abnormalities. Similarly, Elisa kits were used to determine serum ALT, AST, ALP, and AFP levels, as well as p53 protein levels, MDM 2, Bax, Bcl-2, Bcl-xl, cytochrome C, Caspase-3, Caspase-9, and PARP levels. In comparison to rats treated with DEN, Celastrol dramatically decreased liver index, tumor nodule count, and mortality in rats treated with Celastrol. Celastrol also lowered elevated levels of ALT, AST, ALP, and AFP and appeared to improve liver pathological abnormalities. Celastrol, on the other hand, inhibited anti-apoptotic Bcl-2 and Bcl-xl and activated pro-apoptotic Bax, cytochrome C, PARP, and caspases by repressing MDM2 protein expression, activating the p53-induced intrinsic mitochondrial apoptotic pathway (Chang et al. 2016).

Tetilla dactyloidea

Gowri Shankar et al. (2017) investigated the zoochemical status, antioxidant capability, and anti-cancer efficacy of *Tetilla dactyloidea* crude methanol extract (CMETD) in Sprague Dawley (SD) rats treated with DEN. Nodule formation, body mass, hepatic marker enzymes, enzymatic and non-enzymatic antioxidants, Phase-I metabolizing and hepatic macromolecular enzymes, and immuno-histopathological alterations were evaluated in the DEN and DEN + CMETD treated groups. Following oral administration of 400 mg/kg body weight of CMETD, all parameters in the DEN-treated groups were restored to normal levels. The recovered biochemical levels were in accordance with histological findings, indicating that CMETD has a dose-dependent hepatoprotective effect. Six chemicals were detected in CMETD after GCMS screening. In DEN-induced HCC, the results demonstrated that CMETD reduced liver damage, protected the antioxidant immune system, and exhibited anti-cancer activities (Gowri Shankar et al. 2017).

Ajwa Dates, *Phoenix dactylifera*

Khan et al. (2017) conducted another study in Wister rats, this time looking at the anti-cancer properties of Ajwa dates (*Phoenix dactylifera* L.), where HCC was

caused by DEN administration. The liver architecture of the DEN-treated rats was reversed from partial to complete, while the liver architecture of the AD-treated rats was reversed from partial to complete. Antioxidant enzymes like SOD, GR, GPx, and CAT elevated, whereas liver enzymes like ALT, AST, and ALP, as well as LPO, declined in AD treated rats compared to DEN. Antitumor cytokines like IL-2 and IL-12 were found to be elevated in DEN-treated groups' serum, while pro-inflammatory cytokines like IL-1 and GM-CSF increased in AD-treated groups' serum. Furthermore, gene levels of Alpha-Feto Protein (AFP) and IL-6 were up-regulated in DEN-treated groups, but down-regulated in AD-treated groups. AD extract helped to restore normalcy to a damaged liver that had been treated with DEN. Following AD treatment, antioxidant enzymes, liver enzymes, cytokine balance, and gene expression all restored to normal, proving that AD enhances the function of liver and protects it against HCC (Khan et al. 2017).

Garcinia mangostana

In a rat animal model of DEN-induced HCC, Priya et al. (2018) revealed a protective mechanism of *G. mangostana* fruit extract (GME). In rats treated with DEN, the levels of HCC indicators such as AFP, CEA, TNF-α, hepatic hydroxyproline and total protein were determined by ELISA. Immunohistochemistry was used to detect the expression of vascular endothelial growth factor in liver tissue. Serum AFP, CEA, hepatic hydroxyproline and total protein levels were significantly higher in the DEN-treated rats than that of the control group. Treatment with GME at low or high dosages resulted in significant decline in AFP, CEA, hepatic hydroxyproline, and an elevation rise in total blood protein levels in the DEN-treated rats. Interestingly, treatment with GME resulted in significant improvements in the histological architecture of the liver and down-regulated tumor necrosis factor alpha levels. GME thus exhibited its chemopreventive potential against DEN-induced HCC by reducing the expression of tumor promoting growth factor (Priya et al. 2018).

Wedelia calendulacea

In Wistar rats and HepG-2 and HuH-7 cell lines, Verma et al. (2018) investigated the hepatoprotective potential of 19--Hydroxyurs-12 (13)-hypertensive method of 28 oic acid-3-O-D-glucopyranoside (HEG) eluted from *Wedelia calendulacea* against DEN-induced oxidative stress, hyperproliferation, inflammation, and apoptotic tissue damage. To cause liver damage, single dose of DEN (200 mg/kg) and two doses of phenobarbitol were given. This was followed by a 22-week HEG treatment. Hepatic nodules were confirmed by macroscopic examination, and serum and hepatic samples were subjected to additional biochemical and histological analyses. Inflammatory cytokines such as TNF-α, IL-6, IL-1, and NF-kB were also evaluated, as were hepatic and non-hepatic Phase I and II antioxidant enzymes (NF-kB). To examine the changes that have occurred in the liver of both DEN and

HEG treated rats, histopathological changes were identified. HCC induced by DEN in all groups was significantly altered in a dose-dependent manner by HEG. Likewise, tumor growth and DNA synthesis was reduced by HEG in both cell lines. Pro-inflammatory cytokines were found to be decreased and membrane-bound enzyme activity was altered by HEG. HEG inhibited phase I, II and antioxidant enzymes in an active dose-dependent way, and proved HEG as a precursor in combating HCC. According to alterations in phase I, II, and antioxidant enzymes, HEG suppressed inflammatory responses and oxidative stress that either explicitly or implicitly decreased NF-kB expression. HEG inhibited the growth of HCC by inhibiting the NF-kB pathway (Verma et al. 2018).

Cynanchum auriculatum (Baishouwu)

Ding et al. (2019) investigated the effect of Baishouwu extract (BE) on DEN-induced HCC as well as the potential mechanisms involved in its treatment. Animals were treated simultaneously with BE, which was administered daily by oral gavage for 20 weeks to investigate its preventive benefits, and multistep hepatocarcinogenesis was commenced by injecting DEN. To assess the effect of BE on hepatic carcinogenesis, a serum sample was taken at a predetermined time and organ samples were taken from each group. BE co-treatment significantly reduced the liver damage caused by DEN in rats, as seen by lower levels of serum biochemical markers (AST, ALT, ALP, TP, and T-BIL). From the inflammatory phase until the HCC stage, BE decreased the fibrosis-related index in blood and tissues, which was produced by DEN. In a dose-dependent way, BE dramatically reduced the incidence and frequency of DEN-induced HCC development. Pre-treatment with BE for 20 weeks appeared to be successful in avoiding inflammation produced by DEN, liver fibrosis, and HCC, according to macroscopic and microscopic findings. Furthermore, BE reduced TLR4 over DEN expression, resulting in considerable downregulation of MyD88, TRAF6, NF-B, p65, TGF-1, and -SMA in hepatitis, cirrhosis, and HCC (Ding et al. 2019).

Echinacoside

A phenylethanoid glycoside called as echinacoside (ECH) is obtained from the Chinese herb Cistax'nches salsa. Ye et al. (2019) explored the impact of ECH on HCC as well as the mechanisms involved. ECH decreased DEN-induced HCC in mice and had anti-proliferative and pro-apoptotic effects in the HepG-2 cell line, according to the findings. ECH suppressed AKT (p-AKT) phosphorylation and increased p21 and Bax expression in HepG-2 cells in a dose-dependent manner. ECH reduced p-AKT and cell proliferation generated by insulin-like growth factor-1, demonstrating that PI3K/AKT signaling was involved in ECH's anti-HCC effect. It was shown that the activation of receptors expressed in myeloid cells 2 (TREM2) expression in HCC tissues was positively linked with the PI3K pathway. TREM2

protein levels in HepG-2 cells and DEN-induced HCC in mice were both dramatically reduced after exposure to ECH. Overexpression of TREM2 also substantially inhibited ECH-mediated proliferation inhibition and AKT signaling inactivation. Eventually, the ECH suppressed tumor growth by inhibiting TREM2 expression and PI3K/AKT signaling (Ye et al. 2019).

Ginger

In Wister rats, Hamza et al. (2021) studied the mechanisms of ginger rhizome extracts against DEN-induced HCC. At dosages of 75, 150, and 300 mg/kg/day, ginger was found to have chemopreventive potential in the liver damage caused by DEN and 2-acetylaminofluorene in rats (2-AAF). After 22 weeks of cancer induction, ginger decreased the quantity of placental GST in the liver of the DEN/2-AAF treated groups, as well as the number and incidence of hepatic dyschromatic nodules and positive focal regions. In addition, ginger reduced the levels of myeloperoxidase, malondialdehyde, and protein carbonyl in the liver via inhibiting oxidative stress with DEN. The restoration of SOD, CAT, GST, and glutathione was used to determine this. Ginger decreased the proliferation of Ki-67 cell counts, cyclooxygenase-2 (COX-2) and NF-B p65 in rat liver, as depicted by immunohistochemical staining. In mice treated with TUNEL DEN/2-AAF, M30, and caspase-3 liver tissue, ginger lowered the number of cancer cells. Ginger has a significant chemopreventive potential against liver cancer, as per this study, by slowing cell growth and increasing apoptosis. Ginger protects the rat liver from cancer by lowering oxidative damage and inflammation (Hamza et al. 2021).

Cow Ark with *Allium sativum*

In a study by Nithya (2021), the ability of Cow Ark to control and regulate cancer activity in Wister rats induced with DEN + 2AAF. The weight loss in rats showed that HCC was caused by DEN + 2AAF in the experimental group. On the other hand, Cow ark significantly reduced MMP in mitochondria of HCC hepatocytes in a time-phased way. *Allium sativum* extracts independently showed an insignificant effect on MMP of liver mitochondria, isolated from the control group. Likewise, production of Hydrogen peroxide in the liver mitochondria of the HCC cells was found to be enhanced in rats induced by DEN + 2AAF, whereas the H_2O_2 activity in plant extract did not have significant effects in control group treated only with plant extract. Significant increase in H_2O_2 production was observed in rats treated with Cow Ark and plant extract, due to their synergistic effect. Thus the enhancing potential of Cow ark and plant extract was established by the radical scavenging into the antioxidant activity. In addition, the synergistic action of Cow Ark and plant extract treated mitochondria increased cytochrome c release by cleaving the mitochondrial membrane integrity in liver mitochondria from DEN + 2AAF treated rats, but no such effect was seen in the control group (Nithya 2021).

Conclusion

Based on previous findings, it could be concluded that animals can be considered to be the exact models for drug testing against HCC. Today, the emergence of new therapies that target the immune system and the tumor microenvironment emphasizes the importance of the host, conditions of chronic inflammation, and fibrosis. Hence, use of animal models for a wide range of cancer research will help us to discover new drugs to combat HCC.

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Chorioallantoic Membrane (CAM) and *In Ovo* Models as Potential Platforms for Testing Cancer Agents

23

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Abstract

The construction of clinically related models for cancer metastasis and anticancer testing plays a significant role in evidence-based translational cancer research. Most of the vivo experiments have been conducted on cost- and time-intensive animal models. The CAM has demonstrated successful engraftment of over a dozen tumor subtypes, demonstrating its suitability as a model for patient-derived xenografts. In many investigations, xenografts resembled the unique patient tumor and CAM, increased vascularity subsequent engraftment, mesenchyme micrometastasis was observed. A number of standard and experimental cancer therapies have been applied to xenografts, with the detection of both positive correlations and combinational effects between clinical outcome and drug assays. CAMs allow DNA- and RNA-based patient tumor sequencing, as well as testing of several targeted treatments on fragments from a similar cancer in a short period of time from 5 to 10 days. The CAM chick embryo may provide an excellent model for evaluating antitumor agents which will target the metastatic capability of tumor cells. We have discussed the use of chick chorioallantoic membrane (CAM) as a cost-efficient, fast model for in vivo screening of antitumor drugs.

Keywords

Tumor angiogenesis · Tumor grafting · Cancer model on CAM · Micrometstasis

Introduction

"Precision medicine" in oncology reduces the morbidity and mortality with subdivisions of patients that are further predictable respond to treatment schedules (Landis et al. 2013). Patient-derived xenografts (PDXs) are increasingly used in translational cancer research as they retain key genetic and histologic features of their donor cancer cell (Hidalgo et al. 2014). Before therapy, the PDX platform is potentially useful for characterizing the pathophysiology, molecular features, and drug responsiveness of an individual tumor. With the use of immunodeficient rodents in traditionally favored PDX models, the chick chorioallantoic membrane (CAM) assay is complementary to the method presenting the unique advantages in some areas. The review only describes the advantages and limitations of rodent versus CAM-based PDX models, efforts to engraft patient-derived tumor tissue onto

the CAM, and the current and potential future applications of the method, whereas the history of cell-line-based work in CAM models is not extensively focused due to the limitation of a "complete" cancer model that has contributed enormously to preclinical research and cancer biology.

Practical Benefits of Patient-Derived Xenografts in Oncology

Preclinical research and drugs have historically developed from the cancer cell line (CCL) usage. While the propagation of these immortalized cell populations in simple media is straightforward and invaluable for experimentation, they possess inherent shortcomings of the limited translation of findings into patient benefit. CCLs undergo new mutations during adaptation to growth in culture and generally fail to hold the molecular profile cellular heterogeneity and morphology of the original donor tissue (Landis et al. 2013). Furthermore, the positive drug performance in xenografts from CCLs is not typically highly predictive of clinical efficacy. The use of fresh tissue for xenografting more closely captures patient features by maintaining heterogeneity and pathophysiology of the original tumor (Tentler et al. 2012). PDX models are particularly useful in assessing therapies for cancers driven by rare populations of highly aggressive cells or those with a high variance in molecular alterations between patients (Sapra et al. 2013). The success of new drugs for cancer treatment depends on understanding the pathophysiology and heterogeneity of tumors in preclinical models.

Hence, the PDX models are considered to more accurately mimic drug effects in humans in comparison with CCL models (Rosfjord et al. 2014). In fact, patient-derived xenografts in rodents have been used to establish most of the current models in pediatric oncology and also useful for developing drugs like topotecan and irinotecan for solid tumors (Tentler et al. 2012). The study on the genetics of acquired resistance explores the advantage of PDX models in that alterations in tumors actually occurred clinically (as opposed to developing resistance models from cell lines) (Crystal et al. 2014). The study emphasizes that the PDX models ultimately have the potentiality to accelerate the development of new therapeutic compounds in oncology once promising candidates advance beyond preclinical testing (Malaney et al. 2014).

In addition to accurately representing tumor biology, PDX models also have the theoretical advantage of efficiency (Fig. 1). PDX models are useful for patients who are ineligible for clinical trials due to deteriorating health or other disqualifiers (Malaney et al. 2014). There are, however, some theoretical limitations of the PDX platform that need further assessment. Nonideal tissue selection could conceivably be a limiting factor, based on the untested hypothesis that all tumor foci are not equally lethal (Toivanen et al. 2013). Representation of metastasis has also been a challenge for certain malignancies. Finally, intratumoral heterogeneity has led to a lack of concordance among several models of pancreatic cancer (Li et al. 2016). These observations highlight the challenges of utilizing PDX as a preclinical discovery model for cancer therapeutics. Currently, the assay for the detection of

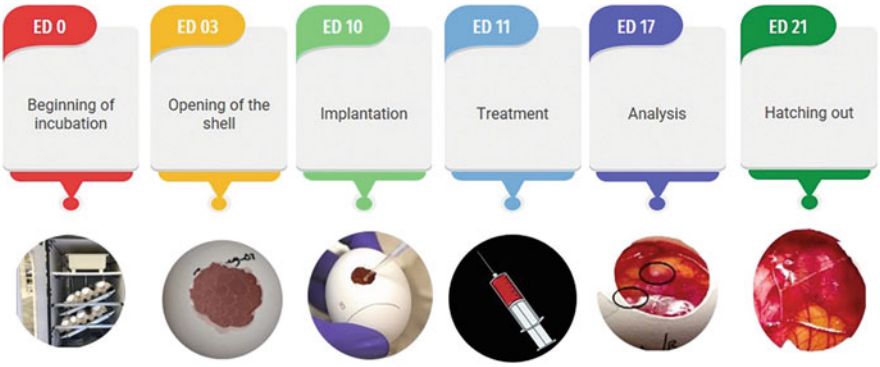

Fig. 1 Graphical representation of the implant timeline of standard experiment representing incubation day

cancerous cells requires lowering of the membrane while forming an air pocket between the membrane of the separated shell region and the CAM itself.

The perfect time for inoculation is postulated as the ninth day of embryonic development and the 16th day (7 days after inoculation) for the harvest (Sys et al. 2013). Another paradigm is to simply harvest on the day before tumor rejection would likely occur on the 18th day (Sys et al. 2012). The "in ovo" method is more popular, whereas the "ex ovo" method is also a possible way for monitoring the extrusion of the egg content and the modification to enable easy in vivo documentation of the effects and the increased embryonic survival rates from ca. 30% to over 50% (Dohle et al. 2009). Ribatti reviewed the "in ovo" and "ex ovo" methods to find out the advantages of each method. The study concluded that the "in ovo" experimentation has a high survival rate, reflection of physiological conditions, the ability to reach hatching, and easy methodology, whereas the "ex ovo" method has the larger CAM area that allows for direct visualization of the entire embryo, evaluation of several samples at a time, and testing at different times within a single embryo.

Focus of Studies Grafting Fresh Human Tumor Tissue on the CAM Angiogenesis

The literature review involved in the engraftment of fresh human tumor tissue onto the CAM highlights the study of angiogenesis. Klagsbrun et al. cultured glioblastoma and meningioma cells (including both cell lines and fresh samples obtained by trypsinization of brain tumors) to test the ability of their supernatant solutions to produce a hypothetical tumor angiogenesis factor (TAF) (Klagsbrun et al. 1976). Then all the tumor-derived cells produce CAM vascularization, with the fresh tissue samples exerting the most potent effect. The first identification of a specific TAF molecule did not occur until 1984, when basic fibroblast growth factor isolated from

a chondrosarcoma was found to be mitogenic for capillary endothelial cells (Shing et al. 1984).

Mostafa et al. induced vascularization with neoplastic lymphoid cells both from cell lines and fresh biopsies on the CAM. Angiogenic activity was dose-dependent on cell volume, and a previously unknown relationship between host monocyte chemotaxis and generation of vascularization was observed. This was one of the initial recognitions of the role played by the developing avian immune system in the CAM assay. Later, Balciuniene et al. reported both enlargement and increase in vascularization in the area of the CAM directly under the transplant. This phenomenon was theorized to be a common response to neoplastic transplants and not an indicative of a more complex interaction such as anastomosis among the existing vascular networks of the graft and host vessels (Balciuniene et al. 2009). Thickening of the mesenchyme and increased of both the growth factors from the implanted tumor and the nonspecific inflammatory reaction of the CAM were previously attributed to the vascularization under the implant (Valdes et al. 2002). Laryngeal squamous cell carcinoma (LSCC) implants caused both thickening of the CAM and increased vascularization of the membrane (a higher mean number of blood vessels per constant length) compared to the control group (Uloza et al. 2015).

Petruzzelli et al. studied vascularization on the CAM stimulated by head and neck squamous cell carcinoma (Petruzzelli et al. 1993). An important contribution from this study was a unique, subjective method of quantifying angiogenesis using blinded evaluators, as per Vu et al. (Vu et al. 1985). Survival rates of tumor explants did not appear as related to angiogenesis scores of individual tumors and also suggested that take rates were not significantly enhanced by a tumor's ability to stimulate new blood vessels (Petruzzelli et al. 1993).

Marzullo et al. modified a morphometric method of "point counting" originally devised by Elias and Hyde. Their study reported that the modified morphometric method of "point counting" has a highly quantitative measurement of angiogenic response to tumor grafts on the CAM (Marzullo et al. 1998). Ribatti et al. subjected biopsies of neuroblastoma and endometrial adenocarcinoma to fenretinide treatment on the CAM and computed vascular response with a previously developed planimetric method of point counting, which involved analyzing serial sections from a specimen through a fine mesh inserted into the eyepiece of the microscope (Ribatti et al. 2001). Their second method of quantifying angiogenic response involved evaluating FGF-2 and VEGF receptor expression and highlighting endothelial cells (and thus blood vessel formation) with a polyclonal anti-factor VIII antibody (Ribatti et al. 2001). Staining intensity of receptor immunological reactivity was graded from 0 to 3 which represented a modification from that of Takahashi et al. (Takahashi et al. 1995). This represented the most nuanced attempt yet to measure angiogenic response.

Sys et al. scored a total of 77 CAMs for macroscopic angiogenesis towards xenografts of sarcoma, using a scale of 0–2 originally developed by Knighton (Sys et al. 2013). Biotinylated *Sambucus nigra* (SNA) bark lectin, which binds exclusively to chick endothelium and causes avian blood vessels to appear brown, is another effective method (Uloza et al. 2017). Visualization techniques such as these

enable a more accurate quantification of angiogenesis, independent of the specific counting method. A recent technique has perfused vessels penetrating breast CCL tumor grafts with certain polymers and then tracked blood flow with micro-CT scans. This method has the advantage of confirming the functionality of vessels and also approximating the average vessel diameter and total vascular density within the graft (Ames et al. 2016).

As an easily visualized model, the CAM has facilitated highly reproducible studies of aggressive malignancies such as glioblastoma and pancreatic adenocarcinoma (Dumartin et al. 2010). Many of the researches attempted to quantify angiogenesis in response to fresh tumor material (Table 1); the dilemma remains whether angiogenic response alone may have translational significance with regard to tumor behavior in the patient. With regard to investigations of antiangiogenic drugs, quantification of the primary response can be difficult due to a secondary vasoproliferative response from nonspecific inflammatory reactions following grafting (Ribatti 2014a). This highlights the need of the development of both an enhanced understanding of the chick immune system's role and an agreed-upon method of quantifying angiogenesis.

Concordance with the Parent Tumor

After the discovery that the CAM could support foreign tissues, Stevenson attempted the first engraftment of fresh human tumor onto the medium in 1918. No growth or mitotic figures were observed following the inoculation of tissue from eight tumors (Stevenson 1918). Hurst et al. grafted 17 human tumors onto both duck and chicken eggs and found that neoplastic cells were able to survive and multiply on the CAM (Hurst et al. 1939). Although a comparatively high proportion of healthy grafts (take rate of 53% for surviving embryos) was reported for its time period, "tumor take" was said to have been achieved merely on the preservation of some of the cellular elements, even if a majority of the grafts had undergone necrosis by the time of sectioning. Hurst et al. noted that while cells typically appeared as healthy as those in the parent tumor, there was no growth approximating that in previous experiments with other mammalian tumors (Hurst et al. 1939). These initial experiments underscore the difference between describing subjective with the histologic appearance of the parent tumor and establishing true concordance via molecular markers or genetic profiling techniques available in the modern era.

Earlier studies typically had a brief mention of histologic similarity to the original tumor, even if primarily focused on other parameters such as drug sensitivity or angiogenic change (Petruzzelli et al. 1993.). The first study exclusively focused on establishing concordance done by Balciuniene et al., who transplanted fresh glioblastoma samples and sectioned specimens at 24-h time intervals up to 7 days post-engraftment. This allowed the researchers to continually monitor interaction between the membrane and neoplastic tissue (Balciuniene et al. 2009). The glioblastoma fragments survived with all cytologic features intact; however, expression of intermediate filaments diminished alongside tumor growth, and Ki67 (a strong

marker for proliferation) was found in only a few transplants. Transplanted tumors only survived up to 6 days and were limited by drying of the nourishing membrane, with more numerous necrotic zones in the graft as incubation progressed (Balciuniene et al. 2009). The finding of nutritional limitation was a significant departure from the expectation of most CAM experiments that tumor proceeds to grow until the chick immune system became mature (Ribatti et al. 2006).

Balke et al. demonstrated the first "in vivo" model for giant cell tumor (GCT) of the bone, whereas the previous attempts failed. Although tumor samples cultured on the CAM displayed components of GCT, giant cells were less numerous and contained fewer nuclei than in the original tumors (Balke et al. 2011). The successful generation of PDXs on the model highlights its utility in the study of rare tumors that currently suffer from lack of suitable models. A recent novel model on the CAM to study bone metastases in prostate cancer found that the metastatic cells preferentially colonized bovine trabecular bone xenografts that were artificially coated with the extracellular matrix protein tenascin-C, which is known to be deposited in the reactive stroma response (San Martin et al. 2017). Recapitulating the exact features of a parent tumor may not always be an essential part of the assay, so long as the method can reliably replicate key biological processes in the patient that may have value as a therapeutic target.

Sys et al. evaluated the xenografted tumor tissue of musculoskeletal on the origination of hallmarks of the original tumors. A variety of tumor fragments were grafted onto CAMs while retaining their original morphology however, largely putback the tumor-associated stroma from the human samples with chicken-derived stroma (Sys et al. 2012). It was first reported that the grafting of human stroma alongside tumor tissue is a possible strategy for replicating the original tumor microenvironment in PDX models (Malaney et al. 2014). The replacement of human stroma suggested that human stromal supplementation may be critical for truly representative models. In comparing tumor types, metastatic types were significantly more viable, infiltrative, and less necrotic than the benign samples, both samples of primary malignant tumors treated with chemotherapy, and samples of primary malignant tumors not treated with chemotherapy. Furthermore, viability was statistically associated with patients' disease progression (Sys et al. 2012).

These results demonstrated the model's ability to assess differences between tumor subtypes and even between treated and untreated tumors with chemotherapy. While this relationship can't be assumed for every cancer type, it highlights the possible prognostic use of the model as an "in vivo" tool to assess tumor aggressiveness. This would be particularly beneficial in determining when a particular patient's cancer might require more aggressive therapy than initially thought.

Fergelot et al. used the CAM as an experimental model for the clear-cell subtype of renal cell carcinoma and its interactions with the surrounding stroma. Penetration of chick vasculature into the grafts was visible on the fourth day after implantation via injection of India ink into a CAM vessel (Fergelot et al. 2013). Formerly, penetration of avian vessels was either identified by IHC of chick erythrocytes as a marker in human vessels or not consistently observed (Table 1). Vessel phenotype was characterized by confocal microscopy, detecting human vessels with antibodies

for human CD31 and CD34 and chicken vessels by the SNA lectin stain. The authors concluded that tumor vessels are maintained, anastomosed to host vessels, and then perfused (Fergelot et al. 2013). This was the most detailed indication so far of a hybrid vessel formation between host and human capillaries in the CAM-based PDX model. Uloza et al. similarly injected the CAM's vessels with fluoresceinated anionic dextran and used biomicroscopy to confirm vascularization of the xenograft in vivo (Uloza et al. 2017).

Nasopharyngeal carcinoma (NPC) is significantly linked with Epstein-Barr virus (EBV); most of the developed cell lines were rendered EBV-negative in prolonged culture and thus have unknown relevance (Gullo et al. 2008). Several PDX lines that retain morphology and harbor the EBV genome have been developed; however, transplantation of primary tumor tissue into nude mice is not efficacious due to rapid replacement by murine stroma (Giovanella et al. 1995). Xiao et al. established an "in vivo" CAM model from a total of 35 NPC primary tumor biopsies. Transplanted tumors retained morphology and poor histologic differentiation; notably, the EBV genome was also maintained (Xiao et al. 2015). This was the first description of the successful preservation of a cancer-associated virus in PDX culture on the CAM highlighting the potential use of this assay to study viral-associated tumor biology with relatively simple visualization and verification. Future studies on the CAM-based PDX model could incorporate tissues harboring other cancer-associated viruses that collectively contribute to 10–15% of cancers worldwide together with hepatitis B and C viruses, human papillomavirus (HPV), Kaposi sarcoma-associated herpes virus (HHV-8), and human T-cell lymphotropic virus (HTLV-1).

The laboratory routine cultures of this study on both cancer cell lines and primary patient-derived tumors on CAM, and the unpublished data closely recapitulate histologic hallmarks of four different types of head and neck cancer. The study of head and neck cancers on xenograft models has been constrained by low take rates and availability; for example, slow tumor growth and engraftment rates of medullary thyroid cancer (MTC) present serious challenges to the development of murine PDX models (Vitale et al. 2017). After obtaining informed consent, this research has successfully established patient-derived xenografts from frozen sections of various head and neck cancers, including MTC, with take rates of 70–80%. Each of the stained sections of four different types of head and neck cancer in the panel has been scored by a pathologist and found to represent maintenance of the parent tumor on the CAM.

Micrometastasis

In CAM-based PDX model, macroscopically visible tumor colonies cannot form in the secondary organs due to the short timeframe of the assay, which forces the detection of "micrometastasis" into local areas in order to assess metastatic potential (Zijlstra et al. 2008). Mostafa et al. assessed tumor growth and expansion in the CAM-based PDX model and found that tumor implants grew poorly in general with evidence of cell survival and mitotic activity in only a few instances (Mostafa et al.

1980). Petruzzelli et al. first visualized metastatic prospective in head and neck squamous cell carcinoma (HNSCC) on the CAM [50]. Morphologically, its basement membrane simulates that of the oral mucosa, making it well suited for studying invasion in epithelial cancers (Inglehart et al. 2014). That micrometastasis is a potential indicator of aggressiveness for parent tumors of PDX grown on the CAM was an important milestone. For at least some human cancers, engraftment onto the CAM seems to allow genuine expansion rather than mere survival.

Subsequent studies found less-than-convincing examples of micrometastasis. Marzullo et al. found that viable tumor cells either adhered to the chorion without invading the mesenchyme or were enclosed within the mesenchyme (Marzullo et al. 1998), in contrast to the previous demonstration of "pushing borders" by HNSCC on the CAM mesenchyme (Petruzzelli et al. 1993). Balciuniene et al. also demonstrated poor progression for glioblastoma xenografts on the CAM. The use of nucleated chicken erythrocytes as a marker, hematoxylin and eosin (H&E) stain, and IHC revealed that the tumors survived as isolated units, with no invasion by either cells or vessels into the mesenchyme or graft, and the tumor cells likely survived only by diffusion of oxygen and nutrients from the CAM (Balciuniene et al. 2009). Micrometastasis was also not visualized in the "in vivo" model for GCT in which tumors appeared to grow on the membrane in a flat pattern rather than invade. Ki67 staining revealed a significantly lower proliferative fraction ($<1\%$ of cells) than in the original tumor, which was attributed in part to the short 6-day time span of tumor growth (Balke et al. 2011). While simulating the early phase of tumor seeding for practical utility, it has the advantage to use serial passaging methods on the CAM-based PDX model to assess the rate of increase in proliferation for subsequent generations (Ismail et al. 1999).

Despite early difficulties, micrometastasis was demonstrated in four of the five most recent studies on the CAM-based PDX model (Table 1). Sys et al. observed revascularization of sarcoma grafts and infiltration of both the graft itself (by chick fibroblasts) and the CAM mesenchyme (Sys et al. 2013). Xiao et al. quantified micrometastasis of an NPC cell line on the CAM by making observations with confocal microscopy after 48 hours of inoculation. Their technique involved visualizing fluorescently labelled intravasated tumor cells within large vessels and then assessing invasion depth of micro-tumors in a 3D pattern; qPCR amplification of the human beta globin gene from frozen heart and lung tissues was also performed in order to quantify micrometastasis to distant organs (Xiao et al. 2015). Daily and noninvasive quantitative monitoring of microscopic spread of engineered tumors from prostate cancer and osteosarcoma cell lines has also been achieved with bioluminescence imaging (BLI) in the CAM assay (Jefferies et al. 2017). Although macroscopically visible metastasis to chick tissue has not yet been achieved due to the short assay time, these proven methods to assess micrometastasis could potentially overcome the difficulty of measuring metastatic potential in CAM models. The fluorescent labelling, qPCR amplification, and BLI methods should be replicated in future studies with the use of primary biopsy tissue and then compared to retrospective analyses of parent tumor metastases in order to determine translational significance.

Assessment of Cancer Therapies

Despite the CAM's versatility as a xenograft model, only five PDX studies evaluated its ability to predict response to cancer therapies (Table 1). Shoin et al. assessed its efficacy as a chemosensitivity prediction model for two established drugs (ACNU and MCNU). A high degree of positive correlation was found between the chick embryo assay and clinical outcome in malignant gliomas. (Shoin et al. 1991). While it is difficult to quantify the faithful dosage administered to the tumor grafts represented that administered to the patients, the reasonably close tracking of clinical outcome suggests that dosage approximation is satisfactory. These results highlight the potential role that relatively cheap, fast assays such as the CAM can play in the screening of new anticancer agents (Bailey et al. 1984).

Photodynamic therapy (PDT), which involves the use of a photosensitizing drug and activating light, is used as adjuvant treatment for patients with residual tumors or peritoneal metastasis following surgery. Ismail et al. used methylene blue (MB) as a photosensitizer in PDT of freshly biopsied malignant ovarian tumors cultivated on the CAM and visualized real-time changes in vasculature. Some treated tumors were transplanted onto new CAMs as a second generation on the eighth day after PDT, and complete remission (if not) was observed on the fourth day after implantation (Ismail et al. 1999). This was another successful instance of serial passaging of PDX onto the CAM 40 years after the study of Kaufman et al. which passaged both sarcoma and astrocytoma onto three consecutive embryos (Kaufman et al. 1956). In both cases, the short timeframe of the assay was overcome in order to continue the morphological study. The later study also concluded that PDT using methylene blue has the potential to achieve complete eradication of visible ovarian tumors in patients with superficial lesions. Methylene blue was used continually as a photosensitizer in studies assessing treatments for ovarian cancer (Xiang et al. 2014). Treatment approaches for other cancers using photodynamic therapy with various photosensitizers have been studied recently (Yokoyama et al. 2016). They might greatly benefit from preclinical testing in the CAM-based PDX model.

Ribatti et al. continued the trend towards assessment of possible clinical benefits of an experimental synthetic compound on a CAM-based PDX model for neuroblastoma (NB). They revealed that an extraordinary vascular index in NB correlates with deprived prognosis creating the CAM predominantly suitable for this trial as an established model for angiogenesis (Ribatti et al. 2001). Fenretinide (HPR) is a synthetic retinoid (a class suggested to have antiangiogenic activity) that has been shown to inhibit carcinogenesis in animals and had also been used to treat cervical carcinoma cell lines in culture (Oridate et al. 1974). Findings from its application to the CAM assay supported the notion that fenretinide might provide new opportunities for neuroblastoma therapy (Ribatti et al. 2001). At least one Phase I trial of this compound was completed over a decade later (Maurer et al. 2013), and attempts to make HPR more efficacious through targeted delivery are ongoing (Di Paolo et al. 2013).

Marimpietri et al. evaluated a synergistic antiangiogenic effect on NB of a low dose of vinblastine (VBL) and established rapamycin (RAP) compounds in

oncology and transplant rejection prevention. Treating fresh biopsies or cell line xenografts with combination treatment, compared to single-drug exposure, significantly enhanced angiostatic activity (Marimpietri et al. 2005). This comparison is notable, because fresh NB tumor biopsies responded similarly to NB cell lines with respect to drug treatment. The researchers later replicated this synergistic effect in mice (Marimpietri et al. 2007). In an early testament to the predictive power of the CAM-based PDX model, a Phase I clinical trial nearly a decade later showed the safety, reduction in the circulating angiogenic factor VEGFR2, and existence of clinical responses with this drug combination (Morgenstern et al. 2014). The CAM has also been proven as a suitable model for acute drug toxicity screenings (Kue et al. 2015). These findings are an indication that engraftment techniques for many tumors are well established and that the CAM-based PDX model as a whole is prepared for the assessment of more specific clinical benefits.

Ferician et al. made the most recent attempt to study therapeutic compounds in the model with a comparative analysis of tumor cells with blood vessels from renal cell carcinoma (RCC) on endostatin-treated and control CAM implants by assessing endoglin, VEGF, and smooth muscle actin expression (Ferician et al. 2015). While endostatin is a well-known endogenous inhibitor of angiogenesis in the CAM assay, its use as an inhibitory agent in a CAM-based PDX model for RCC was previously unreported. Given that therapeutic resistance to targeted therapies for patients with this type of cancer often begins early (Fericianet al. 2015), the CAM assay is an ideal model for the early quantification of tumor resistance to antiangiogenic drugs.

With regard to particular therapeutic methods, the easy accessibility of the CAM has enabled both the topical and intravenous administration of anticancer drugs (Kue et al. 2015), derivation of the optimal irradiation conditions in photodynamic therapies (Honda et al. 2015), and the potential for testing drug candidates in multiple tumor samples following exome sequencing (Garralda et al. 2014). Studies using the CAM-based PDX model to investigate established or experimental therapies have been promising. While further investigation is needed to determine the degree of concordance with clinical outcome for various malignancies and therapies, a retrospective analysis of drugs that have achieved remission in patients on an individual basis would be a way forward. This strategy has already been used to support superior predictability of clinical outcome in rodent PDX models (Rosfjord et al. 2014). In comparison to rodent models, the CAM-based model also has the potential for significantly lowering drug testing costs and lengthy development times (Taizi et al. 2006). Its rapid readout could improve treatment by shortening the time interval between tumor engraftment and the results of chemotherapy becoming available.

The short timeframe of the assay imparts inherent limitations. For example, tracking the drug resistance or performing toxicology studies in only 1 week is difficult (Taizi et al. 2006). This is compounded by the fact that chick organs are fundamentally less similar to human organs than those of other mammals. However, drug resistance may arise at higher frequencies on the passaged tumors serially from one CAM to another. This would theoretically allow the assessment of acquired resistance but also require verification that resistance is independent of changes

resulting from subsequent grafting. Analyses could also be performed to quantify toxicity in chick organs for compounds known to have adverse effects in humans. Finally, the closed system of the CAM allows for experimental molecules to have a half-life relatively longer than in rodents, allowing efficient use of compounds that may be expensive or available in small quantities (Cimpean et al. 2008). As clinically relevant dose (CRD) has not typically been established for drug delivery onto the CAM; further work needs to be done to ensure that representative dosages are being used to elicit effects on tumor growth.

Embryo Survival and Take Rate

While embryo survival and take rate may not carry direct implications for patient outcome, they are helpful metrics for determining the efficiency of any PDX model. The procedure used to graft fresh tumor tissue onto the CAM has evolved over the past century with the notable result that take rates (the fractions at which successful engraftment of viable tumor occurs) have steadily increased (Table 1). An early attempt to increase the rate of graft survival by irradiating eggs prior to inoculation was made by Sommers et al. While this technique did not improve take rates (23% in normal embryos versus 21% in irradiated embryos), the observation was made that the injection of cell suspensions directly into the CAM resulted in a proportional increase in embryo mortality with increasing number of injections (Sommers et al. 1952). Subsequently, investigators have generally used less invasive techniques for inoculation to minimize the embryo mortality (Table 1).

Sommers et al. also hypothesized that since the first-generation growth was generally the most difficult to obtain, prior heterotransplantation of tumor graft in other media might facilitate improved tissue adaptation and take rates (Sommers et al. 1952). This was later disproven by Kaufman et al., who transplanted fresh biopsies after maintaining the tissue "in vitro" for 10–21 days. Compared to a cumulative take rate of 50% for PDX transferred immediately from the parent tumor, only 14 of 66 (21%) xenografts that were first maintained in vitro survived on the CAM after a period of 3–11 days (Kaufman et al. 1956). Interestingly, both Sommers et al. and Kaufman et al. described that tumors of connective tissue origin were transplanted more successfully; in the case of the former, one sarcoma even survived a third transfer and 27 days total on the CAM assay (Kaufman et al. 1956). This congruence was one of the first indicators that take rates for particular neoplasms depend more on intrinsic properties of their unique tumor biology than differences in experimental techniques. There has been an increased efficiency in the grafting process with tumor take rates of 100% recently reported for nasopharyngeal carcinoma (Xiao et al. 2015) and renal cell carcinoma (Ferician et al. 2015). Thus, the CAM-based PDX model represents a potentially extremely efficient method of making the most scientific use of each patient's tumor tissue following the biopsy or resection.

Embryo mortality, the fraction of eggs that perish at any time during the cultivation period following engraftment, is a function of the gross number and

type of inoculated tumor cells as opposed to procedural variation. For example, in Shoin et al.'s experiment, all glioblastoma specimens had a take rate of 100% on the CAM, and no embryos died during the incubation period either from tumor cell dissemination or from toxicity at the dosage of anticancer drugs tested (Shoin et al. 1991). While this initially appears to be an impressively high take rate for its time period, it is likely explained by the fact that the quantity of implanted tissue was a miniscule 100 μL. Similarly, while tumor fragments derived from glioblastoma (Balciuniene et al. 2009), sarcoma (Sys et al. 2012), and renal cell carcinoma (Ferician et al. 2015) have produced high take rates of 80% and above, those from neoplasms such as head and neck squamous cell carcinoma (Petruzzelli et al. 1993) and giant cell tumor of the bone (Balke et al. 2011) have only approached 50%. High mortality rates have been speculated to be initiated by the secretion of coagulative factors or tumor cell dissemination resulting from hypoxia and serum deprivation (Balke et al. 2011).

The CAM is capable of supporting "in vivo" models of most types of malignancies from fresh tumor material (Table 1). Even tumors that have been historically proven difficult, such as those of the musculoskeletal system, have been recapitulated on the CAM (Sys et al. 2013). There are few reports of inabilities to culture particular tumor types onto the CAM, although failures include attempts for acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (Taizi et al. 2006). This model notably may not be appropriate for cancers in which a loss of tumorigenicity is observed on cultured as in the case with Lewis lung carcinoma in previous experiments. Ideally, engraftment of these neoplasms will be attempted again with the use of fresh tumor tissue in order to reevaluate their use. While the contribution of rodent models to precision medicine should not be understated, the features of the CAM-based PDX model give the potential to fill unique gaps. Unsatisfactory take rates in immunodeficient rodents across a range of malignancies represent a major barrier to cost reduction and efficiency (Malaney et al. 2014). In contrast, engraftment of viable tumor has been achieved at progressively higher rates in the CAM-based PDX model over the past four decades. Tumor fragments are generally grown more effectively on the CAM as compared to the low take rates seen in some rodent models.

Tumor Heterogeneity in the CAM Model

Many cancer hallmarks demonstrate that tumor heterogeneity is widely established (Dagogo-Jack and Shaw 2018). Any experimental model intended at precision medicine should reflect underlying cancer heterogeneity at least partially. As a consequence, in any preclinical model, preserving diversity is crucial. CCLs and PDXs significantly impact the maintenance of CAM model tumor heterogeneity and have both benefits and drawbacks. According to many investigations, tumors generated in the CAM from CCLs have been shown to transcend beyond the original characteristics to some level. Tumors generated by inoculating cell lines onto the CAM in melanoma exhibit shapes comparable to actual cancers (Sys et al.

2012). In the CAM tumor model, the composition of the tumor microenvironment was indistinguishably fabricated by the lung and ovarian CCLs, which contained extracellular matrix, cancer cells, tumor vasculature, collagen, and stromal cells (Komatsu et al. 2019). However, in these CCL-derived malignancies, the typical difficulties of in vitro CCL culture before grafting onto the CAM are still evident. Due to the lack of biological challenges under culture settings and the achievement of additional molecular modifications throughout the culture formulation phase, the CAM tumor graft would eventually develop heterogeneity not noted in actual patient malignancies.

The CCLs' growth environment in culture medium is considered essential in contrast to samples of in vivo. It might not be a tumor microenvironment contemplative. Moreover, different immortalized CCL subpopulations were favored by diverse culture microenvironments. Second, during cell culture adaptation, a sudden change in the gene may occur, changing the configuration of the cell and form, possibly leading to differing investigational results across passage (Fiebig et al. 2004). After selection with unanticipated genetic instability, cells may have intrinsic abnormalities. Nonetheless, immortalized CCLs generated on CAMs are still valuable in studying molecular processes, preclinical testing, and screening drugs because they are ubiquitous.

PDXs faithfully replicated the primary malignancies' heterogeneity, etiology, and critical anatomical and cellular characteristics in CAM. In a study (Rovithi et al. 2017), PDAC (pancreatic ductal adenocarcinoma) tumors were transplanted against the CAM. Immunohistochemistry (IHC) labelling of numerous PDAC markers, which includes mucin-1, CK19, and cytokeratin 7 (CK7), showed that morphology and cellular heterogeneity were preserved (MUC1). More significantly, the genetic characteristics of PDAC were maintained, including frequent mutations like SMAD4/DPC4, KRAS, CDKN2A/p16INK4a, and TP53. In a study with recurrent pulmonary papillomas, other molecular characteristics such as proliferation markers like Ki67 were also maintained (Uloza et al. 2017). After grafting, the original tumors' cellular heterogeneity and morphology were preserved, indicating that they did not lose their fundamental features. Cancer-related viral genomic information is maintained in transplanted patient biopsies, including Epstein Barr virus in throat carcinoma, suggesting that pathophysiological characteristics may be preserved in CAM-PDXs.

Additionally, the tumor microenvironment is maintained, including the vasculature tumor and stroma. There have also been reports of immuno-infiltration. Even though CAM-PDXs have been shown to maintain the features in both histologically and morphologically of parent tumors, several differences have been identified. Even though the retention of essential characteristics such as immunohistochemically features of the novel tumors, Sys et al. (2012) revealed that in the CAM-PDXs, stroma with human tumor was effectively superseded by stroma acquired from chicken comparable to what was seen in the murine PDX model. On the whole, since it maintains the preponderance of tumor characteristics, CAM is a consistent approach for tumor heterogeneity modelling.

Improving the Take Rate of Xenograft in the CAM Model for Different Tumor Types

One of the most important benefits of the CAM model over the rodent model is the greater tumor take rate (Ribatti 2014b). In immune-deficient mice, low engraftment rates have been seen in a variety of tumor forms. Zuo et al. (2017) showed that only 24% of mammary tumor grafts were taken via the NOD/SCID mouse model, whereas Li et al. (2015) found that head and neck cancer has a take rate of 29%, tumor grafts employing both B10.LP/Cpb and BALB/c nude mice. Although employing NSG immunodeficient mice increased the take rate significantly, the comparatively expensive expense of maintaining these colonies in an animal capability might be prohibitive in stumpy source circumstances. During pioneering research, 28 out of 59 (47%) person malignancies were successfully transplanted in the CAM system, albeit with varying levels of success. Over the years, the CAM test's grafting take rate has improved significantly, reaching almost 100 percent in instances like glioblastoma and renal clear cell carcinoma. The CAM test for PDX has achieved a widespread adoption in many tumor types because its take rate is higher. Various factors of the CAM model might impact take rates. If less intrusive inoculation procedures are used, graft take rates and chick embryo survival rates will improve. Various take rates have been seen in cells with different intrinsic characteristics. Increased take rates of 80–100 percent have been related to sarcoma, glioblastoma, renal cell cancer, and nasopharyngeal carcinoma. On the other hand, several malignancies, namely, giant cell cancers and squamous cell carcinoma in the head and neck, may merely achieve a take rate of about 50%. Despite this, the CAM model has a greater tumor take rate than the models of murine.

The CAM Model of Metastasis

The tumor spread in CAM can be analyzed using natural and experimental models. The CAM surface covered with grafting tumor cells or patient-derived samples and tracking metastases are the characteristic feature of the spontaneous model. This experiment's goal is to investigate cancer metastasis through injecting malignancy cells directly into the allantoic vein. These two unique settings are utilized to distinguish between nonmetastatic and metastatic cells because tumor cell metastasis is a series of processes.

The spontaneous model may be used to investigate tumor cell penetration via the CAM and cell intravasation (tumor cell invasion into blood vessels). Docking, extravasation, and tumor cell growth in tertiary organs are all studied using this experimental paradigm. Both models may be used to estimate tumor cell survival and vascular arrest. Surprisingly, tumor cells prefer to halt in the CAM microcirculation, where their survival and extravasation rates are high. Mice may die when spreading cancer cells and have substantial cell damage, with a lower incidence of extravasation (Massague and Obenauf 2016). In the CAM system, the urokinase

plasminogen activator receptor may be a leading responsibility in the proliferation of cancer cells and cellular arrest or dormancy activation of the p38 and ERK pathways. The CAM model may potentially be used to establish colonization at different metastatic locations. On the surface of CAM, metastasized epidermoid carcinomas set to the chick embryo's brain, eye, and heart, in addition to the liver, according to Dagg et al. (1956). The CAM model is superior for examining organ-specific metastatic mechanisms since it can replicate metastasis to many embryonic chick organs. Human beings' leukemia cells have the potential to metastasize to the chick's brain, indicating a further consistent result in a human being leukemia development than in mice, where metastasis sites are frequently restricted.

Many methods for evaluating tumor morphology in metastatic sites have been developed. CAM tumor grafts are involved in assessing metastasis through traditional mouse techniques or hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC) staining of the tumor section. The acceptance of numerous cell shapes and kinds and the assessment of implanted cell penetration into the underlying CAM tissue. In the CAM model, fluorescent tags on cells would make it much simpler to examine metastatic phases. Real-time imaging sophisticatedly reveals intravasation, dissemination, and extravasation of cancer cells with high (HT-hi/diss) and low (HT-lo/diss) disseminating potential using fluorescence-tagged human HT-1080 fibrosarcoma cells and concurrent labelling of vasculature with fluorescence-tagged Lens culinaris agglutinin (Deryugina and Quigley 2008). Researchers can differentiate between endothelial cells and intravasation tumor cells during metastasis using this two-color labelling method, which allows them to study the dynamic interaction with the environment. According to the results, HT-hi/diss cells show vasculotropism, which involves tumor cells being wrapped in blood vessels rather than disseminated haphazardly throughout the mesoderm of CAM. Intravasation is partly due to the attractiveness of HT-hi/diss cells to the arteries of blood. A minimum of HT-lo/diss cells seemed to flee from the original injection location, preserving a comparatively clean tumor-stroma border.

Additionally, assessing metastatic capacity, the CAM test may be used to quantitatively evaluate each stage of the metastatic cascade (Zijlstra et al. 2002). It may be accomplished by following the mRNA levels of metastatic tumor cells in chick embryos or identifying Alu sequences of humans in metastatic locations. The association of fraction of human cancer cell intravasation and spreading into the chick embryo via the CAM can be achieved through measurement of human Alu sequences and PCR-mediated amplification. Increased circulating malignant cells and metastasis interconnected genes epitomize the host, and malignant tissue connections lead to gene expression alterations at the early phases of metastasis.

Angiogenesis of CAM Models

Angiogenesis is defined as the formation of new blood vessels from existing blood vessels. Tumor cells from adjacent blood capillaries cause angiogenesis. Cancer cells may enter the circulatory system via the developing neovasculatures, enabling them

to spread. Because it is enclosed with thick vasculature, the CAM is the first in vivo platform for evaluating angiogenesis. In 1976, the CAM's host-mediated vascular response was utilized to evaluate the effectiveness of extracellular chemicals produced by malignancies in influencing tumor vascularity (Klagsbrun et al. 1976). The primary fibroblast growth factor was discovered, the first tumor angiogenesis factor. Following tumor transplantation, TAF secretion has been demonstrated to enhance CAM mesenchyme growth and thickness, as well as an increase in the CAM's vascularization area. As a result, the CAM model is being utilized more often to explore the anticancer effects of antiangiogenic medicines. Hormones, antibodies, growth factors, gases, antibiotics, chemical compounds, organometallic compounds, and small-molecule medications have all been tested using the CAM assay for their antiangiogenesis properties. Through various imaging procedures, quantifiable or qualitative evaluations of the CAM vasculatures have been attained to determine the applied medicines' antiangiogenesis impact correctly.

The CAM's vasculature can be studied with the help of a microscope; in addition, detailed information about vascular remodelling can be reached through light microscopy and transmission electron microscopy. Many analytical techniques have been developed to offer quantifiable readouts for angiogenesis on a quantitative basis. Vacuum density and vessel branching points (Woloszyk et al. 2019), vascular length (Seidlitz et al. 2004) Pyrio Digital images allow for objective monitoring of the vasculature on the CAM utilizing these quantitative methods. Not all quantitative techniques, however, will be able to identify small angiogenesis changes. Assessing methodologies and imaging techniques may deliver an ideal ideology and cross-reference with the outcome assessed by diverse endpoints for future examinations.

When it comes to researching tumor angiogenesis, the CAM model has several drawbacks. Endothelial cell proliferation and neovascularization cause vascular alterations during embryo development. As a consequence, differentiating between tumor-related neovascularization and preexisting embryonic neovascularization may be difficult. This confusing scenario may affect data interpretation. The assessment of vessel measurements may stop damage in data interpretation should be performed while embryonic neovascularization is at its lowest. From ED10/11 to ED14–15, the endothelium mitotic index of CAM drops, indicating that this is an excellent period to evaluate. The CAM organization on ED13–14 increases with a reduced intricacy, whereas the CAM network on ED13–14 gets bigger with less difficulty (Komatsu et al. 2019).

Using the CAM Model to Establish Ovarian Cancer PDX (Ovarian Cancer Patient-Derived Xenografts): A Case Study

We utilize the case study of ovarian cancer to reveal the platform's know-how and applicability and how the CAM model may be used to construct the PDX. Ovarian cancer (OC) is considered a fatal gynecological malignancy through histopathological and molecular heterogeneity of the tumor. Based on gene expression profiling,

the heterogeneity of ovarian tumors may be classified into different molecular subgroups (Kroeger and Drapkin 2017). In testing and validating subtype-specific therapies, OC PDXc has been recognized as an excellent platform to improve precision management. H&E slides from five of the transplanted tumors showed papillary structures, squamous proliferation, and tumor cells. Even though the success rate is just 50%, it is a hopeful initiative towards grafting patient tumors onto CAMs which will preserve the uniqueness of a patient's novel tumor. Consequently, a case study of ovarian cancer transplanted onto the CAM and the following tests to provide further information about the chick CAM model's application in cancer research. Below is a summary of the primary technique for generating the OC PDX in CAM and the crucial tumor evaluation.

CAM Procedure for Patient Samples: A High-Level Overview

Fertilized eggs from a nearby farm were labelled with the embryonic day 0 designations. Eggs were instinctively washed and placed flat in a Rcom Max 50 incubator at 37.5 °C and 60% humidity. ED3, a syringe, and a needle were used to collect 3 mL of albumin, and a 1 cm^2 window was created in the egg's center, which was subsequently sealed with a semipermeable adhesive membrane. The tumor was removed from the patient on ED6, cleaned in PBS, and cut into 3 × 3 × 3 mm pieces for grafting on top of the chorioallantoic membrane (CAM). Before being embedded in paraffin, the patient's tumor fragments were placed in snap-frozen, freezing media and formalin-fixed. The vascularity (%) and volume of transplanted patient tumor were then assessed by 3D power Doppler ultrasonography. The cancer was permissible to develop until ED13 previous to the changes in volume and vascularity, was investigated. The transplanted tumor subfragments were frozen, snap-frozen, or treated (formalin) and implanted (paraffin). Before and after grafting into the CAM, it was compared to the FFPE samples.

The OC CAM Xenograft General Protocol

Incubation of Eggs

By using dry paper towels, the eggs are wiped. Cleanup with ethanol (70%) or any other washing chemical dramatically decreases the endurance time of embryos and should be avoided at all costs. Before being placed flat on one side, the top surface of the eggs is branded and labelled. The incubation temperature and humidity of the incubator – Rcom Max 50 – should be 60% and 37.5 °C, respectively. The incubator's autorotation feature is disabled, and the ventilation is set at partially opened.

CAM Preparation

In a biosafety cabinet, the chick CAM preparation will take place. After ED3, the egg membrane should not be opened since it tends to attach to the shell and damage the CAM. Before opening the egg, a small needle is used to remove 3–4 mL of albumin from the apex. After cutting the egg, a 3MTM Tegaderm™ transparent film layer prevents shell particles from dropping onto the CAM. In the shell, a 1 cm^2 window is produced. On the surface of the egg yolks at ED3, the beating heart of the chicks and the supporting arteries might be seen. Following that, I resealed the glass with a transparent film dressing of 3MTM Tegaderm™. On CAM, tumors are grafted at ED6 or ED7 because the embryos of the chick are more resistant to agitation.

Fragments of OC Tumor Grafted on CAM

In the operating room, tumor fragments of a late-phase OC patient are obtained and instantly positioned in a 50 mL Falcon tube in serum-free DMEM, which is kept cold until engraftment on CAM. Following that, the fragments are split into small chunks. They used a scalpel in sterile condition and cut about $3 \times 3 \times 3$ mm^3 (macroscopically) homogeneous tumor portions with no apparent indications of necrosis on a clean Petri plate using a biosafety cabinet. Few fragments were formalin-fixed paraffin-embedded (FFPE) for IHC or H&E staining, while others by liquid nitrogen in snap-frozen condition or molecular profiling in dry ice. The remainder was frozen. With a pair of sharp forceps, the fragments are grabbed and transferred to the CAM, along with some PBS. At a blood vessel's "Y" bifurcation, tumor fragments are put on the CAM. Minimizing bleeding or apparent capillary rupture can be achieved through gently tapping a glass rod (autoclaved) on the periderm while ensuring a minimum harm to the CAM. With the blunt-end forceps, tumor pieces are snuggled in even with light pushing on the CAM. The subfragments are grafted onto the CAM (ED6) and allowed to extend and develop until ED13.

Chick CAM is grafted with ovarian cancer patient segments. A sample of the patient's tumor was removed from the operating room and split into 12 parts. The top row was snap-frozen, on ED6, the next row was grafted onto the CAM, and the bottom row was treated with FFPE. The CAM on ED6 in the second row was grafted with subfragments 1–6. A picture (C) of tumor subfragments obtained from the CAM was shown on ED13. Subfragments in the blue box (b) tumors were implanted on ED6 into the CAM and left until ED13, scanned with ultrasounds (3D), and removed. This image shows the CAM tumor subfragments that were excised. At the National University Hospital of Singapore's Department of Pathology, the tumors were set in formalin (10%) and entrenched in paraffin previous to being stained with H&E. After grafting onto the CAM for seven developing (embryonic) days, fragments 1 (top left) and 5 (bottom left) from an ovarian cancer patient were H&E stained, as well as subfragments 1 (top right) and 5 (bottom right). The post-grafted

subfragments on the CAM exhibited related histology and morphology as the early stage of patient fragment due to ovarian cancer. Immune expression of p53 in the patient's original fragment 5 (left) and subfragments 5 (right) after grafting onto CAM, with scale bars. The subfragments post-grafted onto the CAM had comparable p53 immunoexpression to the fragments of the original ovarian cancer patient.

Ultrasound Scan Imaging of the CAM Xenograft

With a 40-MHz center-frequency MS-550D transducer coupled to an automated 3D motorized slider, the VisualSonics Vevo® 2100 Imaging System performs ultrasonic scans. The translucent film dressing from 3MTM Tegaderm™ not involved, and the exposed tumor is covered with a cling wrap. A blunt-end tweezer is gently tapped to establish appropriate contact between the tumor and the cling film. The MS-550D transducer is lowered until it comes into contact with the warmed Aquasonic® 100 Ultrasound transducer gel positioned on top of the cling wrap. Ultrasounds are recorded in 3D power Doppler mode at different cross-sections using an automated 3D motorized slider at a low 2D gain (5 Hz) and power Doppler gain (10 Hz) and then reconstructed into a 3D image. The CAM's vascularization, in addition to the tumor grafted onto the CAM's vasculature, is shown by the red areas. The cyan lines point out diagonal cross-sections of individual measurement sites of the transplanted tumor. All tumor subfragments should be feasible after a week after being implanted onto the CAM.

Ultrasound Using Three-Dimensional Power Doppler

The Tegaderm membrane was removed on embryonic day 13, and Aquasonic gel in warmed condition was applied to the cling wrap that was suspiciously put over the CAM tumors. A transducer (550D) coupled to an Acquisition motor (3D) was utilized to capture ultrasound pictures of cancer cells grown or grafted onto the CAM using the VisualSonics Vevo 2100 Imaging System and to examine the tumors transplanted onto the CAM using Vevo Lab 1.7.0 software, as shown in the image (top left: tumor's location (in cyan) about the CAM's surrounding blood vessels (orange)). Every cyan line indicates a cross-section that was considered to create a 3D picture of the tumor. The axes of the measured area are represented by X, Y, and Z (purple) (top right: tumor's axes are displayed at their center, both longitudinally and width-wise). Measuring the grafted tumor leads to achieving a guaranteed precise picture that might be rotated. The contour of the transplanted tumor with regard to the CAM's surrounding vasculature (in red) is depicted at the bottom left. At the bottom right: the axis' center, as well as the individual cross-sections (in cyan) and surrounding blood arteries, is detailed, as in the top right (in red). The % vasculatures and tumor volumes were determined using the Vevo Lab 1.7.0 program and indicated on the top left in cyan after the parallel 2D cross-sections were further rebuilt to create 3D pictures of the tumors.

Aside from ultrasonic scanning, several additional imaging modalities may be used. Bioluminescence imaging was used by Jefferies et al. (2017) to evaluate cancer cells produced with luciferase reporter genes. CAM cell grafting in conjunction with basement membrane extract or gel foam scaffolds allows for noninvasive daily monitoring of created tumor cells in live chick embryos. Herrmann reported that the high-resolution MRI scrutinized CAM tumor growth. Although on the CAM there are numerous ways for seeing and quantifying tumor development and vasculature, we opted to use ultrasound imaging for the 3D Doppler mode because it is more quantitative than fluorescence or bioluminescence imaging, which only allows for 2D viewing of cancer cells. In contrast, a high-resolution MRI would provide more precise data for tracking tumor progression, but it would almost probably raise the trial's cost significantly. Furthermore, if time and finances allowed, PET-CT scans might be used to investigate the drug and molecule pharmacokinetics and tumor development.

The 3D Ultrasound Images Have Been Analyzed

The pictures are analyzed using the Vevo Lab 1.7.0's 3D analysis software. At a minimum, 100 2D sections are gathered and rebuilt into a 3D image, from which the cancer volume and vascularization percentages are intended. The software estimates the volume of the tumor and the percent vasculature at different time intervals without harming the tumors by carefully drawing the tumor circumference for each section. Before putting the CAM back into the incubator, remove the cling wrap, and cover the window with a transparent film dressing of 3MTM Tegaderm™.

PDX Tumor Harvesting and Storage

Following the last ultrasound scan on ED14, the host chick embryo is quickly killed through decapitation; the CAM with accompanying tumor is discarded using a circular cut with scissors and relocated to a 12-well plate packed with PBS for every cancer. Excess CAM is removed from the graft save for the initial attachment site if the malignancy is regrafted onto another CAM. The tumors are photographed and sliced into various parts before being FFPE-treated, with liquid nitrogen (snap-frozen), or molecularly analyzed in dry ice, stored in a glacial media, or transferred on the next set of CAM.

CAM-PDX and Histopathology

With the patient's original morphological profile, histopathological investigations are essential to verify CAM tumor pieces. Irregular glandular gaps and darkly pigmented nuclei are symptomatic of high-grade serous carcinomas in the papillary shapes of actual patients. Correspondingly, after a week of grafting, subfragments of

tumors transplanted onto the CAM retained their morphological characteristics. The virtual patient parts and tumor subfragments of CAM were also subjected to a p53 stain (DO-7, Dako) that might be employed as a substitute marker in ovarian cancer for TP53 mutations. Surprisingly, tumor subfragments transplanted in a week onto CAM preserved the overexpression and, immune-expression of p53 as seen by the dark stain.

Conclusion

The CAM of chick has been demonstrated to be a reliable model for inoculating grafting and cell lines of human malignancies for the cost- and time-effective assessment of pharmacological treatments and the future functioning of target genes/pathways. Furthermore, the turnaround time for medication efficacy testing is approximately 2 weeks, compared to 3–6 months in mice. As a result, the CAM model can be used as a rapid screening technique to identify mice models that need exact mouse handling knowledge. The CAM model was used to demonstrate that the AXL inhibitor BGB324 has better efficacy in the mesenchymal subtype. The CAM was used to identify a possible approach by targeting AXL for treating the mesenchymal subtype of OC and then tested in mice models. Furthermore, we demonstrated therapeutic synergy in which the tumor suppressor protein OPCML inhibits AXL and other RTKs like EGFR and HER2 and AXL inhibitor BGB324, sensitizing the mesenchymal subtype. The CAM model is currently being used to screen additional therapies for distinct OC subtypes to identify specific targets of subtype for future preclinical validation in animal models.

In addition, CAM's capacity to retain the histology of engrafted tumor pieces of the patient can give further therapeutic benefits. In both tumor of the original patient and the subfragments engrafted on the CAM, staining (H&E) revealed stained nuclei (dark in color) and irregular glandular gaps of papillary areas. The initial tumor of the patient fragment and the transplanted subfragment were shown to have p53 overexpression, which is typical of high-grade serous carcinomas. Next-generation sequencing is required to resolve the actionable mutations of tumor grafts and the medicine or mixture of treatments that may be more suited for the patients. These patients would also have PET-CT scans, allowing us to engrave tumor subfragments to explore drug pharmacokinetics onto the CAM and perform micro-PET-CT studies. Given the CAM model's flexibility, it has the potential to be a concurrent platform for tracking cancer patients' treatment progress. It may forecast medication efficacy on tumor subfragment grafts and evaluate actionable mutations to design more tailored treatments.

In CAM assay, which is a viable and versatile platform for malignancy research, some general constraints are to be aware of while planning an experiment. Although chick embryos are inherently immunodeficient, nonspecific immunological responses may arise throughout the development of the chick immune system. Moreover, despite its utility as a model for studying metastasis, the short surveillance period makes it hard to create macroscopically able-to-be-seen metastases in chick

tissues or to consider treatment response. Furthermore, because chicks have a different medication metabolism and immune system than mammals, they may have varied reactions to treatments. As a result, the CAM assay may be beneficial in screening drugs; nonetheless, after promising medicines have been found using the chick CAM assay, mammalian models will still need to be evaluated during preclinical research.

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Engineering and Studying Syngeneic Animal Tumors and Large Animal Endogenous Tumor Models

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K. Suganya, Sreya Babu, and Indranil Chattopadhyay

Contents

Abstract

In this review, we discussed mice model and canine model which are used to study different types of cancer. Mice models are the most commonly used animal model in research. Genetically engineered mouse models, cell line derived

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models, syngeneic models, Xenograft models, and chemically induced mouse models are discussed. The study of naturally occurring malignancies in companion (pet) animals to find their translational significance to human cancer are known as comparative oncology. These studies, which are usually carried out in pet dogs, allow for the evaluation of novel anticancer drugs and combination therapies in a veterinary clinical setting that allows for pharmacokinetic/pharmacodynamic relationships. One should choose the most suitable model for research since each model has its pros and cons.

Keywords

Canine cancers · Immunotherapy · Canine vaccine · GEMM · Xenograft · Syngeneic model

Introduction

The real prevalence of spontaneous cancer development in companion dogs is critical knowledge for including these canine patients in cancer biology and therapeutic development. Dogs tend to acquire both bone and soft-tissue sarcomas at a greater rate than humans, with a lower prevalence of epithelial malignancies such as lung, colon, prostate, and pancreatic cancers. Pet dogs are an appealing supplementary animal model of human cancer because of many features of naturally occurring malignancies in dogs. Over induce cancer models, identical body size, a shared home environment, and a complete immune system, spontaneous development of tumor and natural co-evolution of tumor and host stroma are clear benefits (LeBlanc and Mazcko 2020).

Mice (*Mus musculus*) are the most common animals to imitate human disease. Murine models have an advantage because their physiology and molecular signaling pathways are similar to humans'. A vast collection of mouse strains is dedicated to researching various diseases and their molecular processes (Tudrej et al. 2019). Using mouse cancer models, researchers can learn about tumor biology in complex and dynamic physiological systems. In an ideal mouse system, tumors that are genetically and morphologically similar to human tumor cells could be generated fast. Using mice models to accomplish this has provided an invaluable tool for investigating tumor start, maintenance, development, and treatment response (Zhang et al. 2011). Humans are a widely diverse population with varying genetic backgrounds, diets, and environmental exposures. Any of these factors can impact how cancer expresses itself and so can be a source of confusion while studying human malignancies. Researchers may control these variables in mice studies, making it easier to simplify procedures and ask well-structured questions. Mice have many anatomical, cellular, and molecular traits similar to humans linked to cancer, including the immune system, maternal effects in utero, gene imprinting, and alternative splicing. Mice are a model organism that may be used in experiments and have a

variety of developed research tools for understanding the fundamentals of cancer (Walrath et al. 2010).

Canine Model

Lymphoma

Human and canine sarcoma exhibit a variety of morphological and immunophenotypic variations. Tumors are classified as low grade, intermediate grade, and high grade by the Kiel and Working Formulation (WF) system. Canine lymphoma (CL) is considered an intermediate or high grade. Large cells and rapid mitotic rate distinguish intermediate to high-grade cancers. Human and canine sarcoma exhibit a variety of morphological and immunophenotypic variations (Richards and Suter 2015). Diffuse large B-cell Lymphomas (DLBCL) comprise the majority of CL (79%) while marginal zone lymphomas (MZL) are a distant second (Ponce et al. 2010). Follicular lymphoma (B-cell) in dogs is relatively uncommon compared to human lymphomas. Peripheral T-cell lymphoma-not otherwise specified (PTCL-NOS) (High grade) and T-zone lymphoma (TZL) are the two primary subtypes of T-cell Lymphoma 3,4. Hematoxylin and Eosin (H&E)-stained sections were examined in combination with standard immunohistochemical procedures employing CD3 and CD79a markers to confirm the diagnosis of lymphoma. The tumors histological subtype, grade, and mitotic index were determined using standard morphological criteria based on the World Health Organization (WHO) categorization system for human lymphoid neoplasia, which has been proved to be very accurate and reproducible in canines (Jaffe 2001; Thomas et al. 2011). Microarray detection can be done by isolating DNA from representative tumor tissue using Qiagen DNeasy Kit. A bespoke microarray including cytogenetically mapped bacterial artificial chromosome (BAC) clones from the CHORI -82 dog collection (BACPAC resources, children's Hospital Oakland research institute, Oakland, CA) was used for ACGH analysis. The array incorporates clones spread at 1 Mb intervals throughout each dog autosome and the X chromosome (mean interval 1.10 Mb, range 0.28–3.28 Mb), as well as clones representing canine orthologues of 53 human genes linked to a variety of malignancies (Thomas et al. 2008). At the molecular level, canine lymphoma is quite relevant to human lymphoma. The results of comparative genomic hybridization (aCGH) array were examined between canine and human lymphoma to access DNA copy number variations comprehensively. Between canine BCLs and TCLs, there were considerable disparities in the degree of genomic stability; TCLs had more gains and losses than BCLs (Frantz et al.) demonstrating that GEP (Gene expression profiling) could distinguish between low-grade T-cell lymphomas, high-grade Tcell lymphomas, and B cell lymphomas with more B-cell lymphoma samples. The team has managed to divide canine B-cell lymphomas into two groups, comparable to human ABC/GCB subtypes, within the ABC-like subtype having a poorer prognosis, similar to human DLBCL and also

discovered that, similar to human DLBCL, the ABC subtype displayed greater expression of B cell receptor and NF-kB pathway genes (Frantz et al. 2013). The underlying basis of cancer phenotypes are anomalies in oncogenic pathways. *FLT3* drives mutation in human acute myelogenous leukemia (AML) and acts as a negative prognostic marker. But in the case of canines, AML is rare. Mutations in the *FLT3* gene have been identified in canine ALL (acute lymphocytic leukemia) (Small 2008; Suter et al. 2011). Gene expression profiling studies in dogs and humans, comparing normal and DLBCL-bearing lymph nodes, report NF-kB-related genes. Expression levels of NF-kB target genes are able to distinguish normal from tumor samples in canine models. Furthermore, immunohistochemistry for P52 and P65 has been demonstrated to activate both the alternative and traditional NF-kB pathways in both canine and human DLCBL. By immunohistochemistry, the canine lymphoma samples had greater P52 (non-canonical route) levels than P65 (canonical pathway), but this was inverted in humans. In vitro, drug sensitivity tests with these cell lines revealed that they were sensitive to an IKK inhibitor in both human and canine cells 2, 11. In two clinical trials of NF-kB Essential modulator (NEMO)-binding domain peptide, a medication that inhibits NF-kB signaling, the functional importance of NF-kB activation in CL was demonstrated (Gaurnier-Hausser et al. 2011; Habineza Ndikuyeze et al. 2014). Tcell lymphoma, notably canine peripheral Tcell lymphoma (PTCL), which is frequent in boxer breeds, may also provide chances for comparative modeling. Activation of PI3 kinase pathways, loss of *PTEN* (Phosphate and TENsin Homologue deleted on chromosome 10), and the tumor suppressor *CDKN2* are all common aspects of canine and human PTCL. There is currently insufficient evidence to evaluate if canine PTCL displays the GATA3-TBX21 dichotomy reported in humans. CHOPsystemic chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) is the first-line treatment for an advanced stage of CL, identical to human NHL. Following CHOP-based chemotherapy treatments, dogs with high-grade T-cell lymphoma (PTCL-NOS), which is frequently linked with paraneoplastic hypercalcemia, suffer poorly.

Urothelial Carcinoma

Urothelial carcinoma (UC), also known as transitional cell carcinoma, is the most prevalent bladder cancer in both human and canine populations (TCC). After prostate cancer, it is the second most common urogenital cancer. Previous research has shown that canine UC mimics and acts like invasive human UC, making it a useful in vivo model for cancer (Shapiro et al. 2015). In terms of cellular and molecular traits, immune response patterns, and location of metastasis, canine invUC (Invasive urothelial carcinoma) closely resembles human muscle-invasive bladder cancer. In dogs, the majority of InvUC is located in the trigone region of the bladder, with an extension down the urethra being prevalent, but in humans, the cancer is distributed more evenly over the bladder. Bladder cancer accounts for around 2% of all naturally occurring malignancies in dogs, which is similar to human rates. Histological analysis of tissue samples in dogs and humans is used to diagnose

InvUC. These tissues are obtained in dogs by catheter biopsy, cystoscopy, and surgery. The majority of TCC in dogs is intermediate to high-grade invasive TCC (Valli et al. 2013). Canine urothelial carcinoma, in particular, is known to exhibit several molecular targets, including BRCA2, CDKN2A, CDKN2B, epidermal growth factor receptor (EGFR), HER2(ERBB2), PIK3CA, and NF-kB (Shapiro et al. 2015; Dhawan et al. 2018). Through local instillation into the urinary bladder, EGF–conjugated anthrax toxin was effectively utilized to decrease over-expression of EGFR in urothelial cancer of dogs. Can225, a canine-specific anti-EGFR IgG antibody, has also conjugated to the radionucleotide 99mTc and a photo absorbing dye, providing both diagnostic and therapeutic uses in EGFR–expressing canine malignancies. Use of Can225 would be a fascinating opportunity to test novel ways for treating EGFR- over-expressing tumors in both humans and dogs (Fazekas-Singer et al. 2017; Nagaya et al. 2018). Numerous clinical studies have been done on dogs. In particular, Cyclooxygenase (COX) inhibitors have exhibited significant antitumor effects and enhanced chemotherapeutic effects in invasive urothelial carcinoma. The biological changes associated with COX inhibitor-induced remission in dogs were reported in humans receiving celecoxib in a neoadjuvant study administered between cystoscopic diagnosis and cystectomy (Dhawan et al. 2010). The majority of canine InvUCs possess a mutation in the MAPK signaling pathway (dog homolog of $BRAF^{V600E}$), which has been found in various human malignancies, which is an important molecular distinction between canine and human InvUC, while BRAF mutations are uncommon in human InvUC, additionally, MAPK pathway activating mutations are found in about 30% of patients. The identification of the BRAF mutation opens the door for the translational significance of bladder cancer in dogs and humans (Decker et al. 2015; Mochizuki et al. 2015).

Carcinoma of the Breast

Mammary gland tumors in dogs have been identified as models for human breast cancer. Mammary tumors are the most prevalent form of tumor in healthy female canines, accounting for more than 40% of all tumors in this patient group. Exposure to ovarian hormones is involved in the formation of mammary tumors in dogs, and the risk of tumor development can be considerably decreased by conducting an ovariohysterectomy (OHE) at a young age. The mammary glands were thoroughly inspected, and all tumors were documented and quantified. The surgical excision was carried out according to standard procedure, to completely remove the tumor. The type of surgery such as lumpectomy, simple mastectomy, or regional mastectomy, was determined by the size, location, and a number of tumors present in each dog. In dogs with many big tumors, the procedures were conducted in two phases if necessary to avoid excessive tissue tension and wound dehiscence. Histopathological examinations were performed on all of the tumors. The assessments were completed by two independent pathologists (MHG and JT) who were blinded to each other and all of the clinical information regarding the case. The tumors were classed as malignant or benign based on the kind of tissue present (epithelial,

myoepithelial, and/or connective tissue). For this study, malignant simple epithelial tumors were classed together as carcinomas, which comprised papillary and tubular adenocarcinomas, solid carcinomas, and anaplastic carcinomas (Sorenmo et al. 2009). Dogs have lately been classified into 'human-like' mammary tumor phenotypes such as luminal A, luminal B, and triple-negative (basal-like) (Varallo et al. 2019). Mutations in *ESR1* and *BRCA2* genes and frequent losses of key tumor suppressors such as *CDKN2A, CDH1,* and *TP53* are all found in human breast cancers (Lutful Kabir et al. 2015). The pharmacokinetics of iniparib (PARP inhibitor) in conjunction with carboplatin was examined in mammary carcinoma of dogs (Saba et al. 2016).

Melanoma

Melanomas in dogs are most usually detected in the buccal cavity, although they can be found on the skin, eyes, and fingers. Melanoma in the skin (cutaneous), eye (ocular), footpads and nail apparatus (acral), and other mucocutaneous locations are substantially less common in dogs. In contrast to most oral/mucosal and acral melanomas in dogs, cutaneous melanocytic neoplasms have a usually positive prognosis. Due to the protective hair covering, UV is not expected to have a significant effect on canine cutaneous melanoma. Because anatomic location appears to be linked to the biological activity of canine melanocytic neoplasia, it is thought to be a valuable prognostic factor. Mutations in RAS family members, *PTEN*, and *TP53*, as well as amplification of MYC and MDM2 and deletions of CDKN2A, have been reported in recent efforts to define the genetic landscape of canine melanoma. Furthermore, mutations in PTPRJ, a rarely documented suspected tumor suppressor gene that encodes a receptor-type protein tyrosine phosphatase, have been found in 3% and 19% of cases, respectively, in two canine melanoma cohorts. Canine can serve as a model for BRAF wild-type human melanoma, which needs an understanding of new treatment. When exposed to interferon-, canine melanoma tumor cell lines and tumor-infiltrating macrophages elevated PD-L1 expression, indicating a key mechanism of tumor-mediated T cell suppression. This provides the way for the clinical use of PD1/PD-L1 inhibitors as new cancer therapeutics in canines (Hernandez et al. 2018).

Osteosarcoma

The discovery of canine osteosarcoma is an intriguing opportunity to use a common canine cancer to increase information about rare pediatric and adolescent/young adult cancer. Array-based comparative genome hybridization studies revealed similar type copy number alterations of *CDKN2A, CDKN2B, MYC, PTEN, RB1, RUNX2,* and *TP53* in both human and dog osteosarcomas. Clinical studies are frequently enrolled in pet dogs with osteosarcoma to investigate innovative medications added to the standard of therapy for their potential to delay or prevent the

development of metastatic disease (LeBlanc and Mazcko 2020). Canine osteosarcoma differs from human osteosarcoma in a number of ways, one of which being the lack of histone methyltransferase SET domain containing 2 (SETD2), a protein that controls epigenetics and acts as a general tumor suppressor in human cancers.

Immune Cells and Immunology of Canines

With a few major exceptions, the immune system and immunological response of dogs are extremely similar to those of humans. In both dogs and humans, the ratio of CD4 to CD8 T lymphocytes in blood and lymph nodes is comparable (2:1). Both species' blood contains equal numbers and percentages of neutrophils and monocytes. Recent reagent development for canine NK cells, on the other hand, might increase our capacity to quantify responses of NK cells in dog. Dogs contain circulating gamma-delta T cells as well, however little is known about how their numbers alter in illness. Regulatory T cells (CD4 + FoxP3+) in dogs have also been identified, and their levels in both blood and tumor-draining lymph nodes are considerably higher in dogs with cancer. Similar to human IgG subclasses, canine IgG molecules are divided into four functional subclasses (A–D), with two subclasses capable of binding Fc receptors and two subclasses that are Fc functionally negative. Many of the costimulatory or co-inhibitory molecules are found in dog T cells, including CD28, PD 1, OX40, TIGIT, TIM3, and LAG 3. Canine Tcells have low levels of MHCII, which can be increased during activation of T cell. The expression of CD4 by dog neutrophils differs from that of human neutrophils, which is a unique trait. But it was unclear how CD4 was expressed in dogs.

Cancer Vaccine

Dogs acquire several extremely metastatic malignancies that are closely linked to human diseases, such as osteosarcoma and melanoma. As a result, these malignancies in dogs provide a chance to test novel cancer vaccination techniques in immunologically relevant environments. Studies in dogs with osteosarcoma, for example, are underway to see if a newly conditionally authorized canine osteosarcoma Listeria-vectored vaccination targeting HER2/neu may successfully prevent tumor metastases and regulate the progression of macroscopic metastases (Mason et al. 2016). Another example is a tumor vaccination based on plasmid-DNA that targets the TERT antigen and has been tested in dogs with lymphoma in conjunction with CHOP treatment. In small-scale trials in dogs, ACT (adaptive cellular therapy) with CAR T cells has been investigated, including CAR T cell studies with CD20 targeted CAR T cells in dogs with B cell lymphoma (Panjwani et al. 2016). In dogs with melanoma, we discovered that directly transfecting the TME with a powerful T cell activating molecule such as a bacterial super antigen might drive T cell infiltration and activation, as well as considerable tumor remission (Dow et al. 1998). We recently discovered that the ARB losartan has a strong anticancer effect by reducing

the migration of inflammatory monocytes into tumor tissues, resulting in overall tumor macrophage depletion, via blocking signaling via the CCR2 chemokine receptor (Regan et al. 2019).

Immunotherapies

As novel checkpoint molecule targeted medications become accessible in dogs, there are chances for the dog model to provide critical new information, particularly in terms of sensible immune targeted drug combinations administered with checkpoint inhibitors. Other immunotherapies such as systemic administration of IL12 nanoparticles in soft tissue sarcoma, adoptive transfer of non-specifically activated T cells, depletion of regulatory T cells by using toceranib, and delivery of viral vectored cytokine in brain tumors are used (Dow 2020) (Tables 1 and 2).

Mice Model Categories

The pathogenesis and malignancy of many tumors are studied using various mouse models. The mouse models include genetically engineered mouse models, cell line-derived models, xenograft models, environmentally induced models, and chemical carcinogen-induced models, and syngeneic models.

Genetically Engineered Mouse Models (GEMMs)

GEMMs have substantially contributed to cancer research. In a natural immune proficient microenvironment, GEMMs generate de novo tumors. Advanced GEMM tumors resemble their human counterparts in histological and molecular characteristics, exhibit genetic variability, and spontaneously develop metastatic disease. GEMMs have been used to verify prospective cancer genes and therapeutic targets, evaluate efficacy of therapy, examine the effects of the tumor microenvironment, and investigate drug resistance mechanisms. The development of several types of transplantation-based and genetically altered tumor models to investigate cancer biology has been aided by advances in mouse genome engineering (Kersten et al. 2016). In the mid-1980s, the first GEM tumor models were simple transgenic mice with randomly integrated oncogenes controlled by a tissue-specific minimum promoter. Because of locus-specific gene-targeting methodology, the production of global tumor suppressor gene (TSG) knockout animals was made possible. GEM models not only accurately reproduce the molecular and histological characteristics of human illness, but they also have high predictive power for therapeutic response and resistance. GEM models can be employed for immunotherapy research and preventative investigations. The major disadvantage of GEM models is the inactivation of potent oncogenes and TSGs; the second disadvantage is due to a substantial multifocal initial tumor load; GEM models must be sacrificed before acquiring the

Table 1 Trials and results of immunotherapy in canine tumor

Trial	Delivery	Tumor type	Study primary endpoints	Secondary endpoints	Result
Her2 neu	Listeria vectored (IV)	Osteosarcoma	Metastasis	T cell responses	Increase OST vs historical control (Mason et al. 2016)
TERT vaccine	AAV vectored (IM)	B Cell Lymphoma	Progression	TERT antibodies	Increase OS (Peruzzi et al. 2010)
CD20 CAR T	Transduced autologous Tcell	B cell lymphoma	Safety	Tumor regression	Safety, tolerated, partial tumor responses (Panjwani et al. 2016)
NK cell ACT	Intertumoral administration	Osteosarcoma	Safety, tumor regression	Tumor infiltrates	Improved DFI, NK localization (Canter et al. 2017
Local superantigen immunotherapy	Plasmid DNA, intertumoral	Melanoma	Tumor regression	Immune infiltrates	Increased survival; CTL activity (Dow et al. 1998)
Liposomal MTP	IV, repeat infusions	Osteosarcoma	DFI and OST	Activation of Macrophages	DFI and OST significant increased (MacEwen et al. 1999)

Table 2 Different types of mouse models

Nude mouse models	Athymic Nude Mouse BALB/c Nude Mouse CD-1 Nude Mouse NIH-III Nude Mouse NU/NU Nude Mouse
SCID mouse models	Fox Chase SCID Mouse (C.B.17 SCID) Fox Chase SCID Beige Mouse NOD SCID Mouse SCID Hairless Congenic Mouse (SHC™) SCID Hairless Outbred (SHO) Mouse SCID/NCr Mouse
Triple immuno-deficient mouse model	NCG mouse
Germ free mouse model	C57BL/6
Inbred mouse model	B6 Albino Mouse BALB/c Mouse BALB/c Nude Mouse BALB/c-Elite Mouse C3H Mouse C3H SOPF Mouse C57BL/6 Mouse C57BL/6-Elite(SOPF) Mouse C57BL/6-Germ-Free Mouse CBA Mouse 129 Mouse 129-Elite (SOPF) Mouse
Humanized mouse model	HuCD34-NCG Mouse HuPBMC-NCG Mouse

metastatic disease. Because of the immunocompromised background required for PDX engraftment, they can't be used to examine immune cell function or evaluate immunotherapeutic strategies (Gengenbacher et al. 2017).

Genetically Engineered Mouse Models of OSCC

One of the most common human cancers is oral squamous cell carcinoma (OSCC). It accounts for 40% of all head and neck squamous cell carcinoma (HNSCC) cases. The tumor-bearing mice model provides a preclinical study platform and contributes significantly to discovering the OSCC cure. GEMMs allow for the editing of particular genes, leading to the over-expression of oncogenes and the silencing of tumor-suppressor genes. These GEMMs have pathological alterations similar to primary OSCC in people, such as hyperkeratosis, aberrant hyperplasia, carcinoma in situ, and invasive carcinoma. C57BL/6 is the first mouse strain to have its entire genome sequenced, and it is considered a "standard" inbred line capable of providing a genetic background for a wide range of mutant genes. As a result, C57BL/6 is commonly utilized as a transgenic mouse model in genetic investigations to imitate human genetic abnormalities. The presence of a recombinase controls the specificity

of conditional GEMMs. Conditional GEMMs have temporal specificity attributable to inducible promoters regulated by exogenous drugs like tamoxifen and RU486. The promoters are triggered when the substances are present or absent, and the expression of downstream target genes is altered. Cre transgenes expressed from tissue-specific promoters determine the spatial specificity or tissue specificity of conditional GEMMs. The keratin 5 (K5) and keratin 14 (K14) promoters are the best promoters for the oral cavity. K5 is expressed in the tongue's basal layer and the stomach's stratified squamous epithelia, whereas K14 is expressed in the oral mucosa and tongue's basal layer. ED-L2, an Epstein–Barr virus promoter, has also been found to target genes in oral and esophageal squamous epithelial cells. Several studies have now been conducted to induce oral carcinogenesis by altering the expression of oncogenes or tumor-suppressor genes and identifying the linkages between genes and cancer. The simple GEMMs can be utilized to uncover new pathways of tumorigenesis. In the future, they will be a crucial tool for researching proteins or gene alterations connected to oral cancer. However, its usage in highly aggressive OSCC is limited due to the substantial mortality induced by unanticipated primary tumors (Li et al. 2020).

Cell Line Derived Models

Cancer cell lines are useful in vitro model systems utilized in cancer research and medication development (Mirabelli et al. 2019). The development of long-term in vitro cultivated tumor cell lines and their in vivo inoculation in mice accounts for most of our present understanding of cancer. Due to their low cost, synchronous tumor growth, and ease of technological manipulation, these models can be employed for fast identification and validation of cancer-relevant genes as well as preclinical evaluation of treatment candidates (Gengenbacher et al. 2017). The disadvantage of cell line models is rapid non-autochthonous growth, perturbed tissue architecture, cell line-derived tumors, and loss of genetic heterogeneity. Two categories of cell line models are allografts and xenografts.

Xenografts

The human tumor xenograft is one of the most extensively used models. Human tumor cells are transplanted into immunocompromised mice, either under the skin or into the organ type where the tumor originated. Tumor will develop 1–8 weeks after the cells are injected. The different types of xenograft models are; Ectopic xenograft model, Orthotopic xenograft model, Metastasis model, and Patient-derived xenograft model; they are classified on the basis of transplant site. Mouse strains with decreased immunological response are used in the xenograft models, nude mice Foxn1 Nu/Nu, Balb/c, SCID mice (Severe combined immunodeficiency), NOD mice (Non-obese diabetic mice), and NOD/SCID IL2R$^{\gamma null}$ (NSG) mice. In the ectopic tumor xenograft model, human cancer cells are injected into the transplanted

site that differs from the origin of cultured cells. In the orthotopic tumor xenograft model, human cancer cells are transplanted into the same origin site of the tumor. Tumors that cause metastasis are studied in metastasis models. Tissues from the cancer patients are directly transplanted into the immunocompromised mice in the patient-derived xenograft model (Jung 2014). PDX model has been used in many cancer studies such as breast cancer, cervical cancer, ovarian cancer, pancreatic cancer, and endometrial carcinoma.

Patient-Derived Xenograft Model in Ovarian Cancer

BALB/c-nu/nu mice of age 6–8 weeks are used for the Patient-Derived Xenograft (PDX) model in and they are maintained under specific pathogen-free conditions. Specimens from ovarian cancer patients were collected from ovarian lesions or metastasis at the time of primary tumor reductive surgery. The specimen was kept under 0 °C for 2 h. The mice were anesthetized, and at the dorsal surface of the mouse, a 1 cm paramedian incision was made, then the tumor tissue was injected and sutured into the ovary of the mouse. The weight of the mouse was measured weekly. After 2 months, the tumor was collected, and the size of the tumor was measured, ascites and metastasis were observed. Then the portion was used immediately for in situ ovarian cancer model (Wu et al. 2019).

Patient-Derived Xenograft Model in Prostate Model

Prostate tumors are difficult to establish since it is heterogeneous disease; the PDX model is of great importance since it can retain the key features of the primary tumor. Non-obese diabetic (NOD)/SCID mice of age 6–8 weeks are used in the PDX models. Specimens are obtained from prostate cancer patients for xenografting. Tumors are cut into pieces and into the subrenal capsule of the mice model. Mice were sacrificed after 3–6 months of tumor growth. The tumors were extracted and regrafted under the kidney capsule of the NOD/SCID mouse. Serial SRC transplantation maintained the rapidly growing tumor. The static xenograft pieces can be maintained for up to 3 years (Lin et al. 2013).

Syngenic Models

Allografts immortalized from mouse cancer cell lines are engrafted back into the same inbred immunocompetent mouse strain as syngeneic models. Tumor rejection does not occur since the host, and cell line strains are similar, resulting in an immunocompetent model for immunotherapy testing. Syngeneic mouse models are widely used to demonstrate novel anticancer immune therapies. Mice with

functional immune systems are used in the syngeneic model, so it provides an effective method for studying the action of immune systems in cancer, unlike the xenograft model in which immunocompromised mice are used. BALB/cANHsd, C57BL/6NCrl female mouse of age 6–8 weeks are used in this model. The syngeneic panel model offers information on the cell lines, tumors' RNA sequences, immunophenotyping, and biomarker identification; this information may be integrated with the results of Vivo efficacy benchmarking profiles from popular checkpoint medicines. Tumors can be grown quickly in the syngeneic model. Metastatic modeling is aided by orthotopic bioluminescent syngeneic, which provides real-time, in-life simulation of metastatic invasion, lesions in secondary organs, and disease progression to the late stage. Only murine immunity can be studied in the syngeneic model, so there will be difficulty in comparing and predicting mouse immune responses to human responses. Due to the scarcity of cancer cell lines, not all cancers and cancer subtypes can be represented in the syngeneic model.

Chemically Induced Mouse Model

Chemically induced mouse models are the mouse models in which cancer is induced by chemicals. Chemically induced mouse models are used to study oral squamous cell carcinoma, ulcerative colitis, and Crohn's disease.

OSCC Mouse Model Induced by 4NQO

In oral squamous cell carcinoma (OSCC) carcinogenesis is induced by chemicals like 4-nitroquinoline-1-oxide(4NQO),Benzo[a]pyrene(B[a]P),NNN,Dibenzo[a,l]pyrene (DB[a,1]P) and combination of 4NQO and arecoline or ethanol. Most of these are the chemicals present in tobacco that are characterized to induce DNA adducts; the covalent binding of the chemicals to the DNA leads to mutation and causes cancer. 4-nitroquinoline-1-oxide (4NQO) is an aromatic amine heterocyclic compound that mimics tobacco since it induces cancer by causing oxidative stress, DNA adduction, mutagenesis, and tumor induction. It produces oxidative stress by generating reactive oxygen species (ROS), which attach to the nucleophilic part of the DNA, and causes mutations. At first, 4NQO is reduced to 4HAQO by NADH and NADPH, which will be acetylated by seryl-tRNA synthetase to form seryl-AMP enzyme complex; these are carcinogenic metabolites that will induce the formation of DNA adducts. 4NQO-induced OSCC mouse model is very similar to human OSCC at genetic and molecular levels. C57BL/6, BALB/c, CF-1, and CBA immunocompetent mice of age 6–8 weeks are used in the 4NQO- induced mouse models. In the OSCC mouse models, 4NQO is administrated by adding it to the drinking water or by topical application. This is the most widely used and best mouse model available for OSCC (Li et al. 2020) (Fig. 1).

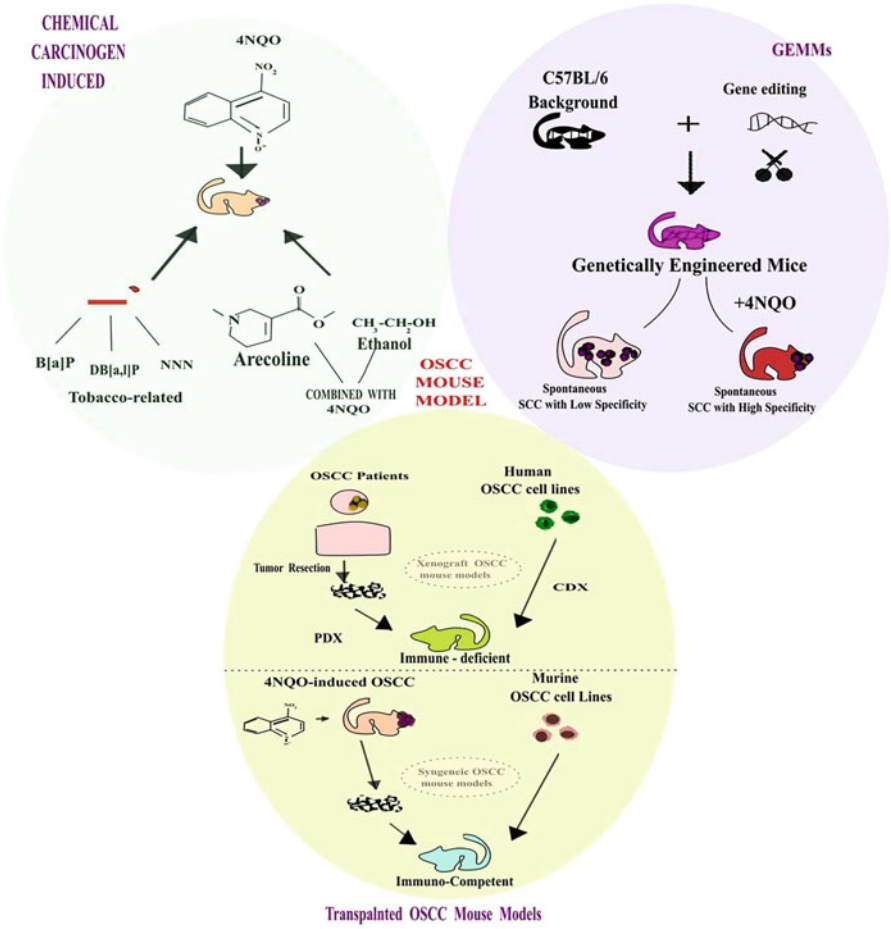

Fig. 1 Different approaches such as induction of chemical carcinogen, genetic engineering, and transplantation of tumor tissue or cancer cell lines utilized to develop mouse models of OSCC. CDX cell derived xenograft, PDX patient derived xenograft, GEMMs genetically engineered mouse models, SCC squamous cell carcinoma

Other Carcinogen-Induced Mouse Model

Benzo[a]preen (B[a] P) is a procarcinogen; when administered to B6C3F1 mice, it can cause the development of tongue lesions. When administrated in high doses, B[a] P can induce tongue cancer. Dibenzo[a,l]pyrene is also present in tobacco. It is administrated to B6C3F1 mice to induce cancer in the lung, skin, and mammary gland. Administrating 4NQO with alcohol increases the chances of oral cancer (Li et al. 2020).

Humanized Mouse Models

The humanized mouse is a new preclinical model for researching the human disease that is created by transplanting viable human cells or tissues into immunodeficient $Il2rg^{null}$ mice. The technology of humanized mice is rather immature as a novel model, and the present humanized immunological mouse model system has undoubtedly many flaws. Human cells produced from humanized bone marrow, infiltration of human T and B cell populations, lymphangiogenesis, cytokine production, and a dynamic microenvironment were all observed in tumors of a xenochimeric mouse (XactMice). The technology of humanized mice is rather immature as a novel model, and the present humanized immunological mouse model system has undoubtedly many flaws. The immunological response in the humanized mouse, for example, could be due to tissue incompatibility. The transplanted human immune cells, primarily T cells, will trigger GvHD and an immunological attack in recipient mice, resulting in their mortality, hence the experimental window is limited. Nonetheless, because the humanized mouse is perfectly in line with the necessity to create a replica of the disease, it is a promising avenue for future mouse model research (Li et al. 2020).

Conclusion

In the last 10 years, we've seen a significant increase in our collective understanding of companion animal malignancies, as well as the importance of these animal patients in therapeutic development and optimization efforts for human. Furthermore, novel treatment techniques for companion animals are being developed as a result of this research. The phase of potent cancer immunotherapy represents a significant shift in cancer treatment, and the dog cancer model offers a unique chance to contribute to this field's development. With their shared tumor forms, dogs and humans can both benefit from this study. The mouse has many similarities to human which make them the most suitable animal model in oncology research. Different types of mouse models are used in cancer research to understand the pathogenesis and malignancies of cancer which includes cell line-derived models, xenograft models, environmentally induced mouse models, syngeneic models, and chemically induced mouse models. They help researchers to discover drugs, understand the cancer genes' therapeutic efficacy and drug resistance in different carcinoma. Mouse models used in prostate cancer, oral squamous cell carcinoma, and ovarian cancer paved the way to better understand cancer.

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Orthotopic PDX and CDX Mice Model for Cancer Stem Cell Research

25

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Abstract

The advancement of cancer research definitely enlightened the survival of cancer patients in the United States; however, more translation and preclinical research are needed to prevent the death of cancer patients in the USA and worldwide. Both preclinical and translational research required patient-derived xenograft (PDX) and cell line-derived xenograft (CDX) models to design and screen rapidly anticancer drugs against drug-registrant cancer stem cells of different cancers. There are increased applications of different cancerous cells or tissues from the tumor of cancer patients that are implanted in immunodeficient mice to simulate human aggressive tumor growth in vivo, which are aimed to intervene by designing specific anticancer drugs. The PDX and CDX models are extensively used in cancer research in current years. These models are able to reproduce stably the patients' tumors in relation to gene mutation, gene expression, reprogramming of drug resistant heterogenic cancer stem cells, inflammation, histopathology, genetic mutations, and therapeutic efficacy of different types of cancers, namely, pancreatic cancer, serous carcinoma, glioblastoma, lymphocytic leukemia, brain cancer, gastric cancer, lymphoma, colorectal cancer, and hepatic carcinoma. Therefore, both PDX and CDX models permit precious evaluation in tumor biology, preclinical study, finding therapeutic signaling pathways, and evaluation of anticancer drugs against different cancers.

Keywords

PDX · CDX · hu-PBL · Hu-SRC · hu-BLT

Abbreviations

CTCs	Circulating tumor cells
FGS	Precise fluorescence-guided surgery
HCC	Hepatocellular carcinoma
NSCLC	Non-small-cell lung cancer
PDX	Patient-derived xenograft

Introduction

Immunodeficient mice are used for PDX and CDX models for different cancer research. Therefore, the highly immunodeficient mouse strain, namely, athymic nude mice, NOD-SCID, SCID, and recombination-activating gene 2 /Rag2-knockout mice, are mostly used to establish cancer cell line xenograft PDX or CDX model. The efficient engraftment in immunodeficient mice is performed by using primary cancerous cells or tissues. The genetic features of the patient tumors also showed in serial transplantation of the xenograft lines in mice. The close relationship of 3D in vitro heterogenous cancer stem cells spheroids (Das et al.

2022, Das et al. 2021a) and in vivo tumor cell heterogeneity of PDX and CDX model gives an ample scope of investigation for new drug discovery and cancer stem cell signaling studies in cancer research. Methodology to establish PDX and CDX model in cancer stem cell research. Use of Immunodeficient mice. The patient-derived tumor cells or human cell lines are maintained in laboratory and cultured in proper cell culture media to make the 3D tumor spheroids (Das et al. 2021b, c). These spheroids are implanted with Matrigel as subcutaneous (Das et al. 2018) or intracranial implantation as CDX or PDX model. Both innate and hematopoietic immune systems played vital role during transplantation of human-derived peripheral blood mononuclear cells (PBMCs) and hematopoietic stem cells (HSCs) in the humanized mouse model (de la Rochere et al. 2018; Flerin et al. 2019; Meraz et al. 2019). Scientific studies showed the significant comparability of CDX and PDX tumors biological reaction as well as cancer patients, because of the steady growth of these xenograft models in the humanized mice (Zhao et al. 2018). The contest of immune rejection has been resolved by the modifications of specific genes to make immunodeficient and lack of any T, NK, and B cells in these mice. Therefore, the creation of PDX and CDX into humanized mice models is significant tread to introduce diverse applications including onco-immunology, cancer pathogenesis, drug-registrant, and anticancer stem cells' therapeutic effects (Ny et al. 2020; Wang et al. 2018; Buque and Galluzzi 2018). The establishment of immunodeficient mice was improved over the time by specific genes editing process by genetic engineering to allow immunodeficient mice to receive the transplanted patient-derived tumor or cell line-derived 3D tumor-spheroid cells (Table 1).

The human malignant tumors are xenografted in nude mice where tumors can grow as lack of T lymphocytes in the nude mice; however, severe tumor rejections are observed due to B cells and innate immune cells (Fujiwara 2018; Flanagan 1966). There was a spontaneous subgene mutation of Prkdc resulted the C.B-17-Prkdcscid (C.B-17 scid) mice strain (Bosma et al. 1983) and the mutant Prkdc directed to the nonexistence of T and B lymphocytes, which showed the severe combined immune deficiency (scid) syndrome (Bosma and Carroll 1991). Eventually, the necrotic NOD scid mice strain was developed by scid mutation of nonobese diabetic (NOD) mice that showed better compatibility of human immune system with faulty levels of NK as well as myeloid cells (Shultz et al. 1995; Pflumio et al. 1996; Greiner et al. 1995). The expression of murine cytokines, including IL-2, 4, 7, 9, 15, and 21, was observed due to the knockout mutation of interleukin-2 receptor common γ-chain ($IL2rg^{null}$) in humanized mice (Shultz et al. 2005; Ito et al. 2002; van Rijn et al. 2003). The BRG (Balb/c $Rag2^{-/-}IL2rg^{-/-}$), NSG (NOD.Cg-$Prkdc^{scid}IL2rg^{tm1Wjl}$), and NOG (NOD.Cg-$Prkdc^{scid}IL2rg^{tm1Sug}$) were developed as severely immunodeficient mice strains due to $IL2rg^{null}$ mutation together with scid mutation (Wege 2018). The highly accepted immunodeficient models, namely, NPG (NOD-$Prkdc^{scid}IL2rg^{null}$) and NCG (NOD-$Prkdc^{em26}IL2rg^{em26}$Nju), were developed by Rag knockout (Liu et al. 2015; Bai et al. 2015; Yu et al. 2017). Therefore, these new mice generations have revealed a dramatic enhancement in the rate of CDX and PDX.

Table 1 Establishment of different strains of immunodeficient mice

Mice strains	Year	References
Nude mice	1966	Flanagan (1966)
C.B -17 scid mice	1983	Bosma et al. (1983)
Rag-/- mice	1992	Mombaerts P (1992)
NOD-scid mice	1995	L.D. Shultz et al. (1995)
NOG mice	2002	Ilto M et al. (2002)
BRG mice	2003	Van Rijn et al. (2003)
BRG	2004	Traggiai E et al. (2004)
NSG mice	2005	L.D. Shultz et al. (2005)
C.B17-scid-IL4	2008	Okuma K et al. (2008)
BRG-IL-4	2008	Okuma K et al. (2008)
TK-NOG	2011	Hasegawa M et al. (2011)
NOG-Iab KO, HLA-DR 0405 Tg	2012	Suzuki M et al. (2012)
NOG-DLL1	2012	Ito R et al. (2012)
BRG-nude	2013	Suemizu H et al. (2013)
NOG-JAG1	2014	Negishi N et al. (2014)
NPG mice	2015	Kang Liu et al. (2015)
NCG mice	2017	Yu Y et al. (2017)
NOG-EXL, IL5 mice	2018	Ito R et al. (2018)
NOG-IL5	2018	Ito R et al. (2018)
NOG-IL6	2018	Hanazawa A et al. (2018)
NOG-IL15	2018	Katano I et al. (2018)
NOG-IL1b/IL23	2019	Ito R et al. (2019)
NOGF-IL15	2020	Katano I et al. (2020)
NOG-ΔMHC	2020	Ka et al. (2020)
NOG-mdx	2021	Nalbandian M et al. (2021)

Primary Tumor Tissues or Cells

The successful tumor engraftment into immunodeficient mice generally depends on the viability and sterility of the patient's tumor. The patient-derived tumors are implanted into immunodeficient mice either in the form of small tumor fragments or the cell suspensions resulting from the patient's blood or after digestion of tumors into the single-cell suspensions. The heterogenous cancer cells or tissues are mixed with basement membrane protein matrix (Matrigel) before injected into recipient mice for CDX or PDX model, which allows efficiently the growth of tumors, without losing of the primary tumor phenotype. The tumor cells can also be co-injected with other cells, namely, fibroblasts, stromal cells, astrocytes, glial cells, and endothelial cells, according to different experimental aims.

The Heterotopic and Orthotopic Implantation

In both CDX and PDX models, the heterogenous cancer stem cells and patient's cancer cells or tissues are implanted heterotopically or orthotopically and observed

for tumor development. The heterotopic implantation is a simple technique of cell implantation and precise monitoring of the tumor size compared to orthotopic implantation. Generally, subcutaneous and intravenous PDX models are extensively used for solid tumors and leukemia cancer research. The orthotopic implantation is more complex and requires for ultrasound examinations or exploratory laparotomies to check the existence of tumors internally. However, the orthotopic tumors are more close to human tumors. The orthotopic implantation also amplified the chances of metastases of xenografts. The small quantities of magnetic assorted cancer stem cells (MACSC) of spheroid cells (Das et al. 2015) from patient-derived tumors or cancer cell lines are able to develop primary subcutaneous tumors in F1 mice. Then these tumors orthotopically move into succeeding generations of mice.

Development of Next-Generation PDX and CDX Models in Humanized Mice

Recently, the immunotherapies showed an important application to control tumor progression, which need PDX and CDX models to enable the learning of preclinical assessment and immunity-cancer interactions for cancer immune therapies. Therefore, these human immune system or human hemato-lymphoid chimeric mice models are required first to create a human immune conditioned system. Then peripheral blood or tumor-infiltrating lymphocytes are transplanted into immunodeficient mice. In another method, the CD34+ human hematopoietic stem cells alone or in combination with human thymic tissue are transplanted into immunodeficient mice. The CD34+ human hematopoietic stem cells produce the several lineages of human blood cells in mice. There are several genetically modified immunocompromised mouse strains, namely, NSG-SGM3, NOG-GM3, and MISTRG are developed which advanced the integrity of engrafted human immune system (Byrne et al. 2017). Therefore, the both next-generation PDX and CDX models will advance the field of cancer research as well (Fig. 1).

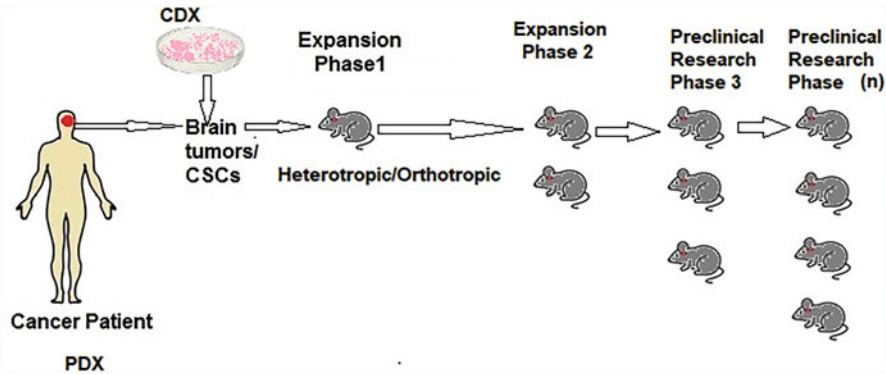

Fig. 1 Methodology to establish PDX and CDX model in cancer stem cell research

The mutual repulsion is caused by innate immune system in humanized immune mouse models. The development of sever immunodeficiencies mice minimized the innate immune repulsion and the metrologies of generation of such mice and corresponding characteristics are mentioned as follows: (**i**) Hu-PBL, (**ii**) Hu-SRC, and (**iii**) Hu-BLT.

Hu-PBL Mouse Model

The hu-PBL mouse model is well accepted as low expenditure and having the simplest establishment process. The majority of engraftment that comprised of T cells is done by intravenous injection (I.V.) during transplantation of human PBMCs into immunodeficient mice (Flerin et al. 2019). The microenvironment for the development of T cells may be hardly affected by absence of specific cytokines which is necessary for survival of hu-PBL mouse and the absence of cytokines prohibits the growth of B and NK cells in vivo (Shultz et al. 2007). Therefore, the hu-PBL mouse model could recreate the higher levels of human mature T cells and the gaining of CD3+, with in 4 weeks (de la Rochere et al. 2018; Walsh et al. 2017). For the development of an ideal humanized mouse model with IgA nephropathy, the administration of IL-18 is necessary to engraft the human CD4+ and CD8+ T cells (Senpuku et al. 2002). Harui A et al. mentioned another combination with donor-matched dendritic cells (DCs), which will represent the better development for antigen responsiveness in the absence of antigen-presenting cells (Harui et al. 2011). Therefore, these discoveries showed that recombinant human prolactin (rhPRL) stimulation could augment the human T cells engraftment into the spleens, thymus, and lymph nodes, which is susceptible to re-formation of the human immune system of hu-PBL mice (Sun et al. 2004). The development of severe graft-versus-host disease (GVHD) after PBMC injection limited the use of hu-PBL mice (Pyo et al. 2019). However, scientific studies also showed that the predepletion of CD4+ T cells could improve GVHD symptoms, even with the decrease in application opportunity (Li et al. 2020). The general method to increase the survival period of grafted mouse is to give the interracial immune rejection for genetic knock-out of the murine major histocompatibility complex (MHC) (Brehm et al. 2019).

Hu-SRC Mouse Model

The Hu-SRC mouse model has also been developed as a more complete immune re-formation, to understand different human diseases. In this model, the CD34+ HSCs got from the human umbilical cord blood (UCB), bone marrow, fetal liver (FL), and granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood (MPB) are used to grow the multilineages of hematopoietic stem cells (Watanabe et al. 2009). The human CD45+ T cells developed 25% in peripheral blood of this mouse model (Meraz et al. 2019) and the host mouse did not show any immunological rejection up to 12 weeks (Danisch et al. 2019). The main factors for the differentiation and maturation of immunocytes are principally associated with the route of injection, HSC source and age of the recipient mouse. Lepus et al. showed that the significant development of human CD45+ cells is more from FL or UCB-derived HSCs than from MPB and bone marrow cells (Lepus et al. 2009). Surprisingly, the newborn immunodeficient mice

implanted with showed the enhanced T-cell maturation than adult mice (Katano et al. 2014). This mouse model only showed the problem by developing H2-restricted T cells, which is caused by the maturation of human T cells in the murine thymus (Watanabe et al. 2009). The sublethal γ-irradiation is necessary to facilitate human HSC engraftment in this mouse model (Audige et al. 2017).

Hu-BLT Mouse Model

The hu-BLT (bone marrow, liver, and thymus) mouse model has been developed to encourage the T-cell maturation in human thymus, to remove the murine H2 restriction, and to avoid the unlikelihood of antigen-specific responses (Lan et al. 2006). The advancement of the protocol with this mouse model showed the concurrent implantation of human fetal thymus (FT) and FL pieces into the subrenal capsule, with the injection of CD34+ HSCs (Brainard et al. 2009). For the investigations of immunopathogenesis and hepatotropic virus infections, Jinglong Guo et al. mentioned that the hepatocyte engraftment showed a good re-form for the immunocytes and chemokines in the liver (Guo et al. 2018). This methodology showed the highest level of B- and T-lymphocytes, macrophages, and dendritic cells and served for investigating mucosa-associated diseases, namely, Ebola virus infection and human immunodeficiency virus (HIV) infection (Escudero-Perez et al. 2019). Therefore, the hu-BLT mouse model is used for HIV infection and prevention research (Karpel et al. 2015). Prominently, this model is an ideal model in immunology research including chemotherapeutic drug response, tumor immunotherapy, and the prediction of cytokine release (Yan et al. 2019; Akkina 2013; Kaur et al. 2018). Kerry J Lavender et al. showed that the other strains with the genetic inactivation of CD47 on the C57BL/6 $Rag2^{-/-}$ $\gamma c^{-/-}$ are able to survive 15–20 weeks more than hu-BLT model (Lavender et al. 2013). The hu-BLT model showed that the presence of the incompatible HLA between immunocytes and tumor tissues is susceptible to immunologic rejection, signifying that the engraftments for generation should be derived from the same donor or HLA-matching. Therefore, the intricate methods and ethical issues showed main difficulties for the extensive use of hu-BLT model. The following table and figure show the outline of features of different mice strains (Table 2 and Fig. 2).

Usefulness of PDX and CDX Model in Cancer Research

The tumorigenesis, tumor characterization, and metastasis in cancer research, improved understanding of cancer, anticancer drug design, and investigation of signaling mechanism of cancer stem cells are valuable, which require to use of PDX as well as CDX models.

Usefulness in Tumorigenesis Research

The PDX and CDX models are extensively used to investigate the molecular mechanism of cancer stem cells (CSCs) initiation, reprogramming, proliferation,

Table 2 The differences and features among Hu-PBL-SCID, Hu-SRC-SCID, and Hu-BLT-SCID mice

	Hu-PBL-SCID	Hu-SRC-SCID	Hu-BLT-SCID
Source of immunocytes	PBMCs	FL; MPB; bone marrow; UCB	FL; FT
Immune reformation	Mostly T cells	Human hematopoietic lines	Immune system totally functional
Advantages	Mature efficient T cells; rapid formation	Injection to newborns increase reconstitution; long experimental window without GVHD	HLA-restricted T cells; development of mucosal human immune system
Restrictions	Absence B or myeloid cells; expansion of GVHD within 4–6 weeks	H2-limited T cells; extended period of cell differentiation; essential sublethal γ-irradiation	Extended period of cell differentiation; essential sublethal γ-irradiation; increased opportunity of GVHD; erudite technique and obligatory material

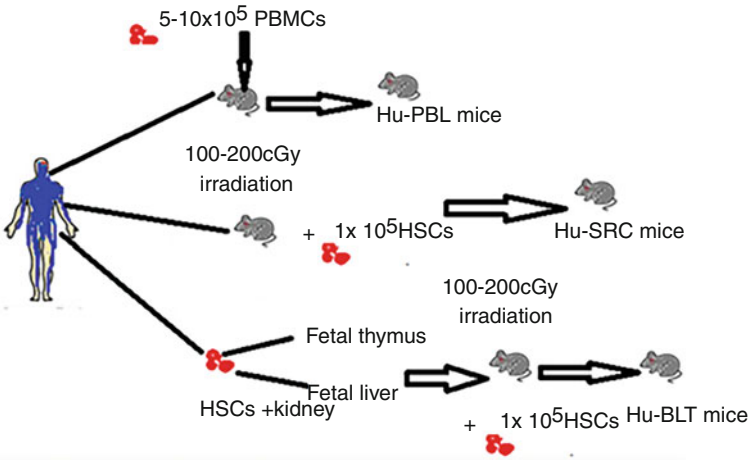

Fig. 2 The outline of features of different mice strains

and progression. The heterogenic CD133$^+$ human brain CSCs that recruit heterogenic tumor cells in vivo, supporting the understandings of human brain tumor pathogenesis by Xenograft assay, evidenced CSC hypothesis for investigating many solid tumors of different cancers (Singh et al. 2004). The fundamental molecular mechanisms of 3D in vitro spheroid tumorigenesis are well investigated in different cancer cell line xenograft (CCLX) in vivo models. For instance, the role of leucine zipper-containing ARF-binding protein (LZAP) in inhibition of heterogenous tumor cells' growth and vascularity was studied by cancer cell line xenografts (Wang et al. 2007); Notch- and Hedgehog-dependent CSCs were found in prostate cancer CCLX models (Domingo-Domenech et al. 2012); the role of noncoding

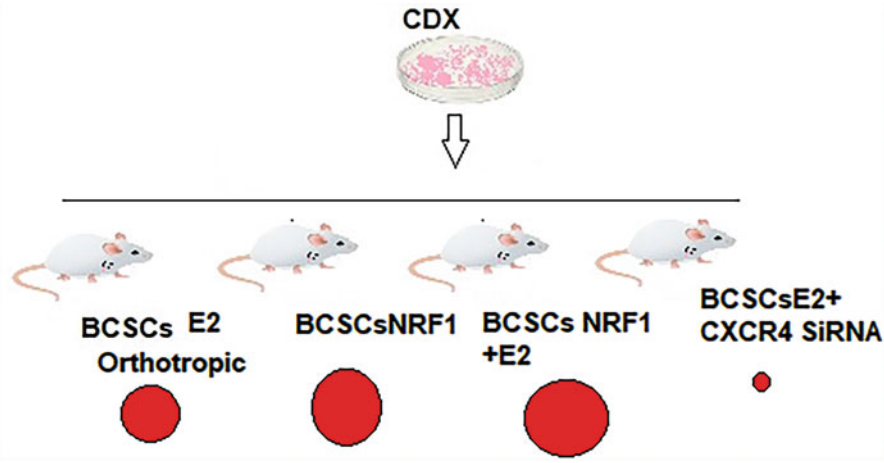

Fig. 3 The different heterogeneous breast cancer stem cells (BCSCs; CD44 + CD49f + CD133 + CXCR4+) showed different tumor sizes drive by NRF1 as oncoprotein induced with estrogen in CDX Xenograft model (Das et al. 2018)

RNAs (lncRNAs) in castration-resistant prostate cancer cell lines was found to inhibit tumor xenograft growth in vivo (Yang et al. 2013). The expansion of cancer stem cells of spheroids and patients' tumors are used to expand to find molecular target by inhibitors, agonists, or small molecules (Das et al. 2021c). The commencement of prostate cancer is determined by N-Myc and AKT1 signaling, as demonstrated by the in vivo study in prostate epithelial cells of NSG mice (Lee et al. 2016). The role of NRF1 as oncoprotein in estrogen-induced heterogenous CD44 + CD49f + CD133 + CXCR4+ breast CSCs was reported in CDX Xenograft model (Das et al. 2018) (Fig. 3). The MiRNA-126 alleviates B-ALL in a proliferative B cell by aiming cell cycle or apoptosis and p53 response genes and antagonizing miRNA-126 in human B-ALL reduce tumor growth in its PDX model (Nucera et al. 2016). The PDX models, helped also to investigate the role of several somatic mutations for inducing tumor formation and the role of resistant cancer stem cells for cancer therapy (Berger et al. 2016). The proliferation of heterogenous cancer stem cells always associated with the growth of cancer cells in both PDX and CDX mice (Das et al. 2018). The metabolic genes related to aggressive breast cancer and stemness were ascertained in vivo tumorigenesis by recognizing novel cancer targets with negative-selection RNAi in breast cancer xenograft model (Possemato et al. 2011).

Usefulness in Preclinical Cancer Research

The necessity of new anticancer drugs is anticipated against drug-resistant cancer stem cells for the development of therapeutic approach for different types of cancers. Both PDX and CDX models help to find the molecular relationships of therapeutic

targets for specific molecules in preclinical research. The PDX models are also used for the clinical treatment. Importantly, PDX models are used to design appropriate therapy for cancer patients (Fig. 2), also used to recognize chemotherapy-resistant patients with different types of cancers (Dobbin et al. 2014), and showed the close relationship between drug resistance cancer stem cells and genetic alterations (Lohse et al. 2015). The PDX and CDX models are very appropriate to discover new therapeutic approaches, designing anticancer drugs with exiting chemotherapies, surgery, radiation, and beneficial with genetic and nanoparticles therapies. The PDX model showed the effectiveness of BYL719 with P-selectin-targeted nanoparticles against tumors of head and neck cancer (Mizrachi et al. 2017). The PDX models of glioblastoma (Bago et al. 2016), pancreatic cancer (Yano et al. 2015), and colon cancer (Hiroshima et al. 2014) are proved effective for preclinical evaluation of fluorescence-guided surgery (FGS) of patients. Both PDX and CDX models are definitely helpful for preclinical drug designing against different types of cancers. The PDX models have been used in preclinical drug assay for many different cancers, namely, melanoma, (Fiebig et al. 2007) breast cancer (Marangoni et al. 2007; de Plater et al. 2010) colon cancer (Jin et al. 2011), pancreatic cancer (Rubio-Viqueira et al. 2006), non-small-cell lung cancer (NSCLC) (Zhang et al. 2013; Merk et al. 2009), and prostate cancer (Yoshida et al. 2005; Tentler et al. 2012). The use of CCLX or CDX models is also appropriate by using 3D spheroids for preclinical anticancer drug screening (Das et al. 2021c) and development against CSCs of different types of cancers.

Evaluation of Novel Anticancer Drugs

The PDX models are important preclinical tools for the evaluation and designing of novel anticancer drugs in vivo which required chemotherapy against different types of cancers. The PDX models have been shown to investigate many small molecules (Das et al. 2021c), namely, kinase inhibitors, against different cancers. The kinase inhibitors are evaluated using PDX models in NSCLC (Martin et al. 2016) chordoma (Siu et al. 2013), gastric cancer (Li et al. 2013), and cholangiocarcinoma (Wang et al. 2016a). The PDX model showed the effectiveness of FP3, a VEGF blocker in gastric cancer (Jin et al. 2012). The CXCR4 has major role associated with migration and progression of T-ALL cell leukemogenicity (Passaro et al. 2015) and breast cancer (Das et al. 2018). The PDX models have been used in aiming CXCR4 by small-molecule antagonists against tumors of T-ALL (Pitt et al. 2015) and for inhibition of MDM2–p53 interaction against the tumor growth of NSCLC (Hai et al. 2015). The antibodies therapy against various cancers (Wang et al. 2016b; Wu et al. 2012) is well accepted and the effectiveness of these antibodies has been evaluated by PDX models. For example, the PD-1/PD-L1 antibodies have been evaluated using NSCLC PDX models (Lai et al. 2017). The PGE2-neutralizing antibody in PGE2 signaling was identified using PDX model to design drug celecoxib against CSCs of bladder cancer (Kurtova et al. 2015).

Finding of Cancer Stem Cells and Cancer Biomarkers

The PDX models were extensively used in preclinical cancer research to find the cancer stem cells and cancer biomarkers. The tumor and stromal biomarkers have been discovered by using PDX models (Bradford et al. 2016). The PDX models have been used to find biomarkers in colon cancer (Metildi et al. 2014), stem cell biomarkers in hepatocellular carcinoma (HCC) (Zhao et al. 2016), biomarkers in bladder cancer (Skowron et al. 2016), epigenetic biomarkers (SLFN11 and EZH2) in SCLC (Gardner et al. 2017), and the IGF-1 receptor biomarker in multiple myeloma (Lai et al. 2017). The PDX model of human breast cancer was examined to find and separate breast cancer stem cells (BCSCs) by fluorescence-activated cell sorting (FACS) (Das et al. 2018). The CSCs have significant role in tumor metastasis and have been investigated and isolated from patients' heterogenous tumors. These CSCs have been established in the PDX models of prostate (Lai et al. 2017), breast (Das et al. 2018), and pancreatic (Lai et al. 2017) cancers (Table 3).

Next Generation Humanized Mice

The use of genetic engineering and advance technology propelled to develop humanized mice model with several key boundaries. Therefore, conclusive removal is required before HSC transplantation that will provide enough time for HSC cultivation in the bone marrow. The NBSGW mice with the mutation of w41 in c-Kit showed the HSCs with the expression of c-Kit (CD117) and resulted in the better HSC engraftment. The NBSGW mice also showed increased amount of erythropoiesis and platelet development than irradiated NSG mice to investigate the human HSC differentiation and pathophysiology.

The absence of cross-reactivity of cytokines showed the minimal production of functional lymphoid cell differentiation and this was caused by the absence of IL-15,

Table 3 Different types of cancers with associated cancer biomarkers were observed and PDX or CDX model used in these studies

Types of cancers	Biomarkers	PDX/CDX used
Breast cancers	BCSCs (CD44 + CD49f + CD133 + CXCR4+)	Yes
Colon cancer	CD44, CD133, CD24, EpCAM, LGR5, ALDH	Yes
Hepatocellular carcinoma	CD24, CD133, CD44, EpCAM	Yes
Bladder cancer	CD44, CD67LR, EMA, ALDH1A1, SLFN11, and EZH2	Yes
SCLC	CD133, Sall4, Oct4, Nestin, NCAM, S100β, vimentin, CD44, and CD105	Yes
Multiple myeloma	IGF-1, CD117	Yes
Prostate	EpCAM, EZH2, PSA, ALDH1, CD133	Yes
Pancreatic	CD 19 + 31 + 45 + 133+	Yes

IL-7, IL-3, IL-4, thrombopoietin (TPO), and stem cell factor (SCF). Therefore, the replacement of the coding genes of mice with human genes possibly will enhance the targeted expression and will overcome any limitation. The human IL-7 and IL-15 double knock-in NSG mice engrafted with human HSCs showed the amplified amount of human NK cells (Yin et al. 2020). The gene knock-ins of diverse cytokines and growth factors, namely, TPO, macrophage colony-stimulating factor (M-CSF), IL-3, granulocyte macrophage colony-stimulating factor (GM-CSF), SCF, or signal-regulatory protein alpha (SIRPα), into the humanized mouse models, namely, MISTRG, NSG-SGM3, and NOG-EXL mice, showed the growing amount of lymphoid cells (Table 4). The immature T and B cells of the humanized mice engrafted with HSCs barely showed any IgG antibody response to antigen. Qingfeng Chen et al. showed that the treatment of GM-CSF and IL-4 can fix the fault by exciting the Ag-specific CD4+ T cells and by initiating the B cells response for investigating antibodies against clinically related targets. The cytokines, namely, IL-1β, IL-6, IL-23, and TGF-β, induced the differentiation of IL-17-producing T-helper 17 (Th17). It was found that there was a high development in interferon-gamma (IFN-γ)-producing pathogenic Th17 cells and IL-17 in the skin of the mice exhibiting human IL-1β and IL-23. Ryoji Ito et al. reported that in a pathogenesis investigation for airway inflammation, the introduction of human IL-33 triggered Th2 and mast cells in the humanized mouse model caused asthma (Yin et al. 2020). Therefore, it could be concluded that during several difficulties in the differentiation and development of immunocytes, the cytokines played one of the most important roles to avoid the bias for the immunocyte development process.

The disparity of MHC among the humanized mice species showed some limitations, and in case of hu-PBL mice, this showed unembellished GVHD as well as the imperfection of T cell role. Similarly, the NOG-MHC double knockout

Table 4 Different humanized mice model and transgenic platforms

Mice strain	Major molecules	Benefits	Restrictions
NBSGW	c-Kit	Deprived of irradiation; expansion of erythropoiesis and platelet	Not described
NSG-SGM3	IL-3, SCF, GM-CSF	Steady HSCs engraftment; amplified monocytes, DCs and macrophages	Decreased stem cell function, short-term re-formation
NOG-EXL	IL-3, GM-CSF	Unchanging HSCs engraftment, augmented monocytes, DCs, and macrophages	Augmented anemia
MISTRG	TPO, IL-3, GM-CSF, M-CSF, SIRPα	Unchanging HSCs engraftment; augmented monocytes, macrophages, and DCs; enhanced NK cells growth	Amplified anemia, shorter lifespan
NOG-dKO	$IA\beta^{null}$, $\beta2m^{null}$	Reduced GVHD	Fewer T-cell differentiation
NSG-HLA	HLA class I and II	Antigen-specific IgG responses, HLA-limited B- and T-cell functions	Predetermination of HLA in donor cells

(NOG-dKO) mice for murine class I and class II MHCs ($\beta 2m^{null}$ and $IA\beta^{null}$) exhibited slighter xenograft denunciation and GVHD, in a long-term experiment. Therefore, NOG-dKO mice were likely to use compared to NOG mice for the precise assessment of antitumor T-cell response in immune checkpoint therapies due to absence of the GVHD-induced nonspecific T-cell formation. As the appropriate model for studying vaccines and immunotherapies, the advanced transgenic engineered humanized mouse model was used for the development of HLA class I and/or HLA class II molecules by human HLA-restricted B- and T-cell functions and antigen-specific IgG retorts (Yin et al. 2020).

Generation of TME in Hu-PDX Mice for Cancer Immunotherapies

The tumor development and therapeutic effectiveness have been measured to be tremendously connected to tumor microenvironment (TME), which consisted of lymph vessels, blood vessels, stromal cells, fibroblast, the extracellular matrix (ECM), and immunocytes. In onco-biology research, the complex tumor microenvironment is significantly investigated for the tumor diagnosis, prevention, prognosis, tumor development, and metastasis. Therefore, the necessity of humanized mice with cancer cell lines has been attributed to personalized cancer patient treatment as well as immunotherapeutic evaluation. Both the PDX and CDX models showed the gene expression outlines and drug responses of individual patient-derived tumors, that reiterated the TME to design drugs for the genomic diversity, and showed as the most dependable tumor model. Therefore, the heterogenic cancer stem cells of tumor in PDX and CDX into immunosuppressed mice showed the challenge to establish the stable existence of human tumor-infiltrating lymphocytes (TILs) for the exact assessment of immunotherapies. The humanized PDX and CDX mice model moderately replicates the TME alike to humans, and the cytokines and chemokines produced by TILs, tumor cells, and stromal cells regulate the angiogenesis, immune responses, and metastasis. The myeloid-derived suppressor cells (MDSCs) exhibited a heterogeneity with adequate immunosuppressive function in TME and showed the T cell inactivation, which resulted to the induction of regulatory T cells (Tregs) in the presence of IFN-γ and IL-2 as well as the release of MDSC-induced reactive nitrogen species (RNS). The cancer-associated fibroblasts (CAFs), CD8+ cytotoxic T lymphocytes (CTLs), immunocytes, MDSCs, and tumor-associated macrophages (TAMs) promoted the generation of cytokines in TME. Recently a research study of murine and clinical trials showed that the suppressive signals of TME were due to dysfunctional states of intratumoral T cells through the metabolic factors, soluble mediators, and hypoxia. Therefore, the character of TME in tumorigenesis and development might offer new approaches for the tumor therapies. Thus, the concurrent implantation of PDX and humanization reiterates the interplay between the tumor and immunocytes, which showed the growing dimensions for each hypothesis conclusions (Yin et al. 2020). Applications of the humanized mice model for immunotherapy are shown in Table 5.

Table 5 Applications of the humanized mice model for immunotherapy

Different diseases	Origin of tumors	Immune reformation	Therapies
Leukemia	CDX	HSCs	WT-1 TCR-T therapy
B-ALL	PDX	HSCs, FT, FL	Anti-CD19 CAR-T therapy
Melanoma	CDX	HSCs, FT, FL	F5 TCR-T therapy
Gastric cancer	CDX	PBMCs	chA21-4-1BBz CAR-T therapy
Pancreatic cancer	CDX	PBMCs	PSCA CAR-T therapy
Colorectal cancer	PDX	HSCs	Anti-PD-1 therapy
NSCLC	CDX	PBMCs	EGFR CAR-T therapy
NSCLC	CDX&PDX	PBMCs, HSCs	Anti-PD-1/PD-L1 therapy
TNBC	PDX	HSCs	Anti-PD-1 therapy
Osteosarcoma	CDX	PBMCs	Anti-PD-1 therapy
Mesothelioma	CDX	PBMCs	CAR-T + anti-PD-1 therapy
Ovarian cancer	PDX	TILs	Anti-PD-1 therapy
Lymphoma	CDX	HSCs, FT, FL	Anti-PD-1/CTLA-4 therapy
HCC	PDX	HSCs	Anti-PD-1/CTLA-4 therapy

The Genetic-Modified T Cells Used in Humanized Mice

The adoptive cellular therapy (ACT) is used for the development of the immunocompetent cells in vitro and is reinjected back those cells to patients themselves, that excite the immune response for targeted killing tumor cells of patients. The ACT infusion technique is chiefly relied on engineered T cells that develop the affinity with tumor-associated antigens (TAAs), direct chimeric antigen receptors (CARs), and transgenic T cell receptors (TCRs). Therefore, the humanized mice were preferred to immunodeficient mice because of the mechanism and safety of TCR-T/CAR-T cell therapies and for the improvement of experimental recipients in vivo by using ACT as the benefits for cancer patients. The TCRs have natural antigen receptors that arise on the surface of T cells and bind its similar tumor antigen through MHC-peptide complex. Therefore, in TCR-T cell therapy, the TCR gene sequence specified TAAs of T cells helped to destroy tumor cells and the transgenic TCRs have been investigated in humanized mice. Yuho Najima et al. mentioned that the growth of transgenic Wilm's Tumor-1 (WT-1) with TCRs is observed in HLA-I transgenic NSG mice transplanted with HSCs and the WT-1 specific CTLs showed the ability for increasing the antitumor function, proliferation, and antigen-specific cytokine response. Francesca Giannoni et al. and Dimitrios N Vatakis et al. reported that in melanoma immunotherapy, the hu-BLT mice with transgenic CTLs specific for MART-1 (melanoma antigen recognized by T cells) exhibited the development of tumor-targeted mature T cells and long-term function (Yin et al. 2020). Eventually, the CARs with T cells helped to recognize the target proteins on the exterior of cancer cells, without any constraint of MHC. Therefore, CAR-T cell therapy has gained amazing results for the treatment of hematological malignancies, B-cell lymphoma, and leukemia, with the limitations for solid tumors. Chun-Hui Jin

et al (2019). developed a genetically modified CAR with primary acute B-lymphoblastic leukemia (B-ALL), aiming CD19 in the hu-BLT mice model that showed the possible efficacy to illustrate the host immunological variations related with CAR-T cell therapy. Additionally, the CD28 and CD137 (4-1BB) initiate the ideal signaling to induce CAR-T cell response. Pratiksha Gulati et al. showed that the Δ-CD28/CD3ζ CAR-T cells were able to eliminate tumor growth and to continue a durable antineoplastic activity in the hu-SRC mice model. The CAR-T therapy showed excellent result in immunodeficient mice against epidermal growth factor receptor (EGFR), various tumor CARs specific for prostate stem cell antigen (PSCA), and chA21-4-1BBz but has only limitation against solid tumors. The wellbeing of CAR-T cell therapy is commonly linked with treatment-related adverse reactions, including neurotoxicity and cytokine-release syndrome (CRS). Marco L Davila et al. explained that adverse reactions are chiefly resolute by IL-6 and IL-1. The hu-NSG mice are perfect for preclinical studies and the identification of CRS is necessary for hu-SGM3 strain, but not in hu-NSG mice, for the requirement of GM-CSF (Norelli et al. 2018).

The Immune Checkpoint Blockade Therapy Using Humanized Mice

In the immuno-oncology, multitactics are needed to recognize and to help the effectiveness of immunocytes on target cells of the tumors. The immune checkpoints, namely, T cell immunoglobulin-3 (TIM-3), programmed cell death protein-1 (PD-1), lymphocyte activation gene-3 (LAG-3), and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), should be active for inhibitory receptors and signaling pathways to avoid the incidence of autoimmune effects. The PD-1 and CTLA-4 inhibitors showed the distinctive variances between mouse and human DNA in the immune checkpoints. However, the humanized mice are used in the investigation of immune checkpoint inhibitors for the evaluation of the effectiveness of individual clinical therapies (Kametani et al. 2019). Instantaneously, the human PD-1xLAG-3 knock-in was developed using the mice homologous recombination with CRISPR-Cas9, in which the mouse Pdcd1 gene was substituted with human PDCD1 to prompt the expression of human PD-1 protein (Yin et al. 2020). The possible consequence from the different humanized mice models was to authorize the valuation of human immune response, namely, cytokines, lymphocyte proportion, and the analysis of tumor volume (Table 6). The use of antibodies against PD-1 and CTLA-4 with the mono- or combination treatment discovered the important tumor inhibition in humanized mice with CDX or PDX triple-negative breast cancer (TNBC), hepatocellular carcinoma (HCC), non-small-cell lung cancer (NSCLC), colorectal cancer (CRC), lymphoma, osteosarcoma, and ovarian cancer (Zheng et al. 2018; Rosato et al. 2018; Lin et al. 2018; Capasso et al. 2019; Ma et al. 2016; Gitto et al. 2020). Leonid Cherkassky et al. found that the concurrent initiation of the PD-1 obstruction with CAR-T cell therapy increased the usefulness of monotherapy, endorsing the onco-immunotherapy to the comprehensive therapy (Cherkassky et al. 2016).

Table 6 The drug therapies with immune checkpoints in humanized mice

Drugs	Marks	Mice strains	Tumor features	Valuations
Atezolizumab	PD-L1	NSG, engrafted with HSCs	Caki-1, A375, A549, H1299, H1975, etc.	Reduction of tumor growth curve; upregulation of hCD3+, hCD4+, and hCD8+ T cells; inconstant appearance of PD-1 on T cells and PD-L1 on tumor cells and macrophages
Pembrolizumab	PD-1	NSG, engrafted with HSCs	Dedifferentiated liposarcoma	Reduction of tumor growth curve; augmented number of hCD3 + hCD8+, hCD8 + IFNγ+ T cells, and hCD56 + Ki-67+ NK cells
Nivolumab	PD-1	BRG-SIRPα, engrafted with HSCs	TNBC, CRC	Reduction of tumor growth curve; augmented appearance of IFN-γ + and HLADR+ on hCD8 + T cells
Nivolumab Ipilimumab	PD-1 CTLA-4	NSG, engrafted with HSCs	Nasopharyngeal carcinoma	Reduction of tumor growth curve; upregulation of IFN-γ, IL-6; augmented appearance of HLA-DR+ on hCD8+ T cells; a reduction in hCD4+/hCD8+ ratio
Sintilimab	PD-1	NOG, engrafted with PBMCs	NCI-H292	Inhibition of tumor growth curve and tumor weight; upregulation of IL-2; increased number of hCD3+, hCD8+, hCD8 + IFNγ+ T cells; an increase in hCD8 + T/hTregs ratio

Use of Humanized Mice for NK Cells Therapies

The NK cells are used as one of the important parts of tumor immune investigation. The antitumor functions were observed through stimulation of NK cells chiefly the cytokines and other ex-incentive. Therefore, the exertions of onco-immune investigation have prominently observant on the NK cells function as well as the cytokines therapies. Anja K Wege et al. mentioned that during IL-15 treatment, the HCS-engrafted NSG mice transplanted with the human breast cancer cells showed the development of NK cell accretion in all lymphoid and extra lymphoid tissues. The use of hIL-7 and hIL-15 double knock-in enhanced the NK cells engraftment in NSG mice injected with HSCs. The human IL-15 and SIRPα knock-in mice (injected with HSCs based on the $Rag2^{-/-}$ $IL2rg^{-/-}$) or the humanized SRG-15 mice showed the similar appearance of inhibitory receptors on NK cells to humans. For the preclinical investigation of NK-cell targeted therapies, the dramatic maturation and tissue residence of NK cells were observed in the humanized SRG-15 mouse model (Yin et al. 2020).

Conclusions

The PDX and CDX models are valuable for investigation in cancer biology, finding novel cancer biomarkers, anticancer drug designing, and the preclinical evaluation for therapeutic strategies. This chapter delivers the outline of the key importance of both PDX and CDX models in both translational and preclinical cancer stem cell research and a comprehensive discussion of important usefulness in advancing the field of cancer biology and anticancer drug discovery research. Currently, cancer therapies showed groundbreaking advancement that leads to increased therapeutic efficacy and endurance in cancer patients. The humanized mouse model demonstrates the exact human immune microenvironment for simulating the process of tumor occurrence, development, and metastasis that is an active tool for immunotherapeutic evaluation for cancer patient therapies. Therefore, the introduction of human immunocytes to the PDX mice enhanced the chance of possible preclinical therapies for individual patient. However, the creation and application of both PDX and CDX model showed many challenges, including the residual murine innate immunocytes, the MHC incompatibility between tumors and immunocytes, and the absence of specific-specific cytokines. To overcome these challenges, numerous alterations for transgenic MHC or tissue implantation have been made to enhance in functionality level and to complete the subsets of the human immune system with more erudite technology and the application assessment of targeted immunotherapy profited from the humanized mice. Still, the outcome of immunomodulatory therapy desires to be more explored. Therefore, these humanized PDX and CDX mice models definitely offered an extraordinary platform in cancer immunotherapy.

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Animal Models for Small Cell Lung Cancer Research: Current Status and Future Perspectives

26

Suganthy Natarajan

Contents

Abstract

Small-cell lung cancer (SCLC), an aggressive neuroendocrine tumor characterized by enhanced proliferation rate and rapid early metastasis with low recovery rate, accounts for 15% of total lung cancer cases. Major risk factor for SCLC is tobacco smoking. Most of the patients were diagnosed at metastatic stage, and only one third in early stage of disease are amenable for curative multimodal therapy because of absence of precise symptoms and enhanced proliferation rate with metastatic nature. SCLC genomic analysis indicates prevalent rearrangements in chromosome with extensive mutation. Genomic analysis of SCLC revealed extensive chromosomal rearrangements and enhanced mutation rate leading to substantial intratumoral heterogeneity which limits the efficiency of therapy and relapse of tumor due to development of therapeutic resistance. Unraveling the mechanism of pathogenesis such as initiation, progression, and metastasis of SCLC is essential to identify the novel vulnerabilities amenable for targeted therapeutic approaches.

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Construction of several animal models such as chemical induction models, patient-derived xenotransplantation, gene programming harboring mutations associated with SCLC not only facilitated to unravel the biological pathways responsible for incidence and prognosis of SCLC, but also to explore the anticancer drugs and gene therapy. This chapter focuses on several animal models depicting the characteristic features of SCLC, combined with latest progress and future research of animal model used for SCLC.

Keywords

SCLC, Genetically engineered model system (GEM) · Patients xenograft model system (PDX) · Cell lines originated from SCLC · Zebra fish

Introduction

Increase in life span, control of infectious disease, increased pollution due to urbanization and industrialization, and modern life style increased the incidence of cancer, which turned to be socioeconomic burden to society (Wild 2019). Among the cancer, lung cancer is the prevalent cause of cancer-related mortality claiming more than one million lives worldwide. Epidemiological survey and WHO statistics 2001 to 2019 reveal that lung cancer is one of the top ten causative of mortality globally, with incidence rate of two million new cases and 1.8 million deaths annually. Prevalence rate of lung cancer is very high in developed countries like United States, Europe, and East Asian countries (Barta et al. 2019). Lung cancer has developed into serious health issue due to its highly invasive and metastasizing nature. Cancer originating in the lungs is termed as primary cancer, while the cancer that spreads to lungs from different body parts are called secondary lung carcinoma. The major risk factors leading to lung cancer is tobacco smoking responsible for 70% of total lung cancer cases. Other etiological factors include passive smoking, air pollution, exposure to arsenic, chromium, nickel, radon gas during occupation, previous radiation therapy, low immunity, and family history of lung cancer (Malhotra et al. 2016). Clinical symptoms in lung cancer vary with site of tumor incidence such as symptoms due to primary tumor includes cough associated with bleeding in the respiratory tract, syndromes such as Horner's, superior vena, and paraneoplastic which leads to inappropriate level of antidiuretic hormone secretion, while distant metastases cause bone pain. General complications include shortness of breath, appetite loss, insomnia, tiredness, pleural effusion, persistent cough, and pain in the ribs (Latimer and Mott 2015). Lung cancer is incurable disease; however, treatment can diminish symptomatic effect increasing the life span of affected people. Treatment strategies for SCLC and NSCLC involve multimodal approach/combinatorial therapy including surgery, radiotherapy, chemotherapy, and immunotherapy, which showed successful outcomes for early-stage of disease (Indini et al. 2020). Despite advances in therapeutic strategies such as site-targeted chemo/radio/immunotherapy

and constant supervision of patients, the life span was observed to be less than 5 years, which might be due to diagnosis at later histologic stages and aggressive heterogeneous nature of cancer. Hence, comprehensive understanding of genetic alteration responsible for commencement and successive development of lung carcinoma is necessary for the development of effective therapeutic and timely diagnosis. Genomic analysis of patient sample led to identification of several candidate genes with pivotal role in lung carcinoma, opening up new channels for targeted therapy. Identification of EGFR domain mutations in human lung cancer promoted the use of tyrosine kinase inhibitors (gefitinib and erlotinib) for lung cancer therapy, which showed positive response in patients revolutionizing targeted therapy in lung carcinoma (PaoW and Kris 2004). WHO categorized lung carcinoma into small-cell lung cancer (SCLC 15%) and non-small-cell lung cancer (NSCLC 85%), which was further subcategorized as Adenocarcinoma, Squamous Cell Carcinoma and Neuroendocrine Tumors (Travis et al. 2015a; Inamura 2017). SCLC also termed as oat cell carcinoma is deadliest form of lung carcinoma with high mortality rate and survival period of less than 5%. Absence of definite symptoms, quick proliferation rate, and metastatic nature acts as hindrance for premature diagnosis of SCLC (Yang et al. 2019). Therapeutic strategies available for SCLC are ineffective with limited outcomes due to complex genetic alteration and poor diagnosis. Although in vitro studies elucidate the mechanism of lung cancer, it cannot replicate the complicated pathways of carcinogenesis and the treatment response as observed under *in vivo* condition (van Meerbeeck et al. 2011; Dutt and Wang 2006). Hence, designing *in vivo* model system associated with genetic mutation linked with lung carcinoma can provide an insight in understanding the mechanism of SCLC progression from precancerous tumor to highly metastatic stage and reason for failure in chemotherapy, thereby providing new targets for effective control of SCLC. This book chapter highlights the various model systems representing small cell lung carcinoma, which will help in the advancement of chemotherapeutics for effective lung cancer therapy

Small Cell Lung Carcinoma

Small-cell lung cancer (SCLC) is the most lethal form of lung cancer, marked by its exceptionally enhanced proliferative rate, rapid metastasis potential and poor diagnosis. It constitutes 15% of total lung cancer cases affecting male population predominantly the current and former smokers when compared to female population (Franco et al. 2021). Global epidemiological survey indicates prevalence of 250,000 cases with 2,00,000 deaths per year prevalently in developed countries with high intake of tobacco-related products. Although African-American men and women have high smoking rate, the incidence of SCLC is less in African-American men and women when compared to white Americans. Patients with high smoking histories have less than 10% 5-year survival rate. Prevention and cessation of smoking are the major interventions to reduce mortality due to SCLC (Rudin et al. 2021).

Etiological Factors Leading to SCLC

Major risk factor for SCLC is smoking and consumption of tobacco with less than 2% incidence in nonsmokers. Other risk factors include air pollution, exposure to radon, occupational exposure to mining, asbestos industry, heavy metal compounds in agricultural, paint, stainless steel and welding industry, silica dust, consumption of drinking water containing arsenic, and family history. Another key factor leading to SCLC in smoking person is chronic obstructive pulmonary disease (Lamichhane et al. 2017; Rodríguez-Martínez et al. 2018). Genetic changes affecting oncogenes/tumor suppressor genes are another major driving factor leading to SCLC. These genetics changes are not inherited but acquired during life time on prolonged exposure to smoking and other environmental factors (Wang et al. 2017). Alteration in these genes affects the regulation of cell growth and division leading to cancer. Mutation observed in SCLC includes null mutation of retinoblastoma gene (RB1) in 90% SCLC, p53 gene in 75% cases, gene clustering the receptor kinase/P13 K pathway, Hedgehog and notch signaling pathway, and genes involved in DNA repair mechanism (Denninghoff et al. 2021; Jin et al. 2021). Enhanced protooncogene expression of L-MYC, C-MYC and N-MYC, SOX2, and SOX4 was observed in SCLC (Table 1) (Kim et al. 2018). Diverse lesion in SCLC necessitates development of fast track mouse model system with various combinations of oncogenic mutations in order to assess the target pathway for treatment of SCLC (Zhao et al. 2019).

Diagnosis of SCLC

Diagnosis involves characteristic histopathological examination of tumor tissue (hematoxylin and eosin staining) aided by cytology. High circulating tumor cells (CTC), although negative prognostic factor for SCLC patients, are used for evaluation of tumor characteristics including intratumoral heterogeneity. Chest X-ray indicates the tumor mass of tissue in lungs, and PET scan along with CT helps to diagnose the lung tumor and metastasize to distal body parts, while magnetic resonance imaging (MRI) reveals the cross section images of particular organ and bodily tissue to assess if the extent cancer has spread to the brain (Austin et al. 2012; Wang et al. 2019). Due to the central location of tumor, biopsies obtained by bronchoscopy, video thoracoscopy, and mediastinoscopy were used by the physicians to evaluate the spread of cancer in the chest, breast bone, and lymph nodes (Fruh et al. 2013). Radiological findings help to identify the location and advanced stages of SCLC. Metastatic spread is radiologically evident, which includes pleural and pericardial effusions (Travis 2014; Travis et al. 2015b). About 5% of SCLC presents peripheral nodule without associated lymphadenopathy acquiescent to surgery.

Clinical Features

Clinical features includes primary tumor in the major airways with extensive extra pulmonary metastatic spread. Rapid tumor growth with high metastatic rate SCLC

Table 1 Genetically engineered mouse model system **for SCLC**

S. No.	Mouse model	Induction method	Target cell	Latency period (months)	Phenotype	Reference
1	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$	Intratracheal Ad-CMV-Cre	Deletion of Rb1 and Trp 53 in lung cells	7–15	SCLC with metastasis, minor AC and LCNEC	Meuwissen et al. 2003; Sutherland et al. 2011; Cui et al. 2014
		Intratracheal Ad-CGRP-Cre	Deletion of Rb1 and Trp53 in NE lung cells	10–18	SCLC, metastasis	Sutherland et al. 2011; McFadden et al. 2014
2	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $p130^{lox/lox}$	Intratracheal Ad-CMV-Cre	Deletion of Rb1, Trp 53 and p130 in lung cells	4–6	SCLC, LCNEC metastasis	Schaffer et al. 2010
3	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ invCAG-Mycl1-Luc2	Intratracheal Ad-CMV-Cre	Deletion of Rb1 and Trp53 and activation of Mycl	4–5	SCLC, metastasis are rare	Huijbers et al. 2014
4	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Pten^{lox/+}$	Intratracheal Ad-CMV-Cre	Deletion of Rb1 and Trp53 and one allele of Pten in lung cells	6–10	SCLC with minor NSCLC-NE	Cui et al. 2014
5	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Pten^{lox/lox}$	Intratracheal Ad-CMV-Cre	Deletion of Rb1 and Trp53 and Pten in lung cells	3–5	NSCLC-NE, AC, SCLC	Cui et al. 2014
6	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Pten^{lox/+}$	Intratracheal Ad-CGRP-Cre	Deletion of Rb1 and Trp53 and one allele of Pten in lung cells	7–11	SCLC	McFadden et al. 2014
7	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Pten^{lox/lox}$	Intratracheal Ad-CGRP-Cre	Deletion of Rb1, Trp53, and Pten in NE lung cells	6–7	Mainly LCNEC, minor SCLC and NSCLC-NE	McFadden et al. 2014
8	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Pten^{+/+}$		Tamoxifen-based deletion of Rb1 and Trp53 in all NE cells of mice	6–7	SCLC	Song et al. 2012

(continued)

Table 1 (continued)

S. No.	Mouse model	Induction method	Target cell	Latency period (months)	Phenotype	Reference
		Cross to a CGRP $^{CreER/+}$ mouse model				
9	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Pten^{lox/lox}$	Cross to a CGRP $^{CreER/+}$ mouse model	Tamoxifen-based deletion of Rb1 and Trp53 in NE cells of mice	2–3	SCLC	Song et al. 2012
10	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Rosa26^{+/LSL\text{-}SMOM2\text{-}YFP}$	Intratracheal Ad-CMV-Cre	Deletion of Rb1 and Trp53 together with stimulation of hedgehog pathway in cells	Not determined	SCLC	Park et al. 2011
11	$Rb1^{lox/lox}$ $Trp53^{lox/lox}$ $Rbl2^{lox/lox}$ $Smo^{lox/lox}$	Intratracheal Ad-CMV-Cre	Deletion of Rb1, Trp53 and p130 associated with stimulation of hedgehog pathway	Not determined	SCLC	Park et al. 2011

patients is symptomatic at presentation with less than 3 months duration. Most common sign is mediastinal tumor. Symptoms associated with intrathoracic local growth includes cough with bleeding in the respiratory tract, shortness of breath, compression in superior vena cava, esophagus, and laryngeal nerve leading to dysphagia and vocal cord damage. Distal spread is associated with loss of appetite affecting body weight, tiredness, and neurological complications (Kalemkerian et al. 2013; Raso et al. 2021). Paraneoplastic syndromes include endocrine anomalies such as abnormal level of vasopressin, hypercortisolism, neurologic syndromes caused by onconeural antibodies like Lambert–Eaton myasthenic syndrome, limbic encephalitis and subacute sensory neuropathy, cerebellar degeneration, opsoclonus-myoclonus ataxi, hematological syndrome, ophthalmologic syndrome, and glomerulopathy. Dermatomyositis, hypercalcaemia, and gynecomastia are uncommon signs observed in SCLC due to enhanced immune response (Kanaji et al. 2014).

Different Stages of SCLC

Tumor node metastases (TNM) are classified based on Veterans Administration Lung Study Group (VALSG) into limited disease SCLC (LD-SCLC) and extensive disease SCLC (ED-SCLC) (Table 1). LD-SCLC exemplifies disease confined to hemi-thorax with regional lymph node metastase, encompassed within the single radiation field with the 5-year survival rate of 30–35%. Patients with distant metastasis, that is, tumor in pleural/pericardial nodule, contralateral lung, are categorized as ED-SCLC insensitive for radiation therapy and effective for combinatorial, that is, chemo/radiation therapy (West 2019). TNM categorization is useful for effective clinical trials treatment strategies. In SCLC stage by stage diagnosis is very poor when compared to NSCLC. Most common clinical symptom of SCLC is brain metastasis representing with less than 10% incidence during initial diagnosis with subsequent development of 50% of brain metastasis. VALSG staging system that differentiates patients based on therapy as ED-SCLC (chemotherapy), LD-SCLC (chemo/radiotherapy), and symptomatic metastasis (Radiotherapy) plays a vital role in scheming clinical trials and data presentation (Carter et al. 2014). Based on histological examination, WHO classified lung carcinoma as SCLC (20%), NSCLC (80%), and large cell carcinoma, with SCLC further classified as SCLC (oat cell cancer) and combined SCLC (Zheng 2016; Raso et al. 2021).

Animal Model System for SCLC

SCLC is distinctive malignancy with high death rate due to its enhanced proliferation rate, rapid metastasis with delayed diagnosis, which necessitates an urgent need for novel and efficacious diagnosis, as well as therapeutic options. SCLC research can be accelerated by using short-term animal models, which can recapitulate the pathological features of SCLC pinpointing the therapeutic targets and appropriate interventions for the control of SCLC. Animal models widely used for clinical

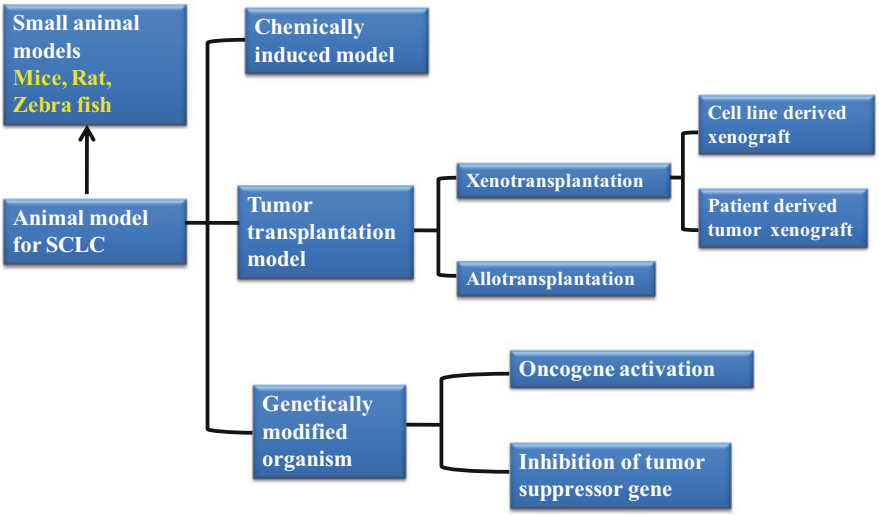

Fig. 1 Animal models used for study of SCLC

research of SCLC involve chemical-induced tumor model, genetically modified and transgenic model system, and cell lined and patient -derived xenograft model (CDX, PDX) (Fig. 1). Probability of development of single model system depicting the entire pathway is very less. Model system that depicts the various stages of lung cancer such as initiation, rapid cell multiplication and localization, invasion, angiogenesis, distal spread, and therapy is essential to understand the complex stages of SCLC (Gazdar et al. 2016; Malyla et al. 2020). To reproduce the required biochemical pathways under investigation, the model system should undergo some deviation from human disorder and the result should be interpreted utilizing model systems with caution understanding their limitations.

Mouse Model System

Mouse have been considered as suitable candidate for the study of underlying mechanism and therapeutic strategy of lung carcinoma owing to its anatomic, biological function, and genomic homology with human. Convenience in feeding, low cost, easy to handle, and gene modification made mouse model a valuable platform for cancer drug discovery. Mostly mouse model for lung cancer has been generated by mutating the specific gene in the mouse using embryonic stem cells whose major drawback is prolonged time period to induce specific mutation within single genome and extremely costly. Limitations can be overcome by genetically modifying embryonic stems of existing mouse tumor model using adenoviral and lentiviral vectors to generate complex model faster (Xia et al. 2012).

Genetically Engineered Mouse Model System

Genetically engineered mouse models (GEMMs) with mutation in driver genes have been developed as valid system to study SCLC, which exhibited the neuroendocrine nature of tumor and cytological characteristics of human SCLC (Kwon and Berns 2013). Inactivation of tumor suppressor gene Rb and Trp53 observed in SCLC formed the basis for the design of mouse model system with Cre/LoxP-based deletion of conditional alleles for retinoblastoma (Rbf) and Trp53 gene achieved via intratracheal installation of engineered adeno-Cre virus leading to formation of tumor mimicking the human SCLC histology and metastasis. Inactivation of P130 gene along with Rbf and Trp 53 showed poor survival, indicating that deletion of P130 (Rb family member) together with Rb1 and Trp53 accelerated the tumor development retaining the same histological and metastatic features similar to human SCLC (Schaffer et al. 2010). SCLC patients sample revealed mutation in PTEN gene, and interestingly in Rb1/Trp53 mouse model, system loss of chromosome 19 encoding PTEN was observed (McFadden et al. 2014). Triple Rb1/Trp53/Pten conditional knockout mouse model was generated to evaluate the function of PTEN in SCLC, which showed drastic progression of tumor revealing the significant role of Phosphatidylinositol 3 kinase pathway in SCLC.

As amplification of MYC family oncogene was observed in SCLC patients, to validate the significance of MYC oncogenes, MYCL gene was inserted in Rb1/Trp53 mouse model, which displayed features of neuroendocrine tumors indicating MYCL as driver factor (Huijbers et al. 2014). Another driver factor in SCLC is members of Hedgehog pathway, which on constitutive activation induced the commencement and development of SCLC in mouse model system (Park et al. 2011). Similarly conditional overexpression of NOTCH1/2 in Trp53/Rb1/Rb2 mouse model suppressed the tumor increasing the survival rate revealing the Notch as tumor suppressor gene (George et al. 2015). Despite the fact that double mutation Rb1/Trp53 model system resembles human SCLC, combination of mutation of several genes (loss or gain of function) represents the whole spectrum of neuroendocrine carcinoma of lungs displaying a stage to understand mechanism of initiation, development, diversity, and metastasis of tumor. Future perspective must focus on mouse model system for screening new driver genes, which might act as an insight for novel therapies. CRISPR/Cas9-induced inactivation of Ep300 or Crebbp and transactivation of proto-oncogenes like Fgfr1, Sox2, and Pik3ca will facilitate further understanding of SCLC (Hsu et al. 2014). Fast-track mouse models that allow swift sampling of entire spectrum of mutations will help in developing combinatorial therapy and also in detecting the fatal interaction involved in persistent lesion in SCLC (Huijbers et al. 2011).

Patient-Derived Xenografts

PDX model was developed by implanting the freshly dissected tumor tissue in severely immunocompromised mice, which is widely used as a preclinical model to understand molecular mechanism of SCLC and identify the therapeutic targets

(Tentler et al. 2012; Williams et al. 2013). These models retain characteristics similar to patient's tumor from which they are generated and also their sensitivity to chemotherapy as clinically observed. PDX tumor facilitates evaluation of preclinical efficacy of therapeutic agents *in vivo*. Cryopreservation of PDX model system helps to overcome the limitation of repeated passaging such as genomic alteration observed in cell lines and the resulting xenografts (Roschke et al. 2003; Daniel et al. 2009). As SCLC is rarely surgically excised, development of PDX model system using biopsies or circulating tumor cells (CTCs) suffers limitation such as tissue scarcity and poor tumor growth. Fine needle aspiration of primary SCLC tumor material can generate PDX tumors similar to primary tumor both in phenotype and genotype (Anderson et al. 2015). Aspired samples collected in hospitals are shipped to collaborating research facility within overnight facilitating establishment of PDX tumor morphologically similar to primary tumor of origin (Leong et al. 2014; Hodgkinson et al. 2014). PDX model system obtained from tumor graft of patients where adequate tumor tissues are available has developed into promising alternative to autochthonous mouse models. Restoring the texture of tumor with intact stromal components will help us in understanding the cell to cell interaction, which cannot be replicated in studies using primary/transformed cell lines. Major limitation is the heterogeneous nature of tumor and complicated mutation, which minimizes the predictive value. However, PDX model is considered as only model system mimicking the human SCLC to study about intact human tumor (Moro et al. 2012).

Cell of Origin of SCLC

Highly complicated cancer genome of lung cancer was analyzed using primary cell lines/ transformed cell lines possessing every single driver mutation of lung carcinoma, which will be a resource for understanding the pathogenesis of lung carcinoma (Table 2). As SCLC are seldom surgically removed, samples available for laboratory studies are limited as only a small tissue sample from biopsy examination, sample aspirates using fine needle, and malignant pleural effusion are available for investigation. Till date, molecular pathogenesis of SCLC has been obtained from the research in continuous cell cultures and to some extent from PDX and paraffin-embedded formalin-preserved samples. Culturing of SCLC cells was initiated in Japan (1971) followed by USA (Oboshi et al. 1971; Pettengill et al. 1977). Observation revealed that SCLC is nonadherent cells which float as suspension or spheroids. Sato and his colleagues derived chemically defined medium HITES for the growth, development, and preservation of SCLC, which possess growth factors, such as hydrocortisone, insulin, transferrin, β estradiol, and sodium selenite (Barnes et al. 1981). These cultured cells showed similar cytological features and neuroendocrine (NE) differentiation as observed in tumor tissues (Carney et al. 1985). Investigation of various SCLC cell lines depicted the presence of "variant" cell lines, which lacked the cytological characteristics and NE differentiation features of SCLC with enhanced expression of MYC family of oncogenes. Investigation of SCLC biology using various cell lines revealed the role of basic helix-loop-helix transcription factor human achaete–scute homologue-1 and Notch signaling

Table 2 Genomic alteration observed in SCLC

Gene	Occurrence in SCLC (%)	Variation	Main function
TP53 89	89	Inactivating mutation; deletion – LOF	Tumor suppressor, stress, and transcription regulation
RB1	64	Inactivating mutation; deletion – LOF	Suppression of tumor, cell cycle regulation, and transcription repression
KMT2D	13	Inactivating mutation; deletion – LOF	Tumor inhibition, histone alteration, and modifying chromatin
PIK3A	7	Activating mutation – GOF	Oncogene; PTEN-mTOR signaling pathway
PTEN	7	Inactivation mutations, deletions	Tumor suppressor; PTEN-mTOR signaling pathway
NOTCH1	6	Inactivating mutation	Tumor suppressor; cell–cell signaling
CREBBP	5	Inactivation mutations, deletions – LOF	Tumor inhibition; modification in acetyltransferase (histone and nonhistone proteins); chromatin and regulation of transcription process
FAT1	4	Inactivation mutations, deletions – LOF	Tumor suppressor; cell–cell signaling
NF1	4	Inactivation mutations, deletions – LOF	Tumor suppressor; cell to cell communication/signaling
APC	4	Inactivation mutations, deletions – LOF	Tumor suppressor; RA
EGFR	4	Activating mutations	Oncogene; RAS signaling pathway
KRAS	3	Activating mutations	Oncogene; RAS signaling pathway
NOTCH3	2.9	Inactivation mutations, deletions	Tumor suppressor; cell–cell signaling
ARIDIA	2.9	Inactivation mutations, deletions	Suppression of tumor, remodeling chromatin, and regulation of transcription process
PTPRD	2.7	Inactivation mutations, deletions	Tumor suppressor; chromatin remodeling
ATRX	2.4	Inactivation mutations, deletions	Tumor suppressor; cell–cell signaling
TSC2	2.3	Inactivation mutations, deletions	Tumor suppressor; PTEN–mTOR signaling pathway
EP300	2.1	Inactivation mutations, deletions	Tumor suppressor; chromatin remodeling

pathway in differentiation and growth of NE cells (Oie et al. 1996; Sriuranpong et al. 2001). Rb1 and p53 gene inactivation by Adeno-Cre virus in NE cells (calcitonin gene-related peptide promoter), nonciliated bronchiolar secretory cells (CC10 promoter), and alveolar type II cells (SPC promoter) initiates neuroendocrine origin of SCLC (Sutherland et al. 2011; Park et al. 2011). However, in SPC-positive cells, switching of Rb1 and p53 gene developed less efficient SCLC, with tumor incidence

peripherally exhibiting chromosomal aberrations not observed in induced NE cells. Experiment was repeated in mice carrying any one type of allele Rb1 or p53. Switching of Rb1 and p53 gene led to loss of heterozygosity developing SCLC. When compared to other tumor, relapse and metastasis of SCLC was mainly due to enhanced circulating tumor cells (CTCs) (Yu et al. 2015). These CTCs are chemoresistance-inducing secondary lesions and these cells perish in peripheral population, with few cells having tumor-initiating properties (Celià-Terrassa and Kang 2016). Cultured CTC cell lines (BHGc7, 10, 16, 26 and UHGc5) isolated from SCLC patients exhibited characteristic markers of SCLC, which formed tumorospheres, the multicellular aggregates. Staining the section of solid cell aggregates for prognostic tumor marker nuclear protein Ki67 and hypoxia indicator carbonic anhydrase 9 illustrated the hypoxic and dormant state of cells. CTC cells in the tumorospheres showed enhanced resistant to drug used for SCLC treatment, when compared to single cell CTC culture (Klameth et al. 2017). SCLC cell miner combines the drug sensitivity and multiomics data of molecularly characterized cell lines derived from 118 SCLC patients, which acts as a promising resource for this recalcitrant cancer research. SCLC is classified based on the expression of four master transcription factors NEUROD1, ASCL1, POU2F3, and YAP1. Transcription networks analysis showed the connection of SCLC subsets with MYC and its paralogous genes, the NOTCH and HIPPO pathways. Expression of surface marker in SCLC subsets facilitates targeted immunotherapy. Yes associated protein 1 (YAP1) SCLC cell lines differ from other subsets based on the differential expression of the NOTCH pathway, epithelial-mesenchymal transition (EMT), and antigen-presenting machinery (APM) genes and responding to mTOR and AKT inhibitors. For several years, SCLC was treated as single disease despite molecular and clinical heterogeneity with poor prognosis. Classification of SCLC based on tumor expression data and non-negative matrix factorization paved way for understanding the SCLC tumor biology and also provided a framework for future research on SCLC subtype-specific vulnerabilities opening up avenue for personalized therapy (Tlemsani et al. 2020).

Zebra Fish as Model System

Zebra fish (*Danio rerio*) has developed into powerful and genetically modifiable vertebrate model system for the study of human malignancies. Zebra fish has become an attractive model system in tumor research owing to its genetic (70%) and biological similarity with human and also replicate the clinical progression of cancer similar to human (MacRae and Peterson 2015). Unique characteristics of zebra fish such as smaller size, low cost, quick reproduction, transparent embryos facilitating real time observation of various stages of cancer cell progression, development of transgenic zebra fish and immunodeficient zebra fish, and *in vitro* fertilization aiding ease genome modification make it suitable candidate for cancer studies when compared to mouse model system (Tang et al. 2016; Brown et al. 2017). Stemness and underdeveloped immune environment of zebra fish provides optimum condition for the growth of cancer cells (Jiang et al. 2019). Currently several zebrafish cancer

model systems such as transgenic, genome edited, xenograft model, drug induced model are available for cancer studies. Xenotransplantation model of zebra fish was developed by implanting LINC00152 knockout lung carcinoma cells into zebra fish and results revealed that silencing LINC00152 attenuated the rapid cell division rate and distal spread of lung cancer cells, indicating Zebra fish xenograft as suitable model system for functional study of human lung cancer (Shen et al. 2020). Zebra fish model system was also utilized to evaluate the clinical efficiency and toxicological profile of drugs used for the treatment of lung cancer. Zebrafish larvae grafted with H1299 cells for 72 h were used as xenograft NSCLC model system to assess the clinical efficiency of DFIQ (Quinoline Derivative), which showed inhibition of tumor growth (Huang et al. 2020). PC9, PC9-Gefitinib-resistant strain (PC9-GR), H1975, H1975, and osimertinib-resistant strains (H1975-OR cells) cells were microinjected into zebra fish to develop xenograft model system to study the effect of antiangiogenenic and antitumor efficiency of osimertinib. Results revealed that attenuation of cell multiplication in osimertinib-treated zebra fish with mutation in EGFR and T790M resistance mutation is consistent as observed in clinical research. Third-generation EGFR-TKI drug-resistant xenograft model was designed to analyze the mechanism of osimertinib (AZD9291) resistance and follow-up drug therapy for these patients, indicating Zebrafish xenotransplantation model acts as real time drug screening platform for lung cancer therapy (Li et al. 2019).

Conclusion

SCLC is an aggressive form of metastatic malignancy with poor prognosis and therapeutics, which needs a suitable model system to elucidate the molecular targets for effective therapy. This review gives an overview on model system available for SCLC. Genetically engineered mouse model system by inactivating the Rb and Trp53 gene will serve as suitable model system to elucidate the chemoresistance and relapse of SCLC in human. Protein expression pattern in these murine model system acts as early diagnostic marker for human SCLC. PDX implanted murine model system played vital role in unraveling the molecular pathways for onset and progression of tumor in SCLC. Zebra fish model system developed by implanting the LINC00152 knockout lung carcinoma cells and H1299 cells served as model system for the functional study of human lung cancer and for screening anticancer drugs. Overall the current model system available for SCLC assists in identifying the driver markers for early lung cancer diagnosis and to screen anti-lung cancer therapies. Currently researchers are focusing on development of Autochthonous Mouse Models as promising model system for SCLC.

Future Perspective

Autochthonous mouse models have turned into promising intervention approach for SCLC (Singh et al. 2012) due to the following reasons:

- Cancer genome sequencing particularly for smoking-related lung cancers is highly complex due to multiple genomic aberrations associated with damage, difficulty in assessing the phenotypic changes in tumor tissue, and its response to therapy which prompted the development of animal model system to assess the consequence of each lesions.
- Fast track mouse models have the capacity to initiate quick mutations leading to the formation of complex lesions mimicking human tumors, which paves a way to identify cancer driver gene and its response for therapy. These model systems will facilitate understanding tumor drivers and the target gene, which in turn will provoke designing several inhibitors for these target genes.
- In mouse model system, the gene homogeneity does not mimic the human or outbred mice gene complexity; hence, reduction of background noise is essential to evaluate the significance of driver mutation for clinical intervention. Autochthonous models with genetic background similar to human can reiterate the human disease precisely and the observed mutation pattern was similar to human tumors. Large number of mouse must be examined to identify the alleles, which can alter the physical characteristics of tumor (Welsh et al. 2012). Incorporating mutation of all driver genes responsible for tumor will help in understanding combined mutation effect suitable for clinical analysis.
- Autochthonous mouse models used at present act as promising source of tumor cell lines to study the therapeutic response, screening drug resistance, and short hairpin RNA library for RNA interference therapy. Screening dropout ShRNA in cell lines and tumor graft model system led to identification of deadly targets for clinical analysis.
- Performing co-clinical trials in patients and autochthonous mouse models concurrently will provide new insight on the mechanism of drug action and its efficiency (Singh et al. 2012; Chen et al. 2012). In the combinatorial therapies, the mode, frequency, and dose of drug administration act as critical factors to assess its efficiency and determine the safe dose. Mouse model system will help in identifying whether the drugs have the ability to remove the initiating cancer cells, which cannot be done in patients. Designing mouse models acts as promising tool for planning and validation of therapeutic strategies based on the facilities and funding available. Quality infrastructure, expert in animal handling, and sufficient grant are the areas to be focused for designing precise animal model system.

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Melatonin Induced in Cancer as a Frame of Zebrafish Model

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Nithar Ranjan Madhu, Bhanumati Sarkar, Shubhadeep Roychoudhury, and Biplab Kumar Behera

Contents

Abstract

The zebrafish (*Danio rerio*) is an excellent lower vertebrate model to research circadian rhythms, light sensitivity, toxicity, and diseases such as cancer. Numerous research and the authors' own works have demonstrated that lengthy photoperiods were sufficient to reset the population's rhythms in a reasonable number of day. It is possible that there was less selection pressure to build a molecular clock in the zebrafish that was precise and resilient throughout time, as compared to other species. The pineal gland is the location where melatonin is produced in the brain for animals. Melatonin has been used to treat various conditions, including shift work, jet lag, sleep-wake cycle imbalance, primary insomnia,

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sleep difficulties, cancer, etc. The zebrafish is one of the most significant lower vertebrates in terms of scientific significance in cancer research because of its ability to survive in freshwater. Zebrafish have 71% of all human proteins and 82% of all disease-causing human proteins., indicating a high degree of homology between humans and their closest relatives. However, in cancer research, traditional cell culture assays and frequently utilized mouse models, which serve as the workhorse for most in vivo studies, have drawbacks, but the zebrafish model has advantages. For starters, zebrafish produce far bigger progeny than rodent models. Because of the large number of participants in the trial, it may be possible to detect small differences across treatment groups. These two primary strategies are used in the creation of zebrafish cancer models. One possibility is the development of transgenic and mutant lines. Instead, tumor cells from human patients might be injected into zebrafish. The cancer spreads far more swiftly in embryos, with tumors appearing in as little as two days after the embryos are implanted. Zebrafish are a potential option for in vitro cancer testing as a model for cancer metastasis due to their low care needs, rapid turnaround time, and ease of building up metastasis models.

Keywords

Cancer · Melatonin · Model animal · Pineal · Oncology · Zebrafish

Introduction

The zebrafish, a relatively recent creature that serves as a model in scientific study, is swiftly establishing itself as a powerful tool in the area. During the night, when the light/dark cycle is regular, humans and other animals secrete melatonin, a pineal gland hormone that governs circadian rhythms (Madhu and Manna 2021; Madhu 2018; Madhu and Manna 2011). It is during the night that the corpus pineal generates the hormone melatonin (N-acetyl-5-methoxy-tryptamine), which is controlled by an endogenous clock located in the SCN (suprachiasmatic nucleus) of the hypothalamus (Claustrat et al. 2005; Madhu and Manna 2010; Madhu et al. 2010; Madhu and Manna 2009).

Pinealectomy and exogenous melatonin delivery in zebrafish have long been employed to investigate the pineal gland's function and the role of melatonin in regulating seasonal reproduction and daily rhythms. As a result of this diversity, it's not unexpected that research on several fish species has shown mixed findings, ranging from no effect from pineal surgery or melatonin to the loss of annual or daily physiological and behavioral cycles. As a consequence, general inferences concerning the function of the pineal gland and melatonin in fish physiology are difficult to make. The fish pineal gland contains melatonin-producing photoreceptor cells, which may include neurons that transmit photic and/or circadian information to other brain locations. However, this may be misleading since the duration of melatonin's effects depends on the duration of the hormone and the timing of the body's internal clock

(Ben-Moshe et al. 2014; Falcon et al. 2009; Ekstrom and Meissl 1997; Falcon et al. 2010; Masai et al. 1997; Wilson et al. 1990; Yáñez et al. 2009).

Controlling angiogenesis may be a viable technique for avoiding cancer development, since neovascularization is required for the growth and spread of tumors. Melatonin's antiangiogenic capabilities have been studied in detail for some time now (Jardim-Perassi et al. 2014). The formation of new capillary structures (a process known as angiogenesis) during cancer cell growth at the primary tumor site provides not only oxygen and nutrients but also a pathway for a subset of cancer cells with the ability to self-renew and actively migrate (described as cancer stem cells) to penetrate the basement membrane and extracellular matrix and spread throughout the body (ECM). Many malignancies, including breast, uterine, colorectal, and prostate, have been thoroughly examined in vivo and in vitro as oncostatin medicine. Melatonin has been shown to be effective in treating cancers of the breast, uterus, colon, and prostate (Cos et al. 2006; Sánchez-Barceló et al. 2005).

The zebrafish is one of the most often used models in biological research, and it has been around for a long time. Originally from the Himalayan region of South Asia, freshwater zebrafish may be found in various habitats, including Nepal, India, Bhutan, Bangladesh, Myanmar, and Pakistan. Known as zebrafish, they are tropical freshwater fish that prefer tropical settings to survive. It is a teleost (bony fish) member of the Cyprinidae family and the Actinopterygii class of fish, and it is native to Southeast Asia (ray-finned fishes). George Streisinger (University of Oregon) was the first to employ zebrafish as a biological model in the 1970s because it was less difficult to genetically modify than mice. A popular method of investigation into the development of the nervous system among Streisinger's colleagues, particularly Chuck Kimmel, at his university was the use of zebrafish embryos (Khan and Alhewairini 2018).

In research involving nonmammalian lower vertebrates, the use of fish as cancer models is not a recent development. The platyfish, *Xiphophorus* sp., was the first fish to be used as a model for melanoma research. Genetic hybrids of the *Xiphophorus helleri* and *Xiphophorus maculatus* have been demonstrated to be prone to melanoma, which is a kind of skin cancer. This concept developed one of the first animal cancer models (Sarasamma et al. 2018; Schartl et al. 2016). The goldfish (*Carassius auratus*), the world's first model animal, has been used as a model species by scientists for more than two centuries. Goldfish were mostly used in aquatic toxicity testing until recently. Experiments have been conducted on medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), roach (*Rutilus rutilus*), goldfish (*Carassius auratus*), three-spined stickleback (*Gasterosteus aculeatus*), swordtail (*Xiphophorus hellerii*), and pufferfish (*Takifugu rubripes*) (Ribas and Piferrer, 2014). Each kind of fish has its own set of advantages and disadvantages. Goldfish, for example, have been used in studies on stress, growth, reproductive biology, and immunology, among other subjects. The medaka was the most often used fish for development studies, reproduction, and genetics. Because of advancements in molecular biology, for decades, zebrafish have been utilized as a model organism in practically every part of biology, including developmental biology (Teame et al. 2019). In the 1990s, Nobel Prize winner Christiane Nusslein-Volhard in Tubingen, Germany, and

Wolfgang Driever and Mark Fishman in Boston, USA, used the *Danio rerio* as a model organism to create two massive genetic mutants, one of which was named after the late Nobel Prize winner.

It is possible that the carcinogens in the water that fish are exposed to in the wild may cause different cancers in teleosts, including benign and malignant tumors that are similar to those observed in humans. The development of melanomas in *Xiphophorus* hybrids demonstrates that cancer is a genetic sickness in fish, as shown by the development of melanomas. There are several advantages of utilizing *Danio rerio* as a cancer model, including the following: zebrafish may be utilized to do genetic screening for cancer pathways that are highly conserved. The study of the cell cycle phenotype of zebrafish embryos was made possible by the use of mutagenesis screens, which are typically reserved for the elderly. It is possible to explore the origin of cancer in different vertebrate species by screening gene functions for cell cycle, proliferation, and apoptosis genes in zebrafish, as has been done before in yeast, *C. elegans*, and *Drosophila*, among other things (Walter and Kazianis 2001).

An adult zebrafish fish pair may lay up to 500 fertilized eggs in a single week, depending on their size. Until 1 month following fertilization, the embryos' external development and transparency are hallmarks of the developing embryo (Spitsbergen 2007). Adult Casper mutant (roy$^{-/-}$; nacre$^{-/-}$) mice retain their transparent appearance throughout their lives and may be employed for in vivo transplantation studies in the future (White et al. 2008). Because of the exceptional transparency of *Danio rerio* tissues, angiogenesis, cell invasion, intravasation, and extravasation may all be seen in the animals' tissues (Stoletov et al. 2007). Furthermore, several human cancer genes have been shown to be structurally and functionally conserved in the *Danio rerio*, which has a complete genome sequence and can be studied in the laboratory. There are many mutant and tissue-specific transgenic zebrafish fish lines available that may be used to manipulate the genetics of zebrafish (Kari et al. 2007). *Danio rerio* may also absorb tiny molecular weight chemicals straight from the water, making it an excellent candidate for drug screening, particularly in the area of cancer (Parng et al. 2002; Stoletov and Klemke 2008) (Fig. 1).

On the other hand, cancer cells infiltrate the circulatory system and penetrate the parenchyma, where they may then move to other areas of the body via metastasis. A better knowledge of the process of tumor metastasis is essential for the development of novel anticancer drugs and the improvement of treatment approaches. Cancer models based on zebrafish have overcome many limitations that have hindered studies based on in vivo mouse models in the past, enabling researchers to better understand how cancer spreads. When zebrafish larvae reach 14 DPF, they often develop an adaptive immune system, which produces an environment that is optimal for cancer cells to thrive and propagate throughout the body (Traver et al. 2003). Using a microscope, zebrafish may be used to analyze the development of tumor metastasis and how it might be prevented. It is possible to utilize CM-Dil or red fluorescent protein to stain or identify transplanted cancer cells in order to better understand the metastatic process (RFP) (Marques et al. 2009). Using transgenic zebrafish injected with a red fluorescent protein, researchers were able to clearly see the process of cancer cell metastasis and angiogenesis after just 48 hours (Yang et al.

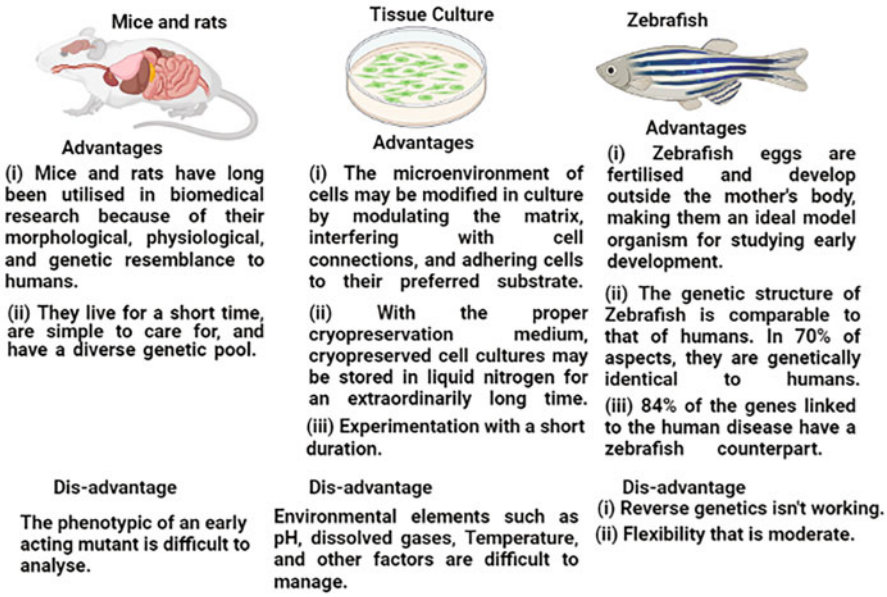

Fig. 1 Model organisms' strengths and weaknesses as tools for scientific investigation. BioRender software was used to make this picture

2013). Identification of the factors that either prevent or promote metastasis in vivo may be accomplished via the use of zebrafish. When RFP-treated U87 glioma stem cells are injected into the yolk sac of a *Danio rerio* embryo, they may clearly demonstrate the numerous invasive stages of GSCs, including approaching and invasion, migration, transmigration, and cluster formation, 48 hours after injection (Khan and Alhewairini 2018; Yang et al. 2013).

Genetics

Science had successfully cloned the first circadian gene from the zebrafish by 1998, which was followed by the discovery of period genes (Whitmore et al., 1998; Delaunay et al. 2000). It seems that the *Danio rerio* has many of the same clock genes found in many other creatures, including humans, according to this research. The circadian timing system of the zebrafish may be fine-tuned to achieve minuscule degrees of control and regulation via a large number of gene replications. According to a recent study, zebrafish's circadian clock may not be as reliant on the SCN as the clock of humans. Cyclops (cyc) mutant *Danio rerio*, which lack ventral brain areas such as the SCN, have a normal period and phase of pineal cycles of melatonin, but wild-type zebrafish have abnormal periods and phases (Noche et al. 2011). Because the amplitude of the melatonin rhythm diminishes with age, the SCN or another ventral brain region may be involved in the modulation of pineal rhythmicity.

The zebrafish genome contains two homologs, one of which is found in the larval pineal gland (melatonin production gland) of the zebrafish genome (Shiraki et al. 2010). Exorhodopsin (exorh) is a pineal opsin that is extraretinal rhodopsin-like, and it was first identified in the pineal of the zebrafish. It is a kind of opsin that is found in the pineal of the zebrafish. It has been discovered that exorh and Rh1.1, both of which are found in the retina, have a 74% amino acid match (Mano et al. 1999). The expression of the pineal exorh gene is high (Asaoka et al. 2002; Mano et al. 1999; Pierce et al. 2008). Exorh has been demonstrated to be expressed in the pineal gland of zebrafish embryos during the ZT18 developmental stage (Pierce et al. 2008).

It has been more than 70 years since the discovery of hereditary melanoma in a hybrid *Xiphophorus* (platyfish) strain that was genetically linked to the expression of a new receptor tyrosine kinase gene was made. The detection of a genetic predisposition to cancer in fish is thus possible by advanced genetic analysis. Mutagenesis screenings in zebrafish were carried out concurrently at the Massachusetts General Hospital (Boston, MA) and the Max-Planck Institute in Tübingen, Germany, resulting in the identification of hundreds of mutants with diverse population range of developmental characteristics at both institutions, the researchers reported. In the framework of these historic genetic screens, we will demonstrate how three unique mutagenesis processes may be used to find mutations in cancer-related genes using three different mutagenesis procedures. For example, forward genetic screenings on haploid and diploid lines, reverse genetic methods such as TILLING, and viral insertional mutation screens are also available (Berghmans et al. 2005a, b; Walter and Kazianis 2001; Schartl et al. 1999; Haffter et al. 1996).

A chromosomal deletion in band 1p36.1 was found in 35% of primary neuroblastoma samples, and nine additional chromosomal sites with allelic loss were revealed in 15 to 44% of primary neuroblastoma samples: 2p, 3p, 4p, and 5p, 9p and 11q23, 14q23-ter, 16p12–13, and 18q. Identifying practically all of the target genes for these chromosomal locations continues to be a mystery (Berghmans et al. 2005a, b; Maris et al. 2002).

Identifying classical recessive or haploinsufficient tumor suppressors may be possible by genetic changes that worsen the cancer phenotype. The loss of a haploinsufficient tumor suppressor, which directly promotes cancer characteristics in heterozygous carriers of the mutation, is hypothesized to be the primary cause of the disease.

A zebrafish with a mutation in the tumor suppressor 53 was one of the first cancer models to be found by an ENU screen, and it was also one of the most successful (tp53M214K). The tumor suppressor gene TP53 is the gene that is most often altered in malignancies of the human ovary and colon. Individuals with malignant peripheral nerve sheath tumors, often known as PNST, have been seen in these mutant tp53−/− fish, which is rare in wild-type (WT) individuals. The zebrafish phenotype mirrors that of human TP53-inactivated patients to a certain degree. Breast and brain tumors, as well as leukemias, are examples of cancers that are not sarcoma-related. There has been a recent publication of an improved model of CG1 zebrafish with the tp53del/del loss-of-function deletion allele. Many zebrafish develop leukemia or germ cell tumors, which are more comparable to those seen in human patients than in zebrafish (Hason and Bartunek 2019; Ignatius et al. 2018; Berghmans et al. 2005a, b).

Zebrafish Tumors from Chemical Mutation

Cancer affects many individuals worldwide and all vertebrates, which is a horrifying reality. Invertebrates such as flies, nematodes, and other invertebrates are susceptible to aberrant cell development. All vertebrates are affected with one of the most horrible illnesses known to man, and it is practically universally present in all animals. Therefore, vertebrate models must be used to better understand the development, growth, and spread of malignant tumors in humans. Because of its capacity to breed, the *Danio rerio* is an excellent model for cancer research when used as a vertebrate animal. Even though humans and fishes are genetically unique, cancer biology in both species is quite similar. Among the many uses of the zebrafish are carcinogenic treatment, the transfer of tumor cells from mammals, and transgenic control (Amatruda et al. 2002; Mizgirev and Revskoy 2010).

In order to produce tumors in zebrafish, the first approach that was used was chemical carcinogenesis. When exposed to waterborne carcinogens in their natural environments, a vast range of benign and malignant tumors may form in practically every organ of the zebrafish, including the brain, having a histology that is remarkably similar to that of human tumors. Zebrafish have been shown to develop a wide variety of benign and malignant tumors in almost every organ when exposed to waterborne carcinogens in their natural environments (Hawkins et al. 1985; Spitsbergen et al. 2000a, b). In *Xiphophorus* hybrids, melanomas demonstrate that cancer in fish is a genetic sickness, similar to that seen in humans (Walter and Kazianis 2001). According to the results of a genome-wide comparison between humans and zebrafish, cell cycle genes, oncogenes, and tumor suppressors are all conserved across the two species. It is possible to apply carcinogens directly to fish water since they are water-soluble, and a variety of carcinogenic chemical compounds prevalent in humans may induce tumors in zebrafish, as well (Goessling et al. 2007). Zebrafish and medaka (*Oryzias latipes*) are susceptible to developing tumors after being exposed to chemicals such as ENU (ethylnitrosourea), DMB (dimethyl benzanthracene), DEN (diethyl nitrosamine), and NMNN (N-methyl-N-nitro-N-nitrosoguanidine) (Beckwith et al. 2000; Okihiro and Hinton 1999; Mizgireuv et al. 2004; Spitsbergen et al. 2000a, b; Lam and Gong 2006; Mizgireuv and Revskoy 2006; Lam et al. 2006). It is estimated that hepatocellular carcinoma accounts for more than half of all cancer cases in the human body. At the same time, it may also occur in other organs and tissues, such as the muscle (rhabdomyosarcoma), skin, testicles (seminomas), pancreas, and blood vessels (hemangiosarcoma). Using a zebrafish cancer model, researchers observed structural and molecular similarities between chemically induced cancer and human cancer, including enhanced cell proliferation and abnormal nuclear morphology, and a low degree of differentiation in the human cancer model. According to recent research, genes that control the apoptosis, cell cycle, and DNA repair have also been shown to be very similar in both fish and human malignancies (Lam et al. 2006; Lam and Gong 2006). These results suggest that the mechanisms of carcinogenesis in humans and zebrafish are very comparable (Stoletov and Klemke 2008; James et al. 2002).

Mutant Lines of Zebrafish

Cancer is caused by an accumulation of somatic events, such as gene mutations that have an influence on gene function that occur over a period of time and accumulate. Tumors may be generated in a variety of organs of the *Danio rerio*, including the liver, pancreas, vasculature, gastrointestinal system, testicles, muscles, and skin, by introducing different gene mutations or activating signaling pathways using chemicals in various organs of the zebrafish. By using oncogene suppressor and enhancer screens, it is feasible to discover the interacting oncogenes that are responsible for the development of a certain kind of tumor. It is possible to study the interactions between a transplanted cancer cell and its host's vasculature using zebrafish after they have been implanted with human tumor cells (Spitsbergen et al. 2000a, b). Both humans and zebrafish contain orthologous oncogene and tumor suppressor genes (TSGs) that are involved in chemically induced cancer (Berghmans et al. 2005a, b). The comparison of gene expression patterns in the livers of humans and *Danio rerio* has shown that gene expression patterns at different stages of tumor aggressiveness are preserved across these two phylogenetically distant species (Mirbahai et al. 2011).

But somatic mutations occur at a low rate because of the intrinsic imperfection of genome processing or exposure to endogenous and exogenous mutagens, which causes them to occur spontaneously at a low rate (Bertram 2000). According to Nowell's view, most tumors have many mutations, which can only be explained by the presence of a high rate of mutation in the tumor (Loeb et al. 2003). DNA replication, recombination, DNA repair, checkpoint control, and chromosomal segregation (Cheng and Loeb 1993; Loeb et al. 2003) may be involved in the associated genomic instability with the mutator phenotype (Feinberg et al. 2002). Since large-scale genetic screening in zebrafish has been possible, researchers have uncovered many mutant *Danio rerio* lines that show greater rates of spontaneous neoplasia and increased vulnerability to carcinogen treatment, among other characteristics (Shepard et al. 2007; Faucherre et al. 2008). In this group, there are two sorts of mutations that have occurred. Mutations in mammalian tumor suppressor genes are well-known in type-1 breast cancer (p53, apc, pten) (Faucherre et al. 2008). Type-2 genetic mutations include those that have not been linked to cancer in mammals (e.g., bmyb, separase, and other ribosomal proteins), as well as those that have been linked to cancer in humans (Wallace et al. 2005). Many of these mutants (apc, pten, separase, and so on) are homozygous lethal, meaning they must be kept in a heterozygous population to be investigated. In comparison to traditional carcinogen-induced mutations, these mutations have a higher rate of cancer occurrence (28% for p53, 17% for APC, and as high as 100% for ribosomal proteins mutants), as well as a susceptibility to specific cancer types, which distinguishes them from traditional carcinogen-induced mutations P^{53} mutants, for example, Bmyb mutants are at increased risk of developing peripheral nerve sheath tumors, while amyb mutants are at increased risk of developing vascular and testicular malignancies (Shepard et al. 2005) (Fig. 2).

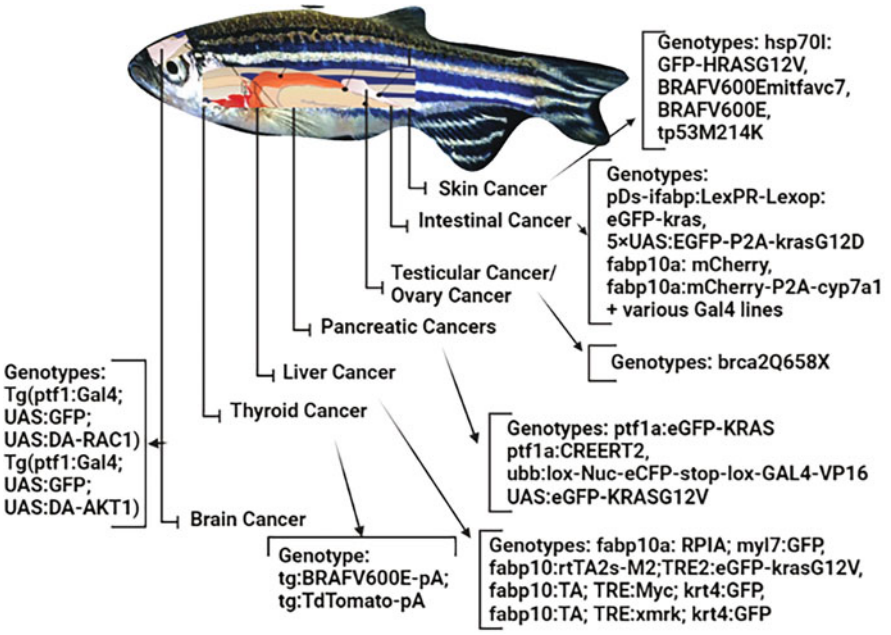

Fig. 2 Some genes are mutated in diverse locations of the zebrafish (*Danio rerio*) as cancer genetic models. BioRender software was used to make this picture

When a cancer gene (such as p^{53} or bmyb) has been found, it is quite simple to do secondary screening for chemical and genetic modifiers of the neoplastic phenotype. Bmyb-induced hyperplasia/cell proliferation issues have been demonstrated to be ameliorated by chemical means, and some of these chemicals have been proven to have anticancer properties (Stern et al. 2005). Until now, the tumor development and metastasis of mutant lines have been recorded mostly by histological evaluation of organ samples, making it difficult to observe these dynamic processes in vivo over time. Carcinogenesis produced by chemicals is analogous to this. The use of ultrasonography has recently been shown to be effective in detecting zebrafish tumors in both the p53-deficient and wild-type conditions. Using this technology, scientists could detect tumors as small as 2 mm in size. Possible future use of anticancer treatment is tracking response over time. This method has the potential to be utilized to monitor tumor growth and treatment effects in live fish at a reasonable cost and without the need for invasive techniques. In addition to the Casper mutant line, which stays translucent throughout adulthood, and transgenic *Danio rerio*, which produce tissue-specific fluorescent marker proteins in numerous organs, other potential crossings include (White et al. 2008; Kari et al. 2007). The use of standard brightfield and fluorescence imaging methods would allow for the direct observation of tumor growth and distant metastasis (Stoletov and Klemke 2008).

Carcinogen-induced carcinogenesis is a multistep process that includes the accumulation of epigenetic aberrations and gene regulatory alterations and the disruption of signaling networks in the tumor microenvironment (Tischoff and Tannapfel 2008). DNA methylation occurs at CpG dinucleotides and affects protein-DNA interactions, which in turn affects gene expression levels (Pomraning et al. 2009). The aberrant methylation of CpG islands (CGT) in the promoter or exonic regions of genes is linked to tumorigenesis and changes in gene expression as a consequence. TSGs, antiangiogenic genes and DNA repair genes are all negatively influenced by the hypermethylation of tumorigenic genes, which is a hallmark of cancerous cells. In addition, tumorigenic genes are often hypermethylated in neoplastic cells (Lopez et al. 2009). It has been shown that carcinogens in the environment may cause cancer in various fish species. On the other hand, zebrafish proved to be the most effective model in terms of embryogenesis, organogenesis, and the effects of environmental carcinogens on cancer formation (Bailey et al. 1996; Khan and Alhewairini 2018).

Xenotransplantation

When human cancer cells are transplanted into zebrafish, it gives a unique method of investigating the interactions between the transplanted tumor cells and their host's vacuole. Additionally, *Danio rerio* have been used in the study of tumor angiogenesis, which is a vital phase in the growth of tumors and a potential target for antitumor therapy.

The capacity to mark or stain cancer cells with a fluorescent dye is the most significant characteristic of xenotransplantation since it allows researchers to distinguish transplanted cells from healthy ones and watch the tumor's progress over time. Transplantation of various tumors into zebrafish, including lung cancer, pancreatic and breast cancer, ovarian carcinomas, retinoblastoma, and prostate cancer, has been effective in the past.

The use of *Danio rerio* models for human cancer has shown that the mechanisms of neoplastic transformation and tumor development are conserved through very vast evolutionary distances. With the zebrafish model, it is also feasible to explore fundamental developmental, transformational, and pathological processes in vivo. Furthermore, this model is especially beneficial in cancer research because of its ability to transition seamlessly between embryonic and adult animal studies. Because of these qualities, zebrafish models of human cancer will continue to be used in the future (Ghagane and Nerli 2021).

When it comes to spreading human cancer cells, zebrafish xenograft models outperform typical rat and chick xenograft models in three important ways. In the first instance, Zunächst combines the superb imaging qualities and power of *Danio rerio* genetics with the extensive knowledge base and complete tool chest obtained from decades of research into the biology of human cancer. This species is particularly well suited for the investigation of new cancer treatments. Zebrafish have a very simple and full vertebrate system that may be utilized to determine the processes by which medications interact with the body in vivo. Zebrafish are also used

to study the effects of pharmaceuticals on the nervous system (Kari et al. 2007; Goessling et al. 2007).

Angiogenesis

Angiogenesis is the most important factor in the formation of tumors, and it is also the most important factor in the process of metastasis. Angiogenesis is crucial in the development of the body, as previously discussed in the section on development. The vascular system assists in transporting oxygen and nutrients to cells, and tumor cells also profit from the movement of blood through the body. This means that the development and ability to create blood vessels inside the tumor determine the aggressiveness of the disease and influence the treatment effects and prognosis of the patient. A fluorescent dye or protein may be used in conjunction with an endothelial cell staining technique to see tumor neovascularization at an early stage and track the progression of metastasizing tumor cells at the cellular level.

Historically, antitumor medications have concentrated on addressing the vascular system of the cancerous tumor. However, research and clinical observation have shown that a combination of angiogenesis inhibitors and chemotherapy may significantly improve the prognosis of cancer patients.

Transgenic Lines of Zebrafish

The ability to express a gene of interest in zebrafish is enabled by using tissue-specific promoters, which may be achieved via the application of zebrafish genetics (e.g., oncogene or mutant version of tumor suppressor). It is also possible to modify the gene to co-express a variety of fluorescent protein isomerases. It is feasible to naturalize stable transgenic lines and in vivo tumor growth monitoring using fluorescence microscopy.

In recent years, many novel techniques for gene editing and transgenic insertion into the zebrafish genome have emerged. The purpose of these reverse genetic procedures is to establish a loss-of-function phenotype or to transfer changed genes from human cancer patients to fish. Developing a *Danio rerio* model with a mutation in a gene orthologous to a human cancer-related characteristic might be a viable alternative. Patton and colleagues developed a *Danio rerio* model of melanoma at the Zon Laboratory, which was the first to show that the BRAFV600E gene could be used to drive the creation of nevi and cancer. BRAFV600E was then expressed in zebrafish under the control of the mitf promoter, resulting in the formation of nevi in the test animals. The researchers then used the TILLING technique (targeting induced local lesions in genomes) to identify mutant tp53 proteins after treating zebrafish with ethylnitrosourea, which caused melanoma. This was the first confirmation that p53 inactivation is responsible for the progression of melanoblastoma development (Barriuso et al. 2015; Beckwith et al. 2000; Patton et al. 2005).

Melanoma-related genes in zebrafish may be transiently overexpressed or knocked out, indicating that they are involved in the disease. The MiniCoopR system was created to achieve tissue-specific gene overexpression in humans (Ceol et al. 2011). Melanocyte proliferation and the development of malignant melanoma are suppressed in Tg(mitfa:BRAFV600E) p53 fish that have been genetically modified to have the mitfa mutation. In these fish, a mitfa minigene is colocated with a putative oncogene that is driven by the mitfa promoter thanks to the use of a transposon-based vector, MiniCoopR (Ceol et al. 2011). Through the use of the CRISPR/Cas9 system modification, it is possible to specifically delete a gene of interest in melanocytes (Barriuso et al. 2015; Ablain and Zon 2016; Ablain et al. 2015).

It was discovered that zebrafish rag2 promoter could govern the overexpression of human c-myc, which was utilized in this investigation. As predicted, the transgenic zRag2-EGFP-c-myc fish line developed T-cell acute lymphoblastic leukemia (ALL) in a short period (21–42 days). It was discovered that the GFP-c-myc cells penetrated various organs and tissues very fast, resulting in the death of the animals. In fact, cancer spreads among these animals at such a rapid pace that the fish line could no longer be kept up to date properly. The next modified their model by using a Cre/lox and heat-shock promoter system to make c-myc expression conditional, which allowed them to test their hypothesis (Stoletov and Klemke 2008; Langenau et al. 2005; Feng et al. 2007).

Role of Melatonin in Cancer

Using more current immune-based treatments such as targeted therapy, cancer cells are targeted and destroyed with greater precision, while side effects are minimized and a reasonable quality of life is maintained. The majority of zebrafish organs, on the other hand, operates in a manner comparable to that of their human counterparts. There are many therapeutic implications and possible applications for melatonin, including the ability to regulate the biological clock (e.g., sleep and mood), immunological function, cancer development and growth, and cancer initiation and progression. For example, in European clinical studies, patients with metastatic non-small cell lung cancer who received concomitant melatonin and chemotherapy outlived those who received only chemotherapy. Furthermore, patients who received melatonin and chemotherapy had higher 5-year survival and overall tumor regression rates than those who received only chemotherapy (Lissoni et al. 2003). The administration of melatonin to patients with advanced cancer who have only a limited projected survival time resulted in the stability of their condition and the improvement of their performance status in certain instances (Bartsch et al. 2002).

The use of zebrafish clock systems, which are comparable to those found in humans, has allowed researchers to better understand the human circadian rhythm (Cahill 2002). Not only does the pineal gland produce melatonin, but it also releases it immediately into the circulation and cerebrospinal fluid (CSF), where it is coupled to albumin and reaches various parts of the central nervous system (CNS) and all other parts of the body. The production of melatonin by the body governs the

migration of neutrophils. While typical circadian clock gene oscillations have been disrupted in aanat2$^{-/-}$-defective fish, neutrophil migration has been reduced in larvae, despite the fact that the fish is still alive. Melatonin treatment causes reduced neutrophil migration in aanat2$^{-/-}$ larvae, suggesting that endogenous melatonin directly regulates neutrophil migration rather than influencing the circadian clock (Da-long et al. 2016).

Conclusion

There are a number of aspects and considerations that must be taken into account in order to correctly analyze the functional properties of melatonin in any studied system or function. Pineal melatonin is a privileged molecule because of its unique qualities. It functions across a range of channels and at practically every level of the organism's physiology due to these characteristics. Furthermore, melatonin is required to pass on the seasonal and circadian rhythms from one generation to the next. Therefore, in the study of circadian rhythms and treatment, zebrafish is crucial because these patterns influence so many different aspects of life on the planet. The results of their investigations and the repercussions of those discoveries will become more understandable to researchers in this manner. To better understand the relevance of the eyes and pineal, two fundamental photosensitive structures in the body of a zebrafish larva, scientists resorted to mutants of the zebrafish that were lacking those organs. Non-receptor-mediated activities include melatonin's amphiphilic ability to permeate cell membranes, nuclear membranes, and organelles, as well as its ability to directly interact with intracellular molecules. The hormone's interaction with membrane and nuclear receptors results in receptor-mediated effects, which are then mediated by the hormone.

In various cancer types, including breast, colon, lung, kidney, and prostate cancers, it has been shown that patients receiving melatonin in conjunction with chemotherapy or radiotherapy have improved tumor remission rates, overall survival rates, and fewer specific chemotherapy side effects. Despite the fact that melatonin is a naturally occurring hormone, it should be emphasized. An excessive dose of medication might be harmful to the patient. It is also useless on its own in terms of anticancer effects, as is the case with the supplementation of other natural products and is only useful when taken in conjunction with the suitable combination of chemotherapy treatments and when operating on a specific tumor.

Zebrafish, which are poikilothermic, prefer a temperature of roughly 28 °C. If this occurs, it might have a negative impact on investigations involving the mammalian homeostatic system's temperature control. On the other hand, zebrafish can survive temperatures ranging from 6 to 38 °C for brief periods (Spence et al. 2008). Teleost genome duplication, which happens when genes are duplicated in numerous copies, affects zebrafish (paralogs). As a result, some genes' functions may become redundant, or their roles may become subdivided from those of the ancestral genes. This trait has been found to be harmful to the genetics of *Danio rerio* (Force et al. 1999; Taylor et al. 2003). The lack of commercial antibodies against *Danio rerio* proteins

is one of the challenges. However, zebrafish lines that are transgenic are widely available from many sources (Hason and Bartunek 2019).

Due to these features, zebrafish are so often used as animal models in scientific research. There are various benefits to utilizing zebrafish as an animal model, including the fact that it has a full genome, is easy to modify the genome, develops embryos in 24 hours, has a short generation time (about 3 months), and may be fertilized outside the body. The transparent structure of the zebrafish embryo allows researchers to explore the multiple phases of embryogenesis, starting with the very earliest stages of development, in great detail. Zebrafish embryos can develop additional organ systems such as the heart, stomach, and blood vessels as early as 48 hours following fertilization. More than 10,000 mutations in protein-coding genes have been discovered in *Danio rerio* transgenic lines, which have been used to study human disorders (Howe et al. 2013). Another advantage of *Danio rerio* is that there are many distinct strains to pick from, which allows for more experimentation. The expense of maintaining a large number of *Danio rerio* in a little amount of laboratory space is also quite affordable in comparison. The prevalence of genomic duplications in zebrafish hinders the study of human disorders such as diabetes mellitus. Researchers want to better understand how the hormone melatonin impacts the development and prevention of human cancer by using zebrafish as a model system.

Conflict of Interest The authors state that there is no conflict of interest between them and their work.

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Part II

Application of Animal Models in Cancer Therapeutics

Role of Telomere and Telomerase Activator in Ageing and Cancer

28

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Abstract

The aging process causes the build-up of free radicals, which causes the cells equilibrium to become unbalanced, finally leading to cellular senescence or cell arrest. Telomere length, a cellular maturation marker, decreases with age and has been linked to aging-related diseases. The rate of telomere shortening, which can be reversed by telomerase, is influenced by environmental factors such as food and manner of life. Telomerase activation by an anti-aging modulator help in the treatment of aging-related diseases. Telomere loss is speeded up by oxidative stress, although it is slowed down by antioxidants. The recurrent telomere-driven tendency is primarily an inflammatory response. Also, considering, the intricacy of the present framework in oncology, the importance of animal *in vivo* model research is important considering the potential to generate effective data with clinical translational impact. The laboratory mouse are commonly used models in biomedical research due to its physiological similarities to humans. The era of genetic engineering techniques has significantly offered powerful tools in designing and developing several mouse strains. The use of induced tumor and aging models has acquired lot of recognition due to availability and ease of various protocols and techniques. Hence, this present chapter will discuss the contribution of *in vivo* models in the development of novel findings toward the biomedical research.

Keywords

Telomere · Telomere length · Cellular senescence · Telomerase activators · Cancer · Biomedical research · Animal models

Introduction

Telomeres, which are complex repeating DNA structures (TTAGGG) at the end of each DNA strand, represent a mechanism that protects chromosomes from damage (Salpea and Hunphries 2010). During cell division, DNA polymerase cannot copy the third terminus of single-stranded DNA, which results in gradual shrinking of telomere and susceptible to DNA impairment (Farzaneh-Far et al. 2008). Shortened telomeres act like an aging clock in every cell. Shorter lengths notify changes in gene expression and change the phenotype of cells to those of older cells. It also alters short telomere genes and can lead to serious complications related to aging (Eisenberg 2011). Aside from aging, oxidative stress; smoking, obesity, and malnutrition reduce telomere length (TL) (Blackburn and Epel 2012; Oeseburg et al. 2010). To recap, telomeres can add, maintain, or augment the TTAGGG telomerase sequence at the end of telomerase-induced chromosomes. Telomerase production declines with age, as well as mortality and morbidity. Telomere length is considered a mitotic clock of aging, which decreases with age. Shortening of telomere is associated with a variety of age-related diseases, including diabetes, Alzheimer's disease, and cancer. Synergistic effects of oxidative stress, inflammation, and repetitive cellular replication are associated with aging and aging related diseases. Telomere length is a significant factor in the prediction of

cardiovascular disease and diabetes. Therefore, to study the in-depth mechanism behind certain diseases associated to telomeres and is of utmost necessity to investigate the disease condition similar to that in human. The possibility to engineer the genome of the mouse has greatly transformed the biomedical research. In the last decade, the gene knockout and transgenic mouse models have become valuable in analyzing genetic disorders, evaluating drugs or even for studying various cancers which aid to answer various fundamental questions. In addition to the increasing demands for more advanced murine models has also become more evident (Lampreht Tratar et al. 2018). Further, in the same line to aging-related diseases, increasing incidence of cancer rates are also attracting the concerns of the researchers. Animal models have contributed various essential information in the field of cancer research. *In-vivo* research is comparable to that of *in vitro* assays, and epidemiological evaluations. Everyone is trying to derive probabilistic interventions in single context that will generalize to humans. The experimental tumors induced in animals, contribute to the most preclinical approach for evaluating novel therapeutic drugs and diagnostic approach (Onaciu et al. 2020). Thus, the topic of animal models for biomedical research is becoming an important area for study, with several investigations to be made in future.

Telomere

Hermann Muller, working with fruit flies (awarded Nobel Prize in 1945) and Barbara McClintock, working with maize (Nobel Prize 1983), hypothesized that telomeres at the end of chromosomes guarantee chromosomal integrity (McClintok 1939). At the end of chromosomes, telomeres comprise a combination of guanine-rich DNA sequences and telomeric binding proteins. The chromosomal peaks are preserved and chromosomal stability is guaranteed by the telomeres because it is necessary for all eukaryotic cells in meiosis. The non-coding DNA strands of telomeres are destroyed during DNA replication during each cell division (Salpea and Hunphries 2010). Cells are seen in somatic cells when they undergo complete cell division through regenerative aging when telomeres are severely shortened (Armstrong and Tomita 2017; Cong et al. 2002). Telomerase is an enzyme that is a complex of RNA subunits, catalytic reverse transcriptase protein, and telomerase binding protein (Wright et al. 1996). The enzyme telomerase can replenish non-coding DNA strands that relax during cell division. The enzyme telomerase is actively expressed in several cells, including stem cells, germ cells, and lymphocytes (Harrington et al. 1997; Palm and de Lange 2008). Telomeres and their role in protecting telomerase and chromosomes were discovered by Carol Glider, Jack Shostaku, and Elizabeth Blackburn. They discovered telomeres in a repeated arrangement, discovered telomerase enzymes, discovered chromosomal capsular structures, such as enzymes that lengthen telomere DNA, and also discovered their role in chromosome protection. In 2009, he received the Nobel Prize in Physiology for the discovery of telomeres and the enzyme telomerase. Recently, it has been discovered that the telomere maintenance pathway by telomerase-related proteins plays a role in various events. Activation and inhibition of telomerase by various significant factors in cancer initiation and aging (Tárkányi and Aradi 2008). The G-quadruplex structure of telomeres and

various telomere proteins serve as therapeutic targets for various cancers, and this secondary structure of telomeres and other telomeres interferes with telomere progression and activity. This G-quadruplex structure can be distorted by other isomeric subunits due to elongation by the enzyme telomerase. It can be seen that the telomerase enzyme has two integrated components, TERT and TERC, which are essential for the therapeutic intervention process. Mutations in these components lead to poor differentiation of stem cells or carcinogens. The enzyme telomerase is one of the main targets in the treatment of aging cancer stem cells. In addition to chemicals to activate and inhibit telomerase, even the active components of plant extracts can be used in a similar therapeutic intervention.

Telomere Structure and Telomere Homeostasis

Telomeres are surrounded by G repeats and are linked to each other by several interacting protein complexes known as telomere shelter complexes (Williams et al. 2013). TTAGGG double-stranded repeating telomeric DNA is single-stranded and has a G-rich overhang with an overhang at the 3′ end called the G (Peterson et al. 2015; Shammas 2011). To prevent recognition as a double-stranded break (DSB), the G tail hides in double-stranded telomeric DNA, forming a translocation loop known as the D-loop (Cong et al. 2002; Takubo et al. 2000). With rich G content, prominent G-rich telomeres (single chains) form the G quartile. The G-quadruplex structure plays an important role in suppressing the expansion of telomerase-dependent telomeres, protecting telomeres, and suppressing genetic recombination (Cawthon et al. 2003). Telomerase and helicase are final treatment enzymes that prevent single-stranded G-tails from being wound at the 3′ end of telomeres. The spread of G-quadruplex requires at least 6–12 nucleotides, which facilitates telomere expansion and alternative telomere prolongation (ALT) substitution mechanism by telomerase (Gong et al. 1999). In humans, telomere maintenance relies on a network of large protein complexes (shelters) found in telomeres. Residential complexes or telomeres are made up of six proteins: TRF1 and TRF2 (telomere repeat binding protein), TIN2 (TRF1-interacting protein 2), RAP1, POT1 (telomere defense 1), and (TPP1 before ACD) (Brouilette et al. 2003). This shelter complex connects single-stranded and double-stranded telomeric DNA with stabilized telomere proteins (Wright et al. 1996). TRF1 was the first double-stranded DNA-binding protein found in telomeres (Palm and de Lange 2008). Telomere length is down-regulated by TRF1 (Vulliamy et al. 2001). The TIN2 protein can identify TRF1 because of its TRFH domain (which is not present in TRF2) (Harley et al. 1990). Like TRF1 (Hastie et al. 1990; Wright et al. 1996), TRF2 plays an important role in down-regulating telomere length. It also helps with the ultimate protection of telomeres. These activities are performed by TRF2 when interacting with multiple factors such as ERCC1/XRF, MRN DNA repair complex, Apollo, FEN1, and WRN (Harley et al. 1990; Lindsey et al. 1991). Telomeres require TRF2 to form an at-loop (loop-shaped structure) (Collado et al. 2007). TRF2 is also required to inhibit ATM activation and non-homologous association (NHEJ) at chromosome ends (Liu et al. 2007). RAP1 is also a protein, but cannot bind directly to telomeres. It relies on TRF2 for telomere

binding and localization (McClintock 1939; Flores et al. 2008). RAP1 is also expected to be involved in the inhibition of NHEJ, but the determinative function of RAP1 is still unknown (Marion et al. 2009). TRF2 and RAP1 also regulate transcription by binding to internal telomere sequences (Brouilette et al. 2003). The RAP1 protein is conserved with the Myb, BRCT, and RCT domains (McClintock 1939; Flores et al. 2008). TRF1 can be used to protect telomere ends and is also required for the synthesis of lagging strands during DNA replication (Blackburn and Epel 2012). POT1 has been shown to regulate telomerase-dependent telomere expansion and protect telomere ends from response to ATR-dependent DNA damage (Zou et al. 2004; Fumagalli et al. 2012). POT1 prevents transcription, protein A (RPA) binding to single-stranded telomere DNA and prevents ATR-dependent DNA damage (Tchkonia et al. 2013). TIN2 is the protein that plays a central role in the terrine complex. It interacts directly with TPP1, TRF1, and TRF2 and acts as a central component (Shay 2016). All disruption of TIN2 significantly reduces telomere localization of all components of the cortex and increases the response to ATR-induced DNA damage (Gems and Partridge 2013). Among the proteins of the telomere complex, TIN2 and ACD play important roles in the recruitment of telomeres to telomeres (Farzaneh-Far et al. 2008). Mammalian telomeres consist of a pair of 10–15 kb for humans and 25–50 kb for mice, and the telomeres of the TTAGGG (Ayyadevara et al. 2009) DNA repeat fragment are 30–400 nucleotides in length of G 3′. It presents the presence of protrusions. G-Strand abundant strands are known for their overhangs. The G-strand protrusion can bend to enter the double-stranded region and form a T-loop to create a transposition or a D-loop. T-loop structures have been proposed to protect the ends of chromosomes from DNA repair and degradation activity, as well as telomerase activity (Kirkwood 1977; de Jesus et al. 2011). Telomere shortens with each cell division due to incomplete replication of linear DNA molecules by traditional DNA polymerase. This is a term known as the replication problem (Bär et al. 2014).

At each cycle, the terminal telomere sequence is lost due to repetitive DNA replication. Telomere shortening is repaired by a number of processes in order to preserve cell homeostasis. Telomerase is a protein that adds a base to the telomere end (Gottlieb and Ruvkun 1994). Cells age when their telomeres shorten. Shortening can cause aging, apoptosis, or malignant malformation of somatic cells, all of which can have a negative impact on a person's health. In sperm and eggs, telomerase is active which then passes from generation to generation. If gametes without telomerase maintains telomere length, the organism's cells would disappear (Campisi 2013). Several studies show that an inverse proportional with telomere length and increased morbidity is associated with increased mortality (Crimmins 2015).

Factors Effecting Telomere Length Determination

Telomere length is a new metric of cellular arrest that is primarily measured in white blood cells. It has a significant morbidity and death risk. With aging, telomere length decreases. Age, lifestyle, genetic structure, social and economic position, weight, smoking, and exercise can all have an impact on telomere length Fig. 1. A high-fiber/

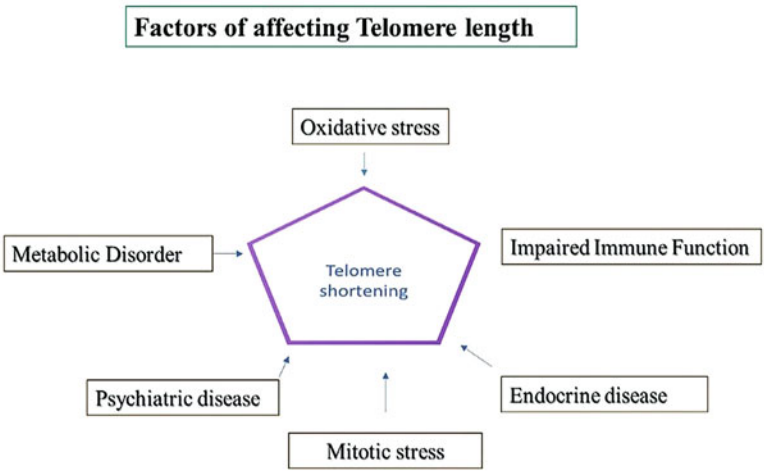

Fig. 1 Picture depicting the factors affecting telomere length

fat diet shortens the length of telomeres. Carbohydrate diets can lower the rate at which telomeres maintain length by relieving oxidative stress (Williams et al. 2013). The telomere shortening rate in human hepatocytes was reported to cause the loss base pairs of DNA each year (Peterson et al. 2015). A similar shortening was also observed in rapidly regenerating gastric mucosa cells. Telomere length is negatively correlated with age because it decreases with the increasing activity of senescent proteins (Shammas 2011). People diagnosed with short telomeres have low survival rates due to high rates of cardiac mortality and infectious disease (Takubo et al. 2000). Gradual shortening leads to aging, the process of dying or forming cancer cells (Cawthon et al. 2003; Gong et al. 1999). Some people are born with shorter telomeres or have a genetic abnormality that causes them to be shorter. These people are more likely to develop early coronary artery disease (Stiewe and Pützer 2001) and age prematurely. Telomerase gene loss shortens telomere length in the genetic illness congenital keratosis and is related to accelerated aging in adulthood, a propensity to malignancy, and susceptibility to infection. Below are certain factors that affect telomere length and homeostasis:

Oxidative Stress

A disrupt balance between the production of reactive oxygen species (free radicals) and antioxidant defenses results in oxidative stress. In 1957, Denham Harman published the "free radical theory"of aging. Reactive oxygen species (ROS) are superoxide (O2-), hydroxyl (OH-), oxygen radicals such as (RO_2-), and non-oxidants. Radicals such as hydrogen, hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), ozone (O_3), etc. are easily oxidized to become radicals. The intracellular reduction environment is maintained by superoxide dismutase (SOD), glutathione peroxidase (Gaps), ascorbic acid (vitamin C) of telomeres by reducing the stress telomere-binding proteins TRF1 and TRF2. Oxidative stress, which causes dysfunction and aging, promotes general

inflammation, oxidative stress, and inflammatory mediators in equilibrium with anti-inflammatory and detoxifying molecules. During illness, this balance changes to oxidative stress and pro-inflammatory sites, leading to DNA, protein, and cell damage, inflammation, and ultimately NF-κB cell death associated with ROS production/RNS and inflammatory cytokines (Brouilette et al. 2003).

Reactive Oxygen Species

Oxidative stress is a major cause of skin damage that causes aging due to the production of reactive oxygen species (ROS). To some extent, ROS fails to achieve its goal of homeostasis and reduces rather than mitigates age-related damage. Free radicals such as peroxyl, nitric oxide, alkoxyl radicals, and superoxide radicals are also the most reactive and toxic hydroxyl radicals. Non-radical oxidizing agents, such as non-oxygen, hydrogen peroxide, hypochlorous acid, ozone, and aldehydes, form free radicals in tissues through a variety of highly reactive chemical reactions and can be converted into active species (Shammas 2011). Few of the genes involved in ROS metabolism are represented in Table 1

Oxidative Stress in Cancer and Aging

The permanent arrest of regenerative aging or growth occurs in cells due to the accumulation of lesions over time during extensive culture. They reduce exposure to apoptosis and malignancies by inducing and maintaining cell senescence and cell stagnation (Epel et al. 2004). Sickle cells are constantly changing, but they cannot proliferate like cancer cells. Crescent cells are wide and flat in shape, with a large number of cytoplasmic granules and vacuoles, and levels of lysosomal beta-galactosidase, p16, and active IL-6. Oxidative stress, mitochondrial degradation, tumor expression, DNA damage, and deprivation of tumor suppressor genes such as INPP4, NF1, PTEN, and RB1 can all lead to cellular senescence. Regenerative aging is caused by stress-induced endogenous stimuli that are different from premature aging. Both processes have identical functional and molecular features, and the extrinsic and endogenous events of apoptosis and cellular senescence depend on the degree of cellular homeostasis disturbance (Liguori et al. 2018). Tissue secreted molecules support the autocrine and endocrine activity of senescent cells at multiple stages including metabolic regulation, epigenetics, and expression of genes. Aging mechanisms lead to activation of mitochondrial-specific pathways that lead to changes in the redox state of mitochondria. Sickle cells secrete molecules involved in the pathology and physiology of various organisms, including tissue repair during

Table 1 Genes involved in ROS metabolism

Genes in Reactive Oxygen Species (ROS) metabolism	*AOX1, BNIP3, MPV17, EPHX2.*
Oxidative stress-responsive genes	*APOE, ATOX1, CAT SIRT2*
Superoxide dismutase (SOD)	*SOD1, SOD2, SOD3*
Genes in superoxide metabolism	*ALOX12, DUOX1, MT3, NCF1, NCF2, NOS2, NOX4, NOX5, CCS, PREX1.*

wound healing, angiogenesis, and tissue regeneration during embryonic development, and are responsible for aging and age-related disorder. Tumor secretion provides cytokines important for the growth of cancer cells and promotes tumorigenesis. Damage to mitochondrial DNA (medina), signaling pathways via p53, Ras, p21, and p16, and autophagy of cellular senescence and possibly a causal relationship between cellular ROS formation (Benz and Yau 2008; Sosa et al. 2013). The irreversible growth arrest that occurs in cells is called regenerative senescence or the accumulation of time-dependent damage during extensive culture. It counteracts apoptosis and confronts malignant progression by inducing and maintaining cell senescence, inhibiting cell proliferation. Sickle cell disease is constantly changing, but it cannot grow like cancer cells. In situ analysis showed that senescent cells showed large, flat shape rich in cytoplasmic granules and vacuoles, with lysosomal beta-galactosidase (SA-beta gal), p16, p21, and IL-6 toll activity showed higher level of expression (Brouilette et al. 2003).

Telomere Length and Age

Telomeres are expressed in the compartments of adult stem cells, but this is not sufficient to compensate for the depletion of telomeres involved in cell division throughout life, both *in vitro* and *in vivo* in age (Brouilette et al. 2007; Vulliamy et al. 2001; Harley et al. 1990). The progressive telomere shortening is a molecular marker of aging that can eventually impair tissue's ability to regenerate. It is an important factor in predicting life expectancy (Flores et al. 2008). Shortened telomeres produce DDR, which induces regeneration and aging as cells attempt to repair damage and are unable to repair DNA damage (Marion et al. 2009). Sickle cell disease gradually accumulates and secretes factors that affect aging-related disorders. Aging has been proposed as an evolutionary defined mechanism against cancer, which limits the promotion of aging-related diseases due to its potential for improving health and extending lifespan.

Aging-Related Genes and Their Roles

Cells that are in constant contact with the harmful environment throughout life, contribute to the factors of dysfunction and induce aging. The deficiency in the mechanism involved in DNA repair which leads to a condition related to aging is known as a progeroid syndrome (Sharpless and DePinho 2007). The genetic factors can act as modulators in the aging process which can lead to developmental studies in the population. The longevity of the individuals is often accompanied by the increased resistance to the disease which leads to early death (Henis-Korenblit et al. 2010). The mutated *AGE-1* gene which was discovered in *C. elegans*, was found to increase longevity in animals, but few researchers believed that effect was induced to the calorie restriction (CR). Later, studies conducted by Thomas Johnson showed that the life-extension is 65% due to the mutation other than the calorie restriction,

the *AEG-1* gene encodes the catalytic subunit of the class-1 phosphatidylinositol 3- (Gottlieb and Ruvkun 1994). In *C. elegans*, mutations in the *daf-2 gene* cause fertile, active young adults which can cause the hermaphrodites to live more than twice as long as the wild type. The genes such as *daf-2* and *daf-16* regulate the formation of the larva and help in the development arrest of larva form which is induced by starvation and crowding. The lifespan extension gets doubled due to the genetic modifications that can be seen in species like *C. elegans* and *D. melanogaster*. In mice, the genetic mutations can be seen in mice which can increase the lifespan up to 1.7 times when combined with calorie restriction (Kenyon et al. 1993). In flies and worms, *SIR2* is capable of extending the lifespan, the *SIR2* homologous in higher organisms have no other role in increasing the lifespan but in human *SIRT1* protein demonstrates to deacetylate p53, *Ku70* with the fork-head family of transcription factors. The other genes can modulate aging by providing resistance to oxidative stress. A protein named superoxide dismutase can protect the effects of the mitochondrial free radicals which can extend the lifespan in the stationary phase when over-expressed (Ayyadevara et al. 2009). Aging is regulated mainly through the insulin/*IGF-1* pathway in higher organisms. The mutation that affects the insulin-like signaling in worms and flies; growth hormone/*IGF1* axis in mice is associated with the lifespan extension. In yeast, nicotinamidase *PNC1* is regulated by the *SIR2* activity. *PNC1* can be transcriptionally upregulated in stressful conditions like heat shock, osmotic shock, and calorie restriction. The activity of the *SIR2* increases in the case of calorie restriction, because the glucose is not available in the cells and more NAD+ can activate *SIR2* (Flachsbart et al. 2009). Large amount genes are associated with the variants associated with age. For example, genetic variation is found in the genes associated with Alzheimer's like *APOE* and *PCDH11X*. Many genes have been identified through conditional senescence studies which include *SOD2*, *TOR/S6K* pathway, Ras/adenylate cyclize pathway, *PKA,* as well as downstream genes (Longo et al. 2012) as a part of the DNA damage mechanism. There are mutations in the proteins that participate in the radical free detoxification that affects variation in the aging the *GPX1* (glutathione peroxidase1) and *rs4880* and *rs1050450* SNP present in MnSOD (manganese superoxide dismutase) genes which are associated with the age-related diseases in mice. In eukaryotes, there are two major oxidative stress regulons, such as dismutase and catalase genes and the other genes mainly involved in the regulation of eukaryotic antioxidant are Rpos, OxyR, and SoxRS regulons (Tatar et al. 1988).

Aging-Related Diseases (ARD's)

The cell arrest considered as an aging hallmark based on two motives: (1) The senescent cells accumulated in the tissue of the organism parallel to the age advancement (2) The depletion of stem and progenitor cells occurs when the senescent cells accelerate the age-related disorder and decrease tissue regeneration (Campisi 2013). An increase in the frequency of aging-associated diseases was found to increase with senescence. The aging-associated diseases are the complications arising from

senescence. Aging involves risk in developing cardiovascular disease (CVD), osteoarthritis, diabetes, cancer, and numerous neurodegenerative diseases. Notably, there was an increase in comorbidities. The research and the observations are trying to find a treatment that can intercept to prevent and delay aging-related disease development, to increase health span and compressing the morbidity. Most of the research work conducted recently mainly focuses on hypertension and cholesterol, the levels of triglyceride, and cancer. There is evidence for an effective strategy to target the molecular mechanisms of age-related disease (Crimmins 2015).

Modern Therapies and Animal Models Involved in Aging Research

There is no proven methodology to stop or delay, the human aging process. Much valid scientific data is not available in the field of anti-science and longevity. It is possible to delay some aging processes by avoiding unprotected exposure to the sun, balanced diet can lower cardiovascular diseases but a single age-related disease cannot scientifically consider as delaying aging. Aging is attributed through the free radical-induced damages, molecular cross-linking, telomere shortening, and changes in the immunological functions. The theories of aging are categorized into three types such as Programmed theory, Combined Theory, and Damage theory (Partridge 2001). In programmed aging theory commonly known as active or adaptive aging theory suggest that there is a deliberate deterioration with age because of limited lifespan.

In the damage theory, the accumulation of the damage in the cells is the spontaneous and entropy derived process occurring due to the absence of the selection of the maintenance. The free radicals are partial intermediates of the oxygen that can be the radical or non-radical molecules generated through a number of inter-related reactions which together cause DNA, lipid, nucleic acids, and protein damage which usually triggers specific mechanisms aimed at neutralizing their effects. In the combined theory unified all the theories of aging carried out following are the postulates that aging is universal, it must be intrinsic, aging is progressive and occurs incrementally and it can be deleterious. Aging is viewed as a highly networked process on the systems level, regulated through the feedback loops between levels of biological organization.

The technological advancement aims at the explicit purpose of curing aging which would prolong a healthy life. There is a multi-factorial process in aging. Following therapies were suggested to delay the aging such as Caloric restriction, Hormonal therapies, Antioxidants, Stem cells, Telomere –Based Therapies, and ALT-711.

The study, conducted by McCay in 1935 showed that the lifespan can be extended by restricting the calories in the laboratory animals. Some have postulated that the reason could be the increased formation of the free radicals within mitochondria, which causes the secondary induction of increased antioxidant defense capacity while other studies suggest that limited availability of the nutrients forces the metabolism to undergo optimization. Additionally, because the Calorie Restriction

can induce various alterations both in the hormone level and proteome level (Robinton and Daley 2012).

The stem cell was the other therapy used for aging-related issues. It demonstrated that the stem cell had the potency to cure health issues ranging from blindness, liver restoration, and nerve regeneration as well as a potent therapy for autoimmune diseases and other age-related diseases, namely, skin carcinogenesis, wound healing, and muscular dystrophies. In somatic stem cells aging, the important regulator is mitochondrial metabolism.

Numerous pharmacological agents have also been studied as blockers for the cross-linking reactions which can lead to the benfotiamine, inhibitors of the rennin-angiotensin system, aminoguanidine, aspirin, and metformin. The compound known as ALT-711 is considered a next-generation anti-aging product, it mainly acts by breaking AGE crosslinks and the research has highlighted its potential in alleviating numerous age-related conditions (Wood et al. 2013).

The hormones are used for the therapy of anti-aging, the patients with the deficiencies of growth hormone (GH) and Insulin-Like Growth Factor-1 (IGH-1) exhibit early signs of aging. In the study conducted, growth hormone was used as an anti-aging medication, GH shows highly beneficial effects on the elderly, and the supplements from the human growth hormone (hGH) have shown an increase in muscle mass and libido, as well as improving the immune system.

There are also few concerns like hGH may stimulate cancer specifically in the patients with existing malignant or pre-malignant tumors. The consensus is that it can be used as an anti-aging therapeutic agent, but more research is required to evaluate the possible deleterious effect and ensure safety as a therapeutic agent.

The antioxidants are also used as the anti-aging therapy, in order to fight the ROS and their effects over lipids, proteins, and nucleic acids which exhibits an array of the endogenous antioxidant systems, which is amplified by the input from the co-factors and by the ingestions of exogenous anti-oxidants. Most of the common antioxidants include vitamin A, E, and C as well as the coenzyme Q10, which was extensively used as advertised face creams which are found to preserve cardiac muscles and the mitochondrial respiratory function in aged rats skeletal. Few studies have revealed that antioxidants do not delay the aging process but rather contribute to the increased longevity.

Antioxidants found in dietary supplements are commonly used as medicines against aging. The high-dosage antioxidants supplements can do more harm than good although, low-dosage of antioxidants have a beneficial effect (Wood et al. 2013).

Telomere-based therapies can increase cell proliferative capacity *in-vitro* and it can reverse the tissue degeneration in the mice. The main concept behind the commercialization, of the telomerase kits, is aimed at estimating the biological age of the individual and for some to estimate the risk of telomerase shortenings associated with diseases such as liver cirrhosis, coronary heart disease, and atherosclerosis. The expression of telomerase has been associated with tumor development and cell proliferation (Wood et al. 2013).

Although, the conventional models have represented in-depth characterization of the molecular alterations occurring during the process of aging, however, limited

number of cell types and model species usually used may limit the interpretation that can be interpreted about the process of basic aging, specifically in long lived species. The advancements in the development of animal models might aid in exploring new areas of genetic manipulation. Advanced molecular techniques will aid in the transformation of the conventional models by genetic modification into new aging model. In a study done by Flurkey et al., reported the development of a mouse model (Pohn strain) for extended female life span (Flurkey et al. 2007). Further, Van Remmen et al., have developed mouse strains with knockouts of particular antioxidant enzymes to analyze the theory of oxidative stress in progression of aging. The profile of lipid membrane of naked mole rates may provide information on their extraordinary longevity (Van Remmen et al.,2004). Therefore, further studies and potential application is required to investigate the variation in the signaling pathways and mechanism which may yield to the delay in the aging process.

Telomerase

Telomerase has a TERT-enzymatic reverse transcriptase subunit and a linked RNA component (Terc) that serves as a template for other de novo telomere repeats (Zou et al. 2004). In eukaryotes. Telomerase is an RNA-based enzyme that catalyzes telomeric DNA elongation. Telomerase activity is low in somatic cells, but it is high in mitotically active cells. It has reverse transcriptase activity. The recruitment of telomeres into telomerase appears to undergo several stages of activation. Telomere shortening is reversed by Telomerase.

Role of Telomerase Activator

Activity of telomerase is measured in vitro by repeating the telomere amplification assay in cell lysates. This repeated telomere amplification assay has been shown to specifically measure a broad spectrum of telomerase enzyme expression levels in different cell types (Fumagalli et al. 2012). The activity of telomerase is absent in the majority of somatic cells during differentiation. Telomerase expression is relatively high in male and female reproductive organs called the testes and ovaries. This may allow lengthy telomeres to be stably transmitted to the upcoming generation (Tchkonia et al. 2013). Telomerase expression was not detected in neonatal stem cells. Its expression begins to decrease in the late blastocyst stage. However, telomerase retains its function during the early stages of embryogenesis (Farzaneh-Far R et al. 2008). Although telomerase activity is weak in stem cells, it extends the proliferative capacity (Gems and Partidge 2013). The expression of the enzyme telomerase explains the proliferative capacity and homeostasis of cells (Farzaneh-Far R et al. 2008). Extension of telomeres from the 3rd end of a DNA sequence occurs in the presence of the enzyme telomerase and occurs in multiple steps rather than a one-step process. Telomerase is actively active in the presence of telomerase-related proteins. This process is precisely and strictly regulated. It includes C chain co-synthesis, substrate recognition, assembly

of ribonucleoproteins, post-translational conversion of hTERT, transport, processing, and nuclear processing. Telomere length homeostasis can be maintained when the active telomerase enzyme is efficiently acquired in all short telomeres (de Jesus et al. 2011). Shorter telomeres in heterozygous TERT mouse cells have been shown to carry short fragments of telomere DNA binding proteins. This negative control paves the way for the mobilization of telomerase. The same mechanism also occurs in yeast (de Jesus et al. 2011). In the S phase of cell division in human and other mammalian cells recruitments of telomerase occurs (Bär et al. 2014). The POT1-ACD protein complex is known to bind to telomerase immediately after recruitment by telomeres (Gottlieb and Ruvkun 1994). This POT1-ACD complex improves telomerase activity and processing capacity, promoting template translocation, and slowing the rate of degradation of RNA primers (Campisi 2013; Crimmins 2015). One of the complex proteins of serotonin, TIN2, aids in the recruitment of telomerase (Kenyon et al. 1993). During the S phase cell cycle, phosphorylation of ACD or TTP1 is aided by Cdk1 and kinases. It is believed that the phosphorylation of TPP1 promotes a generalized stabilization of its interaction with hTERT (Ayyadevara et al. 2009). However, the mechanism of this phosphorylation interaction is controversial. TIN2 likely increases telomerase mobilization (Farzaneh-Far R et al. 2008). TRF1 and TRF2 are proteins of the abortion complex that negatively control telomere elongation by telomerase. TZAP is an alternative telomere DNA binding protein and is also exclusive for binding to TRF proteins (Flachsbart et al. 2009). The TZAP protein stimulates and breaks the T loop of telomeres. Processing and storage of telomerase are provided by the TEN domain of telomerase (Farzaneh-Far R et al. 2008). The telomere TIN2 protein binds to heterochromatin proteins and is recruited to regulate telomere aggregation and condensation (Ayyadevara et al. 2009). The 3′ overhang of the terminal single-stranded telomere DNA indicates the telomerase substrate as the telomerase reaches the telomere. Telomere RNA, repeat DNA fragments at the 3′ single-strand end of telomeres. In this process, the RNA telomerase sequence acts as an additional template for telomere DNA. Telomere DNA elongation occurs when the template region present in the RNA telomerase hybridizes with the substrate formed in the telomere DNA, results in the formation of a DNA / RNA hybrid. The telomerase enzyme then migrates more across the substrate through the RNA template to add additional telomeres repeats at the 3′ end of the telomere. Known as RAP (repeated addition), this process means the ability to simultaneously add repetitive DNA sequences until the telomerase enzyme and binding protein reach the endpoint without fragmentation. Dissociation from temperament is formed from tell-Roman DNA. Appropriate mechanisms for telomere relocation need to be elucidated in the future. Many factors are involved in telomerase processing, telomerase activation, telomere telomerase recruitment, 3′ overhang synthesis, and RNA telomerase reorganization (Farzaneh-Far R et al. 2008). When telomeres become long, it is necessary to stop the activity of telomerase. This is done by the CST assembly. The telomere complex CST (TEN1, STN1, and CTC1) is known to inhibit telomerase progression and telomere elongation (Bilinski and Zadrag-Tecza 2014). Many other factors inhibit the progression of telomerase. For example, (i) lack of connectivity or interaction during telomere elongation affects telomere fibrosis and delayed telomerase progression. (ii) G-quadruplex formation that inhibits telomerase

activity on telomere DNA. The POT1-ACD complex plays an important role in blocking G-quadruplex formation when telomere activity requires telomere elongation (Robinton and Daley 2012; Wood et al. 2013).

Activation of Telomerase Activator

The proliferation of telomerase-negative cells can result in progressive telomere shortening. Although cellular arrest mechanisms protect against cancer, their malfunction may lead to tumorigenesis. The binding of the enzyme Telomerase causes telomeres to lengthen. TAT2 (cycloastragenol) and TA Therapeutics was developed by Geron Corp, is a single-molecule telomerase activator. It was discovered to be a potent telomerase activator in neurons when screening Astragalus membranaceous (Collado et al. 2007). It was first coined from extracts for anti-aging properties. Astragalus membranaceous extract lengthens short telomeres and does not elevate cancer but improves the health period of adult mice, as well as being licensed as a dietary supplement known as TA 65. Some phytonutrients, such as resveratrol and genistein, have the ability to activate telomerase enzymes. Soybean genistein is a naturally occurring isoflavone. Genistein inhibits hTERT transcription in MCF10AT benign breast cells and MCF7 cancer cells. In prostate cancer cells, it also inhibits telomerase activity. Moreover, Ginkgo biloba's effects on telomerase function are still being studied. According to a study Ginkgo biloba extract can increase telomerase activity in endothelial progenitor cells. The human telomerase catalytic subunit is encoded by the vector-transfected cells. Telomeres were elongated in telomerase-expressing clones, and senescence symptoms were decreased. Replicative senescence continues to reduce different physiological functions in tissues, allowing chronic diseases to develop (Partridge 2001). Telomerase activity is observed in somatic cells and there are many chronic diseases where telomerase immortalization becomes a viable option. Phosphorylation via the estrogen receptor agonist histone deacetylase inhibitor (hDAC) and Akt (Vera et al. 2012) is among the one. Many drugs with major non-telomerase targets affect hTERT either at the transcriptional or post-translational stage. Signaling pathways increased hTERT expression and include MAPK / ERK1 / 2, PI3 / Akt, and Wnt / β-catenin pathways. Clonal expansion stimulates the telomerase function of lymphocytes, usually by phosphorylating enzymes to induce nuclear translocation. This role diminishes with age and depletes memory cells, but can be restored by direct interaction with the activating telomerase signaling pathway or the single enzyme telomerase. The strict importance of telomerase recovery limitations has been addressed primarily in the treatment of diseases characterized by aplastic anemia and loss of function of the telomerase enzyme, such as congenital keratosis. Other benefits include the production of epithelium for wounds and burns, and the production of chondrocytes to treat arthritis. Replacement of vascular endothelial cells, osteocytes for bone defects, hematopoietic cells, and immune cells for marrow transplant (Vulliamy et al. 2001). Given the relationship between telomere length and aging, the production of telomerase activators induces the expression of hTERT and/or hTR, increasing enzyme activity and affecting cell localization. This method

aims to reverse the natural process of cellular aging while treating the symptoms of aging. The combination of telomerase inhibitors and conventional chemotherapy is essential in the treatment of cancer. The activity of the enzyme telomerase promotes normal cell division of stem cells and immune cells, and inhibition of telomerase activity has been shown to adversely affect normal stem cell function and immune response. The generation of both telomerase promoters and inhibitors requires a complete understanding of the control of telomerase enzymes in normal cell.

Telomerase activator and Telomere Length

TA-65 (telomerase activator) is known to be a scientifically validated chemical for activating aging human cells. TA-65 has been studied in mice to see what influence it has on telomere length. In *ex vivo* mouse embryonic fibroblasts (MEF) co-cultured with TA-65, the effectiveness of the telomerase promoter resulted in a dose-dependent increase in telomere length. The effect of telomerase for telomere expansion was studied by *in vitro* experiments in the sufficient telomerase-haploid model. The researchers found that the cross of Terc / - (mutation of the Terc gene/absence of low temperature leads to the decreased enzymatic activity of telomerase and increased telomere shortening) with G2 mice Terc / -males to create an older MEF population / -Or G3Terc- / -. Using the TRAP mechanism, TA-65 can activate telomerase approximately twice as much in the telomerase-have super-efficiency model and shows G3Terc / - treated at 10 mM TA-65, showing an average increase in the length of the legs of telomeres. The effect of TA-65 on reducing the consumption of CMV- (cytomegalovirus) telomeres was studied. When compared to the other groups, the high-dose of TA-65 group improved medium-length, telomere length, although the difference was not statistically significant (Berletch et al. 2008).

Role of Telomere in Tumor Progression

The cancer biology is hypothesized to act as a double-edged sword of the cellular processes that alternately inhibit or induces the development of tumorigenesis, depending upon the situations. The impaired telomere function serves as one of the characteristics that enhances this phenomenon. The effect of telomere impairment is greatly transformed when the checkpoint cascades are disturbed and this event has pivotal inference for cancer biology. The activity of telomerase is repressed in normal cells and telomere erosion is part of normal replicative senescence and apoptosis. Specifically, downregulation of p16/Rb cascade and p53 cascade permits and avoid the replicative senescence and further telomere erosion (Beauséjour et al. 2003). Studies have provided evidence on the potential of SV40 large T antigen in extending the proliferative life span of cells in *in vitro* by disrupting both the p16/Rb and p53 pathway (Chapman et al. 2006; Beauséjour et al. 2003). Interestingly, a current study reported that continuous impairment of the telomere can also cause tetraploidy. The crisis of environment itself aids as line of defense against the cancer

development as the resulting instability of the genome and DNA-damage signals kills the wide population of cells. The scarce population of immortalized clones can arise from the crisis by maintaining the telomere length via up regulation of the telomerase expression or by establishing the alternative lengthening of the telomere (ALT) mechanism. The justification of this property of the cells may be because they may have inherited certain secondary genetic alterations that may induce the tumor advancement (Bryan and Reddel 1997; Huang et al. 2007; Li et al. 2019). The dual function of the impaired telomeres was illustrated in murine cancer, where it utilized an inducible TERT expression to create a model of telomerase reactivation in context to analyze the telomere dysfunction. In a p53/PTEN knockout mice of a prostate cancer, the impaired telomere leads to the development of the cancer, but its progression was hindered by the undergoing DNA- damage response activated by the impaired telomeres. However, the TERT nullifies the signaling for the DNA-damage and implements the progression of the metastatic tumors. Such studies provide rigid evidence for understanding the role of telomere dysfunction in tumor development and progression (Chen et al. 2005).

In vivo Study on the Role of Telomere and Telomerase in Tumor Progression

Several hundred years ago, Boveri have suggested that chromosomal instability is the primary event in the process of oncogenesis. In line to the stated hypothesis, majority of the cancers have qualitative chromosomal alterations, like translocation or deletions, also, an aneuploid genome. Telomere erosion has been suggested as a mechanism for the alteration of the chromosome. Telomere length is reported to be associated with various human cancers. The reports of the short length of the telomere in colorectal cancer cells implied that loss of telomere is responsible for the genetic instability and oncogenesis of the malignant cells.

The available evidence from analysis of human malignancy was experimented in the *in vivo* mouse model. The analysis of knockout models for the telomeric proteins is pivotal to delineate how telomere functions and how it is associated with cancer progression. Both *Terc* and *Tert* knockout mice was generated on the B6 background. In the latter generations of *Terc*-knockout mice with shorter telomeres resulted in instability of the chromosome via end-to-end fusion. Although the mechanism of apoptosis dismissed most of these cells but it could be rescued if the DNA damage was not monitored appropriately. Therefore, in *Terc* knockout mice that was also lacking in *p53*, an array of cancers developed mimicking the human malignant condition (Calado and Dumitriu 2013). In order to investigate further the association of the telomere deficiency, short telomere length and p53 in oncogenesis, various *in vivo* experiments in mouse models were done. In various predisposition genetic mice model, the telomerase inactivation enhanced the cancers onset, as in case of *K5-Trf2* (Blanco et al. 2007) and $Atm^{-/-}$ $p53^{-/-}$ double knockout model (Maser et al. 2007). The telomerase inactivation in $Atm^{-/-}$ $p53^{-/-}$ mice model enhanced the occurrence of thymic lymphomas, which was observed to be similar to that of the human

lymphomas. Moreover, *Tert*$^{-/-}$*Atm*$^{-/-}$*p53*$^{+/-}$, mice had complete inactivation of p53 in the tumor cells, suggesting the oncogenic mechanism of the inactivating p53 in the setting of short telomeres. Confirming the necessity of p53 deficiency for tumor onset and progression, as *Atm*$^{-/-}$*Tert*$^{-/-}$ mice with intact p53 had decreased incidence of thymic lymphoma and delayed onset (Qi et al. 2003). Another hypothesis stated that mice deficient with ataxia-telangiectasia mutant (ATM) can outstand as an example of an *in vivo* mouse model that is dysfunctional in both telomere activity as well as in recognizing DNA damage. This might further unveil that despite these mice have chromosomal abnormality and telomere shortening, which is associated with pathologies of pre-mature aging, they also have a greater prevalence of spontaneous tumors. This is parallel to the evidence that specified for Ku86$^{-/-}$/p53$^{-/-}$ and Terc$^{-/-}$/p53$^{-/-}$ mice, and signifies that ATM and p53 may be the signaling dysfunctional telomere tending as impaired DNA, thus provoking apoptosis or cell cycle arrest (Maser et al. 1977). Shortening of the telomere length delayed the growth rate of tumor xenografts and confirmed the susceptibility of the cells to cisplatinum. Further, the inhibition of the telomere not only impacted on the kinetics of growth, but also on the biological features. The histological evaluation portrayed that short telomere length leads to lesser aggressive phenotype of the tumor. Reports also marked that the sensitivity to ionizing radiation that was observed in *mTR*$^{-/-}$ knockout mice with shorter telomeres and the cells isolated from these mice has signified that inhibition of the telomerase activity might be pivotal approach to enhance the effect of radiotherapy. Hence, it is significant to examine the effect of these findings to human cancer cells (Castella et al. 2007).

In mammals the expression of the telomerase is inversely proportional to the body mass, whereas the length of the telomere is inversely correlated with the life span, hence with small body mass and short-lived mammals like mice have emerged to have extended telomeres and the telomerase is expressed at the expense of abolishing the role of telomere in replicative lifespan, as recognized in humans. Therefore, to delineate and characterize the basic biology of telomerase and telomeres, knockout telomerase murine model is effective. However, for thorough understanding about the parts and mechanism of telomeres and telomerase for any human disease and aging it is more challenging to be hypothesized from murine model. Therefore, advanced techniques like deep sequencing might aid in *ex-vivo* modeling of human disorders related to telomeres or telomerase and surmount the limitations inflicted by murine model.

Cancer Model in Animals

Considering the intricacy of the recent foundations in oncology, the importance of animal models in biomedical research is pivotal in accordance with the potential to generate efficient data. Advancements in our knowledge on genetics, immunology and tumor microenvironment have revealed more possibilities in cancer research. Among various animal models, mouse model is most frequently used for cancer research because of its physiological similarities, potential to adapt to different environmental conditions, and genetic variability as shown in Fig. 2. Besides its similarities in

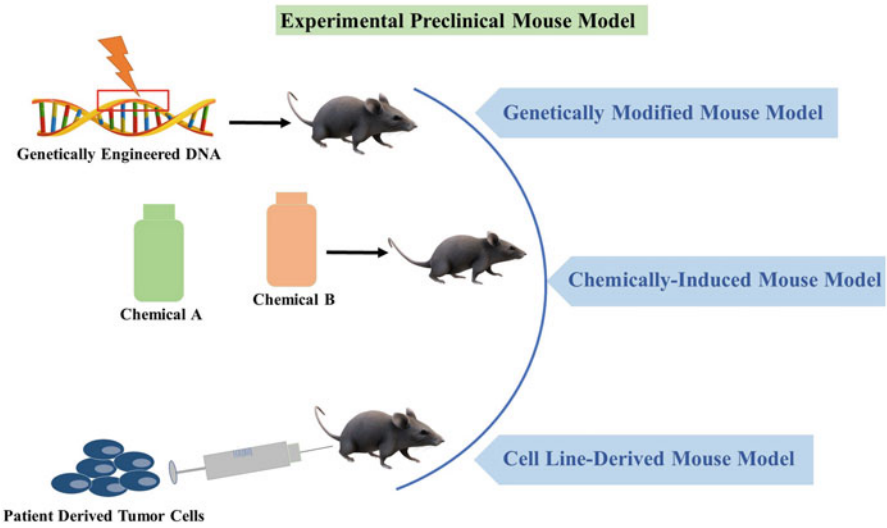

Fig. 2 Schematic diagram representing the major experimental preclinical models for cancer research

biochemical, genetic, and molecular conditions to human, it also contribute similar behavior affected by the emotional status, circadian rhythms, and stress factors.

Specifically, cancer progression follows distinct pathways that comprises of proliferation, angiogenesis, and invasion. To study the molecular mechanism behind each stage of the progression it is of utmost importance to mimic the disease condition and its microenvironment. For instance, recently with the advancement in immunotherapy in cancer research, humanized mouse xenograft models were utilized to specify the relation between human cancer cells and immune cells as depicted in Fig. 3. The mice were transplanted with xenografts derived from patients and also with CD34+ cells that aid in establishing the interplay between tumor and immune cells (Jung et al. 2018).

Animal Model for Colon Cancer

Colon cancer may be sporadic or hereditary comprising for 80% of the total patients affected by the disease. Studies have reported that telomere length (TL) in tumor tissues is shorter than that in the adjacent mucosa. The length of telomere was found to be shorter in lower stage cancer than that in the advanced stage and the shorter telomere length was associated with instability of microsatellite and mucinous tumor histology (Fernández-Marcelo et al. 2016).

For analysis related with the development and treatment of colon cancer or colon and rectum cancer, *in vivo* or *in vitro* models are utilized which act as the representative of the disease condition similar to that of human cancer. In spite of the ethical conflicts involved in *in vivo* experiment, one of the major drawbacks of *in vitro*

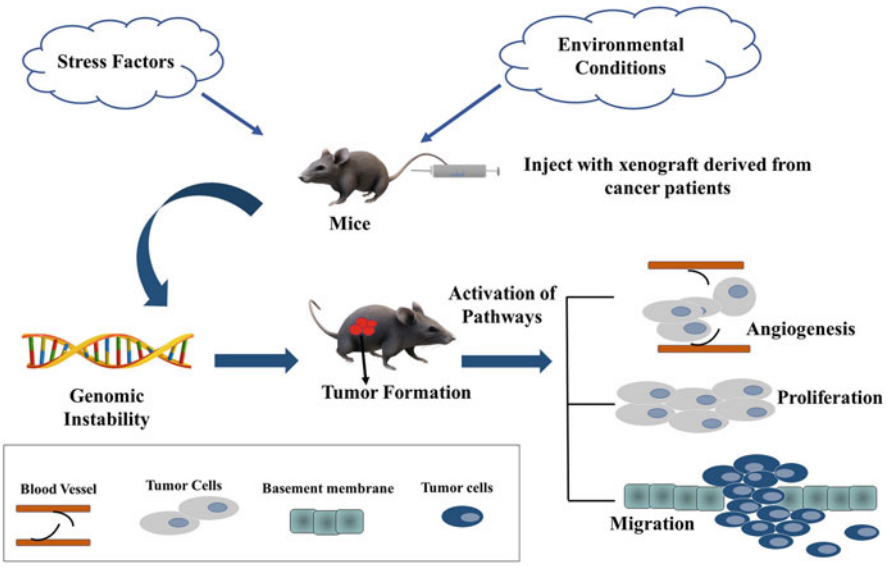

Fig. 3 The diagram representing the overall process for initiation of cancer, in patient-derived xenograft mouse models of cancer

model is that impotent to produce angiogenesis and metastatic condition. Therefore, majorly two types of *in vivo* mouse models are reproduced to represent CRC. One is that induced with chemical compounds and other is genetically modified mice. A study reported that knockout of p53 in mice did not develop into CRC, however the correlation of APC^{min} and *p53* knockout mutations endorsed a surge in aberrant crypt foci count in comparison to APC^{min} animals (De-Souza and Costa-Casagrande 2018). Additionally, Hu et al., evidenced the association of cancer inducer azoxymethane with *p53* knockout mice was capable to induce colon cancer symptoms. Besides mutations in *APC* and *p53* there is also mice models mutated in the codon 12 of the *K-ras* gene (Hu et al. 2005). Another study reported that stated that mice models with *K-ras* mutations causes hyperplasia in the colon region along with aberrant crypts (De-Souza and Costa-Casagrande 2018).

Animal experiments are pivotal for mimicking the pathogenic condition of CRC, also for further evaluation of any specific drug or compounds as therapeutic strategy. Although, certain individual models represent CRC as that of human cancer. However, it is marked that use of at least two compound or methods to induce CRC is mostly accepted, as CRC is a multifactorial disease, and it initiates by the presence of both genetic and environmental factors.

Animal Model for Pancreatic Cancer

Among the several cancers listed pancreatic cancer-related mortality rates is highest and regardless of the efforts from the worldwide to identify noble treatment, only

minor advancements had been made. Hamada and colleagues reported that shorter leukocyte telomere length in pre-diagnostic level was found to be associated with decreased overall survival among with pancreatic cancer patients (Hamada et al. 2019). In a prospective study Luu et al., found that in support to the previously reported studies, longer telomeres of peripheral blood leukocyte were associated with higher risk of pancreatic cancer (Luu et al. 2019). In early years Hiyama et al. reported that telomerase activity was detected in 95% of pancreatic cancer tissues but in tumors was undetectable that indicates and supports telomerase activity was not detectable in benign tumors and normal somatic cells but in primary cancer it is expressed high (Hiyama et al. 1997).

Hence, animal models contribute an appropriate way to evaluate the progression of pancreatic cancer. Several works have been done to delineate the molecular changes that occur in human pancreatic cancer which is one of the major concerns for the researchers for some clinical relevance. Initially, efforts were made to develop pancreatic cancer model utilizing transgenic mice. In which pancreatic-specific gene promoters were used like rat insulin promoter or elastase promoter, to trigger the expression of oncogenes like *SV40, H-ras* in β-cells and acinar cells. However, this model was not able to mimic the disease condition or was unable to develop into PanIN, prior to the development of tumor. Markedly, LSL-Kras^{G12D} inducible knock in mice was the first successful model to demonstrate the development of PanIN to metastatic tumor, similarly to that observed in human PDAC. Genetically engineered mouse model is developed to analyze the frequently found mutations on the background of the knock in mouse model. The SMAD4 model contributes information for the understanding of mechanism behind accelerated tumor development. Further modifications in this model in terms of induction of the transgene also allows for the initiation of mucinous cystic lesions. Involvement of mutations in major genes of signaling pathway was observed to be mutated in knock in mouse models, which has shed light on the signaling cascades associated in the metastasis of the pancreatic cancer. For instance, a study done utilizing LSL-Kras^{G12D}; Ink4a/Arf$^{fl/fl}$ model showed resistance to gemcitabine which was due to the association of hedgehog signaling from the tumor microenvironment (Burchett et al. 2014; Skinner et al. 2012; Hiyama et al. 1997).

Therefore, *in vivo* mouse models of pancreatic cancer are necessary to access the properties of specific drugs or compounds as novel therapeutic or to delineate any molecular mechanism associated in the progression of the tumor. Selection of the proper *in vivo* model for the phase of testing, and also for the experimental intent is crucial for its translation to human clinical studies. Hence, it is possible that in future the genetically modified mouse models for pancreatic cancer might aim on the intricate peroration of the molecular subtypes which can aid for preclinical evaluation which eventually will improve in patient care.

Animal Model for Lung Cancer

Lung cancer is one of the leading cause of deaths around the globe. Lung cancer is a fatal malignancy, basically due to its delay in diagnosis and subsequently has a poor

prognosis. In a study, done on non-small lung cancer cells (NSCLC) obtained from patients found that tumors have shorter telomere length (6.56 ± 0.26 Kb) compared to the control tissues that might cause worse prognosis for post-surgical patients with NSCLCs and altered telomere length might lead to metastasize and recurrent of disease (Fernández-Marcelo et al. 2015). Therefore, there is an immediate need for advanced diagnosis approaches and novel therapeutic options. So, to address these criteria an appropriate disease model is necessary. The highly specific transgenic mice models that firmly resemble the pathophysiological and genetic make-up of the human lung cancer are available. Moreover, due to the reported involvement of the tumor suppressor genes in lung cancer, often these genes are mutated or deleted to induce lung cancer in *in vivo* model. Study done by Wu et al., screened 55 tumor suppress genes to identify its role in tumor progression by utilizing somatic gene editing technique (CRISPR/Cas9) in $Kras^{G12D/+}$ mouse model of lung cancer (Wu et al. 2018). Another study done to analyze the protumor microenvironment, utilized *Kras and p53*-induced lung cancer crossed with *Rag1* gene to exhibit that $Gr1^{+}$ neutrophil deletion tends to increase the tumor progression and deteriorate the efficacy of the programmed cell death-1 immunotherapy. Further a study also reported the association of microbiota, inflammation and cancer by utilizing a *Kras and p53*-induced lung cancer model where it was observed that microbiota triggered the development of adenocarcinoma with elevated cytokine expression (Hosgood III et al. 2009; Jang et al. 2008).

Discussion

A pivotal step in translating telomere research into clinical therapy is by developing telomerase targeted drugs, and therapies. Currently, research in the field of aging and cancer is gaining major concern from the researchers due to which it is attracting more count of project funds for the advancement of novel diagnostic and therapeutic approaches. Therefore, the availability of appropriate animal models to test the new hypothesis prior to its possible clinical translation is necessary. The similarity of the human features of the cancer models is directly linked with the safety and clinical trials. These conditions have direct impact on the social, ethical and economic of our health system, by which a successful preclinical model will govern the rapid translation to the clinical field which will aid in improving the condition of the cancer patients and its surviving rate. Therefore, the study of telomere and telomerase-related human diseases with relevant to using animal models are becoming significant area of analysis, with several other evaluations yet to be done in the future. The major and notable challenges of telomere and telomerase-related researches are accurate measurement methods of telomeric region length and activity of telomerase. A combined analysis of telomere length with telomerase activity may facilitate great exhaustive clinical information compared to the measurement of only a single parameter. In addition, the Telomere Position Effect mechanism can be applied to regulate genes adjacent to human telomeres in Genetic Engineering techniques. Further studies on these aspect aims in throwing light on the treatment

and diagnosis of many human ailments that are linked to dysfunction of telomerase enzyme and silencing of the telomere. The dynamic features of telomerase and telomeres make their structural and biochemical studies difficult. Hence single-molecule approaches facilitate feasible and effective studies of telomerase and telomeres components functioning. Single-molecule methods facilitate an opportunity to know more about the complex dynamics of telomerase and telomeres by allowing researchers and scientists to visualize and manipulate the RNA, DNA, and individual proteins necessary for the normal telomere function. This also paves a way for the production of synthetic or natural drugs from organic sources to halt or stimulate telomerase activity whenever it is required.

Consent for Publication

All authors have approved the final version of the manuscript and gave consent for publication.

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Genetics of Pancreatic Carcinogenesis: Current Molecular Insights from Animal Models

29

Veerabathiran Ramakrishnan and Vajagathali Mohammed

Contents

Abstract

Over the last many years, significant work has been dedicated to imitating the significant characteristics of pancreatic ductal adenocarcinoma (PDAC) in animals, yielding an accurate model of this lethal carcinoma. Carcinogen-treated Syrian models of hamsters create PDAC with hereditary melanomas closely resembling in humans, such as stimulation of the Kras infectious agent, and

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initial research in these organisms affirmed nongenetic health risks for PDAC such as gastroenteritis, overweight, and metabolic syndrome. PDAC study has recently been energized by the advancement of transgenic mouse approaches that rely on tissue-specific Kras modulation and tumor silencer gene deactivation. Amazingly, rodent PDAC develops from excretory acinar cell types instead of stromal cell lines via a phenotypic reprogramming method incited by an inflammatory response. In animal model, scientists found biological processes by which inflammatory endorses and maintains PDAC and objectives for prevention and treatment to repress PDAC in high-risk individual people. The animal model, in specific, has been beneficial in the creation of novel strategies for the early identification and intervention of progressive disease. Several methodologies to be fundamental and preclinical studies on pancreatic cancer, the findings of which could enhance it against presently debilitating illness.

Keywords

Pancreatic cancer · Genetics · Carcinoma · Disease Progression · Animal models

Introduction

Probably one among the deadliest cancers, "pancreatic ductal adenocarcinoma" (PDAC) has a median survival around 6 months to 5-year existence rate of <8% (Siegel et al. 2016). PDAC is the sixth greatest cause of tumor-related fatalities globally, with more than 331,000 deaths each year (Ferlay et al. 2015). For example, both inherent and acquired chemoresistance and significant desmoplasia lead to the poor prognosis of PDAC. Only 9.3% of people diagnosed with pancreatic cancer survived 5 years between 2009 and 2015 in the United States reported by the National Institutes of Health (NIH). It appears thus that pancreatic carcinoma has one of the terrible prognoses of all tumors due to its stealthy onset, high aggressiveness, unusual anatomical location, poor resection rate, high recurrence rate, and absence of typical symptoms associated with other malignancies. Incidence of Pancreatic cancer is predicted to breast and people with colorectal cancer in the United States by 2030, while it is forecasted to be among the world's most frequent cancers (Ryan et al. 2014).

For this reason, to follow the onset and progression of pancreatic cancer in each patient, it is problematic for clinicians to collect samples at altered phases of the disease. So, animal experiments of pancreatic cancer assist physicians in better understanding the incidence, development, and invasion processes of this cancer (Ryan et al. 2014) and can even be utilized to investigate novel treatment methods. For example, an albino rodent was given 2-acetylaminofluorene as a meal in 1941, and Wilson observed that the meal caused pancreatic cancer in albino rats (Wilson et al. 1941). Due to an increase in the incidence of pancreatic cancer, animal experiments began to be studied in the late twentieth century with the help of government agencies. Therefore, it is recommended that an optimal pancreatic cancer animal model should

include the following features: (1) a biological development process that is steady and reproducible, comparable to those of human pancreatic cancer. Precursor lesions such as ductal intraepithelial neoplasia (PanINs) are the most prevalent precursor lesions for pancreatic ductal carcinoma (PDAC) (Hruban et al. 2001). There have been reports of genetic alterations that are significantly associated with this process (Bailey et al. 2016). These mice have been genetically modified to develop pancreatic cancer and are now being tested. According to specialists, the number of mutations in genes such as Ckn2a, Tp53, Kras, Smad4, and others can contribute to the emergence of ductal epithelial neoplasia. The cancer cells are related to human malignancies in anti-apoptotic activity, safe evasion, and metastasis invasion. Pancreatic cancer cell lines come in all sorts of phenotypes and genotypes, each of which represents a subtype of pancreatic cancer. It is possible to deduce the mechanism of carcinogenesis and development by examining how tumor growth, invasion, and metastasis are related to the expression of particular proteins in cell lines (Hruban et al. 2004). Moreover, pancreatic disease models used in experimental trials of customized therapies should be practical and appropriate for large-scale making to deliver tailored therapeutic choices for patients with limited periods.

Impulsive Cancer Animal Models

Impulsive cancer refers to a particular tumor generated in an experimental animal by biochemical, virus-related, or clinical trial genetic means. Tumor transplants are the opposite of this (Hruban et al. 2004). Since spontaneous tumors are more comparable to cancerous lesions in people, animal models of such cancers could extrapolate human outcomes. While large volumes of tumor material can be gathered in a short period, spontaneous tumor incidence can vary. As a result, the examination time is extensive, and the experimentation is overpriced (Longnecker et al. 1992). Figure 1 shows the in vitro studies of myofibroblast-like cells or pancreatic stellate cells separated from malignant or nonmalignant tissues of the pancreas.

Chemically Instigated Animal Models

Rat. Researchers inject azaserine into Wistar and Lewis rats intraperitoneally, and the animals develop cancer in acinar cells of the pancreas and metastases to the lymph nodes and liver (Rao 1987). Although there is no duct-like structure in this model, lesions typically develop with malignancies of organs such as the kidney, liver, and mammary. For example, 4-hydroxyaminoquinoline-1-oxide (HAQO) (Hayashi and Hasegawa 1971), nafenopin (Reddy and Rao 1977), clofibrate (Reddy and Qureshi 1979), and N-(N-methyl-N-nitrosamide)-L-ornithine (Longnecker et al. 1980) (Reddy and Rao 1977) might cause acinar cell damages and other N-nitro compounds (Rao 1987). According to a study by Vesselinovitch et al., benzopyrene, given topically to rats, causes cancer. On average, the rats implanted with the crystal powder of dimethylbenzanthracene produced spindle cell sarcoma and weakly differentiated carcinoma in

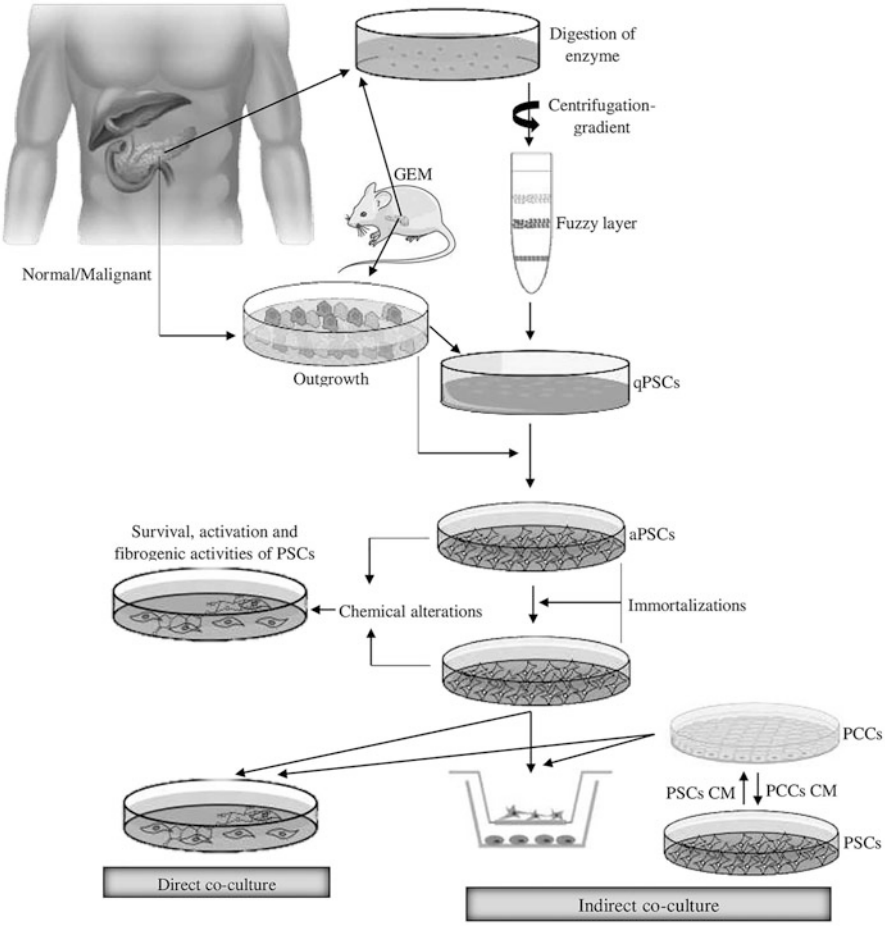

Fig. 1 Isolation and use of PSCs/CAFs for pancreatic cancer desmoplasia studies

around 80% of the cases. Certain research organizations have discovered proliferation in ductal cells, tumors in acinic cells, colon adenocarcinoma, invading ductal adenocarcinoma, and fibro-adenocarcinoma using the same approach (Longnecker et al. 1980).

Hamster. The hamster is one of the most effective model organisms for pancreatic carcinoma research. Cancer-causing chemicals in hamsters do not induce cancer in rats, mice, Dutch pigs, or rabbits. However, despite the reality that its mechanism is unknown, N-nitrosobis (2-oxopropyl)amine (BOP) has the maximum level of selectivity (Gurski 1959; Pour et al. 1979), and it favors the pancreas. Because of its unique characteristics, the N-nitroso-BOP model is related to a well-considered sequence of structural variations that occur in the duct of Wirsung, and it commonly displays single alterations in the Kras gene (codon 12), which is consistent with discoveries in human pancreatic carcinoma (Fujii et al. 2010; Tsutsumi et al. 1993). The hamster's pancreas were caused by

multiplying ductal/ducted acinar cells lose technical characteristics in acinar cells, according to Meijers (Meijers et al. 1989).

Furthermore, concerning form, clinical characteristics, and physical symptoms, hamster tumors are the most comparable to human malignancies. The hamsters had benign and malignant tumors, as well as unusual blemishes. Hamsters, like humans, can develop tumors that enter the perineural region, damage pancreatic lymph nodes near the pancreatic ducts, or cause weight loss, diarrheas, and thrombosis (Pour 1989). In rare cases, jaundice could be created by a pancreatic tumor in the body/tail. Cancers of the hamster pancreatic region had also been discovered to include antigens like glucose, lectin, CA125, TAG-72, TFGR-EGFR, and 17-1A; tolerance has been observed, comparable to human malignancies. As an outcome of this, fetal protein, the embryonal antigen of pancreatic cancer, and carcinoembryonic antigen levels are either less or nonexistent. A hamster pancreatic cancer model, according to the researchers, might be helpful to identify human risk factors and to perform the therapies.

Transgenic Mouse Model of Pancreatic Cancer

Oncogenes were delivered into body cells or embryonic cells of mice using specific promoters of tissues in several recent studies using genetic technology, resulting in pancreatic malignancy. When it comes to genetically modified mouse models (GEMMs), techniques like recombinant DNA, gene knockout, and gene knock-in could be utilized to introduce particular genes into the mice. Protooncogenes of the Kras family are often used in the development of GEMMs. Pancreatic carcinogenesis in mutant Kras-overexpressing transgenic mice resembles mutant Kras (Fig. 2). According to their results, physiological levels of Kras G12D cause pancreatic intraepithelial neoplasia (PanIN) that resemble the whole range of human pancreatic ductal lesions (Hingorani et al. 2003). Since most pancreatic cancers are adenocarcinomas, scientists hoped that the selected promoter would only affect ductal epithelial cells of exocrine cells. However, even the most potent genetically engineered replicas cannot duplicate the complete method of ductal carcinogenesis, and the shift from normal epithelium to tumor cells usually needs four or five genomic alterations (Fearon and Vogelstein 1990).

For example, it is possible to speed up carcinogenesis by inactivating P53 or P16 (or both). This method allows researchers to precisely regulate when and where the mutant gene is expressed, allowing for a more in-depth study of gene function. For conditional gene deletion, Cre/loxp recombinase (Orban et al. 1992) and TET on systems (Gossen and Bujard 1992) have been determined to be the two most commonly used techniques (Schonig et al. 2002). Because of their similarity to human disease, GEMMs for pancreatic cancer have the same characteristics as human disease. However, their metastatic pattern surprisingly mimics pancreatic cancer, according to the research team. Because the model may be used to look at cancer's early stages, researchers can learn more about tumor pathogenesis and treatment effects. However, due to its genetic and biological differences from

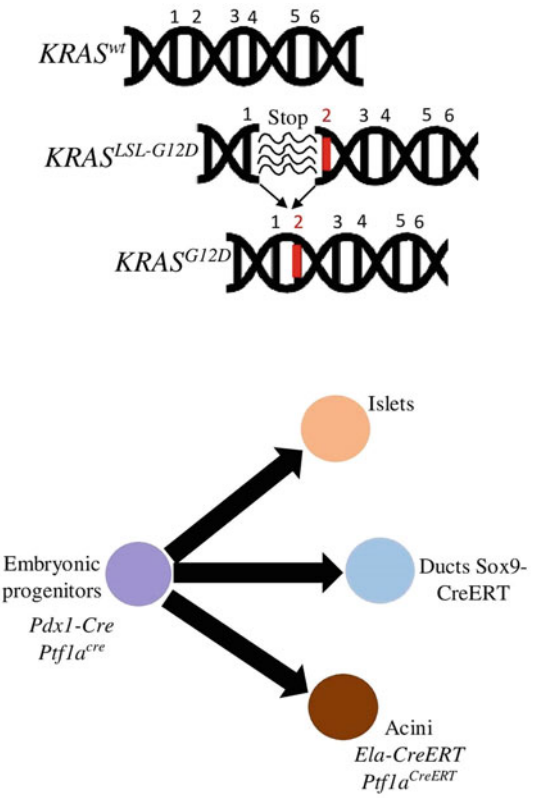

Fig. 2 Inducible Kras mouse model of pancreatic cancer

human tumors, its modeling time is challenging to regulate and costs much money to utilize. Furthermore, meeting experimental needs in terms of quantity might be a challenge.

KIC Model of PDAC

Because acinar and other endocrine cells would display pancreatic duodenal homeobox-1 in response from embryonic stage onward in pre-pancreatic endoderm, Pdx1-Cre may cause gene change in all pancreatic cell types (French et al. 2011). Several spontaneous pancreatic carcinoma types were developed using Pdx1-Cre mice. KIC is one of the most noteworthy models among them. In humans' absence of the cyclin-dependent kinase inhibitor 2A (Cdkn2a, Ink4a) gene, related to cancer syndrome (melanoma-pancreatic), impulsive pancreatic cancer does not arise. However, a mutant Kras allele (KrasG12D) may induce PanIN lesions to grow more quickly, and these neoplasms developed quickly to extremely metastatic and invading malignancy (spleen, stomach duodenum), culminating in a loss in all patients within 80 days (Aguirre et al. 2003).

KPC Model of PDAC

Trp53R172H and KrasG12D have been targeted to the mouse pancreas by Higorani's team, demonstrating the development of aggressive and extensively metastatic cancer that mimics human illness (Hingorani et al. 2005). It takes around 2.5 months for liver and lung metastases to occur in a pancreatic cancer model. The loss of Ink4a/Arf can recapitulate several of the usual structures of virulence in common and pancreatic tumor, in particular, as soon as Kras is activated.

KD Model of PDAC

Smad proteins are hydroxylated and stimulated by cell membrane serine-threonine effector kinases when TGF-beta activation takes place. In pancreatic carcinoma, Smad proteins are frequently inactivated. Various researchers have aimed tumorigenic Kras exclamation and implied Smad4/Dpc4 deletion at the pancreatic progenitor cells of the mouse pancreas (Izeradjene et al. 2007). IPMN lesions were detected in the pancreas of most mice, with a sluggish progression (Fig. 3) (Kojima et al. 2007).

PDAC Model with Kras (TGFBR2 Knockout)

It controls whether cells assigned to pancreatic buds undergo pancreatic organogenesis or revert to duodenal destiny through the pancreas associated with transcription factor 1a (Prf1a). It is believed that the protein has a part in maintaining exocrine pancreas-specific gene expression, such as elastase 1 and amylase, as per the research team. Mutations produce cerebellar agenesis in this gene, and ductal pancreatic tumors lack expression with this gene (Kim and MacDonald 2002). To bind TGF beta, it creates a heterodimeric compound along with TGF-beta receptor type-1. Smad4, which encodes a key signal downstream of TGF-beta, is altered in 55% of human PDAC, while the type 2 TGF-beta receptor (Tgfbr2) gene is rearranged in a lesser proportion. Thus, TGF-beta signaling is critical for PDAC development. When combined with Kras (G12D) expression, Tgfbr2 knockouts resulted in PDAC with 100 percentage penetrance and a medium existence around 60 days (Ijichi et al. 2006). Clinical and histological characteristics of human PDAC are detected in mice with Kras (G12D) expression and a Tgfbr2 deletion. TGF-beta signaling is blocked in these animals, and Ras signaling is turned on, promoting PDAC progression. Therefore, it is preferable to examine TGF-beta signaling in PDAC in humans (Fig. 4).

TetO-Cre Induced by Tetracycline

A Cre gene is triggered when a rtTA or a tTA with transcriptional initiation characteristics connects to a tetO. rtTa or tTa binding to tetO is controlled by tetracycline (or its derived doxycycline, Dox). Dox prevents tTA from binding to TetO; therefore, Cre is not generated when Dox is present (Schmied et al. 2000).

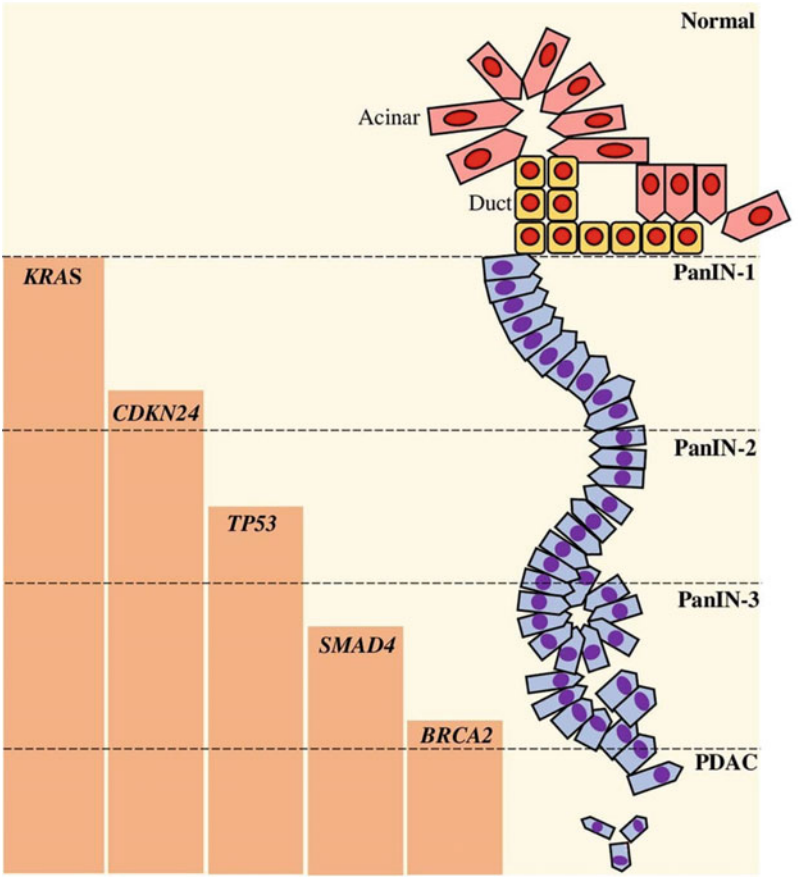

Fig. 3 Pancreatic cancer progression by candidate gene polymorphism

However, rtTA does not function if Dox lacks since it could not interact with teto to promote Cre expression if Dox is present. TetO-Cre and particular tissue rtTA (or tTA) double-transgenic mice with Cre recombinase could be regulated in location and duration by providing or deleting Dox. In particular, the loxp site is detected by Cre, which then splits the sequence of DNA, prompting its arrangement to cross across at the two sites (Fig. 5) (Garofalo et al. 1993).

Formation of Animal Models Based on Cell Lines

In order to examine particular characteristics of human pancreatic cancer, such as the growth of cancer cells, cell cycles, and therapy efficiency, scientists created the athymic (nude) mouse, which is hairless owing to the existence of a pair of

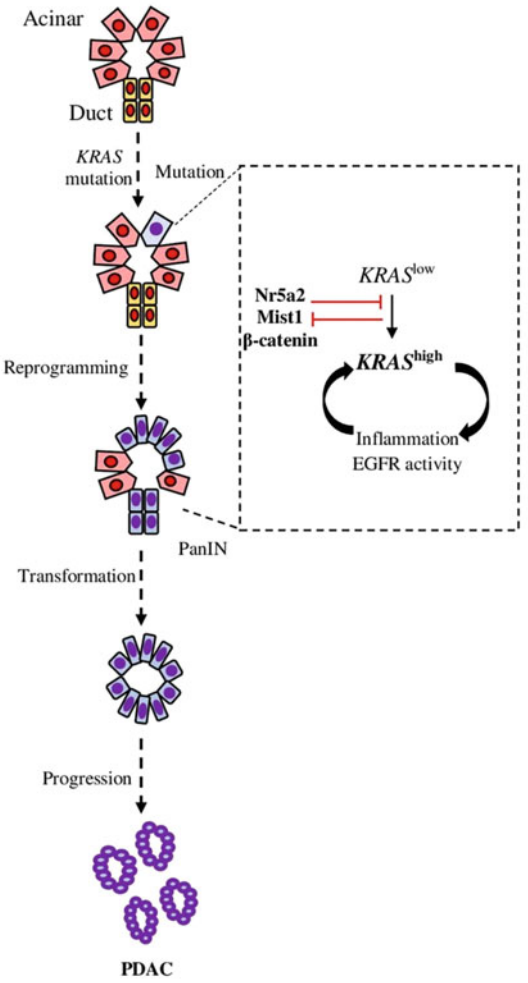

Fig. 4 Acinar reprogramming in the progression of PDAC

genes "nu" (for nude). Therefore, naked mice have no T cells, which are lymphocytes that grow in the thymus. Because they lack T lymphocytes, naked mice cannot reject malignancies or cell transplants from people or animals. Although specific aberrant responses may occur, it is possible to maintain the original tumor's characteristic phenotype by implanting human cancer cells into such models (Loukopoulos et al. 2004). However, in a recent study, pancreatic cancer cells of human origin were given to SCID mice, which had a combined lack of T cells and B cells. There is no difference between mice with different levels of immunodeficiency when it comes to the occurrence of pancreatic cancer (Deer et al. 2010).

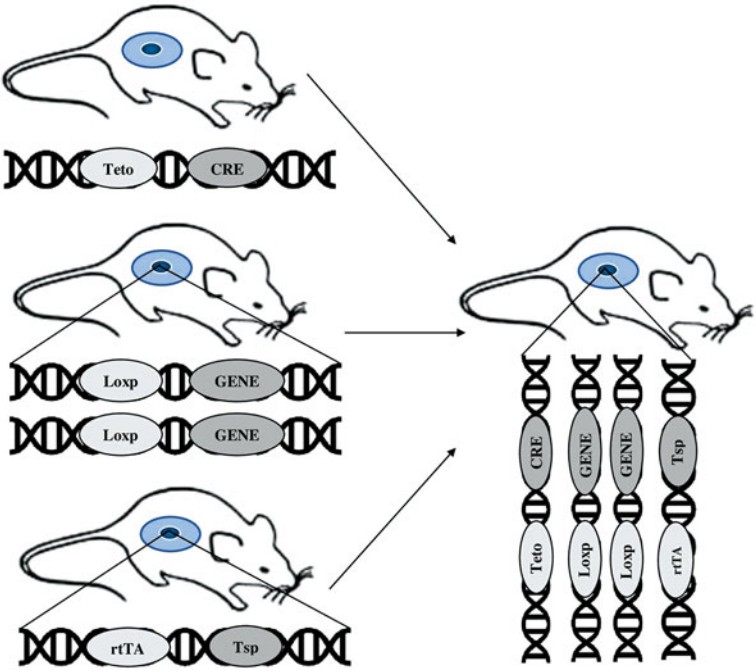

Fig. 5 Tetracycline-induced TetO-Cre for GEMM. Tetracycline actives component controlling Cre mice and its promoter activates the transcriptional factor tTA or rtTA

Cell Line Selection

Because few molecular changes can aid in the diagnosis of pancreatic cancer, it might be helpful for scientists to know their recognized cell lines (Table 1). A precise knowledge of the research direction should influence pancreatic cancer research. A cell's clinical background, in vivo and in vitro morphological characters, and phenotypic, invasion, adhesion (Loukopoulos et al. 2004), and metastatic capabilities as well as genotypic alterations, which most commonly arise in KRAS and other genes, may all be determined (Table 2) (Deer et al. 2010; Alexandrov et al. 2013; Witkiewicz et al. 2015; Kern 2000).

The degrees of differentiation (Sipos et al. 2003) and biological behavior (Monti et al. 2004) of cancerous pancreatic cells were found to be unaffected by mutations within those four genes, according to research (Table 3). However, researchers have found a link between polymorphisms mostly in the p53 gene and tumor metastasis, showing that genotype and phenotype are linked in pancreatic cancerous cell lines (Panayiotis et al. 2004; Moore et al. 2001).

Table 1 Human pancreatic cancer cell lines

Cell line	Tissue origin	Metastasis	Doubling time	Differentiation degree	Morphology	Tumor formation rate (subcutaneous)	Ref
AsPC-I	Ascites	Yes	38–40 hrs	Poor	Epithelioid	–	(Chen et al. 1982)
HPAF-II	Ascites	Yes	42 hrs	Moderate	Epithelioid	–	(Kim et al. 1989)
HPAC-1	Primary tumor	–	41 hrs	Good	Epithelioid	–	(Gower Jr. et al. 1994)
MIA PaCa-2	Primary tumor	–	40 hrs	Poor	Epithelioid	66%	(Yunis et al. 1977)
PANC-1	Primary tumor	Yes	52 hrs	Poor	Epithelioid	86%	(Lieber et al. 1975)
BxPC-3	Primary tumor	No	48–60 hrs	Moderate-poor	Epithelioid	100%	(Tan et al. 1986)
Capan-2	Primary tumor	No	96 hrs	Good		–	(Dahiya et al. 1993)
Capan-1	Liver metastasis	Yes	–	Good	Epithelioid	–	(Fanjul and Hollande 1993; Kyriazis et al. 1982)
SU.86.86	Liver metastasis	Yes	77 hrs	Moderate-poor	Epithelioid	–	(LeDonne 1988)
CFPAC-1	Liver metastasis	Yes	31 hrs	Good	–	100%	(Schoumacher et al. 1990)
Suit-2	Liver metastasis	Yes	29–38 hrs	–	–	–	(Iwamura et al. 1987; Iwamura et al. 1992)

(continued)

Table 1 (continued)

Cell line	Tissue origin	Metastasis	Doubling time	Differentiation degree	Morphology	Tumor formation rate (subcutaneous)	Ref
SW1990	Splenic metastasis	Yes	64 hrs	–	–	100%	(Kyriazis et al. 1983)
Hs766T	Lymphatic metastasis	Yes	6–7 days	–	Epithelioid	–	(Owens et al. 1976)
Colo357	Lymphatic metastasis	Yes	21 hrs	Good	–	–	(Morgan et al. 1980)
T3M4	Lymphatic metastasis	Yes	–	Moderate	–	–	(Okabe et al. 1983)

Table 2 Animal-origin pancreatic cancer cell lines

Cell line	Organism	Carcinogen	Differentiation degree	Gene mutation	Ref
PC	Mouse	BOP	Good	Kras, P53	(Egami et al. 1989; Erill et al. 1996)
WDPaCa	Mouse	BOP	Good	P53	(Chang and Gutman 1982)
PDPaCa	Mouse	BOP	Poor	Kras	(Chang and Gutman 1982)
HPC	Mouse	BOP	Poor	–	(Morita et al. 1998)
HP1	Mouse	BOP	–	–	(Batra et al. 1992)
HaP-T1	Mouse	BOP	Good-moderate	–	(Saito et al. 1988)
H2T	Mouse	BHP	–	Kras, P53	(Townsend Jr. et al. 1982; Sumi et al. 1994)
HPD (1–3)NR	Mouse	BHP	Moderate	Kras, P53	(Saito et al. 1988; Bardi et al. 1997)
Pan02	Mouse	MCA	–	Kras, smad4	(Wang et al. 2012)
6606PDA	Mouse	–	Good	–	(Zechner et al. 2015; Partecke et al. 2011)
AR42J	Rat	–	–	–	(Longnecker et al. 1979; Christophe 1994)

Table 3 Expression of mutant genes in cell lines

Gene	Expression of mutant genes in cell lines
KRAS	Occurred in almost all of the primary tumors of pancreatic cancer, but the BxPC-3 cell line is WT
SMD4/ DPC4	Capan-2, MIA PaCa-2, PANC-1, SU.86.86 without SMD4 gene inactivation
TP53	Its mutation occurs in 50% of pancreatic malignant tumors and is associated with late tumor progression
CDKN2A/ P16	Basically all pancreatic cancer cell lines have inactivation of the P16 gene

Cell Metastasis and Colonization

To better understand tumor metastasis, cancer cell metastasis studies can be performed. According to the Boyden chamber invasion model (Boyden 1962), when chemokine concentrations were varied, cells migrated from one chamber to another. The transwell and scratch tests (Cai et al. 2007) are two more migration experiments that have been conducted (Stahle et al. 2003). Stahle et al. found PANC-1 cells five times more active than BxPC-3 cells in a transwell migration assay. Using

phagocytic cell migration trajectories on a colloidal surface, Li et al. revealed that HPAF-II and BxPC-3 cells were highly mobile (Lin et al. 2005).

Production of Tumor Cells

Schmidt used naked mice to study pancreatic cancer cells. It was only after that that they examined the tumor's size, number, and spread so that they could get an overall picture of the cell line's tumorigenicity. Different ways of causing cancer can lead to varied tumor growth rates and metastatic colonization sites. There are various examples of this, including intravenous injection, implantation metastasis, and in situ implantation. Hypodermic injection of cancer cells remains the most used analytical technique, possibly because it is the easiest to perform. Distinct cell lines cause tumors of varying sizes. In one research, severe combined immunodeficiency (SCID) mice were injected with cell suspensions of MIA PaCa-2, PANC-1, and Capan-1. Biopsies revealed three tumor sizes after 30 days: MIA PaCa-2, Capan-1, and PANC1 (Fogar et al. 2003). MIA PaCa-2 and Capan-2 tumors were developed in donor mice by Eibl et al. (Eibl and Reber 2005). Next, they took out cancer, cut it into a cube of $1 \times 1 \times 1$ mm3, and halved and fixed it in the pancreatic tails of naked mice as a treatment. As a result, they discovered that MIA PaCa-2 tumors formed more quickly and at an incredibly high rate (100%). In this case, however, the tumor microenvironment and morphogenesis are absent because the tumor was originally established under the skin. Pancreatic cancer carcinogenesis and development may be better understood by injecting malignant cells directly into the pancreas. Multiple scientific types of research have examined the effects of injecting various pancreatic malignant cell lines directly into the pancreas of SCID mice to stimulate tumor development (Loukopoulos et al. 2004). The following formula was used to determine the tumor development frequency: AsPC-1 scored 100% (10/10); CFPAC-1 scored 100% (10/10); HPAF-II scored 100% (8/8); Capan-2 scored 90% (9/10); Hs 766 T scored 90% (9/10); HPAC scored 88% (7/8); PANC-1 scored 80% (8/10); and BxPC-3 scored 67% (6/9).

Subcutaneous/Orthotopic Xenografts

Athymic or severe combined immunodeficient (SCID) mice can be used to study the biology of pancreatic cancer using xenografts. T lymphocytes are absent in nude or athymic mice because of defective thymus development (Grippo and Sandgren 2005). SCID mice lack mature B and T lymphocytes because their immunoglobulin gene and T-cell receptor gene rearrangements are faulty (Grippo and Sandgren 2005). Cell lines from pancreatic cancer or primary pancreatic neoplasms are injected into the pancreas either subcutaneously or orthotopically through xenograft transplantation. Pancreatic cancer cells may be studied in vivo for invasion, metastasis, and angiogenesis, as well as the rate of growth and responsiveness to treatments using this technique.

Subcutaneous Model

An injection of pancreatic cancer cells or tumors subcutaneously into mice is a valuable model for assessing tumor growth, either independently or in response to various therapies. To calculate a tumor's volume, it is necessary to keep track of its measurements over time. It is possible to measure tumor mass after tissue/tumor removal. Human pancreatic cancers may be evaluated in vivo using healthy nude or SCID mice, including identifying and conserving cell variants with significant metastatic potential from various human cancers and testing therapeutic medicines against metastatic cell proliferation in visceral organs (Fidler 1986). This approach may also collect human pancreatic carcinomas with varying degrees of histologic differentiation on nude mice (Capella et al. 1999). These xenografts seldom metastasize, even though they usually grow and might be locally aggressive and malignant (Fogh et al. 1980). Subcutaneous xenografts, on either hand, do not show the signs and symptoms of tumor growth in visceral organs (Alisauskus et al. 1995). As an alternative, xenotransplantation of the spleen, liver, or vein has been utilized to make metastatic models, although they bypass many steps in the metastatic cascade (Loukopoulos et al. 2004). The extracellular matrix and the local environment allow pancreatic malignant cells and malignancies, in general, to invade and spread. For this reason, subcutaneous xenotransplantation does not allow for the crucial interactions between stroma and cancer cells, including the exchange of enzymes and cytokines that alter the extracellular matrix, promote cellular proliferation, and boost tumor cell survival (Ellenrieder et al. 1999; Keleg et al. 2003; Addison 2006; Petrulio et al. 2006).

Orthotopic Model

Contrary to popular belief and despite its technical hurdles, the orthotopic model may help to assess local and distant metastases in people. Changes and modifications were made to this orthotopic model to decrease pancreatic injury and intraperitoneal tumor leaking. Direct injection of pancreatic carcinoma cells further into the pancreas is conceivable (Tan and Chu 1985; Marincola et al. 1989; Mohammad et al. 1998). As per some publications, the proximal portion of the pancreas can be treated by injecting 1106 cells in 15 l of PBS (Alves et al. 2001). The cells were injected into the pancreatic tissue such that they may be seen infiltrating it. However, this procedure can result in intra-abdominal hemorrhage, pancreatic capsule rupture, and substantial intraperitoneal tumor leaking (Capella et al. 1999). Surgical sutures can also be used to attach a compact tumor portion into the pancreas. For example, a 1 mm-3 tumor fragment could be secured to the pancreas with a 1 6–0 Dexon II suture (Loukopoulos et al. 2004). The mouse pancreas takes several weeks to solidify into a solid mass that preserves the histological appearance of the original tumor and expresses antigens linked to cancer (Alisauskus et al. 1995; Fu et al. 1992; Reyes et al. 1996). When 10 pancreatic carcinoma cell lines and 12 solid cancers

were orthotopically transplanted into SCID mice, Luokopoulos et al. assessed the results (Loukopoulos et al. 2004).

Hematologic differentiation of xenografts produced by cell lines was complete, and the overall metastasis rate was relatively high. The xenografts generated from primary tumors were identical to the original tumors in question in terms of histology and biology. Kras p53, p16 DPC4/SMAD4, and p16 p53 mutations were identified in 80% of the cell lines and 100% of the primary tumor-derived xenografts. Thus, it was crucial to predict xenograft metastatic behavior, histological differentiation, gene mutation rate, and p53 status. Pancreatic tumors have been regularly generated by this approach (Fu et al. 1992; Reyes et al. 1996; Vezeridis et al. 1989). Implant success rates might range from 66% to 100% with this technique. In addition to this, there is a strong correlation between initial tumor histology and growing mass (Capella et al. 1999). However, there are some drawbacks: sutures are painful and might cause an inflammatory reaction when utilized to bind tumor fragments to the pancreas. In a study (Hotz et al. 2003), Hotz et al. compared intrapancreatic injections of human cancer cells to atraumatic pancreatic implantation of 2 pieces of subcutaneous donor tumors. Fourteen weeks after implantation or shortly after death, this was done to determine the initial tumor volume, localized infiltrate, and systemic metastasis.

According to their findings, all four cell lines had a 100% implantation rate. In addition to the differentiation state of the cells, there was a significant variation in tumor size, metastatic dissemination, and survival. Compared to well-differentiated cells, less-differentiated cells had a more excellent spread score and higher death rate. There was a poor tumor take-up rate and rapid development of the fake abdominal tumor in individuals with orthotopic tumor cell injections, likely owing to microscopic cell loss during the injection. It is possible to analyze the biological characteristics of orthotopic transplantation independently of the method used. There seems to be a 50 to 100% chance of developing cancer in the pancreas. There is no local development with orthotopic implantation, superior to subcutaneous implantation, where up to 60% of tumors disseminate (Loukopoulos et al. 2004; Capella et al. 1999). A variety of organs can be affected by metastatic cell spread. Researchers found substantial metastasis to the peritoneum (43%), liver (58%), lungs (22%), and lymph nodes (33%) in the investigation of pancreatic cell lines (Loukopoulos et al. 2004). The metastatic potential of cell lines varies based on their degree of differentiation in another study (Hotz et al. 2003). Despite lymphatic, bloodborne, and peritoneal dissemination, the implantation of tumor pieces decreased perineural and lymph node invasion (Capella et al. 1999; Reyes et al. 1996).

Imaging of Xenograft Models

These pancreatic cancer models are the most useful for early detection and therapy studies. Following the progression of lesions might act as a translation model toward identifying preclinical disease in people. To track the development of malignancies in animals, researchers have used positron emission tomography (PET) (Samnick

et al. 2004). Before explantation, other researchers introduced the green fluorescent protein (GFP) coding area into the pancreatic cancer cell line (Bouvet et al. 2000). Another research used MIA PaCa-2 cells that had been genetically engineered to generate significant quantities of a red fluorescent protein in an orthotopic, highly metastatic model of human pancreatic cancer (Bouvet et al. 2005; Gingell et al. 1976)59). These methods were evaluated by imaging orthotopically implanted primary tumors and metastases in the same animals at different time intervals with fluorescent protein imaging (FPI) and high-resolution MRI (Bouvet et al. 2005) (58). According to their results, MRI permits identifying tissue structure, whereas FPI allows for high-resolution and rapid picture acquisition. Because FPI does not require anesthesia or a contrasting agent on the animals, it provides various advantages. According to the outcomes of preceding research, FPI and MRI have complementary roles in noninvasively monitoring the development of primary tumors and metastatic lesions and their responses to therapy.

Diagnostic and Therapeutic Use of Animal Models of Improved PDAC

Some studies suggest that mutating the Kras gene within the pancreas can lead to spontaneous pancreatic carcinoma in mice. Human pancreatic ductal carcinoma specimens include more than 90% of the mutated Kras gene (Jones et al. 2008). The constitutively activated KRASG12D allele seems to be the most common mutation. Using Kras mutant mice, researchers can better understand carcinogenesis and progression of pancreatic cancer by this research (Morris et al. 2010). Because pancreatic carcinoma carcinogenesis and development are complex and lengthy processes, animal models that focus on a single mutation of Kras may not be sufficient for studying the landscape of pancreatic carcinoma biology. According to epidemiological statistics, pancreatic cancer's oncogenesis and development are complex procedures. Several risk factors for pancreatic cancer include smoking, eating a high-calorie diet, and having diabetes type 2 (Morris et al. 2010). Interactions between oncogene-tumor suppressor gene-metabolic environment-immune system cause the disease. There has not been a reliable and feasible animal model that can fully imitate the pathological progression of pancreatic malignancy until now (Li et al. 2010). Currently, a hands-on and potential technique is to merge several different models, such as pancreatic malignance pattern with type 2 diabetes induced by injection of STZ into KIC mice with a high-fat diet, pancreatitis-pancreatic cancer model recognized by bombesin injection into KIC mice, and KIC mice mediated with smoking, high-fat, or a high-cholesterol diet. Every one of these models is designed to investigate the important biological event of pancreatic cancer. It is also possible to eliminate particular immune cell subsets by using the iDTR-CRE system in a pancreatic cancer model. With this approach, we can determine which immune subsets are crucial in sustaining immune scrutiny and antitumor activity in the progression of pancreatic malignancy. As a result of the development of CRISPR/Cas9 technology for in vivo screening, it may be possible

to identify gene's novel function in the pathogenesis of pancreatic cancer directly, including a novel animal model for pancreatic cancer that might be developed. Current thinking characterizes pancreatic cancer's development as a biological event: an intraepithelial neoplasm caused by a Kras mutation and Her2 overexpression might develop to pancreatic cancer. Tumor suppressor genes p16, p53, DPC4, and BRCA2 may malfunction in an immunosuppressive microenvironment, leading to pancreatic cancer. For pancreatic carcinoma burden mice, several effective treatments have been found to manage pancreatic carcinoma in animal models and even eradicate tumors. While early detection of pancreatic cancer is still difficult, there is no effective treatment, and the prognosis is dismal. The 5-year overall survival rate for pancreatic ductal adenocarcinoma is still less than 10%. As an outcome of the recent investigation, no effective biomarkers toward pancreatic cancer were diagnosed. Future research must focus on integrating animal models with circulating tumor cell observing technologies, cfDNA sequencing technology, metabolomics, etc. Controlling the architecture of tumors when investigating single gene mutation animal models will be difficult. A valid theoretical basis for therapeutic medical attention of pancreatic cancer can be developed by incorporating numerous pathogenic factors in model organisms and employing multitarget therapy techniques, including microsurgery. Tumor-associated microenvironment indeed plays a prominent role in every tumor growth is driven by the collaboration between cancerous cells and their microenvironment, including appropriate fibroblasts, immune cells, as well as other particular interstitial cells, according to researchers from the University of. On account of its uniqueness, cancerous cells in pancreatic tissue include fibroblasts, pancreatic cells with quite a stellate shape, nerve tissue, immune cells, and vessels. Because of its uniqueness, cancerous cells in pancreatic tissue include fibroblasts, pancreatic cells with a stellate shape, nerve tissue, immune cells, and vessels. Pancreatic carcinoma can elude immune monitoring and even "counteract" the immune system because of these distinct sorts of cells, which afford an existing habitat for cancerous cell multiplication and malignant development; also an important scientific question is how to achieve "mimics" or even "humanization" of pancreatic malignancy's microenvironment in animal models. Animal models of the tumor microenvironment are challenging to humanize, but the maturity of numerous modern technologies makes this a possibility. Examples include using small-molecule compound cocktails to induce the long-term growth of hematopoietic stem progenitor cells in vitro in serious immunodeficient mice, such as NCG/NSG (Morris et al. 2010), which can also be used to explore the modified mechanism of tumor-resistant discharge in a PDX model.

Conclusion

There seems to be no particular early clinical manifestations of pancreatic carcinoma, and the disease has a high death rate. As a result, medical experts find it challenging to investigate early pancreatic cancer's biological activity and internal processes, and our understanding of this helps individuals to receive therapy in the

curable phase when they have been diagnosed early; understanding the genesis, risks, prevention, and treatment of this tumor requires the use of experimental animal models. However, despite the reality that transgenic technology may be used to create various mice models, clinical research is still that pancreatic carcinoma is caused in 70% by carcinogens, including nitrosamine and polycyclic aromatic hydrocarbon (PAH) in cigarette smoke. Chemically generated models are therefore more helpful in inducing carcinogenesis in pancreatic cancer. As a result, the transplanted tumor model has been utilized to research the causes of pancreatic cancer and the effects of food, modifying factors, and certain natural items. Current animal models may mimic most human pancreatic cancers. To better understand pancreatic cancer carcinogenesis and progression, researchers should focus on specific needs while also taking into account the unique characteristics of each model. Consideration of hereditary and nongenetic risk factors is necessary to reduce PDAC mortality. Desperately needed are biomarkers and high-resolution imaging methods to detect individuals with initial-period, high-risk malignancies, as well as pharmacological therapy options to prevent PDAC and increase life expectancy for those who do get the disease. In recent years, improved animal models have steered these regions forward, and these models will endure making substantial involvements in the future.

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Optical Techniques for Treatment and Tissue Evaluation Using Skin Models for Preclinical Studies

30

Michelle Barreto Requena, Mirian Denise Stringasci, and Hilde Harb Buzzá

Contents

Abstract

Cancer is one of the most incident diseases in the world, and skin cancer is the most prevalent type, making it a public health problem. New treatments and optimization of existent protocols are always desired to prolong or improve human life. The better way to avoid complications for patients with skin cancer is by providing efficient diagnosis and accessible treatment. For this purpose, it is indispensable to perform preclinical trials for the new therapies' developments and new diagnosis methods. The standardization of skin tumor models in animals and the techniques to explore treatment response is what can make the translational to humans quicker. This chapter summarizes the main different animal

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models including chorioallantoic membrane and skin lesions induction procedures in this research area as well as the techniques to evaluate and monitor the treatment's efficiency and optical techniques that help the diagnosis and understanding of biological samples.

Keywords

Skin cancer · Tumor model · Normal skin · Chorioallantoic membrane · Phototherapies · Optical techniques

Introduction

Experiments using ex vivo and in vitro models, such as phantoms or cell culture, have been widely used to mimic several in vivo conditions. These approaches are extremely important to prepare the animal experiments always planning to incorporate replacement, reduction, and refinement as much as possible. The reality is that not all animal research or testing can be replaced by non-animal methods, notably when the organism complexity (as an immunological system or the involvement of many organs) is the key in the study. Especially for cancer research, animal models still are essential to understand the fundamental mechanisms underpinning malignancy and to discover improved methods to prevent, diagnose, and treat cancer. There are series of recommendations concerning animal welfare in cancer research, including study design, statistics, and pilot studies, tumor models choice, therapy (drugs and radiation), imaging (covering techniques, anesthesia, and restraint), humane endpoints (including tumor burden and site), and publication of best practice (Workman et al. 2010).

Focusing on skin cancer, several types of animal experimentation models can be performed. There is a model in chicken eggs, which can be considered an alternative method because, if worked until the 14th day of development, it does not cause any pain for the embryo. Due to its high vascularization, it is widely used to study angiogenesis and vascular drug response. Besides its easy access to blood vessels, this is a simple model to use, inexpensive, and easy-to-install method in a laboratory environment (Station et al. 2004). Considering a more direct response for drug discovery and skin cancer treatments, healthy skin of porcine, rabbits, and rats also have been used (Avci et al. 2013). Concerning skin cancer, xenographic tumors by cell induction and skin cancer-derived tumors or induced by ultraviolet (UV) light have been widely used to study treatment response or improvements in the diagnostic mainly using mice as an animal model (Gober et al. 2013).

After the model was chosen, the treatment protocols and techniques for monitoring the response also need to be determined. In this context, optical techniques are attractive options to be explored since the skin lesions allow straightforward access compared to internal cancer types and also for being a non-invasive method. Photodynamic therapy (PDT) and photothermal therapy (PTT) are examples of these types of treatments. In terms of monitoring or diagnosis purposes, techniques

involving fluorescence, ultrasound images, optical coherence tomography, and histology are widely explored.

By establishing the relationships between animal models and humans, it is possible to translate more rapidly through the knowledge of what is the most successful way to improve treatment and diagnosis in patients. Thus, the objective of this chapter is to summarize the main experimental animal models used to study skin cancer, exploring their basic characteristics with optical techniques for treatment and monitoring of the treatment response.

Animal Models

In vivo models have been used in studies with phototherapies as a treatment for skin lesions. In this session, the main models found in the literature will be explored.

Healthy Skin

The skin is a complex organ that is structured for body protection and thermoregulation and provides sensation. The main human skin layers are the epidermis, dermis, and hypodermis. The epidermis is mainly composed of keratinocytes and fibroblasts. There are morphologically distinct epidermal sublayers such as *stratum corneum*, *stratum lucidium*, *stratum granulosm*, *stratum spinosum*, and *stratum basale*. The *stratum corneum* is the most external layer where dead cells are placed, and it is composed mainly of keratin. The *stratum basale* is the dermal-epidermal junction and works as a mechanical support for the adhesion of the epidermis to the dermis. The thickness of the skin layers can vary according to age, body part, gender, and species. In the case of humans, the use of a histometric technique to estimate the mean epidermis thickness considering different regions is reported as 76.9 to 267.4 μm for women and 112.4 to 244.8 μm for men (Oltulu et al. 2018). The dermis is composed of the superior papillary dermis and inferior reticular dermis sublayers. The main constituent of the dermis is collagen which provides tensile strength and mechanical resistance to the skin (Khavkin and Ellis 2011). The hypodermis is the layer lying below the dermis, and it consists largely of fat interlaced with blood vessels and nerves. The main function of this layer is to provide structural support for the skin, aiding shock absorption and insulating the body from cold (Lawton 2019). Figure 1 summarizes the main human skin layers and structures.

In terms of histological and biochemical properties, the healthy skin model of porcine is the most indicated to mimic the human skin in the experiments. In addition to the similarities in the dimensions of the epithelial layers, the arrangement of collagen fibers and the protein composition of the *stratum corneum are* very comparable as well (Godin and Touitou 2007). Considering the similarity of the optical properties of the porcine skin (Zamora-Rojas et al. 2013), this model is useful for comparison with human skin response to treatments involving light, for example (Requena et al. 2019). However, the logistics of the experiments with porcine are

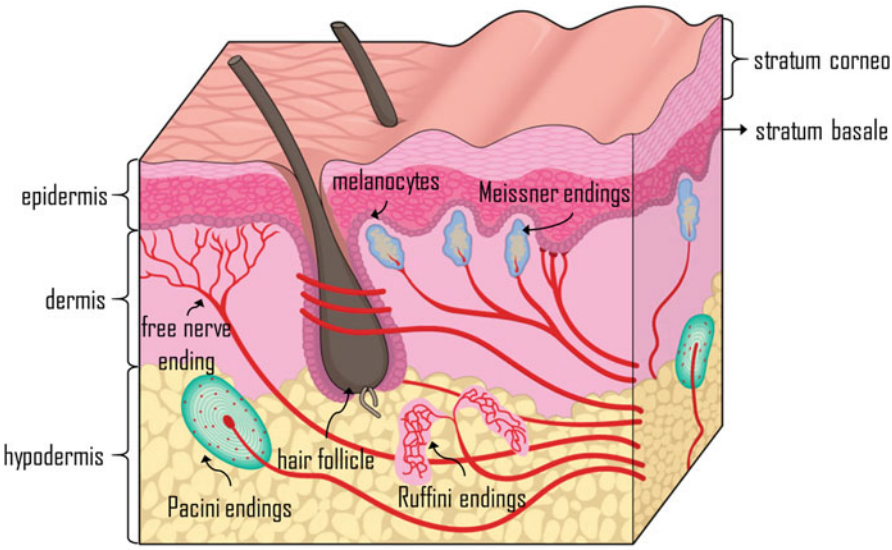

Fig. 1 Scheme of the human skin to indicate the main layers and structures

more complicated than with rodents. There are fewer animal facilities with a production of porcine for using them as an experimental model which turns it more difficult for the standardization of results. Besides that, the infrastructure for maintenance and routine experiments with porcine is also more complex. Seeking for simpler handling, studies are using neonatal porcine to evaluate drug delivery into the skin by microneedles system (González-Vázquez et al. 2017; Permana et al. 2019) or using both neonatal or juvenile porcine to pediatric drug discovery and development (Ayuso et al. 2020).

Even with thinner skin compared to the porcine, rabbits, rats, and mice are widely used for experiments to explore skin response to treatments, considering their easier manipulation and wide literature to base protocols or compare results. In terms of the thickness of the epidermis, rabbit's skin has a relatively thick layer, closer to porcine than rats or mice. Considering preclinical testing of medical devices, Wei et al. recommended experiments to be performed in ex vivo human skin, rabbit skin, and then small porcine skin (Wei et al. 2017). The epidermal thickness reported for rats is about 32 μm, and it is comparable to 46.9 μm of human epidermis (Zendzian 2000; Jung and Maibach 2015). On the other hand, the mice epidermis generally comprises only three cell layers and is less than 25 μm in thickness, whereas the human epidermis commonly constitutes 6–10 cell layers and is more than 50 μm thick (Gudjonsson et al. 2007). For 8 weeks old female nude mice, the estimated value of the thickness of the epidermis with the dermis is approximately 225 μm (Calabro et al. 2011). For this reason, considering the skin cancer research area, the mice model is more used in tumor development than to evaluate the healthy skin treatment response.

In the study of topical treatments, the use of healthy skin is commonly used to explore characteristics such as drug permeability and light penetration, in the case of phototherapies.

Skin Tumors

Subcutaneous and intradermal solid tumors have been used in in vivo studies as preclinical models. There are tumors induced directly with human cells (xenogenic) where immunodeficient mice (e.g., nude or SCID) must be used to prevent rejection. There are other models in which these cells are modified for non-rejection in a murine model (syngeneic), and then, the tumor can be induced in any mice strain. In the animal model choice, the characteristics of each must be evaluated. A syngeneic model, without the need for mice to be immunodeficient, facilitates the maintenance of animals in conventional housing. In other hand, the xenogenic models allow a direct investigation of human cells maintaining their genetic and molecular characteristics, but require immunodeficient animals that need specific care (including isolator cages receiving filtered air, sterilized materials such as food, water, and shavings) (Workman et al. 2010). In this section, some methods of induction of cutaneous solid tumors are described.

There are murine tumor models derived from breast cancer cells that are implanted subcutaneously. One of the main lines explored in the studies is 4 T1. Generally, an amount ranging between 10^4 and 10^6 cells is injected into the flank of Balb/c mice not necessarily nude. However, these cells need to be cultured in vitro through plates (and the specific facilities for cell lab) before being implanted into animals (Jadidi-Niaragh et al. 2017).

An alternative model is the use of Ehrlich cells, a modified breast cancer derivative for murine development. It has the advantage of having a simple culture through injection into the peritoneum of a healthy mouse for cell growth in a fluid form, called ascetic. The ascetic form has an approximate concentration of 10^6 cells in 100 μL of fluid. Then, the tumor can also be developed solidly by being injected into the subcutaneous tissue. This model can be developed in Swiss and Balb/c mice without the need to be nudes (Ozaslan et al. 2011).

Solid tumors can also be developed subcutaneously through the injection of HeLa cells. This model is usually applied in Balb/c nude mice, injecting a volume between 10^5 and 10^7 cells subcutaneously into the animal. The growth of HeLa cells requires in vitro culture using a plate (Kondo et al. 1993).

Squamous cell carcinoma (SCC) is the second-highest incidence of skin cancer. VII/SF and PDV are examples of cell lines that originated in mice (C3H and C57BL, respectively). These solid tumor models have been induced using an intradermal injection of volumes between 10^4 and 10^6 cells (Khurana et al. 2001; Konger et al. 2019). A431 (Fig. 4a) and SCC-13 are human SCC cell lines, so solid tumors can develop when induced in immunodeficient mice through intradermal injections with 10^6 of these cells (Detmar et al. 2000; Hawighorst et al. 2002). Melanoma is the most aggressive type of skin cancer. There are about 5000 cell lines of melanoma. The B16

strains are one of the first syngeneic melanoma models developed and remain one of the most commonly used. In studies in which treatments and diagnostic methods using light are evaluated, the pigmentation of the lesion is extremely important. Then, the strain used must reproduce the darkened characteristic of the skin tumor which is possible with the strains B16 4A5 and B16 F10 (Coricovac et al. 2018).

Skin cancer development can be directly associated with sun exposure. Recurrent exposure to ultraviolet B radiation (UVR-B) can cause cutaneous effects by genetic mutation and also by immunodeficiency. Non-melanoma and melanoma skin cancer can also be induced in rats and mice through different protocols of UV exposure or by chemical induction. However, these lesions induced are non-pigmented and do not recreate precisely the human pathology (Korinfsky et al. 2014; Coricovac et al. 2018).

There are precise ways to measure tumor volumes such as histological analysis and optical coherence tomography (OCT), which will be reported in session "Optical Coherence Tomography." However, a more practical way is to perform measurements using a caliper and estimate the tumor volume. There are some approximation models used with this purpose, and one of the most used is approaching a sphere, as in Eq. 1.

$$V = \left(\frac{d_1}{2} \cdot \frac{d_2}{2} \cdot \frac{d_3}{2}\right) \frac{4\pi}{3} \tag{1}$$

where d_1 and d_2 are the bases diameters and d_3 is the thickness diameter of the tumor (Tomayko and Reynolds 1989). These measures have the main advantage of requiring simple instrumentation, and the animals do not need to be anesthetized for long periods.

Chorioallantoic Membrane

The chorioallantoic membrane (CAM) model in chicken eggs is formed on the fourth day of embryo development and is obtained by the fusion of the chorion and the allantois (Nowak-Sliwinska et al. 2009), becoming responsible for the gaseous exchange of the egg. CAM model has the advantage of not involving animal pain when worked up to the 14th day of development since there are no pain receptors in the embryo until this stage of embryonic development. Therefore, there is a model considered alternative to the use of animals, as it does not cause any suffering to them (Jones et al. 2006).

Because it is highly vascularized, it is a widely used model to study the angiogenesis and activities of drugs or treatments with a vascular target. Figure 2 shows the vascularized environment in the 11th development of the embryo and the access to the vascular network to study different phenomena. CAM is a well-established model that enables easy access to blood vessels, in addition to being a simple, inexpensive, and easy-to-install method in a laboratory environment (Nowak-Sliwinska et al. 2009).

Fig. 2 CAM model and the vascularized environment around the embryo with easy access to several studies

In ovo model is considered an intermediary between in vitro and in vivo models with the advantage of enabling a more complex analysis of the vascular system when compared to in vitro studies, but allowing a more individualized study when compared to in vivo model, with a specific microenvironment (Station et al. 2004).

For these advantages, CAM model has been used for studies of different treatments, either with the function of stimulating vascular growth, for healing processes and inflammation, or for the destruction of blood vessels, as a treatment of cancer and vascular diseases (Ribatti 2016).

The CAM model has an immune system conducive to the introduction of different cell lines and provides, by itself, a source of nutrition for these cells. The tumor obtained, with the insertion of cells or biopsy material, presents the development of regional microvascularization and allows the study of angiogenesis and the processes involved when the tumor becomes viable. It is, therefore, an excellent model for the study of anti-angiogenic and antitumor activities, allowing the assessment of the therapeutic response directly and in real-time (Steiner 1992).

This model has already been used to characterize tumor morphology (Balke et al. 2010) and vascular regrowth (Nowak-Sliwinska et al. 2010) to, among other things, evaluate the tumor destruction promoted by the application of different treatment techniques, such as photodynamic therapy (Yoon et al. 2012).

There is a great diversity of cell lineages that can be used in tumor development transplanted in a chorioallantoic membrane model. Research with in vitro cell culture contributes to the understanding of several cellular mechanisms, including their forms of defense and the action of therapies, and also allows the establishment

of many parameters for the beginning of in vivo studies. It is possible to study different cell lines depending on the purpose of the research, including tumor cells.

The choice of the lineage used is an important step to establish the tumor model in CAM. From this, it is possible to visualize several phenomena in the tumor-blood vessel complex, including therapeutic efficacy and adverse effects of the molecule, as well as its location and kinetics. Therefore, with the establishment of this model, it is possible to elucidate some of the therapeutic response mechanisms in the blood vessels that directly supply the tumor, in adjacent blood vessels, and the direct response of tumor cells.

Optical Treatments

In vivo models have been used in studies of phototherapies for the treatment of skin lesions. Through these models, photodynamic therapy and photothermal therapy have been improved and their mechanisms explored in different types of preclinical studies.

Photodynamic Therapy

Photodynamic therapy (PDT) is a phototherapy that has been increasingly indicated in the treatment of non-melanoma skin lesions. A photosensitizer (PS) is administered locally or systemically, and after a time for biodistribution, PS preferentially accumulates in tumor tissue, and the site is irradiated with light with an appropriate wavelength. PS absorbs light and initiates photochemical reactions that generate cytotoxic products (reactive oxygen species and free radicals) which cause tumor cells death (Wilson and Patterson 2008).

When PS molecules receive light with appropriate energy, electrons are excited from the ground state to higher energy states. Therefore, electrons can return to the ground state by fluorescence or by non-radioactive decays, generating heat by the vibration of the molecules, or it can decay to a metastable intermediate triplet state, through the so-called intersystem crossing, as represented in the Jablonski diagram in Fig. 3. In the metastable intermediate state, the excited PS can interact with adjacent molecules through two mechanisms. The type I reaction consists of removing a hydrogen atom from a molecule or transferring electrons, generating free radicals that can oxidize a large number of molecules. The type II reaction happens when the excited PS molecule transfers energy to molecular oxygen in the ground state (triplet) to excite it to a metastable singlet state, which is a highly reactive form of oxygen, mediating photochemical damage in the cell. Such interactions can result in the destruction of the vascular system or the intercellular matrix, or even in the modification of mitochondrial functions and cell biomembrane systems. Both reaction types preferentially lead to the death of neoplastic cells (Wilson and Patterson 2008).

Topical PDT in skin lesion treatment has the main advantages of being minimally invasive and selective and generally does not present serious side effects. However,

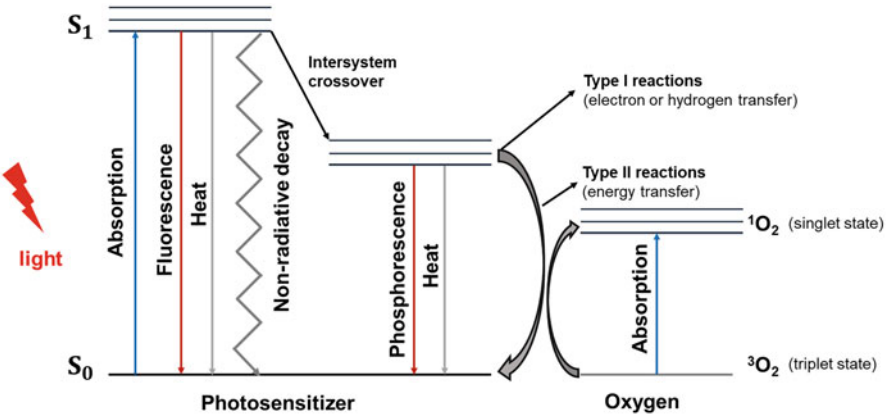

Fig. 3 Jablonski diagram. (Adapted from Langer (1969))

the penetration of PS and light into the tissue is limitations of treatment, making it indicated only for superficial and thin lesions (Ishida et al. 2009). Several studies have been developed to increase the effectiveness of the treatment, as well as to optimize existing protocols to decrease the drug's incubation time and alleviate the pain experienced by patients, increasing treatment acceptance. Furthermore, treatments for more infiltrated and more aggressive tumors have been sought.

Photothermal Therapy

The technique based on increasing the temperature of a certain region is called hyperthermia and has been shown to be efficient in the treatment of neoplasms. The biological tissue temperature variation between 42 °C and 47 °C is already enough to cause several effects, since high temperatures cause, among other effects, the denaturation of proteins (Nadejda and Jinzhong 2009). Hyperthermia can also cause indirect effects, leading to cell death. Specifically in tumors, the increase in temperature can lead to hypoxia of tumor cells; however, to effectively lead to tumor death, the target of therapies using heat is temperatures higher than 60 °C (Kumar et al. 2010).

There are several ways to lead the cell to hyperthermia, ranging from chemical agents to the application of microwaves to tumor cells, involving nanoparticles. Photothermal therapy (PTT) is a more recent concept where light is converted to heat through a combination of non-radiative processes, such as vibrational relaxations (Prasad 2003). PTT has proven to be increasingly efficient with the use of different types of nanoparticles, from semiconductor nanocrystals to carbon-based nanosystems (Jaque et al. 2014).

Given the need for new techniques with greater potential for tumor destruction, PTT comes as one of the promises of cancer treatment, with few side effects, since the use of laser as a light source helps to reduce absorption in adjacent tissues. For

this, several efforts have been made towards the development of nanoparticles with absorption in infrared, for example, due to the low absorption of this wavelength range by biological molecules in the tissue, allowing the light to penetrate more and treat deeper tumors (Jaque et al. 2014).

There is an important relationship among the concentration of nanoparticles, the treatment time, and the light irradiance to ensure that the treatment occurs effectively to the target tissue, depending on its thickness. Furthermore, the treatment time needs to be clinically feasible using accessible light sources, since the irradiance for PTT is normally between 0.5 and 2 W/cm^2 (Su et al. 2021).

Currently, there are a variety of nanoparticles used in photothermal therapies, and depending on the design and absorption peak, this therapy can be used alone, guided by multimodal image, or combined with therapies already used in the clinic, such as chemo, radio, and immunotherapy. These possibilities of using photothermal therapy are being studied, ranging from its use in the in vitro treatment of tumor cells to in vivo studies showing a synergistic effect in the treatment of tumors and metastasis, especially with inhibition of tumor growth (Zou et al. 2016).

A still recent possibility, which has been widely studied, is the combination of photothermal and photodynamic therapies, which use light for their activation, with the possibility of delivering the PS in nanoparticles with photothermal capacity, to enhance the thermal effect with cell death by photodynamic effect (Zhang et al. 2016).

Damage Evaluation Techniques

Studies involving topical treatments that promote tissue damage need effective techniques to evaluate and quantify this damage. In this session, the main techniques used to assess the responses to these treatments are described.

Histology

In the 1700s, the study of detail of the tissues using biopsies started, coining the term "histology" only in 1819. This is an important area in science and has been fundamental for the understanding of the molecular and cellular levels of the body organs (Hussein and Raad 2015).

Tissue fragments to be analyzed can be removed through excisional biopsies, and in the case of tumors, the entire volume can be removed for the study. In in vivo studies, the animal should preferably be euthanized before the tissue is removed.

Tissue fragments are placed in cassettes and then deposited in containers with a buffered formaldehyde solution. After a period (minimum of 24 h), the cassettes containing the tumors can be washed with water and go through dehydration and fixation processes in paraffin. Then, the samples are cut using cryotome, and the sections can be stained with several dyes, depending on the target. After stained, the

tissue is then mounted on a permanent slide, usually to be evaluated in bright-field microscopy.

Stain using hematoxylin and eosin (HE), for example, can be used to allow visualization of the skin layers (epidermis and dermis colored in purple and pink, respectively) as well as visualization of inflammatory cells and damaged processes (Fig. 4c). Masson trichrome is a combination of dye solutions that are used to identify collagen, muscle, cytoplasm, and keratin fibers that are marked in red; nuclei are stained black, while collagen fibers are stained blue. The staining acquired by the Picro-Sirius Red (PSR) solution, prepared with picric acid and Sirius Red, can be chosen for collagen labeling, allowing differentiate collagen I and III when using

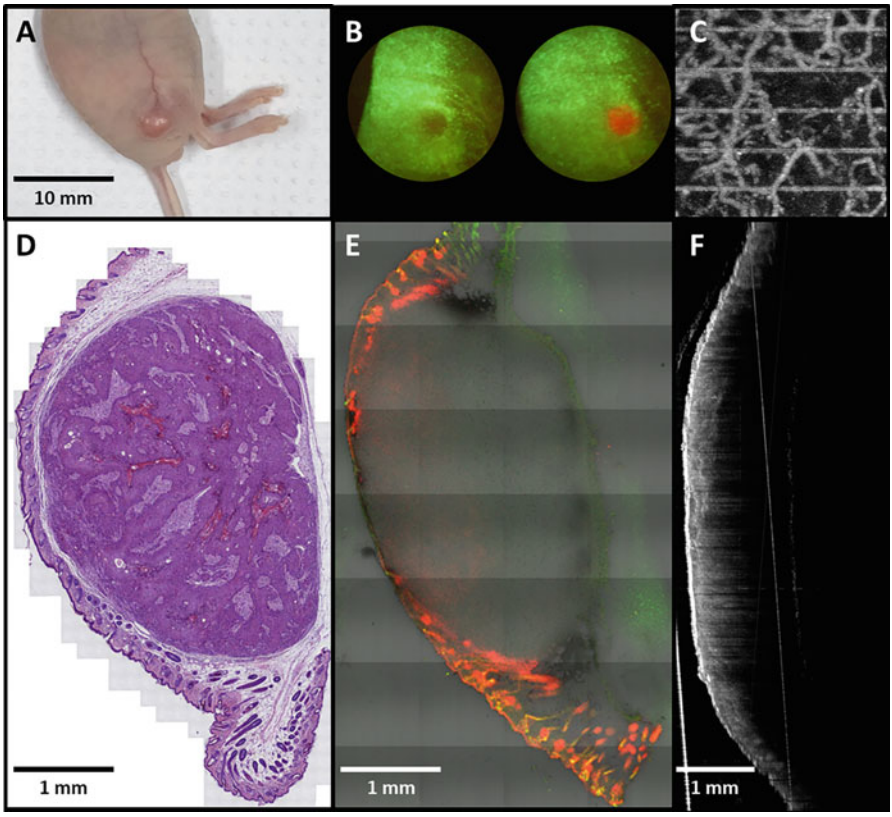

Fig. 4 This is a compilation of images of tumors (xenographic SCC in balb C nude mice) collected/evaluated by different techniques. (**a**) white light image of the tumor induced in the flank of the mouse; (**b**) widefield fluorescence image of the tumor before (left) and after (right) PpIX accumulation; (**c**) OCT speckle variance image from microscopy showing the tumor vessels in the surface (horizontal lines are inherent to the animal's breathing); (**d**) bright-field microscopy of a histological slice of the tumor stained with hematoxylin and eosin confocal fluorescence microscopy image of the tumor with PpIX; (**e**) and (**f**) confocal fluorescence microscopy image of the tumor with PpIX; (**e**) 2D-OCT image of the tumor 2D-OCT image of the tumor

polarized microscopy. In general transmission light microscopy, the skin samples stained with PSR have the collagen marked in red, muscle fibers, and cytoplasm in yellow (Kumer and Kiernan 2010).

It is also possible to evaluate histological characteristics of the tissue using frozen sections. For this procedure, the tissue samples are immersed in an optimal cutting temperature compound (OCT compound) and frozen using liquid nitrogen associated or not to chemical reagents such as hexane and isopentane (Wood and Warnke 1981). Then, the samples are sliced using a cryostat set in negative temperatures. After that, the sections can be stained or observed directly in confocal fluorescence microscopy, if there is an endogenous fluorescent compound of interest in the sample.

Considering fresh or frozen samples of tissues, there is a possibility of incorporating different chemical dyes to measure specific proteins and allow an immunohistochemistry analysis. This step needs to be implemented after the slices are placed in the slide without conventional dyes, using specially labeled antibodies that can bind to the proteins of interest. This test is widely used in tumor samples and is based on the percentage of cells dyed and the staining strength since the samples with more protein will bind more antibodies and will appear darker (Bilous et al. 2003).

Fluorescence

When light interacts with biological tissue, its energy can be absorbed by the electron in the ground state and passes into an excited state (Fig. 3). Some molecules can produce fluorescence as one of the electronic relaxation processes to return to the ground state, which happens when absorbed energy is released in the form of light. This process is called luminescence and can be classified as phosphorescence, with long lifetimes in the excited state, or fluorescence, which has rapid relaxation and short lifetimes in the excited states (Atkins 2006).

Fluorescence depends on tissue conditions such as temperature, pH, and, most importantly, the molecules present. In an altered tissue with a disease, there are biochemical alterations that modify the biological conditions, resulting in a change in the light interaction. Because of differences in the internal light conversion process, tissues with different compositions or structures emit different fluorescence (Richards-Kortum et al. 1991). Thus, there is a specific fluorescence for each biological tissue, or for the same tissue under different metabolic conditions, which can facilitate identification using imaging or emitted fluorescence spectrum. Fluorescence can still be investigated in two ways: steady-state and time-dependent. Studies have been performed in several types of tissues, such as liver and lung, showing the possibility to use endogenous fluorophore spectroscopy for both in vivo and in vitro tests to compare healthy and abnormal tissue or cells (Andersson-Engels et al. 1991).

Thus, fluorescence can be used to help in the diagnosis of lesions, and for this, it is also called an optical biopsy. The spectrum allows for tissue differentiation, but usually has a punctual analysis, and despite showing the condition of healthy tissue or not, it cannot be used in large areas. Widefield fluorescence images are an option that can aid diagnosis, especially of large areas, with, for example, the identification of lesion edges. To improve the visualization of fluorescence contrast, it is also

possible to use markers with absorption and emission at specific wavelengths, being exogenous or endogenous fluorophores (Andrade et al. 2014). Figure 4b demonstrates widefield fluorescence images of an SCC tumor induced in a Balb/c nude mouse after being incubated with a cream containing 20% of methyl aminolevulinate for 1 h. The red fluorescence observed is characteristic of protoporphyrin IX accumulated in the tumor, while the green fluorescence is characteristic of skin endogenous autofluorescence.

Fluorescence can be conjugated with microscopy providing a potent technique in the identification of biological contents, with the fluorescence detection of labeled molecules. Several techniques have been developed using fluorescence, allowing the understanding of complex events of cells and tissues. Fluorescence recovery after photobleaching (FRAP), Förster or fluorescence resonance energy transfer (FRET), fluorescence localization after photobleaching (FLAP), and fluorescence lifetime imaging microscopy (FLIM) are some examples of this application (Ishikawa-ankerhold et al. 2012). Distinct FLIM studies have been used to identify endogenous and exogenous fluorescence assisting in the diagnosis of skin cancer lesions in a preclinical study (Miller et al. 2017). Sometimes, the surrounding tissue can present the same steady-state fluorescence. However, endogenous fluorescence lifetime can indicate differences between them. Studies of ex vivo biopsies of skin lesions have indicated that this lifetime with FLIM technique can help in the identification of non-melanoma skin cancer from healthy tissues (De Beule et al. 2007).

Another type of analysis is fluorescence confocal microscopy (FCM) that allows optical sectioning and 3D reconstruction. In addition, a great advantage of FCM is the use of nonlinear properties of light, which can result in high contrast even in-depth, being able to provide more information compared to other microscopes (Ishikawa-ankerhold et al. 2012). Several studies for in vitro and ex vivo samples in dermatology have used this technique. It commonly uses exogenous fluorophores and can present specificity, identifying different structures, metabolisms, and tissue conditions (Nwaneshiudu et al. 2012). Figure 4e shows the formation of protoporphyrin IX on the tumor surface (red) by FCM imaging. In this case, the tumor was incubated with a cream containing 20% of methyl aminolevulinate for 1 h; then, the animal was euthanized, and the tumor was extracted and passed through the freezing and sliced process to obtain the images.

Ultrasound Image

Ultrasound images are obtained by reflection sound waves in the tissue. Intrinsic variations in tissue (as vascularity and density) make the waves reflect distinctively, making the ultrasound an important tool in assessing borders and interfaces. The imaging technique by ultrasound has been improved in recent years (Kleinerman et al. 2012).

Skin structures can be visible using low-frequency ultrasound. Higher frequencies generate images with better resolution, while lower frequencies allow for greater penetration. Epidermis and the dermis can be seen using 20- to 25-MHz ultrasound, while only epidermis can be observed with 50 to 100 MHz ultrasound.

Despite the limited resolution, ultrasound is increasingly popular in skin application (Kleinerman et al. 2012).

Optical Coherence Tomography

The use of optical coherence tomography (OCT) started in the 1990s as a pure imaging system with micrometer resolution images of skin. Although the images were not very accurate, they were superior to those of ultrasound, and advances in the technique have made it approach clinical usefulness rapidly (Olsen et al. 2018).

OCT utilizes the property of coherence of laser light to detect scattered light from the target tissue. A focused laser beam is scanned across the sample, and the light backscattered from the subsurface is collected. The light detected interfere with a reference beam also derived from the laser. Only backscattered photons that have retained their coherence constructively interfere and generate a signal at the detector. The interferometer also provides depth information these photons backscattered, allowing a 2-D image construction (Olsen et al. 2018).

The human skin normally comprises a thickness approximately of 50 to 100 μm of the epidermis and 2000 μm of the dermis. The wavelength of 1300 nm has been used to obtain satisfactory penetration at least 500 μm into the dermis, in the spectral window with low water absorption (Olsen et al. 2018). Figure 4f shows an OCT image of an SCC tumor induced in Balb/c nude mice.

One of the main advances of OCT has been functional imaging, as angiography is the ability to detect blood vessels. The structural OCT scans produce images with little delineation of vessels and poor sensibility on deeper layers underneath due to the shadow effect. However, the increase in sensitivity and speed of OCT systems has allowed generating images with delineation of vessels using algorithms. Angiography methods are based on the detection of rapid changes of OCT signal intensity due to blood flow. Then, the algorithms build the image, where pixels with fast change signals constitute the vessels (Ang et al. 2018). Figure 4c presents an OCT speckle variance image of vessels from an SCC tumor induced in balb/c nude mice an angiography method.

Therefore, light is widely present in the techniques used to understand biological processes, biopsy identification, and vessel detection. In addition, light can also be used to treat diseases, including tumors, using different mechanisms as photodynamic and photothermal therapies. Thus, preclinical studies have been essential to understanding all these processes involving light and enabling clinical application for the detection and treatment of tumors, especially those of the skin.

Conclusion

The preclinical experiments performed on animals for cancer research are extremely important to promote the continuous advance in the treatment protocols, development, and improvement of monitoring and diagnosis methods. There is extensive

literature exploring each skin tumor models particularity. It is important to choose the model that most fits the purpose of each research. Noteworthy, in vivo models have been shown to be effective in preclinical studies of treatment and monitoring of skin lesions using optical techniques.

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Tumor Models of Retinoblastoma: In Vivo, Ex Vivo, and In Vitro Models

31

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Abstract

In the initial simplistic understanding, childhood retinal cancer retinoblastoma is due to the defect in the retinoblastoma gene. However, the deep insights reveal the complexity of the disease from unilateral single gene mutation to the bilateral two-hit hypothesis proposing two mutations in two alleles of the same gene and more recent studies revealing the role of multigene dysfunction in such cancers without any abnormality in the retinoblastoma gene. Again, retinoblastoma majorly occurs among humans while replicating the same disease among animals with the mutations in Rb gene shows more complexity to the evolution of the disease as the Rb gene mutations alone do not result in the diseased retina in the organism. Thus, it has become vital to develop various alternatives to study disease initiation, development, and treatment. Here we summarize the current understandings of the disease, in vitro, ex vivo, and animal models, and the standard treatment procedures as well as the development of novel therapeutics for such a complex disease.

Keywords

Retinoblastoma · Tumor models · 3D organoids · Cell and gene therapy · CART therapy

Introduction

Retinoblastoma is an intraocular cancer commonly occurring in children, and can affect one or both eyes. The aggressive growth of these tumors can destroy retina quickly resulting in complete loss of vision (Fialho et al. 2020). In last two decades rapid progress, in early diagnosis and treatment of retinoblastoma, has resulted in high ocular survival. However, the vitreous seeds, the tumorous growth from retina to vitreous humor, are still incurable except enucleation of affected eye, thus challenging the modern retinoblastoma disease management.

The impetuous spawn of retinoblastoma is restricted to humans and its imitation with structural and functional properties in mice is difficult. In absentia of adequate animal models, in vitro models provide an opportunity to study a disease in specific cellular environment, and therefore, these models have served as unique initial point of research in biology and medicine. The in vitro studies on retinoblastoma cells have not only led to understanding the retinal cell development and differentiations (Campbell and Chader 1988) but also the cloning studies with retinoblastoma gene have revealed its role in other childhood cancers such as nephroblastoma, hepatoblastoma, and neuroblastoma (Lee et al. 1987). Cell lines for in vitro studies have served vital role in retinoblastoma studies, and commercially available cell lines are listed in Table 1.

These studies have led to the pharmacokinetic and pharmacodynamic characterizations of successful therapeutic agents against retinoblastoma. The inhibitory

Table 1 Commercially available cell lines for retinoblastoma studies

S.N.	Cell line	Organism	Disease	Supplier
1.	PA317 LXSN 16E7	Mouse	Papilloma; retinoblastoma	ATCC
2.	Y79	Human	Retinoblastoma	ATCC, DSMZ
3.	WERI-Rb1	Human	Retinoblastoma	ATCC, DSMZ

constant (IC50) of melphalan was determined using Y79 and WERI RB1, further, combined melphalan and topotecan showed synergistic effects in both the cell lines with effectively reduced IC50 (Schaiquevich et al. 2012a). Melphalan is commonly given in form of melphalan hydrochloride; however, in a recent study it was reported that propylene glycol-free melphalan formulation is also effective without any additional toxicity but enhanced stability (Bogan et al. 2021a). In another study pharmacokinetics of topotecan, in WERI-Rb1 cells, was carried to determine its IC90 that assisted the study of topotecan efficacy in rabbit models (Bogan et al. 2021b). The carboplatin and bevacizumab combination was used to explore the apoptotic effects in Y79 cells, and the authors of this study also reported the successful translation in the animal models.

Recently the development of tri-dimensional animal cell culture techniques has facilitated in establishing in vitro tumor models that mimic the in vivo tumor properties for instance structural complexity and cell-cell interactions (Winter et al. 2019).

Ex Vivo Models of Retinoblastoma

Retinoblastoma is a distinctively human disease (with the exception of a dog being diagnosed with it in 1997). In vitro models do not create a comprehensive environment which helps us to study the environmental cues that lead to the progression of the disease. In vivo models, though being advantageous in the aspects of mimicking the entire environmental cues, are limited by the biological differences between the human tissue and that of the animal model. Ex vivo models began with this as the fundamental basis. To avoid the differences that arise when mimicking the disease in a mice model or when looking at it in a very "simplistic" manner in vitro, the ex vivo models have become popular, particularly in studying therapeutics and disease progression (Assayag et al. 2016). This type of modeling system involves mainly patient-derived xenografts (PDX) and 3D tumor organoid models.

Patient-Derived Xenograft Models

Immunodeficient animals (mostly mice) are injected with the patient's tumor graft and allowed to develop – these serve as models for the tumors. Immunity is silenced in these animals, which means that it cannot mimic the immune response

within the body. Orthotopic grafts are those where the grafts are injected into the retina or the vitreous. Though this is the closest possible way to replicate the disease, it is pretty difficult to do so without disturbing the choroid. They are limited in size by the eye of the animal used and have been shown to study the "invasiveness" of the disease as well. These models demonstrate metastasis and also form the typical rosette structure that is found in the histological analysis of the retinoblastoma tumor (Mendel and Daniels 2019a). Heterotopic grafts are done by injecting the PDXs into the subcutaneous spaces, essentially for experimental ease. The tumors are allowed to grow to a reasonable size and can even be expanded by introducing into other animals. These models can be used to study the disease onset and initial changes within the tumor. But, these models remain at an undifferentiated level and lack the important cues that are present near the eye region (Chévez-Barrios et al. 2000).

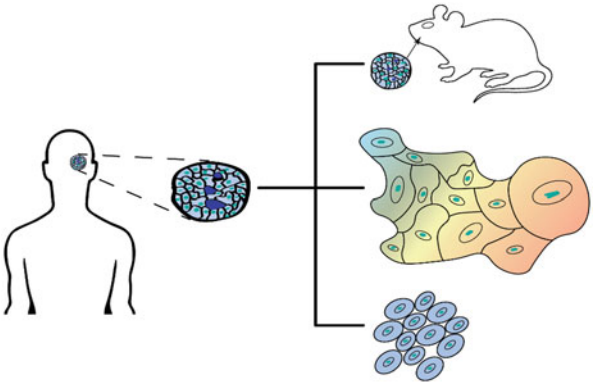
3D Organoid Models

Retinoblastoma organoids are developed from patient tumors that have not been given any sort of prior chemotherapy, most often from enucleated eyes. They maintain the cellular, molecular as well as histopathological characteristics of the original Rb tumor. One of the advantages of Rb organoids is that it forms vitreous and sub-retinal "seeds," which are key to understanding metastasis of the tumor. These models also help in optimizing the antitumor drug dosages, with varying sizes of the tumor. 3D retinoblastoma organoids have shown to maintain the cone receptor gene expression signatures as well as the neural circuitry, which are key for disease progression. Another important aspect that can be studied is the variations arising from germline Rb mutations compared to the acquired somatic Rb mutation (Saengwimol et al. 2018). Organoid cultures have also shown the enhanced expression of fibroblast proliferation and mesenchymal cells which implies that the EMT pathway induction is occurring, which follows the trend of the primary Rb tumor. 3D organoids are currently being actively studied to develop "bio-banks" of the patients which greatly enhance the individual-specific needs of treatment.

Tumorspheres

These are spheroid (aggregate of cells) models developed from a single retinoblastoma tumor cell or a cell mixture. They are allowed to grow in a manner that allows self-adhesion and therefore form a spheroid of similar cells. Organotypic models are developed from a mixture of different types of cells and mimic tissue conditions. Tumorspheres most often include a variety of cells in various stages such as necrotic

cells, nonproliferating cells, and actively proliferating cells. Their maintenance is easier as compared to organoids and they are most often used for high-throughput drug screenings.



In Vivo Models in Rb

I. LH β T-Ag Models:

These models are the most primitive in vivo models of retinoblastoma. The LH β model was developed by constructing a luteinizing hormone beta promotor adjacent to the SV T (large) and SV t (small antigen). The gonadotropic cells of the mice thymus were transfected in vivo. Since these cells released luteinizing hormone, the corresponding promotor LH β would enhance the large and small T antigen which is a part of the viral oncoprotein from SV 40 strain. (Windle et al. 1990) Both large and small T-Ag bind to p53 and pRb inducing tumorigenesis.

I.i.

Success:

I.i.a.

Model serves as an avenue for prognosis of tumor: Can test the effects of anticancer drugs, cryotherapy, and radiation therapy as they would simulate a human model as proximately.

I.i.b.

In vivo tumor assessment: Assessment of retinoblastoma tumors in mice has been done with the help of optical coherence tomography right from the early all the way to the advance stage of cancer.

I.ii.

Failure:

I.ii.a.

Not localized to retina

I.ii.b.

Ectopic Viral Protein

Though the in vivo tumor models have been very helpful in studies related to therapeutics with respect to tumor regression, the main drawback lies in the fact that these models involve transgenic oncogenes recombined from viral system. The integration of the viral oncogenes with the human genome results in vivo recombinant system giving rise to a recombinant protooncogene originating from a viral source. It therefore does not serve as an idle tumor model when compared to a human system where the tumor arises as a result of spontaneous mutation or is familial being inherited from carrier or affected parents. Furthermore, the recombinant oncogene of viral origin would elicit an immune response distinct from the type of immune response generated as a result of inherited or spontaneously acquired mutations in the human system.

I.ii.c.

Origin of Cell

Second drawback lies on the fact that the cell of origin and thus clonal tumors cannot be deciphered using in vivo models. The in vivo models are therefore not suitable for studies pertaining to assess derived tumors. Tumor-derived from progenitor cells and via de-differentiation of stem cells into cancer stem cells cannot be simulated within the in vivo models. Consequently, studying the cell of origin in retinoblastoma becomes challenging with in vivo models. The focal tumors which too are derived from progenitor cells cannot be studied in in vivo models. This therefore renders the in vivo model inefficient in studies pertaining to focal and clonal tumors.

II. Knock Out Models:

In the past researchers had studied the role of the Rb family genes and its association in the development of retinoblastoma. These genes include Rb, p107, and p130. Therefore, by inactivating these genes, in vivo models can be created which specifically lack Rb and/or Rb-related genes. In mice mere Rb deletion does not lead to the development of retinoblastoma (Williams et al. 1994). This is because Rb deletion is compensated by p107 upregulation. This is the major drawback in mice models. The Rb gene deletion exhibits differential effects in mice and humans (Donovan et al. 2006). Therefore, in addition to Rb gene, p107 which is upregulated to compensate Rb loss in mice also needs to be inactivated. Researchers faced another challenge by inactivating both Rb an p107. On deletion of both Rb and p107 the embryonic development in mice suffers fatally. Therefore, research strategically deployed methods to generate chimeric mice with exclusively retinal cells being genetically edited using cre-lox method generating knockout mice (Vooijs et al. 2002). In spite of generating chimeric mice, the histological attributes in these mice did not resemble human retinoblastoma tissues. For instance, cells lacking p53, p107 in addition to Rb did not give rise to features in developing retina in mouse embryos resembling human retinoblastoma characteristically. Researchers therefore developed breedable knockout mice by engaging pax6, Nestin, and Chx10 promotors with Cre lox system and targeted progenitor cells and other cells in the developing retina. With progression of the embryonic development muller glial and horizontal cells marker expression could be detected (Robanus-Maandag et al.

1998; MacPherson et al. 2007; Masland 2001; Johnson et al. 2007). The drawback in this model was that Rb deletion also triggered apoptosis in some of the retinal cells (Chen). This was evident with α-Cre $p53^{-/-}$ cells which manifested an increased sensitivity to apoptosis of retinal cells. On the contrary the Chx10- Cre $p53^{-/-}$ cells did not exhibit upregulation in apoptosis. These differences were due to temporal inconsistency in the phenotype expression manifested corresponding to the specific promoter involved. Therefore, Pax-6 and Nestin-based Cre Lox knockout models were discounted as these models apart from being late onset with respect to expression, also had issues with compromised penetrance and cell non-autonomous factors impacting communication and signaling [Reviewed in Nair et al. (2013)].

There had been studies on the role of an additional Rb-related gene – p130 in retinogenesis, cell cycle exit, and tumor development. Related studies in line deployed chimeric Double knockout (DKO) $p130^{-/-}$ with $pRb^{lox/lox}$ deletion models including α-cre ($Rb^{lox/lox}$)($p130^{-/-}$); Chex10-Cre ($Rb^{lox/lox}$) ($p130^{-/-}$) and Nestin-Cre ($Rb^{lox/lox}$) ($p130^{-/-}$) models (MacPherson et al. 2004, 2007; Dannenberg et al. 2004). In all of these models the developing retinal progenitors had undergone Rb deletion and had manifested the phenotypes of human retinoblastoma-affected retina. The retinal progenitor cells, amacrine cells, horizontal cell, and muller glial cells had developed retinoblastoma attributes resulting in excessive proliferation in the initial stage followed by upregulation in apoptosis. The same pattern of excessive proliferation and apoptosis has also been observed in human retina affected with retinoblastoma. This interprets that the progenitors are relatively resistant to apoptosis and with subsequent proliferation their resistance to apoptosis tappers down acquiring apoptotic sensitivity [Reviewed in Nair et al. (2013)]. This kind of attribute has also been observed in cancer progenitors – cancer stem cells/cancer-like stem cells in humans which apart from being resistant to apoptosis are also multidrug resistant.

In 2007, Aijokia and colleagues generated chimeric mouse models with just a single active copy of each pRb, p107, and p130 to study the effect of each gene in tumorigenesis of developing murine retina. These models included Cre ($pRb^{-/-}$) ($p107^{-/-}$) ($p130^{+/-}$); Cre ($pRb^{-/-}$) ($p107^{+/-}$) ($p130^{-/-}$); and Cre– ($pRb^{+/-}$) ($p107^{-/-}$) ($p130^{-/-}$) knockouts (Ajioka et al. 2007). Of the three knockouts, the one with $p107^{+/-}$ genotype had demonstrated horizontal cells of the retina differentiating normally for initial several weeks. What researchers observed next brought a breakthrough in the understanding of terminally differentiated neuronal cell behavior. They observed that the differentiated horizontal cells re-entered back into cell cycle and then clonally expanded giving rise to bilateral retinoblastoma and these tumor cells also metastasized into the bone marrow. Till then, it was accepted that the terminally differentiated neurons do not reenter back to cell cycle. The other knockout models that included Cre ($pRb^{+/-}$ $p107^{-/-}p130^{-/-}$) and Cre ($pRb^{-/-}$ $p107^{-/-}p130^{+/-}$) could rescue horizontal cells to enter into the cell cycle pointing out the role of p107 in cancer. This also gave a clue about the origin of cell in retinoblastoma being

horizontal cells of the retina (Ajioka et al. 2007). These chimeric models also serve as prognostic avenues to determine the therapeutic efficacies of novel and putative anticancer drugs. The molecular profiles, neuroanatomical aspects, biochemical profiles, and morphometric attributes of these models were in alignment with human retinoblastoma-affected retinal cells.

II.i.

Drawbacks:

II.i.a.

Differential effect of mere Rb deletion in mice and humans.

II.i.b.

A unique and distinct attribute in mice models of compensation effect by upregulation of p107 in mice in response to Rb inactivation.

II.i.c.

Inactivation of both Rb and p107 proves to be lethally fatal in mice embryonic development.

II.i.d.

Chimeric models with Rb and p107 knockout have issues with delayed onset, compromised penetration and cell non-autonomous factors being impacted corresponding to the promotor deployed.

II.ii.

Advantages:

II.ii.a.

Cell of origin could be traced by studies on single copy of the trio (Rb, p107, and p130) inactivated models.

II.ii.b.

The breedable models mimicked human retinoblastoma tumors in terms of the molecular profiles, biochemical profiles, neuroanatomy, and histological morphologies.

II.ii.c.

Serve as idle models to conduct anticancer drug tests for prognosis of tumors with respect to anticancer drug efficacies.

III. Xenograft Models:

These models involve injecting cancer cell lines/cancerous tissues into the animal model which has been immunocompromised. In the past researchers have injected Y79 and WERI1 cell lines and assessed tumor generation in mice models. McFall and colleagues had injected Y79 and WERI cells in the anterior chamber of rabbit eyes and observed tumor formation in two to three weeks. Histopathological studies had confirmed the presence of tumor tissues. Kang and colleagues had injected WERI cell line in the subretinal space of rabbit models and observed that the tumor formed in a week followed by vascularization by fifth week and continuation of tumor progression by eighth week. Though histopathological studies confirmed the presence of tumor resembling retinoblastoma, the tumor was not of metastatic phenotype, and the tumor formation site was confined to optic nerve and vitreous cavity lesions (Kang and Grossniklaus 2011).

III.i.

Drawback:

III.i.a.

Immunocompromised/athymic nude mice required

III.i.b.

The major drawback of xenograft models is that the tumor is developed in the subretinal space rather than developing in retina like in humans. Therefore, the xenograft models developed using WERI cell lines do not mimic retinoblastoma in humans as the tumor development site is anatomically incongruent. This is because the WERI cell line-based xenograft modes do not develop metastatic tumors (Chévez-Barrios et al. 2000).

III.i.c.

When human (primary) cells are injected into mice/rabbit models, the tumor micro-environment changes. The system by and large acts like an interphase between in vitro and native environment. It therefore alters the tumor heterogeneity and disrupts the normal tumor niche.

III.ii.

Advantages:

III.ii.a.

Reproducible: The tumors developed in the xenograft models are reproducible and form tumors congruent to human tumors with respect to histology, morphology, molecular profiles, and biochemical profiles.

III.ii.b.

Originality remains conserved: The tumors from one xenograft model when transplanted to subsequent nude mice would maintain the same pattern of tumorigenesis.

III.ii.c.

Metastatic like Human Rb Tumors: Y79-based models have metastatic potential and develop tumors resembling original retinoblastoma tumors in humans.

III.ii.d.

Therapeutics and Prognostic Studies:

III.ii.d.i.

These models serve as avenues to assess the therapeutic efficacy of anticancer drugs.

III.ii.d.ii.

Imaging: The in vivo models have proved their application in developing noninvasive approaches to assess tumor growth and distant metastasis. Xunda Ji and colleagues injected dual reporter retinoblastoma cell lines in nude mice and demonstrated that Bioluminescence Imaging (BLI) technique can aid in in vivo assessment of tumor progression and metastasis (Ji et al. 2009).

Estimation of Biomarkers of Rb in Various Models

Biomarkers play a pivotal role in the diagnosis of retinoblastoma (RB) and also in the development of appropriate therapeutics to combat it. Biomarkers like proteins or

biomolecular chemical changes are measurable components that imply a definite physiological condition (Huang et al. 2017). They facilitate the cancer study at various clinical stages and offer an insight into therapeutic outcome as well. Accordingly, recent data has shown that long noncoding RNAs, circular RNAs, circulating tumor DNAs, and nonessential amino acids playing a key role in regular metabolism act as biomarkers in cancers. Blood samples play a key role in the design of molecular diagnostic techniques primarily because they play a vital role in the quest for early biomarkers of a few diseases such as hypothyroidism, non-small cell lung cancer (Guo et al. 2019), and Micro-RNAs in RB (Golabchi et al. 2018). Despite the current use of clinical and histopathological examination in the RB risk assessment, a few cases cannot be diagnosed. To ameliorate therapeutic outcome, prevent enucleation, and avoid metastasis, there is a growing need to undertake studies on molecular underpinnings of RB and discover the key biomarkers that initiate tumor progression. Consequently, the study of RB biomarkers facilitates the understanding of the disease intensity, early diagnosis, and exact prognosis evaluation.

Analysis of Biomarkers

Mass Spectrometry (MS) plays a pivotal role in the high-throughput profiling of a plethora of biological samples such as blood, urine, milk, cells, and tissues (Huang et al. 2017). MS-based proteomics facilitated the study of cancer biomarkers thus opening the novel approaches for clinical tests. The analysis includes discovery of candidates; their verification, validation, and clinical validation. In proteomics, the two extensively used gel-based proteomics are bottom-up and top-down methods to detect proteins. While in bottom-up method proteins are isolated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), in a few cases shot-gun proteomics are used in which fractionation step is dropped down, digested in, and further measured by MS (Milman 2015). Despite the use of 2-DE in proteomic research since 1975, a few demerits such as limited dynamic range and low-throughput analysis limit its application.

Phosphoproteomics

It is an exemplary strategy to investigate phosphorylation modifications in proteins. It plays an essential role in the discovery of appropriate biomarkers and therapeutic targets of cancer. Accordingly, global phosphoproteomics of RB have been studied to detect signaling cascade linked to this cancer. Based on the results, more than 350 proteins exhibited variant phosphorylation in RB corresponding to the healthy retina. It has been understood that the stress response proteins were hyper-phosphorylated in RB such as H2A histone family member X (H2AFX) and sirtuin 1. More specifically, Ser140 of H2AFX also called gamma-H2AX was observed to be hyperphosphorylated in RB which suggested the activation of DNA damage response pathways. Based on these findings, discovery of hyperphosphorylated protein kinases such as Bromodomain containing 4 (BRD4), Lysine-deficient protein kinase 1 (WNK1), and Cyclin-dependent kinase 1 (CDK1) in RB shows novel therapeutic interventions in RB (Selvan et al. 2018). Among the numerous methods

used in phosphoproteomic study like antibody-based arrays, mass spectrometry, and flow mass-cytometry, mass spectrometry has been regarded as the vital tool because of the shortage of antibodies for the phosphoproteins. Of the 1393 proteins found to be phosphorylated, around 350 proteins were noticed to be variably phosphorylated in RB compared to control retina, suggesting the preservation of a retina-related signaling signature besides tumor-associated modifications in cell signaling.

Micro RNAs

In a recent study, upregulation of mir17a, miR18a, miR20a, and miR-103 has been observed by microarrays in the merged sera of 14 children suffering from RB corresponding to 14 healthy age-matched controls (Beta et al. 2013). However, these interesting results need to be corroborated by further studies. Another study demonstrated elevated levels of survivin and the TGF b1 in aqueous humor and serum from RB patients corresponding to the healthy controls. Both the proteins showed a reduction after chemotherapy.

Enhanced amount of lactate dehydrogenase (LDH) has been noticed in 21 out of 23 samples of aqueous humor from patients with RB. The study has shown that the amount of LDH was not dependent on clinical conditions like sex, treatment, or family history and on pathological characteristics such as calcification, necrosis, or inflammation. Nonetheless, due to the enzyme's enhanced vulnerability to numerous physiological conditions, these results need additional investigation. Another study evaluated miRNA levels from 65 patients with RB and 65 healthy controls using quantitative reverse transcription-polymerase chain reaction. These findings have shown that miR-320, miR-21, and miR-let7-e were downregulated in plasma received from individuals with RB (Liu et al. 2014). Amazingly, the identical miRNA levels are enhanced in RB tumors compared to normal tissue. Hence, further studies to end this ambiguity play an essential role in RB research.

Cell-Free DNA Analysis in Aqueous Humor

Recent study has demonstrated that the examination of a specific DNA type, called cell-free DNA (cfDNA), within the eye fluid or blood of patients, can be used to identify variations in the RB1 gene or other portions of the genome within an RB tumor. The investigation of cfDNA in the blood of pregnant women is also sufficient to recognize if the baby to be born will be suffered with RB (Gerrish et al. 2021). Aqueous humor of RB patients and the patients with other ocular diseases usually comprise both nucleic acids and proteins. Hence, aqueous humor may be a substantial source of circulating tumor DNA and can substitute the tumor biopsy for patients taking conservative treatment. Although a little volume (less than 100 μL) of aqueous humor is derived within the proximity of the tumor, the closed eye nature and lower secretion of fluid within the region indicated that the limited tumor-derived cfDNA is adequate for a genetic analysis. Quantifiable amount of cfDNA was noticed in aqueous humor samples obtained from RB patients in 2017 (Berry et al. 2017). In addition, the genetic profiling of this cfDNA indicated it was obtained from the tumor. Although this minimally invasive procedure suffices the current needs, biomarkers from blood or plasma may be more appropriate and feasible to study.

Genomic cfDNA Analysis of Aqueous Humor

Liquid biopsies using circulating tumor cells and cfDNA derived from blood or other body fluids play a key role in analyzing therapeutic results. Tumor studies have unraveled somatic copy number alteration (SCNA) profiles with extremely frequent chromosomal increases on 1q, 2p, 6p, decreases on 13q, 16q, and focal MYCN magnification on 2p which collectively are called "RB SCNAs." A study showed that 1q and 6p gain and 16q loss are probably linked to regionally invasive disease (Kapatai et al. 2013). Conversely, another study showed a gain of 6p is linked to fewer differentiated tumors with enhanced rates of optic nerve attack, (Cano et al. 1994) and probably noticed in older patients. On the other hand, these suggestions do not play a role in forecasting eye salvage, tumor diagnosis, or therapy due to the contraindication of invasive tissue biopsy of RB with a probability of extraocular tumor spread.

Blood and Plasma Biomarkers

Although the extraction of aqueous humor involves a significantly less invasive procedure with a reduced complication risk, a liquid biopsy carried out with cfDNA received from blood has numerous benefits over an aqueous humor assay, such as a less-invasive sampling procedure and the facility of a higher sample volume. In a recent study, targeted capture-based next-generation sequencing (NGS) has been used to examine tumor DNA, genomic DNA, and plasma-derived cfDNA from 10 independent RB patients who suffered from progressive intraocular disease, three of which progressed to metastatic disease (Kothari et al. 2020). Plasma cfDNA was examined with capture-based NGS targeted to the RB1 gene. RB1 pathogenic variants have been detected in tumor and genomic DNA with MSK-IMPACT, an NGS panel that aims more than 400 genes linked to cancer, counting all coding exons of RB1.

Corresponding to the study on aqueous humor, it has been shown that in spite of a single cycle of chemotherapy, tumor-derived cfDNA levels within the plasma seem to reduce. Therefore, it can be concluded that a larger study is needed, involving individuals in earlier stages of disease, in addition to pretreatment sampling, to examine the prospect of a diagnostic assay for RB with cell-free DNA obtained from plasma.

Proto-Oncoproteins: MDMX and MDM2

Onset of RB occurs through the cells devoid of the RB gene; however, unlike in other cancer types, p53 gene remains unchanged in RB. The proto-oncoproteins MDM2 and MDMX play a pivotal role in p53 regulation. In contrast to other cancer types, in RB the p53 tumor suppressor is generally wild type, despite the presence of dysregulated regulators, MDMX and MDM2. A wealth of studies have shown that in 75% of cases the p53 pathway is changed, MDMX is upregulated in 65%, and MDM2 in 10% of cases, including those exhibiting a wild-type p53. Currently, studies have observed that only MDM2 initiates retina cancer in p53-independent mechanism by mediating the translation of MYCN, unlike MDMX. A few polymorphisms in the p53 pathway associates were also found to be linked to the RB progression. The significance of the p53 pathway in RB has also been studied in the mice with retina-deficient RB and p107 and p53. Interestingly, these mice manifested bilateral RB with 100% expressivity.

The tumor is initiated in RB-diminished cone precursors and the RB spread relies on the cone precursor characteristics like enhanced expression of oncoproteins like MDM2 (Xu et al. 2009). These are the principal p53 regulators that include proto-oncoproteins MDM2 and MDMX. MDM2 being a ubiquitin ligase can ubiquitinate and diminish p53 via the proteasome 26S during usual cellular functions (Medina-Medina et al. 2016). The MDM2-homologous protein MDMX shows significant similarity with MDM2, and although this protein is devoid of ligase activity, it binds p53 and MDM2 to facilitate the p53 polyubiquitination process. Retina precursor cells lacking the *RB* gene experience p53-mediated apoptosis. The overexpression of MDMX or MDM2 genes provokes p53 inactivation. These findings can also be noticed in blood samples of patients suffering from retina cancer and perhaps the cause of hereditary causes.

Tissue-Specific Biomarkers: Vascular Endothelial Growth Factor

The vascular endothelial growth factor (VEGF) is a peptide mitogen specifically found in endothelial cells and also is an angiogenic factor. In general, hypoxia and innumerable cytokines initiate the VEGF expression (Ferrara and Davis-Smyth 1997). Earlier studies have shown that VEGF was overexpressed in several types of tumors, which provoked angiogenesis both in vitro and in vivo (Viglietto et al. 1996).

Voluminous data have shown the overexpression of VEGF in RB tissues unlike in normal retina tissues. This type of epigenetic variations upsets the genetic expression and perturbs the signaling pathways. These genes probably play a crucial role in the treatment of RB and an insight into these genes uncovered the molecular underpinnings of RB (Areán et al. 2010). Therefore, the expression of these genes and their role in RB tissue played an essential role.

Limitations and Future Perspectives

Despite the potential benefits of a liquid biopsy with blood plasma instead of aqueous humor sample, it is unclear if the genetic analysis of plasma-derived cfDNA is on par with the connection found between tumor DNA and cfDNA obtained from the aqueous humor. Therefore, there is a growing need to develop more appropriate methods to have the genetic analysis that exactly equals the aqueous humor cfDNA analysis. In addition, insight into the development of a few novel biomarkers specifically found in blood or plasma may play a pivotal role in the diagnosis of RB.

Current Therapeutic Modalities: Comparative Analysis of Models

Primary Treatments and Its Limitations

Noninvasive Therapy: Management for Early Intraocular Tumors

Retinoblastoma treatment has evolved over the years and it has been a challenging row to hoe. In the 1960s radiotherapy was extensively performed in treating intraocular malignancy. But due to concomitant radiation-induced complications and added disadvantage of developing secondary malignancies, the advancement of

chemotherapy combined with laser photocoagulation, thermotherapy, and cryotherapy has been widely followed in retinoblastoma (RB) treatment. The advent of intravenous chemotherapy (also known as chemo reduction) in the 1990s happened to increase the survival rate of the eye globe; chemo reduction treatment preceding radiotherapy in patients could massively increase the rate of tumor control by approximately 40% (Kingston et al. 1996), and in several cases reduced in need of enucleation.

1. Photocoagulation: It has been a promising therapy in treating retinal neovascularization, as it targets the vessels supplying blood to the tumor using 520 nm wavelength argon or xenone laser beam generating temperatures around 65 °C that is focused through the pupil coagulating the blood vessels (Mendoza and Hans 2016). Successfully controlling about 70% of malignancy cases (Shields 1994), it is effective only for small tumors ($\leq$4.5 mm base and $\leq$ 2.5 mm thick) without vitreous seeding (Shields 1994), particularly located in the posterior of the eye. Complications for the heat generated from the laser can damage the retina creating blind spots and retinal detachment. This treatment is limited if the tumor extends to the fovea, in which case it can risk compromising central vision.
2. Thermotherapy: Small tumors (i.e., less than 4.5-mm base and 2.5-mm thickness) are directly heat killed using a beam of infrared, ultrasound, or microwaves at temperatures ranging between 45 °C and 60 °C. However, in case of large tumors thermotherapy is combined with either chemotherapy (also known as Chemothermotherapy) or radiotherapy. Nevertheless, the complications implied can possibly cause the iris to shrink, clouding of the lens or cataracts, seeding of the tumor into the vitreous, clotting of the blood vessels, and retinal damage.
3. Cryotherapy: This therapy uses surgical probes for delivering liquid nitrogen to small tumors (less than 3.5-mm base to less than 2-mm thickness) (Mendoza and Hans 2016) located in the anterior of the eye. The process of freeing-thawing ruptures the tumor cell membranes and damages the vascular endothelial supplying blood to the tissues. Although complications are less intense (Shields et al. 1989), the possibilities of retinal detachment, retinal tears, swelling of the eyelids, and vitreous hemorrhage in previously irradiated tumors are added drawbacks associated with this mode of treatment.
4. Plaque Radiotherapy: Radioactive implant (e.g., Iodine^{125}, gold, or ruhenium-106 isotopes) is placed at the base of the tumor, initiating harmful radiations that get absorbed by the tumor cells causing DNA damage and cell death. This treatment is limited to tumor size in 8 mm base to less than 16 mm thickness. It is usually a primary mode of tumor management which is also used for cases with prior failure in radiotherapy or chemotherapy management. Studies reported that in a single application, plaque therapy alone could control 90% of the retinoblastoma (Shields et al. 1993). However complications include radiation retinopathy, cataract formation, and optic neuropathy; however, the risk of developing secondary is unlikely compared to other radiation therapies (e.g., thermotherapy, cryotherapy, or photocoagulation) (Rodriguez-Galindo et al. 2007).

Chemotherapy

Conventional chemotherapy uses combination doses of two, three, or four different drugs administered intravenously. These drugs are well-known DNA-crosslinking agents (e.g., carboplatin, cisplatin), DNA topoisomerase inhibitors (e.g., etoposide, topotecan, teniposide), and alkaloids (e.g., vincristine) (Shields et al. 2014). Intravenous chemotherapy in combination with focal therapies can reduce the need for enucleation as well as radiation therapy and is widely used in treating early RB. However, during vitreous seeding of the tumor, conventional chemotherapy application is limited due to the lack of blood vessels in the vitreous that selectively restricts drug administration at the tumor site (Gupta and Meena 2020). Retinoblastoma management can be satisfactorily controlled by chemotherapy, and unlike radiotherapy, it could salvage the eye from adverse side effects (Shields et al. 2002). Moreover, emergence of intra-arterial and intravitreal chemotherapy has encouraged targeted release of antitumor drugs (e.g., carboplatin, vincristine, and topotecan) into the ophthalmic artery (Suzuki et al. 2011). Yet long-term risks such as choroid toxicity, ophthalmic, retinal or choroid artery spasm, optic neuropathy, headache, and vitreous hemorrhage are well associated (Gupta and Meena 2020). Still, in intra-arterial administration the major drawback is the high cost of treatment, which makes it inaccessible for patients from developing countries (Zanaty et al. 2014). In cases of vitreous seeding, tumor management becomes even more challenging particularly due to the avascular nature of vitreous. In such events drugs can be directly injected into the vitreous in high concentration; however, associated complications such as retinopathy at target-site are common in this treatment.

Attributing to the toxicities allied with the drug regimens in intra-arterial chemotherapy (IAC), animal models mimicking human RB need to be developed for analyzing the novelty of drug management in vivo for better understanding of drug distribution and efficacy in ocular tissue.

External Beam Radiotherapy (EBR)

In advanced stages of retinoblastoma where tumor cells are found to penetrate the vitreous cavity, EBR steps in as the best-suited modality to salvage vision. Using electron and proton beam in standard dose of 20–45 Gray (Gy) over a period of 3–4 weeks, radiotherapy is administered in cases with small recurrent tumors that do not well respond to chemotherapy (Chintagumpala et al. 2007; Gupta and Meena 2020). Although EBR can prevent recurrence of retinoblastoma in long-term, complications like optic nerve damage, vitreous hemorrhage, dry eye syndrome, secondary malignancies, facial bone hypoplasia, and cataract are common.

Enucleation

Enucleation is an intrinsic eye surgery that involves removal of the entire eye globe, transecting the optic nerve while keeping the rectus muscles and the orbit preserved. It is primarily an essential paradigm for treating malignant, blind, and otherwise painful eyes and has been well-known therapeutic management for retinoblastoma particularly when treatments like chemotherapy are a less opted option. In extensive cases of retinoblastoma where saving the vision becomes almost impossible,

enucleation is advised as the best-opted strategy. In 50% of advanced cases of malignancy the eye is enucleated (Suzuki and Kaneko 2004). The first successful enucleation with prosthetic eye implantation was reported back in 1886 and 1887 (Sami et al. 2007), and subsequently, it is efficacious in more than 95% of patients with unilateral RB (Chintagumpala et al. 2007). Biomaterials (e.g., polyethylene, acrylic, hydroxyapatite) are used for implants as they are biodegradable and biologically inert; however, the concern remains with the implant's mobility in postoperative management. Additionally, the complication and cost of the postoperative cosmetic surgery accompanied with the psychological and societal pressure remains a major concern for the child (Leander et al. 2007).

In artificial implants porous materials (e.g., hydroxyapatite and polyethylene) are given advantage over solid implants (e.g., acrylic) as they are light weighted and permit vascular growth from the orbit into the implants through their pores, necessarily imparting more stability with lesser risk of dislocation, reduced infection, and complete development of the implant into mature orbit bone (Shields and Shields 2010).

Extraocular Management

The spread of retinoblastoma goes beyond the eye to the surrounding tissues or even into other parts (e.g., central nervous system, bone marrow, or lymph nodes). Firstly, a bone marrow examination and cerebrospinal fluid (CSF) studies are done to rule out the systemic spread (Gupta and Meena 2020). Regional extraocular tumors (those extending through the sclera into to surrounding orbital tissues) can be administered using conventional chemotherapy and EBR in combination (Rodriguez-Galindo et al. 2007).

Prerequisite for Animal Model Study

Though conservative therapies significantly contribute to saving the vision in retinoblastoma, there is always a concomitant risk of a lingering side effect in the long run. Since retinoblastoma is all human restricted disease, studying the tumor origin and spread becomes critical mainly due to crisis in viable tissue availability owing to limited cases and insufficient clinical trials, wherein the need for animal models becomes indispensable in the study of tumorigenesis and therapeutic approaches (e.g., testing drugs, combination therapy). Emphasis on developing animal models induced with retinoblastoma by far is a successful way to study therapeutic modalities. However, it is a challenging task to develop animals that can mimic all the RB1 mutation in human mainly due to species-specific characteristics. For example, mice do not express Opsin, a hallmark for RB, which is expressed almost more than 95% in Rb tumor cells (Mendel and Daniels 2019b). Again, deletion of RB1 gene alone cannot induce retinoblastoma in mice models, for instance, Rb1/p53/p107 knockout mouse models have three genes deleted using targeted expression of simian virus 40 T-antigen to produce a similar phenotype observed in humans with only single RB1 gene deletion.

In spite of all the odds, several animal models are raised in the laboratory that are comparative to human Rb. For example xenograft models of immunocompromised

mice/rats are developed by either injecting immortalized human tumor cell lines (e.g., Y79 cells, WERI) or patient-derived xenografts (PDX) through orthotropic (in eyes) or heterotopic (e.g., in the flank regions) injections. Nonetheless the in vivo study for intra-arterial chemotherapy or other focal therapies cannot be carried out easily on small-sized eyes, thus large eye-sized animals (essentially in rabbits) are immunocompromised using immunosuppressant (e.g., cyclosporine), as it can primarily inhibit calcineurin phosphatase pathway, inactivating DNA transcription factors for T-cell proliferation (Kang and Grossniklaus 2011). For example, Daniels et al. taking the advantage of large eye size of rabbits developed a model system for intra-arterial chemotherapy and were successful in showing melphalan drug penetration into vitreous and retinal tissues with drug dose efficacy sufficient enough to kill human RB cells, additionally confirming no vascular, neural or retinal toxicity (Daniels et al. 2018). The possibility of treating retinoblastoma xenografts with photodynamic therapy was explored by Kim et al. using pigmented rabbits and cyclosporine was used to facilitate xeno-engraftment (Kim et al. 2017).

Conventional Therapies: Comparative Analysis of Models

Murine models are well considered for intravitreal injections, given the fact that these animals are readily procured and are fast and easy to breed in the laboratory (Zhang et al. 2017). On the other hand the limited vitreous space in murine makes intravitreal injections an intricate task, delivering them as not so excellent choice. Thus rabbit models owing to their large eye size, come up as an efficient choice for such studies (Daniels et al. 2018). Non-albino rabbits are a better choice (Buitrago et al. 2016) in testing toxicity of intravitreal drugs (e.g., carboplatin and etoposide) (Mohney et al. 2017) because in drug testing experiments, when melphalan, the most common clinically used intravitreal injection in humans, is treated into albino rabbits, unlike humans, due to lack of pigmentation, they do not manifest "Salt and pepper" (pigmentation) retinopathy (Mendel and Daniels 2019b).

On the other hand porcine models are used for studying pharmacokinetics of IAC drugs (e.g., melphalan and topotecan) as their eyes are anatomically comparable regarding vascularization and the large eye size made IAC injections easier (Schaiquevich et al. 2012b). However, the great costs of obtaining and housing large pigs ultimately limit long-term experiments, so use of such models is encouraged only in short-term studies of drug kinetics and cytotoxicity. A prominent assessment between porcine and rabbits revealed highest drug localization in the retina and vitreous of rabbit models, despite porcine models (e.g., 70 kg Landrace pigs) were injected with higher intra-arterial drug doses. Also the overall size and weight of rabbits draw a parallel to that of a human baby. However, poor drug delivery in the intraocular tumor has been demonstrated in rabbits due to the tough sclera and high rate of choroidal blood flow, which makes drug penetration in the retina extremely challenging. Interestingly the drug must pass through the sclera, Bruch's membrane, and RPE before gaining access to the retina, hence drug concentrations are found at lowest levels in vitreous even when administered with high doses. Contrasting rabbit

models, in aged nonhuman primates several kinds of vascular atrophies were observed that limited their use in preclinical studies. Of all the animal models of intra-arterial chemotherapy, the rabbit model is the only one in which assessments of efficacy can be performed, as rabbits are the only species in which a retinoblastoma tumor xenograft model exists (Kang and Grossniklaus 2011).

The major disadvantage of xenograft models is that the tumor microenvironment is altered when human cell lines are injected into the rodent/rabbit eyes, which could possibly bring imbalance in the tumor growth (Nair et al. 2013). The first xenografts models of Y79 cells was performed in immune-compromised mouse using heterotopic injections, but the differences between the anatomy of adult mouse flank and its eyes make such models unsuitable for further studies (White et al. 1989). Kang et al. created cyclosporine-induced xenograft rabbit models using WERI cell line to study transscleral drug delivery (Kang and Grossniklaus 2011). The rabbit model developed had added advantage as there was vitreous seeding of tumor cells when retinal tumor was mid-sized, such characteristics are observed only in the late stage in mouse models.

Rhesus macaque used as models for testing anticancer drugs (e.g., melphalan and carboplatin) toxicity have been promising since adult eye size of these models are comparable to human RB eyes, which additionally helped to validate in vivo imaging (e.g., using photography and fluorescein angiography) (Brennan et al. 2011). Although there's parity in eye size with humans models, age-associated vascular complications (e.g. thrombosis and embolus formation) are unavoidable because due to age the blood vessels commonly manifest presence of atherosclerosis (Mendel and Daniels 2019b).

For evaluating current therapeutic modalities (e.g., focal chemotherapy, radiation therapy, cryotherapy, and vascular targeting therapies), LH-β T-Ag transgenic mouse models infected with Simian virus T-antigen are widely used because the viral T-Ag can inactivate both tumor suppressor genes RB and p53 to induce tumor in mouse (Mietz et al. 1992). Controlled tumor growth was reported in the models when combination treatment of intravitreal chemotherapy (using carboplatin drug) and EBRT was commenced (Murray et al. 1996). Using optical coherence tomography (OCT) proper quantification of tumor growth was also observed in these models. Nonetheless, limited knowledge about vial oncoproteins makes them a hard choice for model systems. Also unlike a typical characteristic human RB, these mouse models failed clonal and focal growth.

Xenograft models of thymus-compromised nude mice were developed for chemotherapy and photodynamic studies. Due to the limitations of secondary leukemia and malignancy as side effects of chemotherapy, the advancements in photodynamic therapy are emphasized. It is a combination of photo-chemotherapy using second-generation photo-chemotherapeutics as a prerequisite to develop a promising therapeutic alternative. This therapy implies intravenous application of a photosensitizer activated locally by light of the appropriate wavelength. Activation leads to the formation of free radicals, vascular occlusion, and death of affected cells in the area of irradiation (Stephan et al. 2008). Nude mice models are developed with xenografts (using Y-79 cells) and injected with mannose

functionalized mesoporous silica nanoparticles (MSN) impregnated with light-activated photosensitizer in the flank region. This study was efficient in inducing retinoblastoma cell death and studying the efficacy of camptothecin (chemotherapeutic agent) delivery in tumor cells. Aerts et al. also studied photodynamic therapy in nude mice models using xenograft cell lines and FDA-approved photosensitizers (e.g., meta-tetrahydroxyphenylchlorin (mTHPC); verteporfin) to estimate the efficacy of PDT in the treatment of RB. Recently, Kim et al. used pigmented rabbits to perform photodynamic therapy (PDT) in the treatment of retinoblastoma (Kim et al. 2017).

Advanced Therapeutics

Over the course of the last few decades, numerous models have been developed for the study and analysis of retinoblastoma disease. Today, many different therapeutic techniques are employed to try and cure retinoblastoma. Simultaneously, advanced techniques are being developed, investigated, and tested for future use as improved modalities for treatment. All these therapies are tested on disease models before they can be approved for human testing and for regular use.

Techniques that are relatively new and employ recently developed scientific knowledge are what comprise advanced therapy. These include but are not limited to gene therapy, Chimeric Antigen Receptor-T cell (CAR-T cell) therapy, small molecule drugs, monoclonal antibody, etc.

A comparative list of these techniques has been provided recording the studies and which models they had been conducted on. Also included in the list is the recorded efficacy of the technique. For the scope of this chapter and book, we mainly focus on studies that include animal models.

Gene Therapy

Gene therapy and treatment of retinoblastoma are possible via gene silencing and gene replacement. Studies have been conducted that employ procedures like vector-based method established in experiment models, nanoparticle delivery method, and adenoviral delivery method for small interfering RNA-based therapy. Use of short hairpin RNA for silencing of pathways leading to apoptosis is a reliable method investigated and reported for treatment. Use of oncolytic adenovirus modified to replicate inside cancer or tumor cells has been extensively studied, individually and in combination with chemotherapy for use in eradicating cancer. Early studies using Adenoviral Vector, Herpes Simplex Virus-Thymidine Kinase gene (AdV-TK) working synergistically with prodrug ganciclovir have shown interesting results which have been tested in preclinical and clinical trials (Chévez-Barrios et al. 2005). Other adenoviral therapy caused reduced growth of tumors in animal models when measured using both mass and volume. This reduction in animal tumors was reported as a cumulative effect of cell cycle inhibition to prevent proliferation and induction of apoptosis to cause cell death.

CAR-T Cell

Modification of T-cells by introducing Chimeric Antigen Receptors (CAR) giving rise to CAR-T cells has shown incredible targeting and treatment capabilities of malignant liquid tumors. Multiple clinical trials and results have given rise to FDA-approved therapies like Kymriah® and Yescarta®, yet CAR-T cells show decreased efficacy against solid tumors, mainly because of their inability to maintain the effector functions (D'Aloia et al. 2018). The potential use of oncolytic virus armed with specific T-cell engager for combined therapy with CAR-T cells has shown higher efficacy against solid tumors in mouse models than individual therapies raising hope in use of these modalities for solid tumor therapy in the future.

Small Molecules

Small molecules, usually lesser than 500 Da, are used as therapeutic agents for cancer treatment. Due to their size and dimensions, they are easily able to translocate through the cell membrane and interact with cell receptors, organelles, or nucleic material inside the cells. Studies have shown that multiple small inhibitor molecules are very capable therapeutic molecules. Nutlin 3A, a small molecule, has been extensively studied for its anticancer effect. In vivo preclinical studies have shown Nutlin 3A is capable of impressive tumor growth inhibition. Similar other small molecules have undergone scrutiny for treatment like WP1066 and Galangin molecules, STAT3 inhibitors, and even neddylation inhibitors (Aubry et al. 2020) capable of aiding cancer therapeutics have been investigated. Use of these techniques on animal models has shown between twofold to fourfold decrease in the size of the tumor formed by either inhibiting cell proliferation or causing apoptosis.

mAbs

Use of monoclonal antibodies for treatment of cancer has been under investigation from the early 1990s, with Riechmann et al. analyzing reshaped human antibody and Tarlton and Easty mentioning chimeric mouse-human antibodies specifically for retinoblastoma therapy (Tarlton and Easty 1993).

Since then, many advances have been made in retinoblastoma therapy. Bevacuzimab, an anti- Vascular endothelial growth factor (VEGF) monoclonal antibody to Dinutuximab which enhances natural killer cells anti-cancer activity, has been investigated. Multiple different forms of synergistic therapies which combined chemotherapy with these mAbs and other modalities have also been attempted Table 2.

Conclusion

In this chapter, we cover various aspects of retinoblastoma cancer models and provide deep insights into the various treatment modalities including standard chemotherapeutic options along with new developments in monoclonal antibody-based therapies, CAR-T cell therapy, and oncolytic viral therapies. Even though the current standard treatment procedures are effective in controlling the progression of

Table 2 Advanced therapeutic techniques

Therapy	In vitro	In vivo	Pathway
Short hairpin RNA to silence SYK	Apoptosis in a number of Rb cell lines and cells obtained from patients	Increased apoptosis in xenografted mice tumors	Inhibition of SYK causes apoptosis
Adenoviral vector, herpes simplex virus-thymidine kinase gene (AdV-TK) with prodrug ganciclovir	HXO-RB44 and Y79 cells showed dose-dependent cell death	70% xenografted mice showed a complete ablation of detectable tumor	The prodrug gets phosphorylated and becomes a toxic nucleotide analog due to viral modifications in the cell. The virus also interferes in ERK and MAP-K pathway inhibiting autophagy and increasing cytotoxicity
VCN-01	Virus caused 50% inhibition in proliferation of Y79 and patient-derived cells	Mouse and rabbit xenografts showed much lower tumor load	VCN – 01 produces oncolytic effect due to dysfunctionality of Rb1 gene
Oncolytic adenovirus H101	80% reduction in cell viability in HXO–Rb44 cells	Reduced cell viability and cause reduction of the tumor growth in NOD-SCID mice	Coxsackievirus receptor allowed targeted viral infection which induced G2/M phase arrest
Nutilin 3A	Y79 and WERI-Rb1 induced dose-dependent cell death	Xenograft Mouse models showed decrease in tumor size	p53-induced apoptosis via p21 and HDM2/MDM2
STAT3 phosphorylation inhibitor; WP1066 inhibitor	Decreased cell viability and induced apoptosis in Y79 cells	Fourfold decrease in tumor volume in xenograft mouse	JAK/STAT3 pathway inhibition causes a stop in transcription of many proteins leading to cell death
Galangin (3,5,7-trihydroxyflavone)	Suppressed cell proliferation of Y79 and 2 other Rb cell lines	Antiproliferative activity and threefold decrease in tumor volume in xenograft mouse	PTEN-dependent caspase 3 apoptosis pathway activation
NEDD8 inhibitor MLN4924	14-fold higher toxicity toward Rb cells tested in 7 Rb cell lines	Xenograft mice showed 30–40% cell death within 3 days	Inhibits neddylation, disrupting tumor microenvironment
Bevacuzimab	Snout-Rb1 and Y79; rise in cytotoxicity, increased cell death	Xenograft mice, 3.5-fold smaller tumor on enucleation	Anti-VEGF, ERK 1/2 inhibition, causing rise in cell toxicity and apoptosis
Bevacuzimab + carboplatin	50% increase in Y79 cell death	Xenograft mice, fivefold decrease in tumor size on enucleation	S phase arrest; PI3K/Akt and ERK pathway inhibition

the disease and the life of the affected people, the therapies to salvage the vision for an enhanced life are still at large. The proposed new cell and gene therapy modalities show a glimmer of hope in vision restoration for the affected individuals.

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A Study on the Role of Phytochemicals in the Preclinical Neuronal Cancer Model

32

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Abstract

With more research being conducted to elucidate the various molecular mechanism of cancer progression, the development of chemically synthesized drugs has greatly improved over the past decades. However, many of these drugs still come along with severe side effects that affect the patient's quality of life. Other than these side effects, toxicity and drug resistance of conventional therapy have led researchers to look into the possibility of using plant-derived compounds as treatment strategies. Studies have shown that phytochemicals are capable of interfering with the molecular mechanisms involved in cancer cell growth and proliferation while having minimal toxicity and less detrimental side effects. While a few phytochemical derived anticancer drugs such as the analogues of vinca alkaloids and taxanes have been approved by the Food and Drug Administration (FDA) for cancer therapy, various other compounds are still in the preclinical and clinical trial stages. This chapter is a comprehensive compilation of various phytochemical agents that have been and still are being studied for the therapy of cancer with a specific focus on neuronal cancer research. These experimental beliefs are tapping into a whole new operative perspective for the neuronal cancer model which can be unleashed by further exploratory research.

Keywords

Phytochemicals · Neuronal cancer · Neuroprotection · Cancer therapy · Anticancer properties

Introduction

In recent years, as phytochemicals have come to play an important part in the pharmaceutical industry, scientists have begun to shift their focus into studying the therapeutic and neuroprotective effects of medicinal plants. Due to their diverse range of biological and therapeutic properties and the fact that phytochemicals are generally safer with significantly fewer side effects than synthetic drugs, medicinal plants have gained substantial attention from both the research and medical community as well as the public. In the case of cancer therapy, current treatment strategies primarily involve surgery, chemotherapy, and radiation, each of which brings its own adverse effects and complications. Dietary phytochemicals have anticancer properties and there is influential experimental data and evidence which supports this. This testimony is a

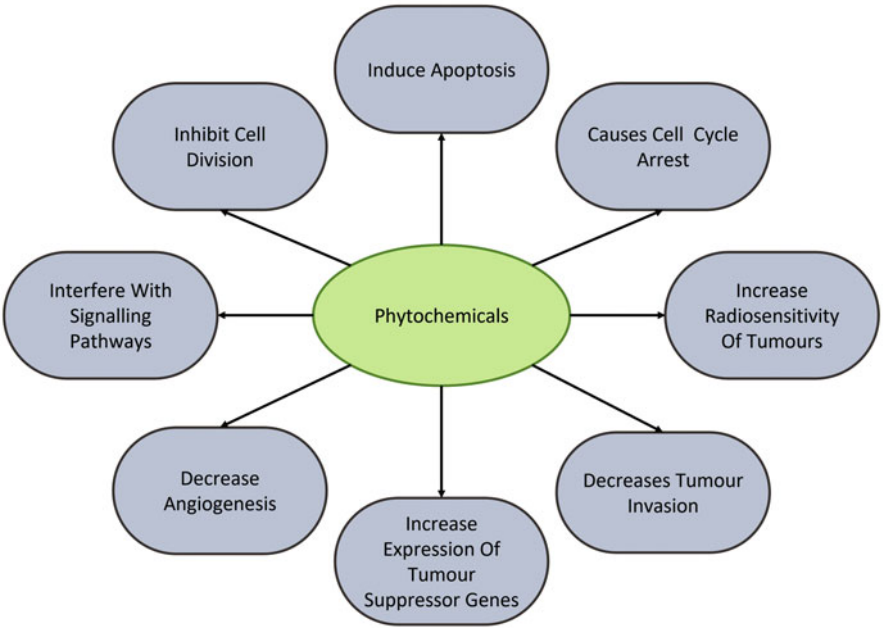

Fig. 1 Role of Phytochemicals in Cancer Therapy

consequence of the enduring association of well-being and dietary models given the abundance of bioactive agents in fruits and vegetables.

Most phytochemicals regulate cancer progression by acting on several molecular pathways related to cancer cell growth and proliferation. The different mechanisms that they follow include carcinogen inactivation, induction of cell cycle arrest and apoptosis, inhibition of cell proliferation, scavenging of free radicals, and even decreasing the invasiveness and angiogenesis of tumors (Lee et al. 2013; Lu et al. 2018) (Fig. 1). As mentioned earlier, there are phytochemicals that target the carcinogens instead of the cancerous cells. They focus on modulating the pathways which lead to apoptosis in the cancer cell lines. An example of such activity was found in capsaicin, an alkaloid that binds with TRPV1 (transient receptor potential cation channel subfamily V member 1). This binding increases the intracellular calcium level which ultimately results in apoptosis of the cell (Ranjan et al. 2019). Apart from the abovementioned ways, there are few polyphenol compounds that are able to target cancer cells and reverse their resistance toward chemotherapy making the process of chemotherapy applicable to the cells. Polyphenols like quercetin modulate p-53, inhibit P-glycoprotein (P-gp), angiogenesis and heat shock factor, as well as induce apoptosis. Fisetin, a naturally occurring flavonoid has also been shown to downregulate NF-kB and inhibit P-gp. Other examples of polyphenols modulating this chemoresistance in cancer cells include baicalein, luteolin, and curcumin (Hussain et al. 2016).

Phytochemicals Being Used in Current Cancer Therapy

While extensive research is still being conducted in the hopes of discovering an effective chemopreventive phytochemical compound with low side effects and unwanted reactions, there have been some compounds that have been approved and are currently being used in cancer therapy.

Vinca alkaloids (VAs), derived from the periwinkle plant *Catharanthus roseus*, were one of the first plant-derived anticancer compounds approved by the Food and Drug Administration (FDA), in 1961, for its use in cancer therapy. VAs are classified as microtubule-targeting agents (MTAs), which are anticancer molecules that function to inhibit abnormal cancer cell growth by inhibiting microtubule formation as well as promoting microtubules depolymerization thus preventing cell mitosis. Presently, five VAs (as mentioned in Table 1) are being used clinically for the treatment of various hematological neoplasms and solid tumors. The main hurdle in the widespread usage of VAs in cancer treatment lies in the fact that cells tend to become resistant to the anticancer activity of VAs and using VAs in combination with other drugs seems to be beneficial. Using it in combination with other drugs also means that a lower dosage is required and this limits the toxicological drawbacks of high dosages (Martino et al. 2018). Vinblastine was administered with an initial dose of 3.7 mg/m^2 body surface area (BSA) and found to be increased

Table 1 FDA-approved phytochemicals for cancer therapy

Phytochemical compounds	Mechanism of action	Cancer type	Reference
Vinca Alkaloids (VAs) Vinblastine (VBL) Vincristine (VCR) Vindesine (VDS) Vinorelbine (VRL) Vinflunine (VFN)	Inhibit microtubule formation, promote microtubules depolymerization, prevent cell mitosis, and inhibit abnormal cancer cell growth	Lymphoma, melanoma, leukemia, glioma, neuroblastoma, small-cell lung, colorectal, breast, renal, and esophageal cancer	Martino et al. (2018)
Taxanes Paclitaxel Docetaxel Cabazitaxel	Bind to tubulin, disrupt mitotic spindle, induce mitotic slippage and apoptosis, inhibit angiogenesis, and increase ROS production	Gastric, prostate, pancreatic, head and neck, non-small cell lung, ovarian, and breast cancer	Mosca et al. (2021)
Camptothecin Irinotecan Topotecan	Forms a complex with topoisomerase I (Top1) and DNA, induces cell death due to large number of double-strand breaks	Colorectal, ovarian, and small cell lung cancer	Venditto and Simanek (2010)

approximately 1.8 mg/m^2 BSA on a weekly basis till the desired therapeutic response was observed or the maximum dose of 18.5 mg/m^2 BSA was reached. Vinblastine, in combination with cisplatin and radiation therapy (VCRT), was used to treat stage IIIA and IIIB non-small cell lung cancer (Waters et al. 2010). Cisplatin, doxorubicin, cyclophosphamide, vinblastine, and bleomycin, known as CISCA/VB, has been shown to be an efficient combination treatment for patients with disseminated non-seminomatous germ-cell tumors (Fizazi et al. 2002). Doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD) were another set of drug combination that was commonly used as a chemotherapy regimen for Hodgkin lymphoma (Schwenkglenks et al. 2010). In adults, vincristine was usually administered at a dose of 1.4 mg/m^2 BSA once a week and capped at a maximum dose of 2.0 mg/m^2 BSA. Vincristine was used in combination drug therapies involving clophosphamide and prednisone (CVP) and cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) for follicular B-cell lymphoma and diffuse large B-cell lymphoma (Dijoseph et al. 2011; Lugtenburg et al. 2012). These combination drugs have also be used alongside rituximab (Pettengell et al. 2012). As a single agent, vinorelbine was intravenously administered at a dose of 30 mg/m^2 BSA weekly, and when used in combination with cisplatin for non-small-cell lung cancer treatment, the dosage and frequency vary based on the dosage of cisplatin. When used along with cisplatin for adjuvant chemotherapy it has shown positive results with acceptable toxicity (Pepe et al. 2007; Reinmuth et al. 2014).

Another class of plant-derived MTAs used in cancer therapy are taxanes, which are extracted from the bark of yew trees. As of now, three different taxanes have been approved by the FDA: paclitaxel, docetaxel, and cabazitaxel. As they are MTAs, they function similarly to VAs by targeting tubulin and interfering with cell division. In addition, it has also been reported that paclitaxel treatment induces apoptosis and inhibits angiogenesis (Mosca et al. 2021). However, the same issue of drug resistance observed in the usage of VAs is also seen when taxanes are used, with various factors causing this resistance (Maloney et al. 2020). Studies aimed at overcoming taxane resistance have focused on modifying taxane structure to enhance drug solubility and bioavailability (Rodrigues-Ferreira et al. 2021). Delivering taxanes via nanoparticles has also been shown to be an effective strategy, and in 2005, the FDA approved nab-paclitaxel, which was an albumin-bound paclitaxel for breast cancer therapy. Various other paclitaxel analogs and delivery methods are currently in clinical trials (Ojima et al. 2016).

Irinotecan and Topotecan are two semi-synthetic FDA approved drugs derived from the pentacyclic alkaloid Camptothecin, isolated from the bark of the *Camptothecin acuminata* tree. Irinotecan was approved in 1996 for the treatment of refractory colorectal carcinoma while Topotecan was approved later on in 2007 for ovarian and small cell lung cancer therapy. Camptothecin and its analogs function by binding to topoisomerase I (Top1) and DNA thus forming a complex which interferes with DNA replication, causeing double-strand breaks, and eventually cell death (Li et al. 2017). Side effects that limit the use of Irinotecan and Topotecan include bradycardia, neutropenia, steatohepatitis, abdominal pain, and diarrhea (Venditto and Simanek 2010). Research is being done to develop new

nanoparticles or polymer conjugates which will aid in improving the clinical utility of the drug. Understanding the genetic background of the patient, that is, polymorphisms in the particular gene or genes that are involved in metabolism of the drug may also provide clinicians with beneficial information on which drugs would be best for the patient (Yang et al. 2018).

Phytochemicals with Anticancer Properties in Preclinical and Clinical Stages

Capsaicin

Capsaicin is a bioactive phytochemical found in various red peppers from the *Capsicum* genus. Cell death was induced in a dose-dependent manner when KB cells (human epithelial carcinoma cells) were treated with capsaicin by decreasing cell division and viability, hence leading to apoptosis. It was found that cell cycle was arrested at the G2/M phase along with disruption and permeabilization of mitochondrial membrane potential. Immunoblotting results also showed that caspase 9, 3, and poly (ADP-ribose) polymerase were activated in KB cells. These results lead to concluding that capsaicin attributes anti-cancer properties (Lin et al. 2013).

Berries

The common berries namely strawberry, blackberry, raspberry, blueberry, and Indian gooseberry are some of the highly known nutritional sources of bioactive agents. Components such as flavonoids, tannins, phenolics along with sugars, fibers, minerals, and vitamins, individually or combined, confer antioxidant and anticancer capability to us. The antioxidant properties function by scavenging the reactive oxygen species (ROS) that causes oxidative damage in cells as well as to nucleic acids. The accumulation of these damaged products encourages tumor formation but when ROS are degraded, chance of carcinogenesis decreases.

Strawberries: Rich source of vitamin C and folate along with minerals like K, I, Mg, Fe, Mn, Cu, and P (Giampieri et al. 2012). Methanolic extract induces the intrinsic pathway for cell death via a mechanism independent of p53 in breast cancer cells, consequently reducing tumor volume and longevity of life (Somasagara et al. 2012). Seeram et al. reported that treating COX-2 expressing HT-29 colon cancer cell line with strawberry extract showed that the extract induced a 2.8-fold effect on cell death over untreated controls (Seeram et al. 2006). In another study, it was indicated that methanol extract (ME) fractions procured from strawberries (*Fragara ananassa,* FA) abbreviated as FA-ME, inhibited benzoapyrene (BaP)-induced cellular transformation in cultured Syrian hamster embryo cells (Xue et al. 2001). In a subsequent study by Li et al. (2008), it was revealed that these extracts from strawberries may attack several signaling pathways causing an inhibitory effect by nuclear factor of activated T cell (NFAT) activation, which is an essential

transcription factor involved in cell respond against environmental carcinogens (Li et al. 2008).

Blackberries: Contain polyphenolic compounds, carbohydrates, and primary natural sugars such as glucose and fructose (Fan-Chiang and Wrolstad 2010). A conspicuous flavonoid extract, quercetin was shown to have a significant cytotoxic effect on actively dividing HT9 and Caco-2 cells (Agullo et al. 1994).

Raspberries: Great combination of ellagitannins, shown to induce caspase 3 activation and cytochrome C flux leading to cell death and further inhibition of pancreatic cancer cell line division and anthocyanins, effective in downregulating cyclooxygenase expression and enhancing antiproliferative impact on cancer cells (Edderkaoui et al. 2008; Rao and Snyder 2010; Seeram et al. 2006).

Blueberries: Source of phenol and polyphenol agents, and stilbene derivatives (Jones 2018). They are a promising carcinogen-protectant against breast, esophageal, and colon cancers. It can inhibit the growth and division of cancer cell lines by regulating the AKT/NF kappa B/P13K pathways. This was checked against a triple negative breast cancer cell line in vitro and in vivo (Adams et al. 2010). By treating ACI rats that were at risk to estrogen-induced mammary tumors with amorphous raspberries and blueberries a 40% reduction in tumor volume was observed (Aiyer et al. 2008).

Spinach

Consumed both in raw and cooked forms, constituents of spinach include water, protein, carbohydrate, and fat. In a case-controlled study of 6888 breast cancer patients and 9428 controls a 45% decreased risk of breast cancer upon consumption of raw spinach (more than 52 servings per year) was reported (Longnecker et al. 1997). This can be attributed to lutein, a carotenoid (Na et al. 2016). This protective impact of spinach can also be conferred by the high chlorophyll content since it works against heme-induced hyperproliferation (Holliday and Speirs 2011). Experimentally supported, spinach extracts did not cause induction of cell death but blocked the G1/S jump (Chen et al. 2016). In 2021, an unprecedented study was sought to prevent cancer in the case of rat colon polyposis models. Rats were fed spinach for 26 weeks and demonstrated substantial antitumor efficacy. In Apc-mutant rats, β-catenin remained positively overexpressed in adenomatous polyps. Enriched gut microbiome diversity after feeding on spinach overlapped with a reversal of taxonomic composition in both wild and Apc-mutant rats. With this, tumor suppression by spinach involved a commendable reshaping of the gut microbiome. This was consequentially observed with modifications in host RNA-miRNA networks. With the help of metabolomics, colon polyps and matched normal-looking tissues were analogized. The anticancer outcomes were linked to spinach-derived linoleate actives which have been investigated on anti-inflammatory/pro-apoptotic mechanism. Parallelly, N-aceto-2-hydroxybutanoate with altered butanoate metabolism was a response to heightened α-diversity of the gut microbiome (Chen et al. 2021).

Aloe Vera

One of the oldest immortal cell lines is the HeLa cell line and the most popular are MCF-7 cells, expressing the estrogen receptor for breast cancer. Cells were treated with loe vera crude extracts of percentages 40, 50, and 60 for 6, 24, and 48 h respectively. Consequently, the cell viability of the cancer cell lines decreased. This happened due to the induction of apoptosis by condensation of the chromatin, fragmentation, and emergence of apoptotic bodies in a pre-stage of G0/G1. During this, there was also regulation of effector gene expression by enhanced CYP-genes (CYP1A1 and CYP1A2) expression and decrement in p21 and bax expression (A. Hussain et al. 2015). A combination of swimming (muscle training) and aloe vera extract diet (300 mg/kg) exerts a shielding anticancer effect with breast cancer affected mice. This occurs by the inhibition of the COX-2 reduction levels in the pathway and production of prostaglandins (Shirali et al. 2017).

Phytochemicals Being Studied for Neuronal Cancer

Camellia nitidissima Chi (CNC)

Camellia nitidissima Chi (CNC) is a medicinal plant, found mainly in Guangxi Province, China whose flower, leaf, and seed oil have been shown to exhibit antioxidative, antitumorigenic effects along with other pharmacological activities (He et al. 2017). The bioactive compounds of the plant have been found to include quercetin, kaempferol, catechin, and their derivatives (An et al. 2020). An et al. reported that treating H_2O_2-induced human neuroblastoma SH-SY5Y cell line with the ethyl acetate fraction of CNC leaves led to a decrease in the H_2O_2-induced changes observed (An et al. 2020). Pretreatment of cells with the ethyl acetate fraction (50–150 μg/ml) significantly increased the viability and reduced the levels of H_2O_2-induced oxidative stress in a concentration-dependent manner. In the same report, it was mentioned that CNC extract increased cAMP-response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) levels in SH-SY5Y cells, both which are downregulated in H_2O_2-induced SH-SY5Y cells, thus indicating the neuroprotective potential of CNC.

Garcinia morella

Garcinia morella, a fruit bearing tree belonging to the Clusiaceae family, is an evergreen tree mainly found in India and Sri Lanka. The numerous metabolites that have been identified and isolated from the various parts of the plant have been reported to exhibit antibacterial anti-inflammatory, antioxidative, and anticancer properties. Phytochemicals that have been isolated from *G. morella* include xanthones, benzophenone/s, flavonoids, and triterpenoids (Murthy et al. 2020). Choudhury et al. demonstrated that garcinol, the main bioactive compound in *G. morella* obtained via

chloroform fractionation, has a significant anticancer effect on SH-SY5Y cells (Choudhury et al. 2017). By treating cells with the chloroform fraction at concentrations of 1.56, 3.12, 6.25, 12.5, and 25 μg/ml for 24, 48, and 72 h, they concluded that the higher the concentration of the chloroform fraction, the lower the rates of SH-SY5Y cell proliferation. It was also reported that the IC_{50} value of the chloroform fraction was found to decrease with an increase in exposure time, thus indicating that a longer treatment duration has the most effect on cell proliferation.

Olive Leaf Extract (OLE)

The multiple phenolic compounds found in olive leaf extract (OLE), namely, oleuropein, hydrohytyrosol, tyrosol, elenolic acid, and rutin, have been shown to be accountable for inhibiting cell proliferation and inducing apoptosis in several tumor models (Goldsmith et al. 2018; Ruzzolini et al. 2018). By treating four human neuroblastoma cell lines (HTLA-230, IMR-32, SH-SY5Y, and SK-N-AS) with 50, 100, 200, and 300 μM of OLE, Morandi et al. established that increasing the OLE dose resulted in an inverse effect on cell viability (Morandi et al. 2021). A time-dependent experiment of cells being treated with 200 and 300 μM OLE showed that prolonged exposure to OLE significantly lessened the cell viability of all four neuroblastoma cell lines, with IMR-32 and HTLA-230 viability being significantly reduced just after 48 h. In the same study, it was reported that OLE was capable of inducing apoptosis and inhibiting cell migration in a scratch assay.

Withaferin A (WA)

Withaferin A (WA), derived from the *Withania somnifera* plant, is a bioactive compound with notable anticancer properties. *Withania somnifera* has been long used in traditional Ayurveda due to its anti-inflammatory, cardioprotective, and neuroprotective activities (Patel et al., 2013). Anticancer mechanisms of WA in various cancers have also been well studied (Sivasankarapillai et al. 2020). Tang et al. studied the effect of WA on glioblastoma multiforme (GBM) by using U87 xenografts in nude mice with tumors of 40–50 mm^3 (Tang et al. 2020b). After 27 days, mice treated with 5 mg/kg WA had significantly lower tumor weight and volume as compared to the control group. In the same study, using human glioblastoma cell lines, U251 and U87, they reported that the underlying anticancer mechanism of WA involves the induction of apoptosis and cell cycle arrest at the G2/M phase in GBM cells.

Luteolin

Luteolin is a naturally occurring flavonoid that has been found to be present in various plants including vegetables and fruits. Multiple studies have demonstrated the antioxidant, anticancer, anti-inflammatory, and neuroprotective properties that

luteolin possesses. Its anticancer properties have been observed in several cancers via mechanisms such as inducing cell death, decreasing cell proliferation or arresting cell cycle. Cell viability of human glioblastoma cell lines U-87 MG and U-251 MG, assessed via trypan blue exclusion assays and MTT assays, indicated that luteolin treatment (0–80 μM) lead to a dose- and time-dependent decrease in cell proliferation (Anson et al. 2018). In the same study, further analysis indicated that luteolin other than having significant apoptotic effects, also arrests cell cycle at S and G2/M phases. In separate research by Wang et al., a deeper study into luteolin-induced apoptosis revealed that intracellular reactive oxygen species (ROS) levels rose in response to luteolin treatment leading to endoplasmic reticulum stress, mitochondrial dysfunction, and eventually apoptosis (Wang et al. 2017).

Zeaxanthin

Zeaxanthin is a carotenoid that can be naturally found in dark green vegetables, yellowish-orange fruits, and egg yolks. In the body, zeaxanthin forms a yellow pigment in the eyes which provides protection from the harmful effects of UV light and oxidative stress (Murillo et al. 2019). Santocono et al. demonstrated that zeaxanthin was efficient at lessening the degree of DNA damage induced by three different reactive nitrogen species (RNOS) donors in SK-N-SH human neuroblastoma cells. Cells were incubated with the RNOS donors along with 20 μM and 40 μM of zeaxanthin. At 40 μM, the protective effect of zeaxanthin was significant against all three RNOS donors (Santocono et al. 2007). In a separate study, it was reported that zeaxanthin was capable of effectively inducing apoptosis in human CHP100 neuroblastoma cells, which are relatively apoptosis resistant. By treating the cells with five different concentrations of zeaxanthin ranging from 0.5 to 10 μM, they deduced that zeaxanthin showed proapoptotic effects in neuroblastoma cells while preventing apoptosis in healthy cells (Maccarrone et al. 2005).

Curcumin

Curcumin, a phenolic compound derived from the rhizome of turmeric (*Curcuma longa*), has been long used as a medicinal compound in traditional Indian medicine due to its natural antiseptic, anti-inflammatory, and wound healing properties. Studies have indicated that curcumin demonstrates its anticancer activity by regulating various cellular signaling pathways thus inducing apoptosis and inhibiting tumor proliferation and invasion (Tomeh et al. 2019). Perry et al. conducted an in vivo study using human glioma U-87 cells xenografted into athymic mice to study the chemopreventive and therapeutic effect of curcumin. For the chemopreventive group, treatment of 60 mg/kg/day curcumin was administered 7 days before tumor cells were injected while to study the therapeutic effect, curcumin treatment was started 3 days after injection. Both groups showed significantly smaller tumors with no other side effects compared to the control group thus indicating that curcumin was capable of suppressing tumor growth both before and after tumor formation. It was also reported that curcumin was able to

inhibit glioma-induced angiogenesis (Perry et al. 2010). In a separate study, using U87 xenografts also reported similar findings of a decrease in tumor size and weight and slight tumor necrosis. They also found that the PTEN and p53 expression were significantly enhanced in curcumin treated mice, hence proposing a mechanism for curcumin induced apoptosis (Wang et al. 2020).

Ginsenosides

Ginsenosides are the main active ingredients of *Panax ginseng*, a medicinal herb that has been used for its various health benefits such as improving blood circulation, promoting immune function and improving fatigue. The anticancer properties of ginsenosides have been studied in several cell lines and xenograft models with relatively successful results (Hong et al. 2021). Mice inoculated with GBM stem cell line 528NS were treated with 200 mg/kg/day Korean Red ginseng extract. Mice were also subjected to concurrent chemoradiotherapy (CCRT) which consisted of 10 mg/kg/day Temozolomide (TMZ) and brain-specific radiation therapy on days 28, 29 and 30. No visible differences were noted in respect to survival time or body weight in mice treated with the extract, however, tissue staining on day 75 indicated that tumor growth was delayed in treated mice (Ham et al. 2019). Ginsenoside F_2 (F2) is a minor ginsenoside whose chemotherapeutic properties were studied in U373MG human glioblastoma cells xenografted mice. Seven days after inoculation, mice were treated with 35 mg/kg F2 daily. MRI imaging on days 15 and 22 showed that F2 treatment resulted in a decrease in tumor mass, density, invasion and necrosis. In the same paper, U373MG cells treated with F2 in vitro indicated that F2 induced cell death by apoptosis. This was deduced from the fact that F2 treatment caused an increase in the sub-G1 cell population (Shin et al. 2012).

Resveratrol

Resveratrol is a natural polyphenolic phytochemical found in several plants such as berries and red grapes. Numerous preclinical and clinical studies have demonstrated its therapeutic potential in diabetes, cancer, neurological, and cardiovascular disease (Berman et al. 2017). Using mice injected with SK-N-AS human neuroblastoma cells, Kenealey et al. showed a treatment of 5.0 mg of resveratrol administered via 5 peri- or intra-tumor injections over 2 weeks caused a significant decrease in tumor volumes. Staining of harvested tumors revealed that resveratrol treatment caused increased cell death, with the treated group only having 35% of viable tumor as compared to the 68% of the control group (Kenealey et al. 2011). Resveratrol was also shown to increase the radiosensitization of glioma stem cells (GSCs) which are resistant to radiation therapy. Wang et al. reported that mice inoculated with human GSC line SU and treated with both 150 mg/kg resveratrol and ionizing radiation had significantly smaller tumors. The level of Bcl-2, an apoptosis regulator, was also significantly lower in the co-treated group as compared to the group which only received ionizing radiation, thus indicating the capability of resveratrol in promoting apoptosis (Wang et al. 2015).

Quercetin

Quercetin is a polyphenolic flavonoid that is commonly found in fruits and vegetables. Quercetin has been extracted and studied for its anti-inflammatory, antioxidant, and antitumor activity. It has also been extensively studied in several cancer models for its potential as a chemopreventive agent (Tang et al. 2020a). Various anticancer mechanisms that have been proposed include promoting apoptosis, affecting the cell cycle and interfering with regulatory proteins (Tavana et al. 2020). By treating C6 xenograft models with 100 mg/kg every other day Bi et al. reported that quercetin can induce autophagy and apoptosis in human malignant glioma cells. They also reported that when used in combination with chloroquine, which inhibits autophagy at a late stage, it may be an effective therapeutic approach to glioma therapy (Bi et al. 2016).

Vomifoliol

Vomifoliol is a norsesquiterpenoid having various pharmacological activities. Vomifoliol can be isolated from the leaves of *Tarenna obtusifolia*, a shrub with small flowers and sleek styles. When different components of the plant were tested for their activity, a recent study showed that vomifoliol can show effective results against amyloid beta-mediated cytotoxicity in neuroblastoma (Tan et al. 2020). The cell lines used in this study were SH-SY5Y neuroblastoma cells. When the plant extract containing vomifoliol was screened against the neuroblastoma cells, it inhibited the aggregation of amyloid beta reducing the toxicity to the cells. Upon using 50 ug/ml, that is, the highest extraction concentration, a cell growth inhibition of 75% was observed. As norsesquiterpenoids are compounds that are derived from sesquiterpenoids by removal of a methylene group, it can be assumed that vomifoliol, a norsesquiterpenoid, might have a similar mechanism of action as certain neuroprotective sesquiterpenoids. Several sesquiterpenoids such as PDA (Podoandin), EHP (1,2-epoxy-10α-hydroxy-podoandin), and ARD (aromadendrane-4β,10α-diol) have been shown to be capable of significantly reducing amyloid beta mediated cytotoxicity by enhancing brain glutathione levels in $A\beta_{1-42}$ mice (Amoah et al. 2015). Glutathione, a major antioxidant within cells, will decrease the oxidative damage that occurs due to amyloid beta aggregation. Further studies on vomifoliol will aggravate our knowledge on the mechanism of action of this compound in a better way.

Andrographolide

Andrographolide, a diterpene lactone, is the active compound extracted from the plant *Andrographis paniculata*. This plant has been used as medicine in parts of Asia for a long time. Andrographolide shows significant antioxidant and anti-inflammatory effects and has been claimed to exhibit neuroprotective effects as well. Recently a study was conducted to conclude the effect of this phytochemical on the SH-SY5Y cell model. There were earlier researches that suggested the

protective effect of andrographolide (Chen et al. 2012; Islam et al. 2018). This study claimed that andrographolide can inhibit NF-κB p65 and JNK MAPK activation. The main take out from the study was that effect of andrographolide treatment differed with the cell and tissue type and the study was unable to come to a conclusion on the therapeutic effect of andrographolide. However, it was able to provide a background to further investigate the complex processes related to the modulation of several pathways (Ketterman et al. 2020).

Polymethoxy Flavones from Citrus

Upon investigation, it was found that several polymethoxy flavones from citrus exhibit growth or apoptosis inhibitory properties. To take the understanding one step further, a study was conducted on nobiletin, tangeretin, and 5-demethyl nobiletin. It was found that when different combinations were used different results were shown by the cell lines. The methyl group present in the fifth carbon position of nobiletin provides the anti-proliferative effect. When tangeretin and 5-demethyl nobiletin were used together the population of apoptotic cells and caspase-3 activity were increased, suggesting that some pathway is being modulated by this combination. However, if this treatment includes nobiletin as well, a reduction in the level of growth inhibition by tangeretin was observed. This study concluded that the combination and the understanding of the combination are necessary to use these flavones in therapeutic activities (Akao et al. 2008).

Tinospora cordifolia

Coming from the family of menispermaceae, *Tinospora cordifolia* has been mostly found in the Indian subcontinent region. This plant has been used as a medicinal plant for a long time for its various positive effects. A study was carried out to determine the anticancer property of the aqueous ethanolic extract of *T. cordifolia* in IMR-32 neuroblastoma cell line. It was found that the treatment was able to arrest most of the cells in their initial growth phase of cell cycle. Besides that, anti-metastatic activity and a decrease in cell migration was also observed. The scientists proposed that the crude extract or active phytochemicals present in the plant can be used as a potential treatment for malignancy in neuroblastoma cells with further references (Mishra and Kaur 2015).

Conclusion and Future Perspectives

Anticancer properties of several phytochemicals have been studied and explored using various cancer cell lines and many phytochemicals are already in the experimental preclinical or clinical trial stages. This new approach of using phytochemicals, if used as an alternative therapy, will be highly beneficial. There are also some

phytochemicals which showed positive results during in vitro experiments but when studied in vivo, did not produce the same effect. For neuronal cancer and brain tumors, the main reason for this seems to be the low blood–brain barrier permeability of the phytochemicals. Thus, studies on how phytochemicals and their compounds can be administered using vectors such as nanoparticles and liposomes may give a better prospect for successful clinical applications. In addition to future work on novel phytochemicals, further research to provide data on the safety, efficacy, usage doses, and even potential side effects of these phytochemicals will reveal more details and hopefully answer the question as to whether this would be a good approach in cancer therapy.

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Perspectives of Stem Cell Therapy: A Promising Therapeutic for Cancer Model and Alzheimer's Disease

33

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Abstract

The most promising therapeutic option is cancer stem cells (CSCs). CSCs, leaders of a cancer hierarchy, possess stem cell-like traits such as self-rehabilitation and the cognition to differentiate abnormally. CSCs do not have to be uncommon in tumors; cancer and non-cancer stem cells can change correctable phenotypic modification, and the phenotype of CSCs can differ significantly between the patients. The function of CSC metabolism and the mechanisms underlying CSC metabolic adaptability have become a key focus of modern cancer research. CSC resistance can develop as a result of radiotherapy and chemotherapy, as well as following chemotherapy secession. Neurodegenerative diseases are involved in continuous damage of neurons structure and/or function which denotes multiple degrees of paralysis and declined cognition and sensation. Globally, millions of people were infected by Alzheimer's disease (AD) which is a complicated, irretrievable, forceful neurodegenerative infirmity. Commonly, the populace over 65 years may acquire this senile disease with characteristic features of destruction, mental function minimization which comprises loss of memory, cognitive injury, and finally declined life eminence of patients. Asymmetrical expression of proteins specifically Beta-amyloid "plaques" and Tau "Tangles" in the brain is measured as pathogenic properties of AD. Worldwide Alzheimer dementia (AD) pervasiveness has been calculated as elevated as 25 million, and it has been forecasted that AD will be twofold every 20 years till 2040. Mutually developed and developing nations had a remarkable collision on Alzheimer's disease had a remarkable collision on infected individuals, caretakers, and civilization. Older age and genetic susceptibility are the major etiological factors for Alzheimer's dementia. Existing treatment protocols facilitate recuperate from symptoms, and there is a deficiency in permanent recovery from the disease. Cell therapy strategies have been considered as an authoritative utensil for treating Alzheimer's disease patients. Stem cell therapy also entitled regenerative cell therapy has been considered an unquestionably influential pioneering tactic in

treating the disease of neurodegenerative for the past 20 years. Stem cells have the competence of recuperating damaged neuronal tissues through substituting the injured vanished neuronal cells with differentiated cells providing an advantageous atmosphere that promotes regeneration and safeguarding existing healthy neurons and glial cells from imminent injury. Therefore, in this chapter, we have elaborately discussed different stem cell types, neurodegenerative disease management through stem cell-based therapies, and current advancements in stem cell rehabilitation. However, improved sympathetic and additional investigation in stem cell methodologies may lead to the sensible and successful handling of ailments of neurodegenerative.

Keywords

Cancer stem cell therapy · Alzheimer's disease (AD) · Dementia · Beta-amyloid · Neurodegenerative disorders · Stem cell therapy

Abbreviations

AChEIs	Acetylcholinesterase Inhibitors
AD	Alzheimer Dementia
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
ApoE	Apolipoprotein
APP	Amyloid Precedent Protein
Aβ	Beta-amyloid
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CADD	Computer-Aided Drug Design
CatB	Cathepsin B
ChBF	Cholinergic Basal Forebrain
CNS	Central Nervous System
ESCs	Embryonic Stem Cells
FAD	Alzheimer's Families Diseases
FTD	Frontotemporal Dementia
HD	Huntington's Disease
IGF-1	Insulin Growth Factor-1
iPSCs	Induced Pluripotent Stem Cells
MSCs	Mesenchymal Stem Cells
nbM	basalis of Meynert
Nep	Neprilysin
NFT	Neurofibrillary Tangles
NGF	Nerve Growth Factor
NSCs	Neural Stem Cells
PD	Parkinson's Disease
PS-1	Preseniline-1
PS-2	Preniline-2

UCB-MSCs	Umbilical cord blood cells
VEGF	Vascular Endothelial Growth Factor

Introduction

Stem cells vary from other cells in that they may self-renew indefinitely, form clonal cell populations from single cells, and develop into a range of cell types. The ability of resident stem cell pools to self-renew is critical for tissue regeneration and homeostasis. The tumor is the complex microenvironment of malignant cells. Solid tumors are made up of malignant cells and stroma, which include endothelium, fibroblasts, mononuclear infiltrate, and lymphatics. Response of stromal elements to tumor cell signaling and factors provides vasculature, extracellular matrices, and structural support among other things. Any technique that targets tumor stromal components and malignant cells has the potential to enhance anticancer treatment. A new line of study has been motivated by discovering particular anticancer genes and finding potent cancer migration and integration of MSCs to execute an effective cancer treatment employing modified stem cells of mesenchymal (Vlashi and Pajonk 2015). Migration of tumor formation and MSC integration were shown in vitro utilizing trans well relocation experiments and in vivo by animal cancer models in various preclinical investigations. MSCs can be homing to virtually all cell lines of humans, including Kaposi's sarcomas, lung cancer, melanoma, breast cancer, malignant glioma, pancreatic cancer, ovarian cancer, and colon cancer. MSCs were produced with genes of anticancer and used in the battle against cancer as a single-edged weapon. Engineered MSCs could act as a continuous source of anticancer drug synthesis in the tumor microenvironment, as well as locally generated and released anticancer compounds that turn on nearby malignant cells, suppressing tumor development or killing them.

Stem Cell Therapy for Cancer Model

Numerous stem cells have different capabilities for migration, proliferation, and differentiation, determining their use in anticancer treatment. Stem cells are classified as "somatic" (SCs) (SSCs) or "embryonic" (ESCs). Adult stem cells (multipotent cells), otherwise called SSCs, and could distinguish into some type of cell from a specific descent. They are stem cells of hematopoietic, stem cells of neural, progenitor cells of endothelial, stem cells of mesenchymal, and others. Cancer or tumor stem cells (CSCs) can induce carcinogenesis and ailment development in certain circumstances (Lytle et al. 2018).

Pluripotent Stem Cells (PSCs)

Except for those in the placenta, embryonic stem cells (ESCs) produced from the embryo's dedifferentiated mass cells in the inner side can give birth to any type of cell. However, because of ethical rules, the use of ESCs in clinical research is modest.

Factors of Yamanaka discovered and from somatic cells are used to produce iPSCs (culture) and constituted a milestone in cell biology. Those iPSCs have similar properties as ESCs while eliminating the ethical difficulties associated with embryo devastation. iPSCs make them potentially more therapeutically relevant than ESCs (Li et al. 2018).

Adult Stem Cells (ASCs)

ASCs may contribute to a wide range of organ- and tissue-specific cell types. In cancer therapy, stem cells of neural, stem cells of mesenchymal, and stem cells of hematopoietic are often used. HSCs, the earliest blood descent cells, are recovered largely in the bone marrow and fabricate adult cells of blood by multiplying and differentiating descent-limited progenitors. Until far, the only stem cell therapy authorized by FDA to medicate numerous leukemia, myeloma, and several blood diseases has been the endue of HSCs obtained from the blood of the cord. HSC transplantation has been used in clinical settings for almost four decades. MSCs execute a captious part in tissue regeneration and repair, which are present in various organs and tissues. MSCs in vitro may rapidly multiply and form multiple particular cell types such as chondrocytes, adipocytes, and osteocytes. MSCs are mesodermal cells that originate from bone marrow and can differentiate into cells of bone, cartilage, stroma, tissues of adipose, muscle, tendon, and connective tissue. MSCs have distinguishable biologic features and have been widely exploited to increase other treatments or present curative substances in the medication of various types of malignity.

NSCs are distinguished by the presence of Nestin, Sox2, and other conventional markers, as well as their enlargement in a culture medium affluent in growth factors of epidermal and fibroblast. NSCs, which are derived from the CNS, have the quality to self-regenerate and develop new glial cells and neurons. They have been largely studied in mice models to treat primary and metastatic breast, prostate, and lung carcinoma. The fundamental drivers of vascular regeneration are EPCs (Nuti et al. 2016). Researchers believe that EPCs might be helpful in cancer treatment after being transected or combined with anticancer medicines or angiogenesis inhibitors. Recent advancements, however, have moved the attention to EPC roles in disease etiology and possible therapeutic advantages. There have been few reports on the use of EPCs in cancer treatment.

Cancer Stem Cells (CSCs)

CSCs, also known as stem-like cells, immature progenitors of cancer cells, or carcinogen-initiating cells, are formed through epigenetic changes in normal stem cells or precursor/progenitor cells. They are found in cancer tissues and play a crucial role in malignant tumor progression, metastasis, and repetition. CSCs express stemness genes, self-renew, differentiate into non-stem cancer cells, and are resistant to standard cancer treatments. Non-stem cancer cells can be killed by traditional cancer treatments, while CSCs cannot. When the residual CSCs multiply and discriminate, tumors frequently relapse. As a result, targeting CSCs may help to alleviate clinical concerns such as medication resistance and recurrence (Najafi et al. 2019) (Fig. 1).

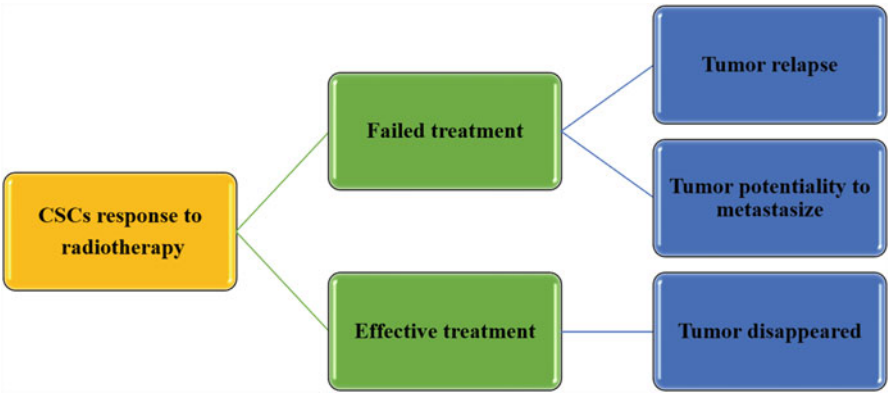

Fig. 1 Stem cell therapy for cancer

Cancer Therapy Model Using Stem Cell Modifications

Cancer stem cell therapy involves modifications of stem cells such as NSCs and MSCs through various processes. Genetically modified NSCs and MSCs move towards enzyme eloquent which gives rise to cytotoxic end products from non-toxic prodrugs. When tumor-bearing mice are implanted with transformed stem cells and they reposition to tumor tissues, where an external enzyme transfers the prodrug into a cell toxic chemical, eventually destroying tumor cells. Consequently, medication release may be precisely regulated in terms of quantity, time, and location. Suicide gene therapy, in other words enzyme/prodrug treatment, was the foremost premeditated NSC curative significance to achieve experimental trials and the first to be approved.

Stem Cell Tumor Therapies Applications

Medicine of Regenerative

Stem cells have the potency to regenerate and to differentiate since they have engaged in treating human cancer tissues next to chemotherapy. HSC transplantation has been frequently used in clinical settings to aid in lifetime hematopoietic recovery after treating cancers through the heavy dosage of radiation or chemotherapy. Replenishing the bone marrow is required in this type of therapy when marrow failure occurs and to recover genetic disorders take place in blood cells (e.g., aplastic anemia). Conversion of stem cells into the appropriate type of blood cell is the mode of action of this therapy. Effective engraftment and only one HSC transplant can restore hematopoiesis in the beneficiary. Vigorous iPSCs can be produced from the tissues of patients that have the potential to repair tumor- or

treatment-damaged tissues. iPSCs may be used to create a variety of tissues in regenerative medicine (Kim 2015). Effectual iPSC remedy is implicated in repairing or replacing cancer patients' iPSCs that have been destroyed by chemotherapy, radiation, or surgery. On the other hand, regenerative treatment with human iPSC necessitates substantial engraftment of iPSC-derived tissues in vivo. Very few human iPSC-derived cells (e.g., hepatic cells) have been engrafted in animal models effectively.

Immunotherapy for Cancer

Following allogeneic HSC transplantation, an immune-mediated anticancer response may be sufficient to treat certain hematological malignancies. Cancer immunotherapy encourages HSCs in cancer therapy which engaged against tumor-associated antigens by genes encoding chimeric antigen receptors (CARs) or T-cell receptors (TCRs). Immunotherapy techniques might benefit from patient-specific iPSCs as well. T lymphocyte-generated pre-rearranged TCR gene was conserved in human iPSCs which was enthused to develop into functionally active T cells. In vitro production of efficient T lymphocytes which was tumor antigen-specific is possible by re-indoctrination chosen T cells into iPSCs, subsequently, discriminate reverse into T lymphocytes for mixture into patients. Nevertheless, it has to be confirmed the protection of T-cell-derived human iPSCs.

Cancer Target with CSCs

Multipotent, self-reparative, and towering proliferative capability of CSCs may contribute to tumor invasion activation in high speed and metastasis. Targeting CSCs is thus critical for guaranteeing high treatment efficiency and limiting tumor reappearance. Since normal stem cells were attracted by CSCs and standard stem cells may be employed to aim CSCs in malignancy treatment. Normal stem cells and CSC communications minimize inflammation and death while suppressing tumor growth, angiogenesis, and metastasis. Modified HSCs may aid in the development of systems of cells capable of inducing under attack CSC death.

Screening and Selection of Anticancer Drugs

Treating cancer cells directly, iPSCs have been utilized to evaluate potential anticancer medicines. Patient cancer tissue-derived iPSC differentiation produces biologically effective cell types similar to human tumors than presently obtainable drug analyzing techniques, such as classical cancer cell lines, models of mice xenograft, and malignancy of mouse. Furthermore, hepatic toxicity inhibits many prospective anticancer medicines from being clinically tested, and it may be detected utilizing hepatocytes derived from human iPSCs with different genetic origins.

Alzheimer's Disease (AD)

Neurogenerative diseases or illnesses namely Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and frontotemporal dementia cause various abnormalities unlikely structural amendment of neurons, loss of neurons function, and destruction in the neural cell count in the brain or backbone. A person who is 65 years older is most likely to suffer from Alzheimer's sickness, the utmost usual dementia connected through more advanced age (Soria Lopez et al. 2019). Alzheimer's disease severely impairs memory, learning abilities, and comprehension. The inability to understand or showing difficulties in usage of words, mental shifts, and loss of coordination are some signs they exhibit. It has been estimated that presently a sum of 35.6 million people were infected with AD, and it may elevate to triple the number in the year 2050. AD is usually divided into two types, namely, sporadic and family disease. The change of the three properties of the amyloid precedent protein (APP), preseniline-1 (PS-1), and preniline-2 (PS-2) are also significantly altered in family AD. Apolipoprotein (ApoE) is the most important outcome of environmental variables and risk factors (Eratne et al. 2018) (Fig. 2).

Pathogenesis of AD

A patient with Alzheimer's disease will display senile plaques and neurofibrillary tangles. There is a segment of a protein called beta-amyloid (Aβ) found in senile plaques that causes neuronal cytotoxicity, whereas tau-protein changes cause neuropathic tangles to form inside nerve cells. In a person with Alzheimer's disease, brain experiences atrophying nerve cells over time. Memory and language are the first areas in the brain to suffer this type of neuronal cell death. But ultimately, the

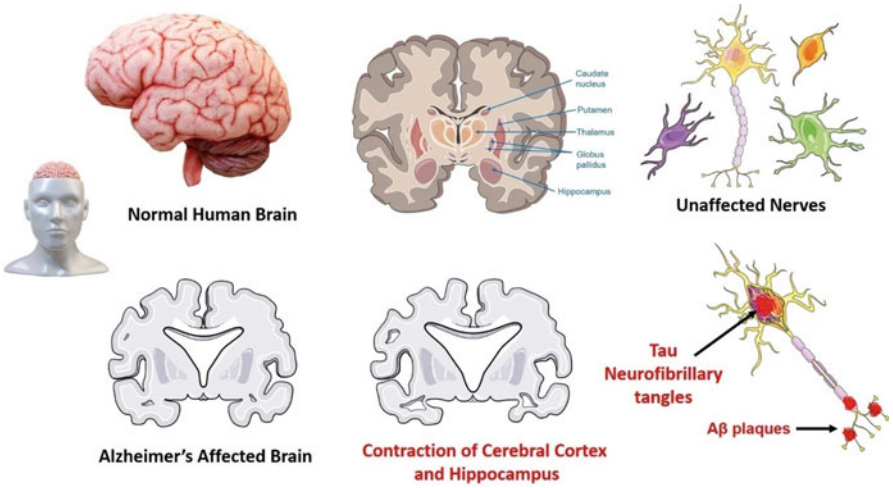

Fig. 2 Schematic representation of unaffected and AD affected nerve cells

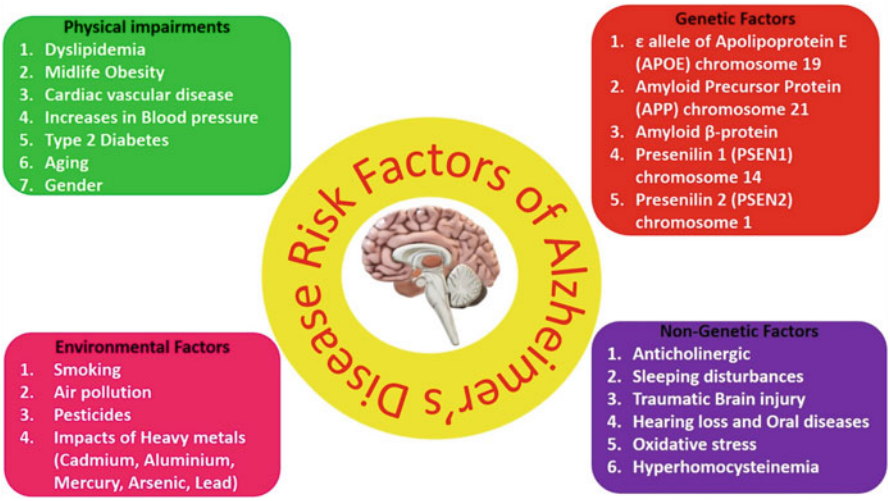

Fig. 3 Depicts different risk factors of Alzheimer's disease

brain as a whole is injured. Alzheimer's disease peoples have lower levels of acetylcholine, an intercellular signaling neurotransmitter, and failures of other neurotransmitters, including serotonin, norepinephrine, and somatostatin (Sery et al. 2013). Gene A encodes for A precursor protein accountable for Alzheimer's families' diseases (FAD) when gene mutation happens in the A gene. FAD aggregation is the main cause of senile plaques. The destruction of neurons occurs as a result of excessive Aβ accumulation. According to newly disclosed studies, gene APOE (apolipoprotein E) seems to be closely connected with AD. In total, there are trio types of APOE: E4 has been linked to Alzheimer's, while E2 and E3 may protect against the disease. A gene called APOE Epsilon 4 is responsible for the heritability of the disease in all of us. There is a consensus that 40% of people with Alzheimer's sickness have the APOE 4 genotype (e4), while more than 50% do not know their APOE genotype (Lumsden et al. 2020) (Fig. 3).

Amyloid and Tau Hypotheses

A collection of harmful forms of "beta-amyloid" (A) plaques, neurofibrillary tangles (NFT), and neurodegeneration causes continual neuron damage and neurotransmitters allied with Alzheimer's disease. Calcium influx and neuron passage are triggered by AB fibrils from pores in neurons, and peptides have been identified as essential elements in the formation of senile plaques (Gourmaud et al. 2020). A protein aggregate containing neurofibrillary protein is formed following uneven hyperphosphorylation of tau, a protein related to microtubules. A central nervous system's safety protection mechanism (CNS) is activated by microglia. Activation of microglia and incendiary factors that are present in Alzheimer's disease have been linked to progress toward neurodegeneration (Majdi et al. 2020) (Fig. 4).

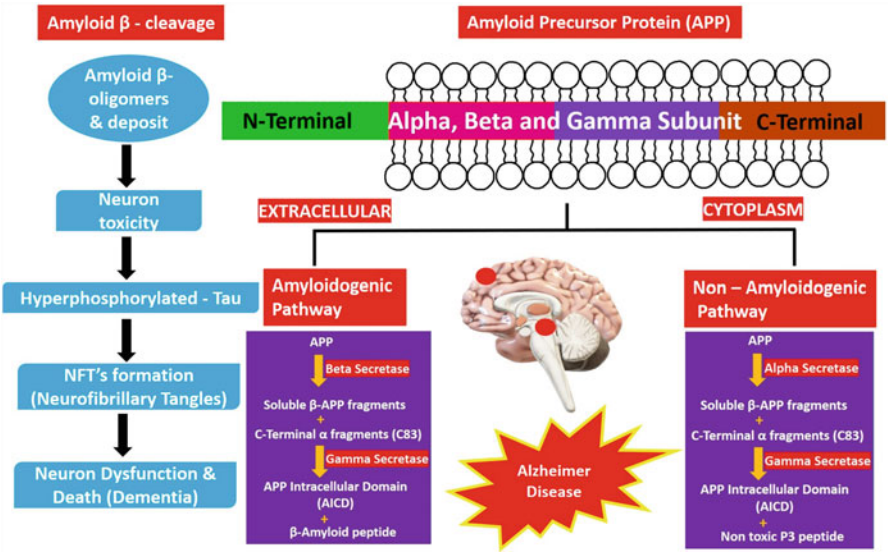

Fig. 4 Symbolizes the amyloid-beta cleavage pathway in Alzheimer's disease

Cholinergic Hypothesis

In addition to providing coordination between sleep, awareness, learning, and memory processing, acetylcholine is also produced by cholinergic neurons. From the nucleus basalis of Meynert (nbM) in the basal forebrain, these neurons project to numerous cortical regions of the brain. Cholinergic neurons are thought to contribute to cognitive decline in AD patients in a direct proportion to their number in the nbM because there are fewer of them. Cholinergic damage was noted in postmortem tissue specimens' neurochemical studies in the late 1970s, decreasing activity in choline acetylcholine, reduced choline assimilation and decreased acetylcholine discharge, and decreasing cortical acetylcholinesterase activity (Hampel et al. 2018). In the late 1970s, the cholinergic system was reported. Cholinergic neurons pass through the most important pathways in the cholinergic basal forebrain (ChBF) for attaining the hippocampus and cerebral cortex, and these neurons are responsible for concentration, memory, and various cognitive functions. Multiple studies have shown that removing cholinergic neurons or treating them with antagonists such as scopolamine or hyoscine impairs memory and cognition. Cholinergic neuron hypofunction in the cerebral cortex and the ChBF weaken Alzheimer's patients' cognitive skills (Francis et al. 1999). There is evidence that the CEIs rivastigmine, donepezil, and galantamine decrease cholinergic damage and improve behavior, focus, social participation, and cognitive abilities. However, long-term use can lead to side effects and drug resistance.

General Treatment for AD

Neurodegenerative diseases cannot be stopped with the current therapeutic options. It is difficult to comprehend the pathogenic processes and, therefore, create effective therapies given the intricacy of the machinery underlying the battering of neuronal cells and the contradictory physiological basis of various illnesses. There is a persistent issue of widespread neuronal cell death combined with an inability to regenerate neurons in the CNS, along with the enormous bulk of medicines (98% of diminutive molecules and 100% of hefty drugs) that help solve the blood-brain barrier (BBB) problems. The build-up of cerebrovascular amyloid-beta (Aβ) and cathepsin B (CatB), a member of the cysteine protease family, can cause Alzheimer's disease to break down peptides and proteins and can be very limitedly connected to amyloid plaques in the AD brains via endocytosis or phagocytosis. It is therefore possible to reduce Aβ using inhibitors of cathepsin B (Weller and Budson 2018). There is a protein called neprilysin (Nep) that is recently identified as a significant A-corrupting protein in the brain, indicating that quality exchange methods may provide a way to develop alternative therapies for Alzheimer's disease. Besides acetylcholinesterase inhibitors (AChEIs), other pharmaceutical options are available for treating AD symptoms, including memantine, an N-methyl-d-aspartic acid antagonist, which can be combined with AChEIs, due to its ability to act as a cell reinforcement and protect against lipid peroxidation. A range of current research work demonstrated that stem cell therapies give a successive outcome in conquering numerous neurological diseases like stroke, Parkinson's disease, spinal cord damage, and amyotrophic lateral sclerosis. Consequently, stem cell therapy for the rehabilitation of cognition has been more intensively studied (Briggs et al. 2016). In this chapter, I intend to examine recent studies that provide evidence that stem cell treatment is effective in regaining cognitive function following Alzheimer's disease or deterioration of cognitive.

AD and Stem Cell Therapies

In recent AD studies, stem cells derived from neural stem cells (NSCs), induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), and mesenchymal stem cells (MSCs) are the most commonly used cell sources. The stem cells can form cells from the ectodermal, mesodermal, and endodermal germ layers of the embryonic blastocyst (at embryonic day 5–6). Umbilical cord blood cells (UCB-MSCs) and Wharton's jelly contain mesenchymal stem cells which are used to develop mesenchymal tissue types. Adult stem cells reside in adipose tissue, bone marrow, and other tissue types (Vasic et al. 2019). In contrast to pluripotent stem cells, which originate from the embryonic origin, mesodermal stem cells can fabricate an extensive diversity of cell categories with a similar origin, the mesodermal germ layer. However, the differentiation potential of MSCs differs depending on the tissue of origin. They also have the capability of forming all types of brain cells, and their multipotency makes them vital to brain development (Penney et al. 2020) (Fig. 5).

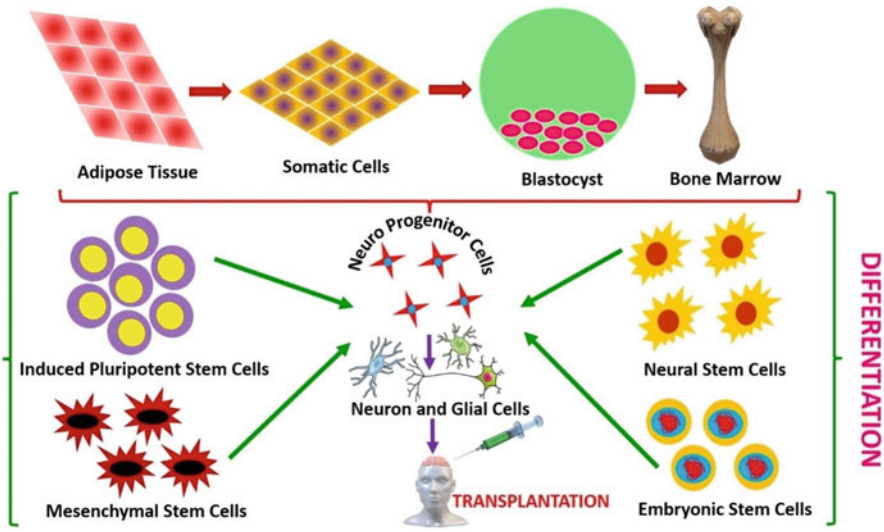

Fig. 5 Signifies applications of therapy with stem cell and AD

Endogenous Repair

A variety of theoretical approaches can be used to develop a stem cell therapy approach for helping patients with premature AD. The neurodegenerative effects of the adult hippocampal enlargement process can be reduced by stimulating resident neuronal stem cells in the adult brain so that regeneration occurs. Neurogenesis in the hippocampal area of the brain is thought to play a responsibility in erudition and memory, which is why increasing it may assist in alleviating the amnestic indication of untimely Alzheimer's disease. Neurogenesis promotes growth factors namely brain-derived neurotrophic factor (BDNF), insulin growth factor-1 (IGF-1), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) (pharmacologically or via gene therapy). However, the technique faces several quantitative challenges (Song et al. 2015). Under disease-free conditions, the number of new neurons produced in the hippocampus slows down as we grow older. In adulthood, there are about 800 new neurons formed every day; by the time we reach later life, it is less than 100. Normal aging is characterized by steady neuronal quantity, making this the bare minimum necessary to maintain neuronal homeostasis due to rapid neuronal turnover. Furthermore, brain cells in the hippocampus are severely affected by Alzheimer's disease. It is expected that one million neurons will be lost from CA1 and five million will be lost from the dentate gyrus (Kerchner et al. 2012). The number of dentate gyri would have to be increased to normal in proportion to AD to compensate. Moreover, it has been determined that adult hippocampal neurogenesis does not influence CA1 neurons, and thus the underlying neuronal insufficiency in early Alzheimer's disease remains unknown. Third, the AD pathological influence

on neurogenesis that is the topic of contradictory results from in vivo studies should be taken into consideration in that method. Neuronal repair methods that use endogenous cell biology for the treatment of early AD are generally ineffective and ignore a key target within the neuron (Regalado-Reyes et al. 2019).

Exogenous Cell Therapy

Increasing cognitive function by introducing exogenous stem cells can be used to restore dysfunctional neural networks. These stem cells could serve as a method of cellular delivery, generating neuroprotective growth factors both naturally and artificially and working via a paracrine "bystander" mechanism. As an alternative method for therapeutic restoration, stem cells may be utilized for differential differentiation and inclusion in repopulating defective neural circuits. Each stem cell type has a different proclivity toward achieving certain goals, so this is a sophisticated and carefully balanced process (Oliver and Reddy 2019) (Fig. 6).

ESCs

Experimental studies done on brain-damaged rats have shown that ESC transplantation can restore cognitive function, but is limited in clinical translation. Their pluripotent nature is partly responsible for this since undifferentiated ESC transplantation can lead to uncontrolled cell proliferation and tumors. NSC pre-differentiation from ESCs mitigates the harshness of AD chiefly cholinergic neurons generating and enhancing spatial memory functions in the animal model. One recent study demonstrated that the neural circuitry of the hippocampus can be functionally integrated with neuronal populations from human ESCs after transplantation. Another recent study showed that ESCs were capable of being converted into a type of transitory stem cell found in the developing brain, called medial ganglionic eminence progenitor cells

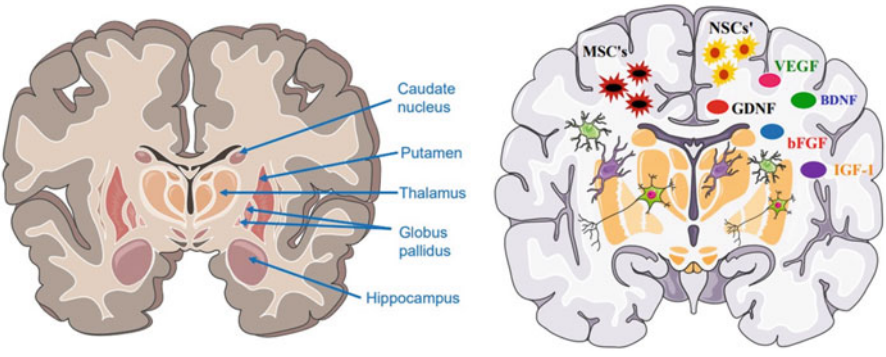

Fig. 6 Represents various growth factors involved in Alzheimer's disease

(Han et al. 2020). Transplanted neurons developed into jointly GABAergic and cholinergic subordinate types and synoptically integrated employing anchor neural networks and took towards the enrichment of learning and spatial memory after transplantation into mice with brain damage. Clinical translation of ESC-based therapeutics is hampered by the inherent ethical, immunogenic, and developmental restrictions associated with using allogeneic donor cells (Sugaya and Vaidya 2018).

NSCs

As a result of their paracrine function, NSCs are known to offer substantial therapeutic benefits. An aged primate brain and a rodent AD model were transplanted with growth factor-producing NSCs and choline acetyltransferase overexpressing human NSCs were found to increase neurogenesis and cognitive function in rats with neurotoxic cholinergic damage. Another in vivo research investigation discovered that NSC transplantation lowers neuro-inflammatory activity, attenuates tau and Aβ AD pathology, and boosts synaptogenesis and integration. Neuronal differentiation occurs in concert with neuroprotective paracrine releases and immune-modulating factors and direct neuronal inclusion, a phenomenon that is likely to limit neuro-replacement strategies (Marsh and Blurton-Jones 2017).

MSCs

Because they are easily accessible, relatively easy to handle, and can produce a broad range of cells, MSCs have become the widely deliberated stem cell types. Based on aged mouse models, MSCs were found to differentiate into brain cell types, boosting concentrations of neurotransmitters, BDNF, and NGF, as well as locomotor enhancement and cognitive occupation. MSC-derived neurons, however, are not known to mature in vivo either in terms of their function or synaptic connectivity. Also, MSCs cannot correctly replace neurons in vivo because they do not differentiate well and produce more glial cells than neuronal cells. With the foreword of MSC-secreted molecules infused into endogenous neurogenic niches and in AD cellular models, MSCs were known to acquire paracrine neuroprotective possessions. MSC transplantation displays reduced A protein deposits and plaque formation; increases neuroprotection, synaptogenesis, and neuronal differentiation; and improves spatial memory and learning insufficiency (Staff et al. 2019). MSCs have been shown to have substantial paracrine anti-inflammatory properties, resulting in increased levels of neuroprotective cytokines like IL-10 and lesser intensity of pro-inflammatory cytokines like TNF- and IL-1. MSC's intravenously administrations have the capability of crossing the blood-brain barrier and successfully migrating to areas of neurological damage without provoking a tumorigenic or immunological reply. Compared to standard brain injection, this technique has some substantial advantages over human clinical translation, although information of MSCs infiltrating various tissues remains a concern (Shariati et al. 2020).

iPSCs

Synaptic networks in iPSC-derived neurons can form electrically active synapses and mature into mature structures. The use of iPSCs as a therapeutic tool is so new that there have been few animal model transplantation studies. Restoring neurological function and reducing inflammatory variables by the bystander effect have been shown in a rodent model of ischemic stroke. Transgenic AD mice treated with iPSC-derived cholinergic neurons developed phenotypically mature neurons as well as restored spatial memory deficits (Lin et al. 2018). In this technology, autologous pluripotent stem cells can be derived that do not pose ethical and immunological rejection issues associated with sources that are not specific to the patient. The use of autologous iPSCs to replace neurons might be limited by neuropathology in neurons derived from AD patients, such as aberrant A levels, phosphorylation of tau, shortened neurite length, and impaired electrical competency. Interestingly, simulating AD in vitro with iPSC-derived neurons has significant etiological research implications and medication screening implications, as discussed elsewhere.

Stem Cell-Based Rehabilitation Challenges for AD Treatment

Numerous complications are preventing full-scale clinical translation of stem cell-based therapy. These include tumorigenicity, immunopathology, contamination, genetic alteration, uncontrolled migration and growth, and accidental trans-differentiation. To achieve the best possible outcomes, further studies are needed to establish protocols for standard cell preparation for transplantation, to understand the mechanisms behind the symptomatic improvement after transplantation, and to assess the immune response following transplantation (Lui 2015). It has yet to be determined whether genetically modified cells are safe and effective for transplantation into humans. In addition, there may be ethical implications surrounding stem cell genome modification.

Guidelines for Stem Cell-Based Treatment

Developing a standardized protocol for the separation and variation of stem cells and developing methodologies to identify their sources will be key to the future of stem cell research. Stem cell transplantation dosages, stages, sources, stages, and routes should be determined in more detail to determine their optimum therapeutic outcomes in AD animal models (Mora et al. 2017).

Immunotherapy for AD

The anti-Aβ peptide has become a promising target with the development of various techniques hostile to Aβ systems (anti-Aβ techniques). By inhibiting the proteins responsible for Aβ aggregates and providing inactive antibodies, these strategies

prevent the formation of total Aβ in the cerebrum and increase its clearance. The combined impact of these strategies prevents Aβ accumulation and the formation of total Aβ by preventing the formation of total Aβ. Taking anti-Aβ monoclonal antibodies into clinical trials showed positive results, including reduced levels of mind Aβ, reduced senile plaque progression, and better recognition of the problem. Bapineuzumab and solanezumab were the leading candidates among the anti-Aβ monoclonal antibodies following passive immunization, prompting an evaluation of their potential in several phase III clinical trials. However, these large clinical trials have failed to produce the anticipated results. A similar class of anti-Aβ monoclonal antibodies similar to bapineuzumab and solanezumab is now in various stages of development (Colpo et al. 2018).

Nanotechnology for AD

The focus on medication conveyance is a crucial part of the nano prescription since general treatment with medications failed to demonstrate any notable effects on the treatment of AD. A study in the Central Nervous System (CNS) tissue, against the Blood-Brain Barrier (BBB), is confused. It has been extensively investigated in the previous decade, including the utilization of biocompatible nanoparticles, such as curcumin, an ingredient of turmeric, and the yellow spice that has been recently identified as efficient management for AD (Formicola et al. 2019).

Gene Therapy for AD

It is very much essential to identify the genetics of Alzheimer's disease, the role of amyloid protein and tau, and the underlying mechanisms that lead to neurodegeneration to develop new medical treatments. To provide maximum support for the remaining neurons in the brain of an Alzheimer's patient, the current treatment mechanism emphasizes maximum stimulation of the remaining neurons (O'Connor and Boulis 2015). Research at DAT focuses on early detection, as early detection can help to preserve a quasi-normal state of cognition for a lot longer, thanks to medication administered when the first signs of memory loss appear.

Identifying Anti-Alzheimer Drug Contenders Via Computational Analysis

Computer-aided drug design (CADD) is a modern computational technique used in drug discovery. CADD has been used in the optimization of many licensed drugs, including captopril, dorzolamide, oseltamivir, and nolatrexed, and several publications report on the successful discovery of leads/drugs with the help of CADD.

Consequently, CADDs are useful in all stages of drug development: from finding targets to validating them, to finding leads, and to optimizing them and in preclinical trials. As a result, CADD could result in a 50% reduction in drug development costs. Visualizing protein structures, ligands, and protein-ligand interactions using computational methods may be the most informative method. In the new drug development process, the basement of all research work is finding an active compound as a lead compound. CADD can therefore provide a lot of benefits to innovative drug research; it serves a crucial role at the beginning of innovation (Ambure and Roy 2017). A CADD-based approach has recently seen exponential growth due to the higher number of novel compounds it can identify than a classic HTS or combinatorial one, as well as the ability to select a much more focused search than traditional HTS. There are three main purposes for which CADD is typically used: (34) select inhibitors to perform the desired activity, which reduces experimental workload; (17) optimize lead compounds, such as increasing affinity or maximizing DMPK properties; and (3) design new compounds by adding functional groups to starting molecules or by combining fragments. A representative example of CADD's use in Alzheimer's disease treatment is beta-secretase inhibitors and gamma-secretase inhibitors, as well as radiotracers. Research into potential amyloid drugs utilizing CADD has developed rapidly over the past few years, and the technique continues to improve (Llorach-Pares et al. 2017). Schein's group described E-pharmacophore modeling, screening of database, and methods of molecular docking as strategies to identify low-toxic compounds to treat AD (Fig. 7).

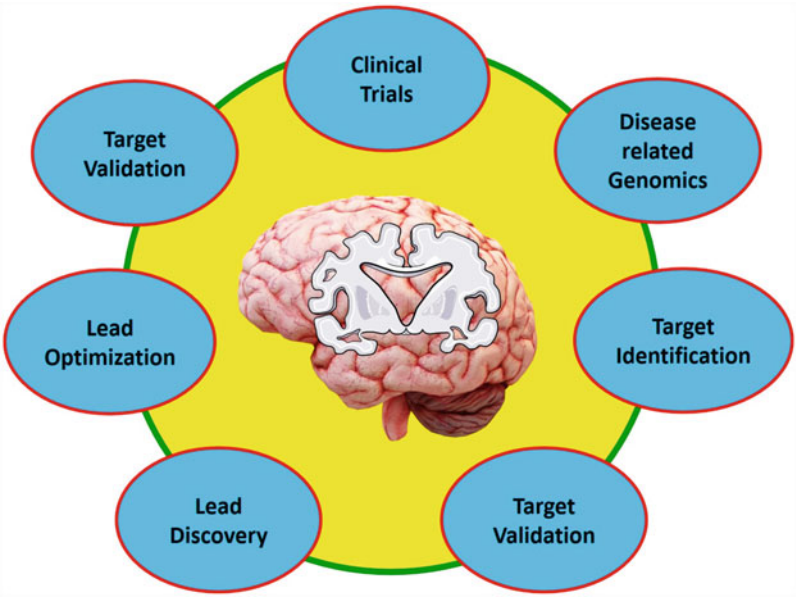

Fig. 7 Designate applications of CADD in Alzheimer's disease

Conclusion

With conservative pharmacological therapies, neurodegenerative diseases have disturbing ramifications. Delaying the development of the disease rather than reestablishing the injured neurons is the Alzheimer's therapies so far. Various therapies for AD, namely, immunotherapy, gene therapy, treatment with nanoparticles, and stem cell therapy, have been deliberated decoratively in this section. Presently, the main focus in the treatment of AD is stem cell-based rehabilitations to eradicate the sources of the ailment. Abundant research outcome ahows that there is twofold advantage in stem cell-based AD therapies in which damaged neurons have been substituted as well as efficient to produce novel neurons. The treatment of neurodegenerative disease is mainly involved in non-functional neuron replacement and also provides neurorestorative and neuroprotective occupations which can be attained through stem cell recuperations. The current stem cell treatments have been intricate in hydrogels and nanomaterials as drug delivery and neuron reconstruction have been considered as effectual therapy for AD. Therefore, reformative therapies and the replacement of neurons through stem cells might be the most efficaciously translated method into the clinical setting. In addition, an efficacious clinical transformation of stem cell-related remedies mainly is subject to responding etiology of AD. Enormous preclinical trials provided a hopeful view for treating AD and paved the way for the following medical application of therapy with stem cells. Stem cell therapy necessitates consistent procedures for both isolation and expansion of stem cells to receive an anticipated healing consequence. Therefore, the upcoming research and investigations should focus on stem cell-based therapies to transfer neurotrophic factors for the neurogenesis modifications in AD representations.

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Animal Model of Inflammatory Bowel Disease Leading to Cancer and Role of Genetic Variation in Colitis-Associated Cancer

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Kavitha R. Thangaraj, Ravi Ramalingam, and Solomon F. D. Paul

Contents

Abstract

Colorectal cancer is one of the most common types of cancer and is associated with chronic inflammation of gastrointestinal tract characterized by inflammatory bowel disease. Individuals with genetic mutation and impaired immune response are among the suspected risk for IBD and CRC. Chemically-induced CRC mouse model and IBD model are widely used for the understanding of molecular mechanism of chronic inflammation, oncogenes, and tumor onset, and can also help in improving the diagnosis and therapeutic targets. Genetic factors play a significant role in the origin of different type of cancers and several genes like

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APC, *TP53*, and *MSH2* have been found to be among the pivotal factors for onset of colorectal cancer. Knockout mice models that mimic the characteristics of CRC is used to study the role of genetic variation in this context.

Keywords

Colorectal cancer · IBD · Mouse model CRC · Genetic variation in CRC · DSS-induced IBD

Introduction

The American Cancer Society estimated that the colorectal cancer (CRC) in human is the third leading cause of cancer and second common cause of cancer death (Bürtin et al. 2020). Worldwide, colon cancer is accounting for 9% of the total cancer incidence (Haggar and Boushey 2009). People with certain hereditary cancer syndrome or family history of colorectal cancer have a high risk of developing CRC. Thus, it is significantly a big health burden, with high incidence and mortality in the advanced stages of the disease. Inflammatory bowel disease is characterized as chronic relapsing inflammatory disorders with unknown etiology that results in mucosal ulceration and inflammation of the gastrointestinal tract. Hence, if this inflammatory condition left untreated may lead to colorectal cancer.

Animal models are very significant and valuable tool to study and validate the diagnosis and therefore it helps to define new therapeutic targets. An ideal animal model must replicate the clinical evolution of the disease and has to mimic the same phases of the cancer. In this chapter, we review the existing mouse colon cancer models with their advantages and disadvantages in the replication of human colon cancer progression and the associated genetic variations. Colitis-associated cancers represent a heterogenous group of conditions in which multiple oncogenic pathways are involved. IBD is a group of idiopathic chronic inflammation of GI tract that mainly affects parts of colon and rectum and characterized by large intestine immune response to gut microflora. While the cause of IBD remains unclear, studies have shown that IBD occurs in genetically susceptible individuals after an inappropriate immune response of the intestinal flora. It has been reported that most of the colon cancer condition emerge from the chronic ulcerative colitis or Crohn's disease. Therefore, there is a strong correlation between chronic colon inflammation and CRC. Colorectal cancer generally emerges in the glandular, epithelial cells of large intestine or rectum (Haggar and Boushey 2009). There is a rapid increase in the colorectal cancer incidence in countries like Eastern Europe, Asia, and South America, whereas reduced CRC incidences are observed in countries like the USA, Australia, and several Western European countries (Rawla et al. 2019). The recent decline may be due to increased early detection and prevention through polypectomy and also due to improvement in perioperative care as well as chemotherapy and radiotherapy.

Animal studies especially mouse model of CRC and IBD throw light on the understanding of the molecular mechanism of inflammation, oncogenes, and tumor

onset, and help in improving the diagnosis and further in therapeutic targets. Therefore, the animal model must be ideal and must replicate the characteristics of inflammation-induced cancer and it must be reproducible. Therefore, it is important to study and select the appropriate mice model to understand the molecular mechanism of inflammation that occurs in tumor growth, polyps formation in IBD, and colorectal cancer.

Colorectal Cancer in Mouse Model

There are number of factors involved in the development of colorectal cancer that include unhealthy life style like low-fiber diet, red meat consumption or tobacco, smoking, alcohol, and obesity. Besides these external factors, a variety of genetic factors like SNPs, indels, copy number variations, and gene expression can increase the risk of CRC. The laboratory mouse is one of the best model systems in biomedical research because of the availability of genomic information on individual murine lines and techniques to construct transgenic and knockout mice. Carcinogen-induced colorectal cancer mice models provide good platform to study the molecular mechanism of colitis-associated CRC. This can be achieved by administering chemical compounds by ad libitum feeding, drinking water, oral gavage, and intraperitoneal, subcutaneous, or intramuscular injections. The first experiment in 1915 by Yamagiwa et al. shows that coal tar application on the ears of rabbits caused carcinogenic effect (Yamagiwa and Ichikawa 1977). In the beginning researchers have used methylazoxymethanol, cycasin, azoxymethane (AOM), and its precursor molecules 1,2 dimethyl hydrazine and methylzoxymetyl acetate to induce colon cancer in mice and rats (Laqueur 1964; Morgan and Hoffmann 1983). These compounds are metabolized to methylazoxyformaldehyde and this is able to alkylate the DNA bases guanine and thymine once it is taken up; these chemicals are introduced into intestinal mucosa and causes carcinogenic effect in the colon. This process is dependent on mouse strain, housing condition, dosage, duration, and the route of administration. Many mouse models have been developed to evaluate the various features of CRC and it is important to choose correct mouse model that mimics all the characteristics of human CRC.

Currently available CRC mice models can be categorized into following groups:

(1) chemically induced CRC models, (2) genetically engineered mouse models, and (3) implantation of tumor cells in the mice colon. However, it is important to use a specific model to address a particular scientific question. Here, we review widely used mouse models for human colorectal cancer and IBD in the scientific community.

Mouse Models of Chemically Induced Colorectal Cancer

Based on the human CRC clinical characteristics, the mouse model of colorectal cancer can be classified as follows: (1) inflammation-induced DSS-AOM chemical carcinogen mouse model; (2) sporadic colorectal cancer induced by DMH/AOM/

MAM; (3) hereditary nonpolyposis colorectal cancer (HNPCC); and (4) familial adenomatous polyposis (FAP).

The study of experimental colon carcinogenesis in rodents was established almost 80 years back. The advantage of studying mouse CRC model includes rapid and reproducible tumor induction in recapitulation of the adenoma carcinoma sequence that occurs in human. Widely used carcinogen are methylazoxymethanol (MAM), 1,2-dimethylhydrazine (DMH), and azoxymethane (AOM), heterocyclic amines (HCAs), such as 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) and 2-amino-33-methylimidazo [4,5-f] quinoline (IQ), aromatic amines, such as 3,2′-dimethyl-4-aminobiphenyl (DMAB), and alkylnitrosamide compounds, such as methylnitrosourea (MNU) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Tong et al. 2011). Walpole et al. in 1952 reported that administration of 4-aminodiphenyl and 3,2 dimethyl-4 amino diphenyl in albino rats caused colon tumors (Walpole et al. 1952). Studies have shown that when rats were fed with cycad flour, adenocarcinoma in the colon was observed. The carcinogen in cycad flour is cycasin, it is a form of methylazoxymethanol (MAM). While DMH, a metabolic precursor of MAM, was widely used in earlier studies to induce tumor in rats (Rosenberg et al. 2009). Therefore, DMH has provided researchers with a reproducible experimental design for studying sporadic CRC. There are many mice strains available for the CRC model, BALB/cHea, SWR/J, A/JP/J, STS/A, and ICR/Ha are mice strains sensitive to DMH. Whereas AKR/J and DBA/2J mice strains are resistant to colon tumor development with DMH (Rosenberg et al. 2009). Route of carcinogen administration in the mice plays a critical role in producing colon carcinoma. Some mouse strains are commonly used for constructing gene knockout and transgenic study. FVB/N, 129/SvJ, C57Bl/6J, and BALB/CJ mice strains are widely used for knockout or knock-in study. These strains act differently for different chemicals exposed to induce tumor. For example, studies have shown that 129SvJ and C57Bl/6J mice failed to develop tumor with AOM injection (Rosenberg et al. 2009).

Transplant Model of CRC in Mice

Inoculation of tumor tissue or cancer cells within the same mouse strain can be done by transplantation, whereas xenografts are derived from a different mouse strain or human donors. Nude severe combined immunodeficient (SCID) mice with functional deficiency of FOXN1 is used for transplantation. Additionally, transplantation can be differentiated into heterotopic and orthotopic models. The *Nu* gene knocked out model was established with hairless thymus-less mice which cannot generate T lymphocytes. Whereas SCID mutation resulted in disrupted lymphocytes maturation and that leads to deficit in circulating, mature, functional T and B cells. But SCID mice possess a completely intact innate immune system, with normal numbers of macrophages, natural killer cells, and granulocytes. However, activation of innate immune system in SCID mice is dependent on the xenografts transplanted in the mice. Importantly, the host-versus-graft response can vary between mice model used

and samples from different tumors. Number of mouse tumor cell lines have been established from spontaneous tumors or carcinogen-induced tumors. There are numerous immunodeficient animal strain available for research. C.B17 SCID has mutation in *Prkdc* gene that results in lack of functional B and T lymphocytes; $NMRI^{nu/nu}$ is another mouse used for xenotransplantation. SCID mice can be crossed with nonobese diabetic (NOD) mice and generate NOD/LtSzscid, NOD/LtSz-scid β2mnull, and NOD/Shi-SCID mice to overcome NK cell function. It has to be considered that more severe immunodeficient the host, the higher are the engraftment rates. When it comes to immune deficient mice it is important to house the mice in a specific sterile, pathogen-free environment, and special care should be given to maintain their environment with specific temperature and microisolator caging (Heyer et al. 1999).

Mouse Model of Inflammatory Bowel Disease

Gastrointestinal tract has the major mucosal surface in the body and in mice it is approximately 30 cm length (Okayasu et al. 2002). Inflammatory bowel disease is characterized by chronic inflammation of the gastrointestinal tract. Types of IBD include ulcerative colitis (UC) and Crohn's disease. UC is the condition that involves inflammation along the upper lining of the large intestine and sometimes extended to rectum. Crohn's disease is characterized by inflammation in the inner lining of the digestive tract. Both UC and Crohn's are diagnosed with the symptoms such as diarrhoea, rectal bleeding, abdominal pain, fatigue, and importantly weight loss. In the mice experimental model weight monitoring is an important criterion to confirm the inflammation in the colon along with blood in the stool. IBD occurs in genetically susceptible individuals after an altered immune response of the intestinal flora. Therefore, the animal model should be ideal and must replicate the characteristics of inflammation-induced cancer. More accumulating evidence suggests that IBD-associated CRC may develop and initiate tumorigenesis pathway that is different from sporadic CRC. IBD mice model is categorized mainly into five types: chemically induced, spontaneous mutation, adoptive T cell transfer, genetically engineered, and microbiome induced.

Chemically Induced Model of Inflammatory Bowel Disease

Chemically induced mouse model of IBD is not homogenous and possesses specific advantages and disadvantages. Most commonly used mice model is BCL6 treated with dextran sodium sulphate (DSS). There are several chemicals available that induce colon inflammation and IBD-induced cancer in mice. Among them DSS is most commonly used chemical, orally administered to mice for the laboratory experiments. Here we focus on chemically induced colitis model which mimics the morphological, histopathological, and clinical features of human IBD. Chemically induced IBD is classified as follows: (1) TNBS-induced IBD, (2) DSS-induced

IBD, and (3) oxazolone-induced IBD. Every chemical-induced IBD model has its unique features that are comparable with human IBD.

2,4,6 Trinitro-Benzene Sulfonic Acid (TNBS) Colitis

TNBS chemical–induced colitis leads to colonic inflammation in susceptible mice strains by intrarectal administration. TNBS is applied with ethanol and it is important to provide access to the intestinal epithelial cells. Ethanol impairs barrier function and facilitates TNBS to penetrate into the mice bowel wall. TNBS administration resulted in haptenization of colonic or microbiota-derived proteins that resulted in generation of TNP-specific CD4+ T cells and subsequent antibodies. Since TNBS is associated with the presence of highly activated T cells, these T cells have a key pathogenic role in TNBS colitis. The colitis formed by TNBS is dependent on intensity and the length of the inflammatory process and it is based on innate immune mechanism. Th1, Th2, and Th17 cytokines activate on specific strains and are also dependent on microflora of the strain used. Chronic TNBS colitis in BALB/C mice is characterized by lamina propria fibrosis, representing that this mode is an important tool for studying the pathological mechanism underlying the intestinal fibrosis that mimics human IBDs (Wirtz et al. 2017).

Dextran Sodium Sulphate–Induced IBD and IBD-Associated CRC

Inflammatory bowel diseases are associated with an increased risk of colitis-associated colorectal carcinoma (CAC). CAC is one of the most important causes of morbidity and mortality in patients with Crohn's disease and ulcerative colitis. Dextran sulfate sodium salt is a synthetic sulphated branched polysaccharide derivative of dextran that has many roles in biomedical and clinical research. DSS model of colitis is one of the widely used models as it can be easily developed owing to the wide availability and low cost of DSS. The DSS research started in 1985 by Ohkusa et al. in hamsters and later it was established in mice, currently there are numerous DSS-induced colitis study in mice (Ohkusa 1985). The mice can develop either acute or chronic colitis or colitis-induced neoplasia. In general, mice show various response and susceptibilities to DSS colitis and severity of inflammation depends on DSS concentration, molecular weight, exposure duration, frequency, and genetic variant of the mice strain used.

DSS colitis is induced by administration of 2–5% DSS in drinking water ad libitum for 4–9 days. Whereas chronic inflammation is induced by continuous administration of low-dose DSS or cyclic administration for 7–10 days. The symptoms of colitis induced in mice is weight loss, diarrhoea, occult blood in stool, and anemia which might lead to death.

C57BL/6JOlaHsd and BALB/cAnNHsd are the commonly used mice strains in DSS-induced CRC model. It is observed that C57BL/6JOlaHsd mice developed lymphoid follicles along with severe colon inflammation which is an important histological feature of Crohn's disease (Wirtz et al. 2017). Studies have reported that in mice intestine the DSS associates with colonic medium chain fatty acids that is present in the chow and it is absorbed and metabolized by the epithelial cells.

When repeated DSS cycle is combined with azoxymethane, colitis-dependent neoplasia is induced that mimics CRC in human. However, genetic factors also play a significant role in DSS-induced colitis. Studies have shown that C3H/HeJ, NOD/Ltj, and NOD-SCID inbred strains are sensitive to DSS-induced colitis (Arnesen et al. 2021). Colitis-associated cancers represent a heterogenous group of conditions in which multiple oncogenic pathways are involved. The AOM/DSS model is considered as successful reproducible CRC model to study the mechanism of colitis-induced colon cancer in mice. The AOM/DSS model involves injection of AOM followed by repeated cycles of 2–3% DSS administration in drinking water. Carcinogenic effect of AOM with combination of DSS has been studied in different mice strains. Among them B6 mice strain is most vulnerable to DSS-induced inflammation and C57BL/6J (B6) has been reported relatively resistant to DSS when compared to other strains (Perše and Cerar 2012). In the AOM-/DSS-induced colorectal cancer mutations in the ß-catenin was observed in codon 32–34. Interestingly, these mutations are not similar that observed on AOM alone treated mice (Chartier et al. 2020). In summary, the AOM/DSS mice model facilitates to study the pathogenesis of human CRC and extensive study of this model may throw light on chemopreventive agents against IBD-related CRC.

Oxazolone Colitis

Oxazolone is a hapten reagent that causes severe colitis in mice after intrarectal administration. The acute model inflammation occurs in distal colon and submucosa and it is characterized by ulceration, neutrophil, macrophage, and lymphocyte infiltration. C57BL/6 mice are more resistant to oxazolone treatment, whereas SJL/J andC57BL/10 are highly susceptible to oxazolone. Oxazolone-induced colitis is known to generate immune response mediated by Th_2 cells (Kojima et al. 2004). The colitis in this model resembles human ulcerative colitis by forming inflammation of mucous membrane, epithelial microulceration, and histopathological changes in distal colon. Oxazolone-induced colitis is characterized by production of IL-13 secreted by natural killer T cells. Thereby, the immune response and IL secretion profile of oxazolone varies from TNBS-induced colitis. The secreted IL-13 reduced epithelial barrier function by increasing apoptosis of epithelial cells (Gerlach et al. 2014).

Role of Genetic Variation in Mouse Model of Colitis-Associated Cancer

Molecular mechanism of CRC comprised of four classes of genes: (1) the growth-promoting proto-oncogenes; (2) the growth-inhibiting cancer suppressor gene; (3) genes that regulate apoptosis; and (4) genes that regulate DNA repair.

Genetic variation contributes to difference in individuals and is also responsible for how they respond to different factors. Genetic variation results in difference in phenotype, an important component for the survival by adapting to the everchanging

environment. There are varied types of genetic variations that can alter structure and function of a gene, mutation and genetic recombination are the major sources of genetic variation. Changes in one of the bases in the genomic sequence result in single-nucleotide polymorphism (SNP). SNPs are also called as single-nucleotide variation (SNV) or point mutation that constitutes a considerable source of genetic variation; SNPs are the genetic variation which occurs in at least 1% of the population. Majority of the SNPS are accumulated in the noncoding region of genome and studies have reported the effect of these variants to alter gene regulation by altering their expression or affecting the splice sites. SNPs in the coding region can result into synonymous and nonsynonymous variation, the latter can be further divided into missense and nonsense mutations. The differences in genome of an individual also decide how they metabolize certain molecule or respond to toxins, and environmental factors (Fig. 1).

Genetic variations can also be classified based on the period of occurrence; germline mutation occurs in the germ cells (sperm and ova) during their development and is inherited from one generation to other. Somatic mutations (spontaneous mutations) can occur in a specific cell type or an organ after certain period of development, these mutations are not hereditary but can be induced on exposure to toxins, viruses, UV light, or radiation.

Advancements in technology have enable us to look broadly into the human genome and identify genetic variations associated with prevalence of disease or reduced efficiency of proteins.

It is well accepted that environmental factors can play a significant role in inducing cancer, these are the mutagens responsible for causing mutation in different gene types. Most of the mutations are corrected by a very efficient DNA repair mechanism, but even though rare escape from this system can cause mutations, few of them can impact the normal functioning of the protein and can also lead to cancer.

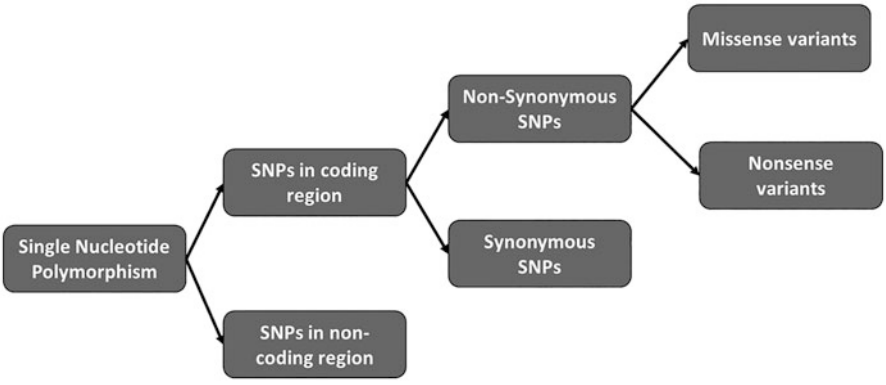

Fig. 1 Diagrammatic representation of different type of single nucleotide polymorphisms

Introduction to the Genetics and Cancer Association

Proteins are complex macromolecules that construct the structure and perform different functions and regulations in a cell. There are diverse proteins that are associated with one or more functions, defects in these proteins can result in different outcome depending on their functionality. Varied protein defects are associated with incidence of cancer and other minor or major disorders. In this section of the chapter, we will discuss about the common protein coding genes that have been studied in colorectal cancer and IBD-associated colorectal cancer; these genes can be categorized as follows:

Oncogenes: *KRAS* and *CTNNB1* (β-catelin)
Tumor suppressor genes: *APC*, *TP53*, *TGFβRI*, *SMAD2*, and *SMAD4*
DNA repair genes: *MLH1*, *MSH2*, *MSH3*, and *MSH6*

The inflammation that occurs in the IBD can be due to infection or changes in genetic components which further leads to colorectal cancer. The genes involved in CRC aid to study the molecular mechanism involved in the disease and help to study relevant targeted therapies. Colorectal cancer can be caused due to not one but series of genetic variations that may accumulate damages difficult for the system to repair. Genetic variations along with environmental factors or dietary habit can also cumulatively increase the risk of colorectal cancer.

Earlier genomic evaluation has identified *APC* gene as a crucial factor in the development of colorectal cancer. Chronic inflammation is one of the factors contributing to the increased stress and induced DNA damage. Earlier studies have reported variation in *APC*, *TP53*, and *IDH1* with IBD-associated colorectal cancers. Mutation in *IDH1* at codon 132 arginine is reported as a hotspot and was significantly common in IBD-associated colorectal cancer (Alpert et al. 2019). Based on genetic evaluation colorectal cancer can be majorly categorized into the following:

- Familial adenomatous polyposis (FAP)
- Hereditary nonpolyposis colorectal cancer (HNPCC)
- MYH-associated polyposis (MAP)
- Hamartomatous polyposis syndromes

Familial adenomatous polyposis (FAP) is rare and contributes to only 1% of colorectal cancer and adenomatous polyposis initiated during childhood may lead to colorectal cancer if left untreated. FAP shows a strong family history with ~80% of cases effected with carrying preexisting conditions. Genetic mutation in *APC* gene has been reported in more than 80% of FAP patients and majority of them lead to truncated or nonfunctional protein (Burt and Neklason 2005). Adenomatous polyposis coli (APC) is a tumor suppressor gene that helps in cell adhesion, migration, and regulation of β-catenin. Defects in this gene is associated with familial adenomatous polyposis (FAP) and subsequent genetic variation are associated with increased risk of colorectal

cancer. *APC* gene encodes for a 2850 long amino acid protein that helps in phosphorylation of N-terminal serine and threonine of β-catenin resulting in accelerated degradation assisted by ubiquitylation. Nonfunctional APC elevates the cytoplasmic levels of β-catenin, which migrates to nucleus and stimulates T cell factor/lymphoid enhancer factor family (TCF/LEF) to transcribe Wnt target gene. Increased Wnt signals activate the associated Wnt pathway and is appeared to be one of the major episodes in colonic polyposis (Taketo and Edelmann 2009). Mouse models are frequently used to study the association of *APC* mutations in colorectal cancer. First mouse model APC^{Min} (multiple intestinal neoplasia) was created using N-ethylnitrosourea (ENU) that carries induce mutation resulting in a termination codon at position 850 and is one of the widely used mouse model for studying gastrointestinal tumors. Heterozygous APC^{Min} mice usually survives for 4 months and develops several polyps in small intestine region, but the homozygous mutant for this truncated mutation usually do not survive. Due to the strong association of APC with gastrointestinal cancer, several mice models have been constructed. $APC^{\Delta 242}$ mutant mouse model produces truncated 242 long amino acid sequence lacking the important armadillo repeat domain resulting in increased rate of tumorigenesis. $APC^{\Delta 474}$ carries duplication of exons 7–10 causing a frameshift mutation leading to immature stop codon and can result in increased polyps mainly in small intestine (Stastna et al. 2019).

Another $APC^{\Delta 716}$ mice model has been observed to have a change in polyp localization shifting to colon similar to human with FAP (Taketo and Edelmann 2009). Another advantage of studying colon cancer in genetic mouse models is that the effects of various diets and food additives on tumor formation and progression can be investigated, whereas feeding $APC^{\Delta 716}$ mice with a high-fat diet increases polyp numbers significantly. Likewise, a Western-style diet (high fat and low calcium) accelerates tumor formation in APC^{1638N} mice. Restriction of caloric intake by 40% reduces intestinal polyp numbers in APC^{Min} mice by ~60%, suggesting that dietary interventions can partially offset genetic susceptibility to intestinal carcinogenesis. Likewise, exercise can reduce the polyp multiplicity in APC^{Min} mice by 30–40% (Taketo and Edelmann 2009).

Hereditary Nonpolyposis Colorectal Cancer

HNPCC increases the risk of colorectal cancer and is transmitted in autosomal dominant manner. A subset of HNPCC patient is also susceptible to tumor in other organs such as stomach, small intestine, endometrium, and ovaries. Depending on the nature of the tumor development and organs effected Lynch syndrome can be differentiated as Lynch I for patients with tumors only in colonic region and Lynch II patients have spread of tumor in additionally in extracolonic regions (Lynch and Smyrk 1996). Around 4% of colorectal cancer cases are reported to be HNPCC with higher prevalence between 45 and 60 years of age.

Additionally, to colorectal cancer HNPCC also increases the risk of other cancers, it increases the risk to 53% in females for developing endometrial cancer. Large number of cancer studies have reported molecular association of HNPCC, with

higher prevalence of microsatellite instability (MSI) and impaired mismatch repair mechanism (Burt and Neklason 2005). DNA repair mechanism involves multiple enzymes and associated pathways to maintain the integrity of the genome. Endogenous cellular process like alkylation, oxidation or hydrolysis, and exogenous damages due to various chemicals, and radiations like ionizing radiation, ultraviolet radiation can result in genomic instability, and impaired protein function. DNA mismatch repair system spots and repairs the damage, and also sends apoptotic cell signals.

Most of the HNPCC patients have impaired proteins associated with DNA damage repair genes (*MLH1*, *MSH2*, *MSH6,* and *PMS2*). Individuals carrying mutations in *MLH1* and *MSH2* genes are at higher risk of extracolonic cancer (Bonadona et al. 2011). Genetic variation in these genes have reported to be familial with two or more members of the family affected with colorectal cancer and also shows family history of the disease. Mismatch repair system helps in retaining the integrity of the mammalian genome and is conserved between prokaryotes and eukaryotes. Several proteins are involved in the mismatch repair mechanism among which *MSH2-MSH6* complex (MutSα) initiates recognition of single-base insertion-deletions, *MSH2-MSH3* complex (MutSβ) involves in the recognition of larger insertion-deletions. The subsequent repair mechanism is activated by MLH1-PMS2 (MutLα), MLH1-PMS1 (MutLβ), and MLH1-MLH3 (MutLγ) protein complexes (Genschel et al. 1998; Edelmann et al. 2000). Mice model with knockout MMR genes has revealed the associated with essential role in mammalian cell division, immunoglobulin recombination, and hypermutation in B cell development. Mice model carrying homozygous mutation in *MSH2*, *MSH6,* and *MLH1* genes with the corresponding knockout is prone to gastrointestinal tumors and leads to premature death due to aggressive lymphoma, whereas heterozygous mutants had delayed onset of tumors. In humans biallelic mutations in *MLH1*, *MSH2*, *MSH6,* and *PMS2* resulted in MMR deficiency, and reduced lifespan due to several malignancies similar to MMR knockout mice models. MMR knockout models have helped to understand the importance of MMR mechanism and resulting tumorigenesis due to their loss of function (Edelmann and Edelmann 2004; Felton et al. 2007).

Multiple *MSH2* knockout mice are created and its inactivation resulted in complete deficiency of MMR process. $MSH2^{-/-}$ resulted in impaired single-base mismatch repair mechanism, reduced survival, and high cancer predisposition. $MSH2^{-/-}$ mice model generally develops T cell lymphoma by 6–8 months, and on survival develops adenomas in small intestine and adenocarcinomas (Taketo and Edelmann 2009).

The $MSH6^{-/-}$ knockout showed similar cancer phenotype as $MSH2^{-/-}$ but had longer survival and delayed onset of tumor. Predominantly due to the role of MSH2 in repair mechanism of two- to four-base insertion-deletion whereas MSH6 is associated with single-base repair mechanism. Indels of two to four base results in higher incidence of frameshift mutations and higher chance of coding impaired proteins. Observations in mice models suggests association of *MSH6* mutations with late onset of cancer. In HNPCC patients *MSH6* mutations are reported with higher rate of cancer onset after 60 years. *MSH3* mutation showed lower incidence of

gastrointestinal tumor and weak association with cancer (Taketo and Edelmann 2009; Edelmann et al. 2000).

Mutation in *MLH1* is reported with strong association with impaired protein function and MMR deficiency. Loss of MLH1 function is reported to have shorter life span and strong susceptibility with cancer phenotype. *MLH1*$^{-/-}$ is reported in several cancer types predominantly including adenocarcinomas, intestinal adenomas, melanoma, and T cell lymphoma (Prolla et al. 1998).

PMS2 is one of the units from MutLα complex and is reported to have strong association with cancer predisposition, predominantly with lymphomas and sarcoma later in life. *PMS2* mutations were not strongly associated with colorectal cancer, possibly due to availability of proteins with similar function and partial defect in repair mechanism. In human *PMS2* mutations were reported to have late onset of Lynch syndrome. *PMS2*$^{-/-}$ mouse model has also reported to have late onset of cancer phenotype. *PMS2* inactivation along with other MMR genes like *MLH3* has reported to increase the levels of microsatellite instability and combined effects of deficit MMR function have reduced survival rate due to increased susceptibility to tumors (Taketo and Edelmann 2009; Chen et al. 2005; Edelmann et al. 1999).

MUTYH-Associated Polyposis

Mutation in *MUTYH* gene can predispose to colorectal cancer adenomatous polyposis and is reported as MUTYH-associated polyposis (MAP). MUTYH contributes to DNA proofreading mechanism and base-excision repair; mutation in this gene is reported to result in increase in the rate of mutations. MUTYH helps in mitigating oxidative DNA damage–induced mutations and prevents tumor development. FAP patients with undetected *APC* mutation are observed to harbor mutations in *MUTYH* gene. Mutant mice model *MUTYH*$^{-/-}$ was reported to increase the susceptibility to spontaneous tumorigenesis. It was also observed that administration of strong oxidative reagents like $KBrO_3$ significantly increased intestinal tumors in *MUTYH* null mice. Mice model with MUTYH/OGG1 deficiency also showed increased incidences of intestinal tumor (Sakamoto et al. 2007).

Hamartomatous Polyposis Syndrome

Hamartomas are prevalent in juvenile polyposis syndrome (JPS), Peutz Jeghers syndrome (PJS), and Cowden disease, however these all contribute to 1 in 100,000 incidences. Autosomal dominant PJS carries hamartomatous polyps all over the gastrointestinal tract with about 30% lifetime risk of colorectal cancer. Autosomal germline mutations in *SKT11* gene are believed to be responsible for 50% of PJS cases (Gryfe 2009).

In JPS patients the risk of developing colon cancer is about 60% with additional risk of developing other gastrointestinal cancer. Germline mutations in *SMAD4* have been associated with increased risk of JPS. *SMAD4* is an important tumor suppressor

gene that mediates intercellular signaling of transforming growth factor (TGF)-beta receptor. In mice model inactivation of one copy *SMAD4* did not increase the incidence of tumors. Mice model with mutations targeting *SMAD4* along with *APC* has been reported to have rapid tumor development as compared to *APC* alone. Various cancers have reported loss of 18q that exhibit SMAD4 gene indicating its complete inactivation, suggesting an important role in cancer development (Gryfe 2009; Tanaka et al. 2006).

Cowden disease are at higher risk of breast and thyroid cancer, and are reported with intestinal hamartomas, facial trichilemmomas, multinodular goiter, fibrocystic breast disease, and oral papilloma. *PTEN* is a tumor suppressor gene, the encoded protein is primarily associated with PIP dephosphorylation. *PTEN* germline mutation is reported to be associated with early tumorigenesis and Cowden disease. Mice model with heterozygous *PTEN* mutation along with APC^{Min} has been reported to have invasive and larger tumorigenesis (Burt and Neklason 2005; Taketo and Edelmann 2009; Gryfe 2009).

Genetic Variation in IBD-Associated Colorectal Cancer

IBD-associated colorectal cancer can be induced due to chronic inflammation in the intestine. Mutations in *TP53* and *KRAS* are commonly observed in IBD-associated colorectal cancer. In IBD-associated colorectal cancer alteration in TP53 function results in intrusive tumor cells, increase genomic instability, and attenuated apoptosis (Stastna et al. 2019).

IBD can increase the risk of gastrointestinal cancer, studies have reported that chronic inflammation can lead to colorectal cancer. IL-23 is a cytokine produced by various immune cells like dendritic cells, macrophages, and monocytes. In the gut IL-23 is downregulated by different pathways and studies have reported that hematopoetic stem cell, progenitor cells, and T lymphocytes are major target for IL-23-directed gut inflammation. It is also reported that the levels *BATF* transcription factor also a known inducer of T-helper 17 cells, mice model has demonstrated the crucial role of BATF in the development of colitis-associated colorectal cancer. Genetic variation of G149R and Q3H in *IL-23R* was shown to increase the risk of Crohn's disease in IBD patients. In mice high levels of IL-23 was observed in small intestine and terminal illium. Lower expression of IL-23 in mice was reported to have lower levels of Reg3b and IL-22, which are associated with maintenance of mucosal homeostasis (Neurath 2019).

IBD patients are at 1.5–2 times higher risk of colitis-associated cancer and risk of colorectal cancer increases with 1.7–2.4 times in Crohn's disease patients. In colorectal cancer, alteration in pathway due to mutation is recorded in Wnt signaling, TGF-β signaling, and RAS-MAPK pathway. Mutations in *APC*, *TP53*, *KRAS*, *DCC*, *SMAD4,* and *NRAS* were some of the commonly reported genes associated with colorectal cancer (Kameyama et al. 2018).

TP53 is a tumor suppressor gene that behaves as a checkpoint and control cell proliferation. Impaired TP53 function is reported in several cancer types and

mutation in this gene is prevalent in colorectal cancer. Majority of *TP53* mutation that leads to impaired function is localized between the 100 and 300 codon span, and codon 175, 248, and 275 contribute to 20% of the mutations. Mutation in *TP53* alone does not initiate colorectal tumorigenesis but along with other mutations increases the risk. More than 60% of colorectal cancer reports *TP53* mutations and additional impaired *APC* gene is also reported to elevate the risk significantly. *TP53* mutations are less prevalent in colorectal tumor related to HNPCC. Mice model with homozygous *TP53* mutation was reported to develop several intestinal tumors and can result in survival of less than 1 year (Alpert et al. 2019; Kameyama et al. 2018).

Anti-EGFR monoclonal antibodies like cetuximab and panitumumab are administered as first- or second-line therapy in combination with chemotherapy. Colorectal cancer patients carrying mutation in *KRAS* or *BRAF* genes are reported to have resistance to anti-EGFR treatment. Unsuccessful anti-EGFR treatment outcome is majorly related to the emergence of variation in EGFR-RAS pathway. $APC^{+/Min}$ mice showed association of EGFR activity for tumorigenesis. $EGFR^{wa5/wa5}$ when treated with AOM/DSS resulted in significant increase in tumor progression, and $EGFR^{wa5/wa5}$ mice with *Il10* deficiency developed tumors in absence of mutagens. *EGFR* mutations and higher expression levels are commonly observed in colorectal cancer, *EGFR* mutation not alone but with additional factors contributes to the initiation of colorectal cancer (Stastna et al. 2019; Corti et al. 2019).

KRAS is an oncogene, a member of GTPase superfamily associated with the regulation of proteins necessary for cellular propagation and cell signaling. *KRAS* mutations are common among colorectal cancer and is also considered an important disease-initiating event. Genetic variation in *KRAS* is reported to increase the RAS-GTP levels, most elevated in mutants G12D and G12C, with a lower level in G12R and G13D cells. $KRAS^{G12D}$ and $KRAS^{G12C}$ were reported to derive widespread of hyperplasia with a significant thickening of mucosa. KRAS mutations has also been reported to interfere with efficacy of EGFR targeted therapy. KRAS has been reported to be mutated at higher rate in pancreatic cancer and is less frequent in other type of cancers, like colorectal cancer. However, variation in *KRAS* gene has been reported to have strong association with onset of colorectal cancer. A mouse model with $KRAS^{G12D}$ mutation leads to hyperproliferation and manifests as chronic intestinal hyperplasia and with *APC* mutation increases the chance of transition from a benign adenoma to malignant adenocarcinoma (Zafra et al. 2020; Wang et al. 2013).

NRAS is a GTPase membrane protein, oncogenic property of *NRAS* has been investigated widely and mutations in this gene have been associated with colorectal cancer, Noonan syndrome, follicular thyroid cancer, and myelomonocytic leukemia. Mutant $NRAS^{G12D}$ has been reported to inhibit apoptotic response of epithelial cells, and it is unclear if this inhibition of NRAS contributes to the initiation and progression of colorectal cancer (Zafra et al. 2020; Wang et al. 2013).

Introduction of mutation in *HER2* gene has been observed to increase the intercellular signaling and induce oncogenic transformation. *HER2* mutations in

few hotspots S310F, L755S, V777L, V842I, and L866M were reported in colon epithelial cells and are indicated as activating mutations. These *HER2* mutations were reported to cause shift in IC50 of cetuximab and panitumumab, and induce resistance to these drugs (Kavuri et al. 2015). Caspase recruitment domain–containing protein 9 (CARD9) is an adaptor protein related to transduction of signals and helps in regulating inflammation and cell apoptosis. Genome-wide association studies (GWAS) have reported *CARD9* polymorphisms in IBD patients are at higher risk for Crohn's disease and ulcerative colitis (Rivas et al. 2011). It has been reported that knockdown mice model for *Card9* gene had increased colitis-associated colorectal cancer (Wang et al. 2018). *BRAF* gene encodes protein of RAF family, this protein is associated with the regulation of MAP kinase/ERK cellular signaling, cell division, and differentiation. $BRAF^{V600E}$ mutation is a commonly reported melanoma and other cancers including colorectal cancer. Colorectal cancer patients of this variant are associated with poor prognosis and show restricted response to the drug. It was deciphered by Anirudh Prahallad et al. in 2012 that $BRAF^{V600E}$ inhibition in colon cancer cells leads to a feedback inhibition of EGFR (Prahallad et al. 2012).

Transforming growth factor β (TGFβ1) helps in cell growth, differentiation, and protect cells from DNA damage caused by inflammatory cells. Due to its multifunctional nature of TGFβ1, it has been studied in different cancer types. Mice model with inactivated *TGFβ1* suffers with autoimmune disease at very early age mostly before 1 month of age. Inactivation of *TGFβ1* in mice results in autoimmune disease and death before 1 month of age. TGFβ1 is involved in differentiation and inhibition of proliferating intestinal epithelial cells, making it an important component in tumor suppression mechanism (Stastna et al. 2019; Taketo 2006).

Concluding Remark

Mice models can help in understanding the importance of each gene and also provides an insight on the importance of coding regions. Various researchers have studied the effects of single-base mutations and multibase insertion-deletions to find the impact of these genetic variations. Evaluation of different mice models has helped to find the genes that are strongly associated with different type of cancers. In case of IBD and associated colorectal cancer, *APC*, *TP53*, and *SMAD4* are among few tumor genes, oncogenes like *NRAS* and *KRAS*, and DNA repair genes like *MLH1*, *MSH2*, *MSH3,* and *MSH6* have been extensively studied.

Genetic variations can be used for targeted therapy, by designing or repurposing drugs based on their affinity toward mutation in cancer cells. Further technological advancements can help in improving the treatment strategies and cater better treatment to cancer patients.

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Selected Methods for Toxicity Testing of Chemical and Biological Compounds Using Animals

35

Maria Walczak

Contents

Abstract

Preclinical studies are an important stage in the development of every drug. They are the primary means of assessing the efficacy and safety of new substances with potential therapeutic activity. Depending on the results obtained, a decision is made on further clinical trials. Preclinical trials allow to determine pharmacological, pharmacokinetic, and toxicity properties of tested compounds. Such experiments are conducted in vitro on cellular models or in vivo on animal models. Experimental pharmacology studies based on the use of experimental animals serve to evaluate the therapeutic efficacy of substances. They are also a useful tool

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in the search for new signaling pathways, which contributes to the understanding of the mechanisms underlying disease development. This makes it possible to identify new drug targets. In general, animal models are expected to mimic the cause and symptoms (phenotype) of a disease in a "human" way. The complexity of pathomechanisms and the lack of complete understanding of the course of many diseases favor the choice of more simplistic systems in which only certain components of the pathological process are induced.

Keywords

Toxicometry · Animals · Safety · OECD · GHS

Introduction

Animal models are of great interest to researchers, often based on invasive procedures that allow research to be conducted in ways that would be impossible in humans. These include genetically modified models as well as non-genetic models. In the latter, a phenotype resembling the disease process under study develops after surgical intervention (e.g., narrowing/closing of a blood vessel), implementation of an appropriate diet (e.g., food with high cholesterol content), or conditions of maintenance (e.g., chronic hypoxia). In the case of so-called spontaneous models, the development of a given disease in selected animal strains occurs spontaneously as a result of specific mutations. For example, preclinical studies on diabetes and obesity use mice with impaired leptin secretion (ob/ob) or lack of active leptin receptors (db/db) for metabolic syndrome programming, Agouti mice, and in hypertension SHR (spontaneously hypertensive rat) or Dahl SS (salt-sensitive) rat. The choice of a model based on its ease of use rather than on the degree of representation of the pathophysiological process, as well as the lack of standardization or the use of different animal models to study the pathogenesis, prevention, or treatment of the same disease, may lead to difficulties in interpreting the results obtained (Gibb 2008).

Toxicometry

Toxicometry is a branch of toxicology that deals with the quantitative assessment of the toxicity of chemical substances using various techniques and test methods. Toxicometric studies are performed primarily in vivo on laboratory animals and in vitro using tests on bacteria, fungi, cell, or tissue cultures (OECD 1998).

A milestone in the field of toxicometric methods was reached in the 1970s when efforts were made to standardize methods and ways of conducting toxicometric tests. Currently, the methodology for conducting a given test, the number and species of animals used, the manner of exposure, and the interpretation of test results have been

developed in OECD guidelines, which are available on the website of this organization (OECD 2000a, b).

Purpose, Scope, and Criteria for Toxicometric Studies

Purpose of Toxicometric Studies

The basic objective of toxicometric studies is to determine the harmful effects of the test compound on the basis of the results of experimental studies on animals and to determine safe levels of human exposure to the test chemical. For this purpose, the results obtained for a particular chemical shall be extrapolated from one species to another, the dose or concentration of the compound in animals to the level of likely exposure in humans, and the frequency, type, and severity of toxic effects observed in a small group of animals to human populations, thus evaluating the dose-effect or dose-response relationship. This is necessary because the mode of exposure of animals and humans may be different, the animal species used may not react as well as humans to the substance, and the doses or concentrations of compounds used in animal experiments may be significantly higher than those to which humans are exposed.

The occurrence of adverse health effects as a result of exposure to chemicals depends on a number of factors, including the ability of the substance to cause damage, the amount (dose) that was administered or absorbed into the body, the route of administration, the length of exposure, the time required to cause the damage, personal factors, and external conditions.

A secondary objective of toxicometric studies shall be to elucidate the mechanism of action of a substance so as to devise an appropriate course of action to prevent the development of adverse reactions or, in the case of existing effects, to alleviate or terminate them.

Toxicometric studies have a functional value in the various branches of applied toxicology, which is reflected in the development of specific parameters relevant to a broad safety assessment (e.g. LD50, MoS, DNEL, NOAEL, LOAEL, SED) (Krewski et al. 2020).

Scope of Toxicometric Studies

Toxicometric studies making it possible to assess the safety of xenobiotics are conducted in several stages, each successive one being carried out when justified by the results obtained in an earlier stage. These steps include:

1. Evaluation of the toxic properties of a compound on the basis of physicochemical properties, examining the relationship between the structure of the substance and its biological properties

2. Acute toxicity – combined with an assessment of skin and eye irritation, sensitization, and studies of metabolism and toxicokinetics
3. Subacute toxicity – determination of toxicity in short-term repeated dose studies (14, 21, or 28 days) with assessment of cumulative effects
4. Subchronic toxicity with repeated dosing for 90 days
5. Chronic toxicity in a 2-year (sometimes 1-year) study
6. Distant effect studies
7. Carcinogenicity studies
8. Teratogenicity studies
9. Studies the effects on fertility, reproduction, and offspring
10. Studies the neurotoxic effects (Andersen et al. 2010) (Fig. 1)

Criteria for Toxicometric Studies

For safety reasons, chemical substances that are raw materials, intermediates, or products that come into contact with humans or are part of environmental pollution should be subjected to toxicometric testing. The testing shall be decided on the basis of such considerations as:

1. The relevance of the human contact – first priority is given to testing: drugs, food, and food-related substances (additives, substances in contact with food during production and storage), household and medical products, materials used in construction (mainly in building).

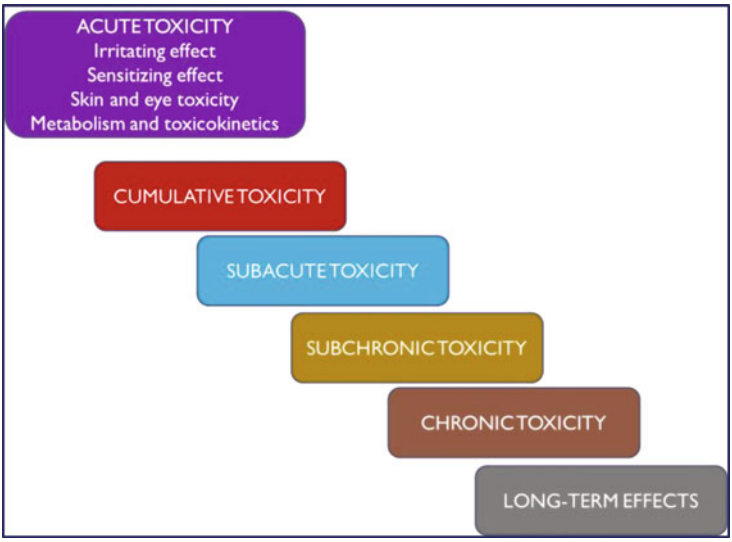

Fig. 1 Schematic representation of toxicometry test stages

2. Information suggesting the relevance of undertaking such studies based on the influence of the chemical structure on the reactivity of substances.
3. Information on the potential to cause acute and chronic poisoning and the likelihood of a carcinogenic effect: possibility of accumulation in the body, persistence of the substance in the environment, and size of exposed population (Gibb 2008; OECD 1998, 2000a, b; Krewski et al. 2020; Andersen et al. 2010)

Stages of Testing

A number of standards must be met in order to obtain reliable and reproducible toxicometric results. Among the important requirements are high purity, of both single substances and their mixtures, and clearly specified composition. The final report shall identify the solvent used and the concentration (dose) at which the preparation was administered. The test animals should be healthy; of approximately the same age, breed, and sex; and of similar body weight. During the study, the animals should be maintained under standard conditions of temperature, humidity, and cage density and given an adequate diet and continuous access to water. It should be remembered that all these variables can significantly affect the rate of onset of signs of adverse effects (OECD 1998, 2000a, b) (Fig. 2).

Acute Toxicity

The purpose of this test is to determine the degree of toxicity of a chemical substance, i.e., to assess the dose-effect relationship, to be able to compare the

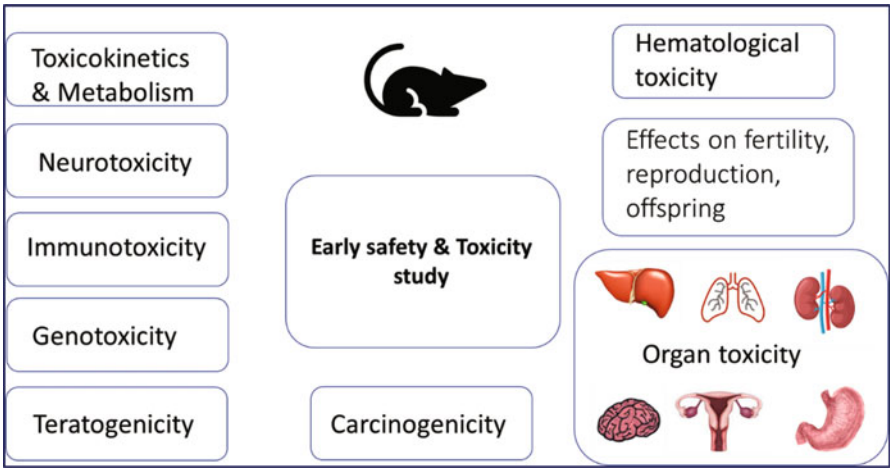

Fig. 2 Schematic representation of toxicometric test endpoints

toxicity of different substances, to gain information about the intrinsic effects of the substance, and to elucidate the mechanism of toxicity.

Acute toxicity refers to the ability of a compound to produce a toxic effect within a short time after a single gastrointestinal administration, a single inhalation exposure, or a single application to the skin at a given dose or several times within 24 h. To examine the likely changes in individual systems, recording the time of onset of symptoms and their duration, the acute exposure effect shall be observed for up to 14 days. The results obtained depend on the route of administration of the substance and the species and strain of animals. This study shall determine the dose of the substance that leads to the death of the animal, identify the organs most liable to adverse effects of the substance, and assess the nature of the effects of the compound, including the occurrence of any allergic reaction or irritant effect.

To quantify acute toxicity, the LD50 parameter (the dose which produces the death of 50% of test animals) is used. Determination of this parameter using the classical procedure involved the use of large numbers of animals and high mortality. To prevent this, the OECD introduced three alternative procedures that make it possible to classify a test substance into acute toxicity class, as required by the Globally Harmonized System (GHS). The acute toxicity alternative tests were developed to significantly improve animal welfare, on the one hand, and to provide results allowing the determination of toxicity classes and the estimation of risks to human health and the environment, on the other hand. According to the 3Rs principle, tests using alternative tests are performed on fewer animals, of only one sex, compared to the classical test of acute toxicity. Females are selected as more sensitive to testing, which allows the number of animals in the experiment to be reduced by half (Diener and Schlede 1999; Hazelden 2013).

Classical Procedure

This procedure involves administering the test substance to a group of animals (five to ten males and five to ten females) at three to six different doses. The highest dose is considered to be 2000 mg/kg body weight. At least three animal species should be used, of which not more than one should be a rodent and not more than one a bird. Substances shall be administered by three routes: intragastrically, intravenously, and subcutaneously. During the study, body weight, behavior, and appearance of the skin, hair, and eyes shall be systematically monitored. Particular attention shall be paid to the onset and duration of toxic signs such as vomiting, salivation, diarrhea, lethargy, and tremors and to the time of death of the animals. The experiment is performed for 14 days. At the end of the experiment, surviving animals shall be sacrificed for macroscopic and microscopic examination of the organs. The results obtained are tabulated giving the number of animals at the start of the experiment, the number of animals showing signs of poisoning and a description of the toxic effects, the number and time of mortality, and the results of the autopsy. The data should be sufficient to describe the dose-response relationship and to calculate the LD50 value.

Due to the need to use large numbers of animals in the classical test and the fact that it is not necessary to determine an exact LD50 value to determine the toxicity

class of a substance, three alternative tests for acute toxicity by the oral route were introduced. Therefore, the classical test was deleted from the OECD Guidelines (van den Heuvel et al. 1990).

Fixed-Dose Procedure

The test is designed to produce clear toxic effects rather than animal deaths. The test is conducted in two stages. In the first stage, the so-called pre-screen test, the effects of different doses (5, 50, 300, and 2000 mg/kg) shall be assessed in single animals of one sex at 24-h dosing intervals. If the chemical structure of the compound or in vitro test results are known, the experiment shall start with 300 mg/kg. The animals shall be observed for 14 days during the study. The pre-screen test shall provide information on the dose dependence of toxicity and allow the determination of a minimum lethal dose.

The dose of compound shall be chosen for the main study at a level which produces clear toxicity, but not animal death. In the main study, the substance is administered intragastrically to a group of five animals of one sex at a single fixed dose (5, 50, 300, or 2000 mg/kg) obtained from a pre-screen test. The animals are observed after administration of the substance. If the administered dose causes the death of the animals or obvious toxic signs, the test shall be continued with a lower dose of the compound. If the administered dose does not produce toxic effects, the test is continued with a higher dose of the test substance. The test does not permit determination of a dose-effect relationship and an LD50, but it does permit determination of a toxicity class of the compound. The dose corresponding to the toxicity class is chosen as that which produces marked toxicity and/or death in one or more animals (Stallard and Whitehead 1995).

Acute Toxic Class Method

This method consists of administering the compound via the intragastric route to three experimental animals at one of four fixed doses (5, 50, 300, or 2000 mg/kg) with the expectation that the selected dose will induce the death of at least one of the three animals. After administration, the animals are observed for 14 days, with particular attention to changes in the skin, fur, eyes, and mucous membranes, as well as those of the respiratory, cardiovascular, autonomic, and CNS systems. The endpoint is death of the animal; hence, the mortality of the animals in the first stage of the study determines the performance of the next stage. The results of the test allow the compound to be classified into toxicity classes according to the GSH (Diener and Schlede 1999).

The Up-and-Down Procedure

The acute toxicity testing by the up-and-down procedure allows for the establishment of a dose-effect curve, thus estimating the LD50 with confidence limits. The procedure involves administering one dose to one animal at a lower dose than the expected LD50. Depending on the effect obtained, a higher or lower dose shall be administered to the next animal, which shall be calculated after taking into account a factor of 3.2. The test shall be carried out until the dose at which an increase in the dose results in death and a decrease in the dose results in survival of the animal.

The procedure shall be carried out in two stages. The first stage is a preliminary test (limit test) which consists of administering the compound to one animal at a dose of 2000 mg/kg. If the animal survives the test, the dose is administered to four more animals. If two out of five animals die, then the LD50 is taken to be greater than 2000 mg/kg. If three out of five animals die, the LD50 is assumed to be less than 2000 mg/kg, and the main study is required.

In the main study, the test will also be started at 5000 mg/kg. In this case, the absence of deaths or deaths of one to two animals suggest that the LD50 is greater than 5000 mg/kg. The death of three animals indicates that the LD50 is less than 5000 mg/kg and indicates that further testing is necessary, i.e., administration of the compound at 2000 mg/kg or 300 mg/kg. In the main study, the compound at strictly defined doses is administered sequentially.

The starting dose in this procedure shall be established based on the physicochemical properties of the substance, the relationship of the activity to the structure of the compound, or other toxicity studies. If these data are not available, the test begins at a dose of 1.75 mg/kg, increased by a fixed factor of 3.2 (a value of 0.5 on a logarithmic scale). The application of this factor produces a dose sequence of 1.75, 5.5, 17.5, 55, 175, 550, 2000 mg/kg. If justified, the dose should be increased to 5000 mg/kg. Note that the test 425 cannot be used for substances exhibiting delayed toxicity (Bruce 1985).

Evaluation of Local Skin Irritation/Corrosion

The purpose of the test is to assess the local effects of a toxic substance following direct contact with the substance. Testing the skin irritation or corrosion of compounds under in vivo conditions is only performed when justified and when the irritant effect of a compound with a similar chemical structure is known. An irritant substance causes reversible damage to the skin after a 4 h exposure, whereas a corrosive substance causes irreversible changes.

For the skin irritation test, the hair is removed from the animal's back, and the next day, the substance is applied to an area of about 6 cm2, and the area is covered with film. After 4 h, the film is removed, and observation is started at 1-, 24-, 48-, and 72 h intervals. The exposed section is observed for redness, swelling, or ulceration. Initially, the test is performed on one individual, especially if strong corrosive effects are expected. More animals are used for the test when results need to be confirmed. The changes observed are interpreted using a three-point scale: 1. corrosive; 2. irritant (at least two out of three individuals tested); and 3. mildly irritant (at least two out of three individuals tested) (Munns et al. 2002).

Skin Sensitization Assessment

A skin sensitization test shall be carried out on guinea pigs. In the first stage, sensitization is induced by intradermal application of the substance (to the dorsal

skin) or by repeated application of the test substance to one side of the animal. In the second stage, after about 2 weeks, sensitization is induced by applying the test substance on the lateral part of the animal's body, after which the skin reaction is assessed. The antigen substance induces the formation of antibodies, which on re-exposure trigger the sensitization reaction. The symptoms indicative of a sensitization are urticaria, redness, and/or swelling of the skin at the site of substance application.

To test the sensitization of the animal strain used and the validity of the test methodology, a pre-screen test using alpha-hexylcinnamaldehyde, which is a known sensitizer, is performed. If in this test at least 30% of guinea pig develop a positive reaction to alpha-hexylcinnamaldehyde, it can be assumed that animals of this strain are sufficiently sensitive and can be used for the evaluation of a substance of unknown sensitization.

Another method is the LLNA (local lymph node assay) protocol, which tests the cutaneous allergenicity of a substance. This method involves injecting the substance into mice topically on the skin or via tail vein injection. As the allergic reaction is associated with an increased proliferation of T-lymphocytes in the lymph nodes, their content in lymph samples is determined. The effective concentration of a chemical required to produce a threefold stimulation of T-lymphocyte proliferation than in the control sample (EC3 parameter) is then determined. If EC3 $>$ 10%, the result is considered negative, while if EC3 $\leq$ 10%, the compound is considered an allergenic agent (Krewski et al. 2011).

Short-Term (Subacute) Toxicity

The subacute toxicity test is not intended to provide a numerical measure of toxicity but to indicate the symptoms occurring after prolonged daily administration of the compound. In this study, the test substance is administered to the animals in several doses over 28 days. Rats or dogs are most commonly used as experimental animals in these studies. The daily dose is usually the maximum tolerated dose (MDT). During the study, the animals are observed, and changes in behavior, locomotion, appetite, and weight are recorded systematically at intervals of 2–3 days. In the middle of the expected test period, blood is drawn for hematological and biochemical tests. At the end of the study, the animals are sacrificed and dissected to detect changes in internal organs. The results of these tests shall be useful to predict the accumulation of the substance in the animals and indicate possible organ effects: nephrotoxic, hepatotoxic, and neurotoxic. The study shall provide results that permit the planning of subchronic and chronic toxicity (Greenberg and Lowrie 2009).

Subchronic Toxicity

In this study, the animals are exposed to the substance for 90 days. During the test, the appearance and behavior of the animals, body weight, daily intake of feed and water,

relative and absolute weight of internal organs of the animals after dissection, hematological and biochemical blood tests, and urine examination shall be evaluated. The study shall allow for the assessment of the substances' potential for accumulation, likely systemic effects, and the identification of critical organs or systems.

This test indicates the likely health risk to the individual as a result of prolonged exposure to the substance and allows the estimation of the highest dose or no-observed-adverse-effect level (NOAEL).

The NOAEL (expressed in mg/kg) shall be converted to the corresponding human equivalent dose (HED) by dividing by the body surface area conversion factor (BSA-CF) which takes into account the difference in body surface area between human and animals. The BSA-CF is read from a table for the relevant species and the specified body weight range of the animal. From the species for which HEDs were obtained, the lowest dose is selected for safety reasons for participants in Phase I clinical trials. Due to the possibility of unanticipated toxic effects and the difficulty in identifying certain adverse effects in the animals, the selected HED is divided by the so-called safety factor (SF) depending on the animal species selected, resulting in the maximum recommended starting dose (MRSD) used for the first time in human (FIH) trials (OECD 2000a; Sass 2000).

Chronic Toxicity

In chronic toxicity evaluation, the exposure of animals (rats, dogs, or monkeys) to a toxic substance shall be carried out for 12 or 24 months. The range of tests is wide and also allows carcinogenicity to be indicated. The route and dose range of the compound shall depend on the clinical indications of the test substance, and the duration of the study shall depend on the expected duration of the drug administration. It is advisable to conduct the study in two different animal species, one of which should be a non-rodent. The size of the dose administered to the animals each day depends on the therapeutic dose of the compound in the same species or strain of animals. Typically, doses in these studies are two to three times higher than the therapeutic dose, and an additional group of animals administered the compound at the MTD. All animals shall be observed daily throughout the study, their weight and reproductive capacity recorded, and blood taken for hematological and biochemical tests. At the end of the study, the animals shall be sacrificed, and anatomopathological and histopathological examinations shall be carried out. As a result, parameters such as NOAEL, maximum allowable dose (NDD), no-observed-effect level (NOEL), and critical organs are determined. Ultimately, the test allows for the assessment of the effects of human occupational and non-occupational exposure to chemicals (OECD 2000a).

Classification of Chemicals

The assignment of a test compound to a toxicity class is made possible by the approach represented by the Globally Harmonized System (GHS) of classification and labeling. This system provides standardized criteria for classifying

Table 1 GHS classification for acute toxicity depending on route of exposure

Exposure route	Category 1	Category 2	Category 3	Category 4
Intragastric [mg/kg b.w.]	$0 \leq 5$	$5 \leq 50$	$50 \leq 300$	$300 \leq 2000$
Epidermal [mg/kg b.w.]	$0 \leq 50$	$50 \leq 200$	$200 \leq 1000$	$1000 \leq 2000$
Inhalation/gas [ppmv]	$0 \leq 100$	$100 \leq 500$	$500 \leq 2500$	$2500 \leq 20{,}000$
Inhalation/vapor [mg/L]	$0 \leq 0{,}5$	$0{,}5 \leq 2$	$2 \leq 10$	$10 \leq 20$
Inhalation/dust/fog [mg/L]	$0 \leq 0{,}05$	$0{,}05 \leq 0{,}5$	$0{,}5 \leq 1$	$1 \leq 5$

substances and mixtures in terms of their risks to human health and the environment and requirements for hazard communication in the form of warning labels and safety data sheets (OECD 1998; British Toxicology Society 1984). Table 1 shows the classification of chemicals in the acute toxicity test according to route of exposure.

Conclusion

Toxicometric studies are performed on young, healthy, sexually mature animals of similar body weight (+/− 10%), acclimatized to the experimental conditions for at least 5 days. Animals of both sexes are used to assess the influence of hormones and different enzyme activities. The animals are kept under stable climatic conditions (temperature 23 °C, relative humidity 30–70%), 12 h light/12 h dark light cycle. The animals must have free access to water and feed. Care should be taken that all groups of animals, including the control group, are housed under the same conditions.

For toxicological studies, compounds are administered intravenously (*i.v.*), intraperitoneally (*i.p.*), intragastrically (*i.g.*), by inhalation (*inh.*), dermally (*p.c.*), intradermally (*i.c.*), subcutaneously (*s.c.*), and intramuscularly (*i.m.*).

In the field of toxicometry, a study is also conducted to determine the maximum tolerated dose (MTD). The MTD represents the highest single dose of a compound that does not induce death in any animal. It is important to remember that the LD50 and the MTD depend on the route of administration of the compound.

Due to the fact that most drugs are dosed repeatedly to patients, it is important, in the early stages of toxicity testing, to determine the ability of the tested compound to accumulate in the body. For this purpose, a so-called cumulative toxicity test (K-LD) is performed, which involves daily administration of the test compound at a dose increasing by 1.5 times every 4 days until all animals die. The test is usually started at a dose representing 9% of the LD50 in a group of at least five animals. The dose at which half the animals die is taken as the K-LD. When comparing K-LD and LD50 values, three cases are possible. In the first case, where the K-LD is equal to LD50, neither tolerance to the compound nor accumulation in the body is observed. In the second case, when K-LD > LD50, tolerance to the compound is seen with no signs of accumulation, while in the third case, when K-LD < LD50, the compound accumulates in the body (Scialli et al. 2018).

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Terpenoids as Chemopreventive Agents and Their Interpretation in Animal Models and Human Clinical Trials

36

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Contents

Abstract

Cancer chemoprevention is described as the utilization of substances of natural or synthetic origin to interfere in the discrete early stages of cancer development and progression. This strategy ultimately targets the reversion, suppression, and prevention of the disease. Representative substances used for this purpose are the terpenoids. Terpenoids are naturally occurring compounds originating either from plants or animals or even bacteria and yeast as products of their secondary metabolism and are often already part of human diet. In this chapter, the chemopreventive action of extensively studied terpenoids will be investigated with focus on their in vivo activity in various animal models and results from clinical trials.

Keywords

d-Limonene · Perillyl alcohol · Vitamin A · Lycopene · Artemisinin · 13-*cis*-retinoic acid · Cucurbitacins

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Introduction

Cancer remains one of the major causes of death globally with the rates of new cases raising year by year (World Cancer Report, 2014). Cancer is a complex disease initiated by deregulation of more than one protein and genes that can even be triggered by external factors like diet, environment, or exposure to cancer-causing substances. The past years, despite the encouraging steps towards the therapy of various cancer types, the multifactorial profile of cancer renders it challenging for treatment. Moreover, chemotherapeutic agents and therapies such as radiation result to serious adverse effects including hair loss, nausea, diarrhea, neuro- and nephrotoxicity, suppression of immune system, and many more (Nurgali et al. 2018). Taking into consideration one of the central dogmas of medicine that prevention should always be preferred over treatment, the field of cancer chemoprevention has received increased research interest (Steward and Brown 2013).

Cancer chemoprevention can be described as the utilization of substances of natural or synthetic origin to interfere in the discrete early stages of cancer development and progression. This strategy ultimately targets the reversion, suppression, and prevention of the progression of the disease from a premalignancy or a single organ localized malignancy into an invasive and metastatic tumor. Additionally, it has been manifested that along with the above-mentioned characteristics, these substances should also potently delay the occurrence of cancer (O'Shaughnessy et al. 2002). Following this theoretical background of cancer chemoprevention, the great success came with the approval by FDA of approximately 10 chemopreventive agents that can be used to treat premalignancies and decrease the risk of cancer occurrence or disease reappearance in combination with the delay in the initiation of cancer hallmarks (Wu et al. 2011). Some of these chemopreventive agents include phytochemicals (terpenoids) showing promising in vitro and in vivo chemopreventive activity and are being evaluated in clinical trials.

Terpenoids or terpenes are naturally occurring substances that originated either from plants or animals or even bacteria and yeast as products of their secondary metabolism and are often already part of human diet which indicates their acceptable toxicity profile. Terpenes display great diversity in their chemical structure which results in a variety of different natural uses. Structurally, the common feature of terpenoids is that they consist of one or more C_5H_8 isoprenoid units (two five-carbon units) and they can be distributed to monoterpenes (two isoprenoid units, C_{10}), sesquiterpenes (three isoprenoid units, C_{15}), diterpenes (four isoprenoid units, C_{20}), triterpenes (six isoprenoid units, C_{30}), and also tetraterpenes (eight isoprenoid units, C_{40}) which are frequently called carotenoids. They exhibit antimicrobial, anti-inflammatory, antifungal, antiparasitic, antiviral, but most importantly anticancer activity (Wang et al. 2005). More specifically, epidemiological data in addition to experimental and mechanistic data regarding the manifestation of cancer have been used for identification of terpenes that result in cancer prevention and treatment with activities against prostate, breast, hepatic, skin, lung, and pancreatic cancer. In the present chapter, the chemopreventive action of well-studied terpenoids will be investigated with focus on their in vivo activity in various animal models and results from clinical trials.

Monoterpenes

Monoterpenes have received the most attention among other terpenoids for their use as potential chemopreventive agents. In their majority, they are products of the secondary metabolism of plants and can be found in many essential oils (90%), scents, and resins. Plants use monoterpenes as defense against insects and pathogens. *D*-Limonene is the most well-studied member of monoterpene family, and along with its hydroxylated metabolite, perillyl alcohol (POH) has shown excellent chemopreventive effects against a variety of cancers (Fig. 1) (Kawata et al. 1994). Both of them are found in essential oils of the citrus family fruits like orange, lemon, and grapefruit peel and tomatoes, whereas POH can be also found in herbs like lavender and mint. It is worth mentioning that POH has also been detected in breast milk (Belanger 1998).

Chemopreventive action of ***d*-limonene** has been demonstrated in mammarian, colorectal, hepatocellular carcinoma (HCC), neoplasia, prostate, and adenocarcinoma cancer cell lines. d-limonene was further subjected into in vivo studies, revealing its great potential (Manassero et al. 2013). Diets containing 10,000 p.p.m. of *D*-limonene generated a 72% tumor regression in rats with DMBA-induced mammary cancer (Elegbede et al. 1984). Extensive studies have been conducted to show the prevention of HCC in a variety of rodent models after treatment with ***d*-limonene**. In detail, in DENA and (2-amino-6-methyldipyrido[1,2-α3′,2′-d]imidazole) Glu-P-1-induced hepatocellular carcinoma in F-344 rats, administration of

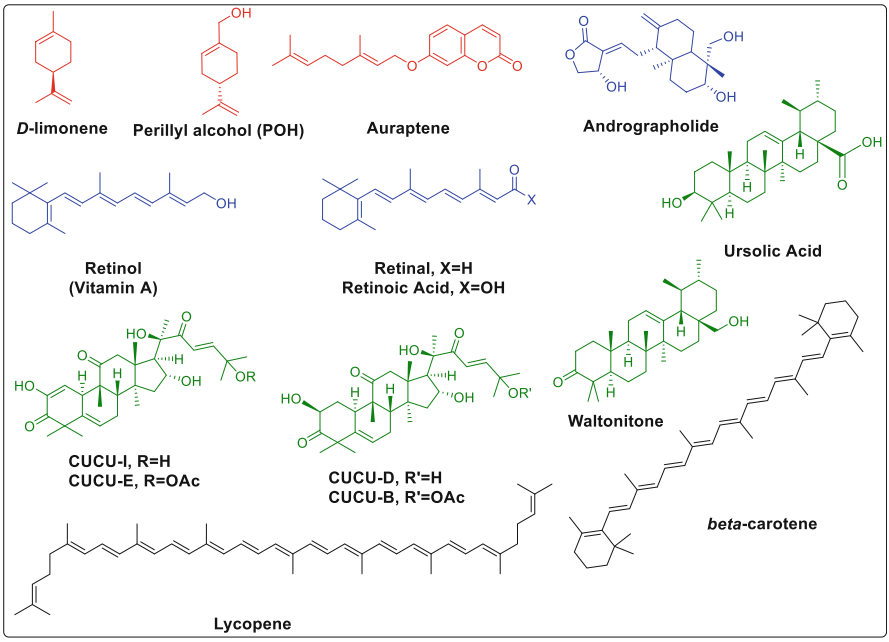

Fig. 1 Chemical structures of selected terpenoids

0.5% *d*-limonene diet inhibited by 32% the glutathione *S*-transferase (glutathione S-transferase is a marker protein for preneoplasia in chemical hepatocarcinogenesis) placental form (GSTp) (Hirose et al. 1995). Mechanistic studies revealed that the chemopreventive effect of *d*-limonene emerges from its action as an oncogene blocker in DENA or PB-induced liver cancer AKR mice models. This combined with the suppression of overexpression of c-jun and c-myc proteins helped in the reversion of the effects of DENA-induced liver cancer (Giri et al. 1999). In NNM-induced HCC Sprague-Dawley rat models, *d*-limonene decreases the cell proliferation rates and reduces the size of GSTp nodes followed by induction of cell apoptosis after a 1–2% dietary administration (Kaji et al. 2001). Bodake *et al*. showed the great chemopreventive effect of orange peel oil (over 90% concentration of *d*-limonene) in animal models. More specifically, male Wistar rats were orally fed for 5 months with orange peel oil and then treated with DENA to induce hepatocarcinogenesis. Results from this study showed a notably lower tumor growth followed by a re-establishment of the WT-phenotype and establishment of several gap junctional complexes and cell polarity (Bodake et al. 2002). *D*-limonene (1–2% in diet) treatment of Wistar rats with MMNG-induced gastric cancer resulted in significant raise in apoptosis with a simultaneous FTPase inhibition and drop of DNA synthesis in gastric cancers (Uedo et al. 1999). A recent study performed in BALB/c xenografts with lung cancer showed again the capability of *d*-limonene to enhance apoptosis. This was achieved via the expression and increase in levels of Bax mRNA protein. Moreover, chemoprevention can be confirmed through the increased expression of several proteins such as LC3-II and Atg5 which are related to the induction of autophagy (Yu et al. 2018). These promising in vivo studies have led to several human clinical trials of *d*-limonene. An open-label single-arm clinical trial in women with early stage or newly diagnosed breast cancer showed that limonene distributes with high predilection in breast tissue unlike its primary metabolite (perillic acid) and was able to decrease the expression of cyclin D1 by 22% in breast cancer tissue. Cyclin D1 is a key cycle regulator found in a number of malignancies. Additionally, this study confirmed the safety and good tolerability of limonene (Miller et al. 2013).

Perillyl alcohol (POH) is a hydroxylated metabolite of *d*-limonene that can be found in herbal essential oils (peppermint, lavender, mint, dill), in citrus family fruits (lemon, grapefruit, orange), or in vegetables and evergreen trees, and its main use is in food industry as additive or as flavoring or fragrance agent (Fig. 1). It exhibits chemopreventive effects in prostate, liver, endothelial, pancreatic, and breast cancer. In pancreatic cancer, POH displays antitumor activity by inhibiting total protein prenylation and H-Ras farnesylation and stimulates apoptosis (Stayrook et al. 1998). In a study conducted by Fisher *et al.* (Lebedeva et al. 2008), pancreatic cancer athymic nude mice xenografts were treated with an intraperitoneal daily dose of POH (75 mg/kg/ 5 mL in tricarpylin) followed by gene therapy with melanoma differentiation-associated gene-7/interleykin-24 (mda-7/IL-24). Thus, treatment with an adenoviral vector expressing the mda-7/IL-24 gene [adenovirus-mediated mda-7 (Ad-mda7)] induces apoptotic cell death and displays broad-spectrum anticancer activities, but pancreatic cells are resistant to this. Remarkably, the

combination of Ad.mda-7 with POH reversed the blockage of the mda-7/IL-24 gene translation, following in MDA-7/IL-24 protein production and growth suppression. Moreover, POH and Ad.mda-7 were used in concentration that can be labeled as safe and non-toxic that could be further evaluated in human clinical trials. In another study, treatment of DENA-induced liver cancer F344 rat model with POH (1–2% for 7 weeks) showed induction of apoptosis of cancer cells and increased levels of TGF-β I-III receptor indicative of the role of POH in prevention of cancer (Mills et al. 1995). Additionally, POH exhibited preventive activity against mammary cancer in rat models. More specifically, it was observed a raise on the levels of TGF-β receptors I and II, a dose-dependent increase in the expression of c-jun and c-fos genes, transcriptional activation of activator protein (AP)-1 reporter gene, as well as c-jun phosphorylation following cell death and apoptosis (Satomi et al. 1999). Similar to *d*-limonene, POH has shown chemopreventive action against prostate cancer. Mechanistic studies revealed the ability of POH to sensitize prostate cancer cells and to inhibit cell growth and function of androgen receptor (AR) and c-Jun. As a consequence, POH resulted in the inhibition of transcription of androgen-responsive genes such as prostate-specific antigen (PSA) and human glandular kallikrein (hK2) (Chung et al. 2006). Despite the large number of phase I and II human clinical trials that have been conducted in order to evaluate the safety, tolerability, and efficacy of POH against various cancer types such as colorectal, ovarian, prostate, or breast cancer, it showed signs of toxicity. Patients mainly had gastrointestinal adverse effects such as nausea, anorexia, and other stomach disorders (Peterson et al. 2014). On the other hand, the efficacy of POH has been confirmed in patients with metastatic prostate cancer that received four doses of 1200 mg/m^2/dose, and cancer was stabilized for 6 months with no reports of tumor regression (Ripple et al. 2000).

Another widely studied monoterpene with very interesting chemopreventive action is **auraptene** (Fig. 1). It belongs to the pharmaceutical class of coumarins, and more specifically, it is a derivative of umbeliferone. Auraptene, like other monoterpenoids, can be either isolated from natural sources such as fruits or vegetables or herbs or easily synthesized in the lab. It showed cancer preventive action against a variety of cancer types (colorectal, esophagus, gastric, hepatocellular, mammary) in animal models. Treatment of AOM-induced colorectal adenocarcinomas in mice with auraptene (0.01%, 0.05%, or 250 ppm in the diet for 10 weeks) revealed an increase in apoptosis of colon cancer cells in combination with a decrease in cell proliferation and cancer-related foci in cells (Kohno et al. 2006). Dietary administration of auraptene (500 ppm, 20 weeks) in NMBA-induced esophageal tumor in rats showed a significant suppression of tumorigenesis correlated with a decrease of cell proliferation in the epithelial tissue of the esophagus (Kawabata et al. 2000). In the meantime, another study conducted in DENA-induced liver cancer in male F344 rats showed that dietary treatment of the animal models with 100–500 ppm of auraptene for 7 weeks resulted in inhibition of hepatocarcinogenesis which was mechanistically correlated with an increase in apoptosis combined with a suppression of cell proliferation (Sakata et al. 2004). Similar mechanisms were involved in the chemopreventive action of auraptene in transgenic

rats with prostate cancer and in MNU-induced mammary cancer rat models. In general, toxicity studies of auraptene (dietary administration with dose range 125–2000 mg/kg) in animal models proved the safety of auraptene with no observed toxic effects (Vakili et al. 2017). Unfortunately, the lack of clinical trials of auraptene has limited its use as a conventional chemopreventive agent.

Diterpenes

Diterpenes consist of four isoprenoid units (C_{20}) and have shown great potential in cancer therapy and prevention, and some agents of the diterpenoid family such as retinol, retinal, retinoic acid (ATRA), and taxol (Trade name: Paclitaxel) have reached the market. One of the most widely studied diterpene is retinol or vitamin A (Bunaciu and Yen 2015).

Vitamin A is a term that usually includes, apart from retinol, all members of the retinoids family (retinal, retinoic acid, retinyl esters) and can be found in almost all types of vegetables. Retinoids consist of a cyclohexene hydrophobic region, a polyene-conjugated linker, and a polar hydrophilic part. Retinoic acid has exhibited its preventive action against various cancer types but in very high concentrations can be teratogenic as it presents adverse effects that can be found in many cell types and systemic toxicities (Tariq et al. 2014). However, retinoic acid, more specifically all-trans retinoic acid (ATRA), has successfully been used in clinic as chemopreventive agent in the treatment of a subtype of acute myelogenous leukemia (AML) and APL (acute promyelocytic leukemia), a disease that has poor clinical outcomes till the 1990s. Treatment of patients with APL using ATRA resulted in a halt in disease progression; however, disease relapsed after the end of ATRA treatment. Combinatorial treatment of ATRA with chemotherapy was performed, and in a recent study, ATRA has shown its chemopreventive effect via elimination of cancer stem cells through a cell-cycle modulation and inductive differentiation (Daver et al. 2015). Additionally, treatment of patients with reoccurred APL using ATRA combined with arsenic trioxide (As_2O_3) instead of any other chemotherapeutic agent showed promising results and is used as a first-line treatment on patients that have family history of APL and are marked as low or intermediate in the scale of risk of APL occurrence (Huang et al. 2014).

13-*cis*-retinoic acid (RA) has shown chemopreventive potential and is a co-administered anti-GD2 immunotherapy for the treatment of neuroblastoma in children (Fig. 1). More specifically, co-administration of these two and granulocyte macrophages colony-stimulating factor results in a stimulation of antineoplastic ability of myeloid effectors (Cheung et al. 2014). Furthermore, neuroblastoma-recovering patients have increased risk of AML occurrence, and administration of 13-*cis*-retinoic acid results in prevention of development of such malignancies. Likewise, 13-*cis*-retinoic acid has underwent clinical evaluation for its chemopreventive effects on patients with head and neck squamous cell carcinoma history (HNSCC). In detail, daily administration of 50–100 mg/m^2 of 13-*cis*-RA for 1 year prevented the second primary tumor occurrence but not HNSCC reappearance.

Additionally, severe adverse reactions such as skin dryness, hypertriglyceridemia, and conjunctivitis were recorded (Hong et al. 1990).

Modifications in cyclohexene moiety, in the polyene side chain, or in the polar terminal group part of retinoids resulted in over 1500 synthetic retinoids displaying various biological effects including great chemopreventive action in mammary gland, prostate, bladder skin, and liver tumor animal models. Yet, the underlying mechanisms have not been yet fully characterized. It is believed that in breast cancer models multiple signal transduction pathways are involved resulting in gene expression (Brtko 2007). Retinoids bind to their receptors (retinoic acid receptors or RAR) which are expressed in healthy and breast cancer cells inducing the regulation of cell growth, proliferation, and apoptosis. A synthetic retinoid derivative, *N*-(4-hydroxyphenyl)retinamide (4-HPR, fenretinide), has showed great chemopreventive action and an acceptable toxicological profile in preclinical models and phase I–III clinical studies (Thomas et al. 2021). For instance, phase III clinical trial data in premenopausal women shows that fenretinide induces reduction of breast cancer relapse (Veronesi et al. 2006). Multiple studies suggest that people with insufficiency in vitamin A have increased risk for cancer development in anatomical sites and especially for prostate cancer. Animal model studies have showed that fenretinide and 9-*cis*-retinoic acid act as prostate cancer preventive agents via inhibition of the invasion of malignant cells in the extracellular matrix and apoptosis induction; however, the complete mechanism remains unclear (Rabi and Gupta 2008). Currently, different formulations and routes of administration of fenretinide are sought to solve its low bioavailability that limits its clinical application.

Acyclic retinoid (ACR) has been reported as another synthetic compound of the diterpene retinoid family that presents chemopreventive action against HCC. HCC is usually associated with disruption in the normal function of the retinoid receptor RXRα after phosphorylation by the Ras-MAPK pathway. In DENA-induced liver cancer animal models, ACR has successfully prevented the occurrence of obesity-linked liver carcinogenesis and inhibited the phosphorylation of ERK and RXRα receptor (Shimizu et al. 2012). This finding is of great importance for the clinical use of ACR, since obese people or people with diabetes mellitus are at high risk for the development of liver cancer.

Another widely studied diterpenoid for its chemopreventive action is **andrographolide**, isolated from the medicinal plant *Andrographis paniculata.* Andrographolide exhibits analgesic, antipyretic, antiviral, anti-inflammatory, hepatoprotective, chemopreventive, and anticancer properties. Studies on various cancer cells (breast and lung cancer, leukemia, melanoma) showed that andrographolide perturbs cell proliferation, and studies in animal models offered great insights regarding the mechanisms involved. The chemopreventive properties of andrographolide emanate from its ability to inactivate NF-kB, AKT, and ERK pathways as well as the activity of MMP2 abolishing the invasion ability from colon cancer cells (Mishra et al. 2015). Studies on nude mice injected with CL1–5 for lung cancer tumor induction that were treated for 6 weeks with andrographolide (4 mg/kg) were able to highlight its chemopreventive action since it was able to reduce tumor sizes and growth significantly (Lai et al. 2013). Same results were observed in A549

(non-small cell lung cancer) injected mice. Additionally, studies in BHC-induced (benzene hexachloride) hepatocellular carcinoma Swiss-albino mice showed that oral administration (5–10 mg/kg/day for 8–10 months) of andrographolide resulted in a significant decrease in liver nodules induced by BHC, combined with increased numbers of antioxidant enzymes such as glutathione reductase, peroxidase, and superoxide dismutase and catalase. Liver restoration ability was demonstrated for andrographolide after the observation of decreased biomarkers that are connected with liver damage (Trivedi et al. 2009). All the promising preclinical studies for andrographolide led to human clinical trials where its efficacy as a combination therapy with capecitabine in patients with colorectal cancer showed great potential (Farooqi et al. 2020). Ultimately, andrographolide can prevent cancer by regulating various signaling pathways, such as JAK-STAT, Wnt/β-catenin, MAPK, PI3K/AKT, and c-Jun/c-Fos that are involved in various cancer hallmark stages (invasion, cell adhesion and migration, metastasis, proliferation).

Triterpenes

Triterpenes consisting of six isoprenoid units (C_{30}) are a group of molecules that are abundant in many plants and animals. Triterpenoids such as steroids and sterols are known for their various medicinal applications exhibiting anti-inflammatory and anticancer action. Examples include cucurbitacins (CUCUs), escin, and ursolic acid.

Cucurbitacins (CUCUs) are highly oxidized tetracyclic triterpenoids, isolated from edible plants of the Cucurbitaceae and Cruciferae families such as cucumbers, bitter melons, pumpkins, and zucchini. They have received great attention for their pharmacological activities since the 1960s. CUCUs exhibit anti-inflammatory, anti-proliferative, anti-angiogenesis, and, most importantly, anticancer activities against several types of cancer such as breast, ovarian, prostate, liver, lung, pancreatic, gastric, and thyroid cancer. More specifically, CUCU-B, CUCU-D, CUCU-E, and CUCU-I have shown great potential in the inhibition of several signaling pathways such as JAK2, STAT3, and Notch; the deregulation of which is strongly related with cancer occurrence. CUCU-B, which is the most abundant CUCU, has shown great potential in the prevention of breast cancer angiogenesis and metastasis after repression of FAK/MMP-9 pathway (Sinha et al. 2016). Moreover, CUCUs have synergistic effects with marketed drugs such as gefitinib and imatinib mesylate with very promising results; however, their low solubility combined with their non-specific toxicities has limited their clinical use.

Patients recovering from colorectal cancer unfortunately have high risk of cancer recurrence or development of chemoresistance due to cancer stem cells that are not completely eradicated after chemotherapy. Both CUCU-B and CUCU-I have shown suppressive effects on Hippo-YAP and Wnt/β-catenin pathways and act as chemopreventives by inhibiting the stem properties of colorectal cancer cells and inducing apoptosis (Shukla et al. 2016). A recent study conducted in nude mice

xenografts injected with HCT166 cells treated with 1 mg/kg of CUCU-B and CUCU-I resulted in reduced tumor sizes and inhibition of cancer progression (Dandawate et al. 2020). In parallel, CUCU-B and CUCU-I induce apoptosis via suppression in the expression of Notch receptors ultimately achieving cell cycle arrest accompanied with a decrease in levels of cancer stem cells that may lead to prevention of colon cancer reoccurrence and drug resistance.

CUCU-D has been reported to show great chemopreventive potential against cervical cancer, linked with apoptotic mechanisms and inhibition of activation of NF-κB and STAT3 pathways (Sikander et al. 2016). To address the chemopreventive ability of CUCU-D against cervical cancer, Jaggi *et al.* used athymic nude mice xenografts with cervical cancer treated with CUCU-D (1 mg/kg). Results not only confirmed the safety profile of CUCUs but also demonstrated its chemopreventive action. Importantly, CUCU-D inhibited cancer metastasis hallmarks such as cell invasion and migration.

HCC is another type of cancer linked with poor clinical outcomes, and even when treated, percentages of reoccurrence risk remain high. CUCU-B exhibited great chemopreventive potency in BEL-7402 and HepG2 mice xenografts treated with 0.1–3 mg/kg/twice a day for 26 days and 25–110 μg/kg/day, respectively, but the exact mechanisms behind its liver cancer prevention properties remain unclear (Thoppil and Bishayee 2011). CUCUs have also been tested in combination with known marketed drugs such as methotrexate, curcumin, and withanone resulting in promising results; however, the clinical use of CUCUs is yet limited due to serious side effects induced after treatment including nausea, diarrhea, skin rushes, etc. Currently, many research groups conduct studies in order to develop targeted CUCU-delivery systems or CUCU-prodrugs aiming for increased therapeutic effects, increased bioavailability, and lower toxicities (Chatzisideri et al. 2021).

Triterpenoids are mostly synthesized in plants via cyclization reaction of squalene. A very potent pentacyclic triterpene is **ursolic acid** (UA) which has shown great anti-inflammatory, antimicrobial, chemopreventive, and anticancer properties. It can be isolated from fruits like apples, bilberries, and cranberries or from herbs like peppermint, basil, and rosemary, and there are multiple in vitro and in vivo reports for the close relation between UA and prevention of cancers such as prostate, liver, colorectal, breast cancer, and leukemia via mechanisms that not only inhibit cell proliferation but also induce apoptosis, suppress angiogenesis, invasion, and metastasis. Chemopreventive mechanisms induced by UA were examined in several animal models, and results showed that UA interferes with cancer hallmarks via the modulation of several signal transducers, transcription factors, growth receptors, and cytokines related to inflammation. In nude mice injected with DU145 cells, treatment for 6 weeks with ursolic acid (200 mg/kg) inhibited proliferation of cells and tumor growth (Shanmugam et al. 2011), while exhibiting chemopreventive action against adenocarcinoma in TRAMP mice model. TRAMP mice xenografts for prostate cancer were receiving UA (1% w/w) through diet for 8 weeks, and it was observed that cancer occurrence was delayed compared to control. Prostate intraepithelial neoplasia did not advance to neoplasia (6 weeks UA diet), and after

12 weeks of UA diet, tumor growth was decreased, while the survival time of the models was elongated. Mechanistic studies revealed the close connection of chemopreventive effect of UA and suppression of various mediators connected with inflammation such as the phosphorylation of NF-κB, STAT3, and AKT. Additionally, studies in orthotopic nude mice models for colorectal cancer showed shrinkage in tumor size and also suppression of cell invasion and metastasis; an effect was also observed when ursolic acid was administered in combination with the drug capecitabine where suppression of proliferative biomarkers was observed accompanied with inhibition of angiogenesis and suppression of NF-κB, STAT3, and Wnt/β-catenin signaling pathways (Shanmugam et al. 2013).

In another study, U937 cells were subcutaneously injected in NOD/SCID nude mice to induce leukemia, and after injection, mice were intraperitoneally treated with UA (50 mg/kg) for 20 days. Increased numbers of death receptors were observed combined with significant increases in suppression of survival proteins expression and JNK activation, while treatment with UA (diet, 54, 106, 266 mg/kg/day) of breast cancer C57BL/6 mice xenografts showed a modulation of Akt pathway followed by induction of apoptosis (De Angel et al. 2010). UA also showed promising chemoprevention potential in HCC animal models and more specifically in DENA-induced and phenobarbital-promoted male Wistar rats. Results suggested that UA acted as a free radical scavenger and decreased various changes related to oxidative stress in rats' liver (Gayathri et al. 2009). The promising chemoprevention results of UA led to studies for its clinical effects, where liposomal UA was tested in order to specify its safety and efficacy profile. A phase I study was conducted with both healthy volunteers and patients with advanced solid tumors, and the maximum tolerated dose (MTD) of UA was established at 98 mg/m^2. The observed adverse effects were liver toxicity and diarrhea. Ultimately, results from clinical trials have marked UA as a potent chemopreventive agent though more advanced trials are required.

Tetraterpenes

Tetraterpenes, also known as carotenoids, consist of eight isoprenoid units (C_{40}) and are one of the most well-studied class of terpenoids for their medicinal properties. Structurally, they consist of a C_{40} polyene chain and can either be hydrocarbon tetraterpenes known as carotenes or highly oxygenated tetraterpenes known as xanthophylls. Xanthophylls are abundant in many dietary products such as tomatoes, carrots (from which carotenoid name is derived), oranges, peas, sprouts, and corn exhibiting anti-inflammatory, antimicrobial, chemopreventive, and anticancer activity. Among the most known tetraterpenoids are *β*-carotene and lycopene which both have shown promising chemopreventive effects in vitro and in vivo, and their use has been tested to the clinic.

β-Carotene can be widely found in carrots, and it is responsible for their orange color. It has been found that *β*-carotene is stored in the liver of humans and is a

precursor molecule of vitamin A which has been mentioned in the diterpenoid section. Interestingly, *β*-carotene displays its chemopreventive action via its ability to interfere in many signaling pathways connected with oxidative stress and can suppress inflammation gene expression in the NF-*κ*B pathway. Additionally, it can block DNA binding to the complex of NF-*κ*B pathway as well as the expression of COX-2, TNF-a, and IL-1b. Carotene acts as pro-oxidant reducing the tumor growth rates via its oxidation to the corresponding carotenoid aldehyde inducing apoptosis. Studies have shown a close relation between *β*-carotene intake and reduction in breast cancer occurrence, an effect related with the conversion of *β*-carotene to retinol and vitamin A which have chemopreventive action. *β*-Carotene has shown a protecting effect against HCC in vivo (Thoppil and Bishayee 2011). Treatment of DENA- and 2-AAF-promoted HCC male Wistar rats with *β*-carotene (70 mg/kg/day) for 2–8 weeks showed an inhibition in preneoplastic foci development and suppression in nodule generation manifested with significant decrease in DNA damage and cell proliferation rates. Additionally, *β*-carotene decreased sizes of nodules in 2-AAF male Sprague-Dawley rats and exhibited chemopreventive action through alteration of levels of proteins related to cancer occurrence and metastasis such as GST, GSH, and GPX reduction and a parallel increase in NADPH, CYP, and vitamin A levels. Thus, antioxidant action of carotenoids and especially *β*-carotenes is responsible for their chemopreventive action due to their ability to act as free radical scavengers preventing any DNA damage in the cellular level.

Another tetraterpenoid with remarkable chemopreventive action is lycopene, an acyclic tetraterpenoid highly abundant in tomatoes, responsible for their bright red color. Its great antioxidant activity arises from its ability to act as a free radical scavenger due to the high structural content of lycopene in double bonds. Multiple studies have shown the anti-inflammatory and cardioprotective abilities of lycopene. The free radical scavenging ability of lycopene leads to modulation of NF-*κ*B signaling pathway by suppressing the formation of NF-*κ*B-DNA complex, and it also inhibits the activation of macrophage and, not unjustly, has been characterized as one of the most promising and efficient natural antioxidants. As evidenced by many clinical trials, the antioxidant activity of lycopene causes a decrease in risk of certain cancer types related to damage from oxidative stress such as liver, prostate, and breast cancer. A large study that conducted for 6 years on over 45,000 men showed that a consumption of 2 to 4 weekly servings of tomatoes reduced prostate cancer occurrence by 26% compared to the "non-treated" subjects. A clinical trial of 26 patients with prostate cancer that received tomato oleoresin extract (lycopene was ~30 mg) showed a significant decrease of tumor size (Kucuk et al. 2002). Another study on a group of 32 prostate cancer patients receiving a diet enriched in tomato products for 3 weeks revealed diminished prostate cancer biomarkers including the prostate-specific antigen (15.5% reduction) (Bowen et al. 2002).

In mammary tumor models, use of lycopene as a diet supplement resulted in significantly decreased activity of proteins related to mammary tumor development. A comparative study between *β*-carotene and lycopene in DMBA-induced

breast cancer tumor rats treated with two β-carotene injections or tomato oleoresin with high concentrations of lycopene (10 mg/kg) for 2 weeks before DMBA-injection and for 16 weeks after showed that both tetraterpenes have great bio-availability in liver and mammary gland. Most importantly, the lycopene-treated group developed a smaller in number or size of breast cancer tumors. In the meantime, β-carotene showed a decreased chemopreventive activity compared to lycopene.

Despite the discouraging preliminary results on the chemopreventive potency of lycopene in HCC animal models, persistence from some groups aiming to prove its action in liver cancer models resulted in interesting outcomes. In DENA-induced liver cancer male Wistar rats treated with 300 mg/kg/day dietary supplements of lycopene for 3 weeks ended up in significant decreases in GGT and GSTp foci of tumors, an action that was initiated by decrease in cytochrome CYP2E1 levels. Similar results were collected after the intraperitoneal injection of 70 mg/kg/day of lycopene for 8 weeks in the same models, and this action was coupled with a decrease in DNA damage (Astorg et al. 1997). In summary, lycopene exhibits great chemopreventive action in vivo, and its safety and efficacy has been proven in clinic where it was able to reduce the occurrence risk of prostate cancer. Unfortunately, multiple clinical trials conducted for other types of cancer such as bladder or oral did not show any promising endpoints and confirmed its safety. Results from placebo-controlled, double blind phase II clinical trials are expected in the forthcoming years to further strengthen the safety and the efficacy of lycopene as a prostate cancer chemopreventive agent (van Breemen and Pajkovic 2008).

Sesquiterpenoids

Sesquiterpenes consist of three isoprenoid units (C_{15}) and are compounds known for their ability to interact with and modulate various biotic pathways in the plant and animal kingdom. Sesquiterpenes, like all terpenoids, have established medicinal use and are widely studied for their pharmacological activities against a variety of diseases like cancer. The last decade research on chemoprevention has led to multiple studies on the preventive ability of various sesquiterpenes with the most of them focused on a marketed drug, artemisinin.

Artemisinin is a compound extracted from the plant *Artemisia annua*; a plant that has been used for thousands of years in the Chinese medicine as an antipyretic. Youyou Tu received the Nobel Prize in Physiology or Medicine for his research regarding the use of artemisinin as a first-line treatment against malaria. Apart from this use, studies proved the activity of artemisinin against other human diseases such as viral infections, atherosclerosis, diabetes mellitus, trypanosomiasis, and cancer. Extensive studies in thousands of patients infected with malaria showed that artemisinin has a relatively safe profile, and it is well-tolerated (Ribeiro and Olliaro 1998).

Chemopreventive effects of artemisinin and its derivatives have been widely studied in animal models in which tumor has been formed either by chemical or viral carcinogens or by tumor cell transplants. Initially, an artemisinin diet supplement of 0.02% in DMBA-induced breast cancer rodents showed that the treated group had a significantly smaller percentage of cancer formation (57% in treated, 96% in untreated models) (Lai and Singh 2006). Furthermore, artemisinin exhibited chemopreventive potential in TPA-induced skin cancer models via mediation of gene expression of TNF-α and NF-κB, ultimately resulting in induction of apoptosis (Wang et al. 2017). On another study, oral administration of artesunate (an artemisinin derivative) (50 or 150 mg/kg/day) in DMH-induced colorectal cancer showed a significant downregulation in the expression of VEGF and MMP-9 along with repression of Wnt/β-catenin signaling pathway (Patyar et al. 2017). The chemopreventive potential of artesunate was further supported by studies in DMH-induced colorectal cancer rat models treated with a daily dose of 6.7 mg/kg of artesunate revealing an increase in antioxidant activities (Abba et al. 2018) (Fig. 2).

Dihydroartemisinin is another artemisinin-based drug that showed promising results in the prevention of viral-induced carcinogenesis. An on-site application of dihydroartemisinin in HPV-infected female dogs did not prevent the viral infection, but it prevented tumor induction a result that designates that dihydroartemisinin might be a chemopreventive agent for tumors as cervical cancer (Disbrow et al. 2005).

The promising results from in vivo studies of artemisinin and its derivatives have led into multiple human clinical trials. The good safety profile and tolerability of these drugs have already been demonstrated via the malaria-based trials. It is known that when they are administered intravenously, they have a very small half-life

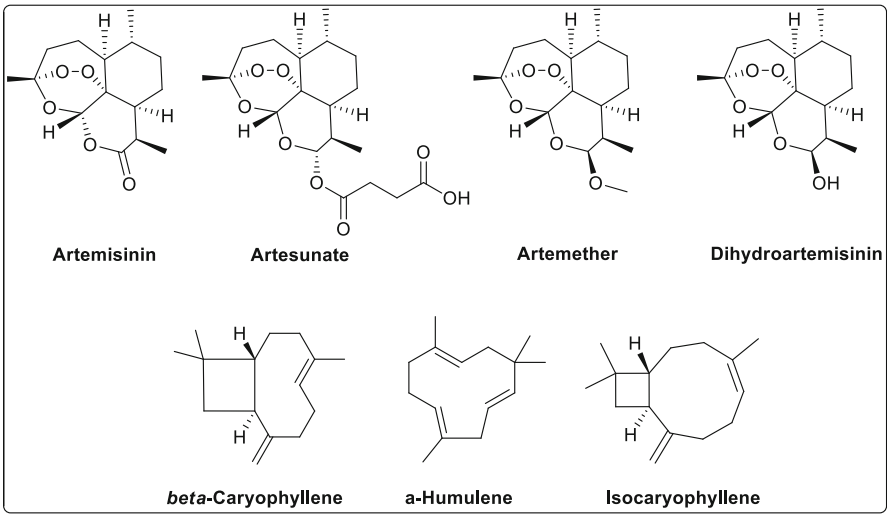

Fig. 2 Structure of artemisinin and its derivatives

(lower than 15 minutes), whereas intramuscular administration results into significantly elongated half-life and higher distribution concentrations. The established safety profile of these drugs has proven helpful to design multiple clinical studies to address the chemopreventive effects of artemisinin-based drugs in colorectal, cervical, and breast tumors and in non-small cell lung cancer. Ultimately, studies in this drug family showed that they prevent cancer occurrence and metastasis combined with low systemic toxicities, a chemopreventive action that mechanistically rises from the ability of these compounds to modulate pathways, and transcription factors that are related with inhibition of apoptosis, metastasis, and angiogenesis in inflammation-derived cancers or with the ability to decrease the viral-promoted carcinogenesis.

Apart from artemisinin, another widely studied member of the sesquiterpene family for its chemopreventive potency is caryophyllene and its derivatives. These sesquiterpenes can be isolated mainly from plants, and the most abundant are *β*-caryophyllene and its corresponding oxide, α-humulene and isocaryophyllene (Di Sotto et al. 2020). Multiple in vivo studies have shown the chemopreventive effect of these compounds. Administration of *β*-caryophyllene (0.15% and 0.3%) in C57BL/6 N mice xenografts of B15F10 melanoma induced with high fat diet showed that it inhibited the incidence of metastasis, cell proliferation, angiogenesis, and induced apoptosis restoring normal levels of glucose in the bloodstream compared to the control group (Jung et al. 2015).

α-Humulene is another sesquiterpenoid that exhibited chemopreventive action. In a study with HepG2 liver cancer mice xenografts treated with intraperitoneal doses of *α*-humulene at 10 and 20 mg/kg every 2 days for a total of 4 weeks resulted in apoptosis, tumor structure disruption, and inhibition of Akt-signaling pathway (Rabi and Gupta 2008). Despite the promising preclinical data, to this day, no clinical trials have been conducted to address the chemopreventive potential of caryophyllene and its derivatives in humans. However, the use of *β*-caryophyllene has been approved by FDA as a flavoring agent indicating its safe general profile (Table 1).

Conclusions

Cancer chemoprevention has turned to a widely studied field with huge potential to decrease cancer initiation or reoccurrence or slow down and stop cancer cells adhesion, angiogenesis, and even metastasis. Terpenoid chemoprevention presented in this chapter is of high importance since terpenoids can be found in dietary products such as food, vegetables, and herbs. So far, many preclinical studies have been conducted highlighting the chemoprevention properties of terpenoids. Some of them like retinoic acid have been approved for clinical use, while for some others clinical evaluation is clearly needed as the collected data exhibit significant benefits to prevent, slow, or treat cancer.

Table 1 Summary of in vivo chemopreventive action of terpenoids and their clinical trials

Compound	In vivo chemoprevention	Clinical trials
Monoterpenes		
D-Limonene	Liver, gastric, lung, breast, prostate	Phase I, phase II
Perillyl alcohol	Liver, prostate, breast, pancreatic, endothelial, ovarian	Phase I, phase II
Auraptene	Liver, colorectal, esophagus, gastric, breast	–
Geraniol	Liver, prostate	–
Carveol	Breast	–
Uroterpenol	Breast	–
Sobrerol	Breast	–
Diterpenes		
Retinoids	Leukemia (AML), head, neck, liver, breast	First-line treatment for AML, phase I, II, III
Andrographolide	Liver, breast, leukemia, melanoma, colorectal	Phase I in combination with capecitabine
Geranyl geraniol	Liver	Phase I
Excisanin A	Liver	–
Calcium glucarate	Breast	–
Triterpenoids		
Cucurbitacins	Breast, ovarian, prostate, liver, lung, pancreatic, gastric, thyroid, colorectal	Phase I in combination with curcumin and waltonitone, phase II
Ursolic acid	Prostate, liver, colorectal, breast, leukemia	Phase I, phase II
Bacoside A	Liver, Dalton's lymphoma, fibrosarcoma, prostate	Phase I
Escin	Liver, colorectal, lung	Phase I
Squalene	Liver, colorectal, lung, skin	–
Waltonitone	Liver	–
Betulinic acid	Prostate, breast, pancreatic	Phase I/II
Tetraterpenes		
β-Carotene	Breast, liver, prostate ovarian	Phase I, phase II
Lycopene	Prostate, breast, liver	Phase I/II
Lutein	Lung, liver, colorectal, breast	Phase I
Sesquiterpenes		
Artemisinin	Breast, skin, HCC	Phase I
Artesunate	Metastatic breast cancer, colorectal, HCC, cervical	Phase I, phase II
Dihydroartemisinin	Colorectal cancer, breast cancer	Phase I, phase II
β-Caryophyllene	Skin, colorectal	–
α-Humulene	Liver	–

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Role of Animal Research to Understand the Prospects for Chemoprevention of Cancer

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Contents

Abstract

Models used in cancer research can be naturally occurring or artificially induced, designed in such a way that they share genetic and phenotypic characteristics with cancer of humans. Animal models serve as templates or experimental setups allowing the ease of studying human cancer. Some of these models are small or large animals having similar or close anatomical setup compared to humans.

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Animal models have revealed cancer disease progression through the discovery of the molecular basis of tumor initiation, growth, and localization. Animal models specifically mouse have been the most explored, playing key role for scientific understanding of molecular basis of cancer and also the role mutation and specific genes plays in cancer initiation and progression. Canines such as dogs have also been recently explored because of the advantages they have over small animal models. Dog's tumor possesses special characteristics similar to that of humans, dogs genetic variations have also contributed to the pace of cancer drug discovery and developments.

Keywords

Animal Models · Cancer research · Small animals · Large animals · Tumors

Introduction

The human cell is born to always grow and divide, maintaining all directives of cell division even while engaging in tissues repair. Cancer is often thought of as an untreatable, unbearably painful disease with no cure. Cancer is no doubt a serious and health threatening issues. Cancer progression is not only restricted to man, it has been found that domestic and wild typed animal also share found the weight of this life-threatening diseases. Cancer is widely regarded as a complex and abnormal process making normal cells grows without restrictions, in other word, cancer cells resist dying, outliving the normal cells and consequentially being transformed to form a new but abnormal cell. From the genetic point of view, cancer progression occurs following DNA damage after several failed attempt from DNA repair mechanisms to correct the defect (Li et al. 2021).

Many of these damages are environmental bound where unguided exposure to radiations and other related substances, however, common agents other than radiation includes; chemicals, and viruses, have all been experimental proven to cause cancer in animals and humans. Substances that are capable of inducing cancer are scientifically called carcinogen (Cooper 2000). In cancer research, dogs, rodents and other experimental animals has been used to study the transformation of normal cells into cancer cells. Experiment animals used in cancer study has yielded significant breakthrough allowing twenty-first century scientist to have a close look on the abnormal cells. The mouse as small and little they appear to be is the most commonly used experimental animals in recent cancer studies.

This little creature has widened the perception and broaden the scope leading to the recent increase of knowledge in cancer research. Laboratory animals used in cancer research help to biochemically and genetically understand various pathways that underpin cancer. Some of these pathways or mechanisms open clearly with evidences surrounding the growth and spread of cancer. Laboratory animals have also contributed greatly to new and safest ways involving diagnose, treatment, and how these diseases can be prevented. Experimental animals, most especially mice

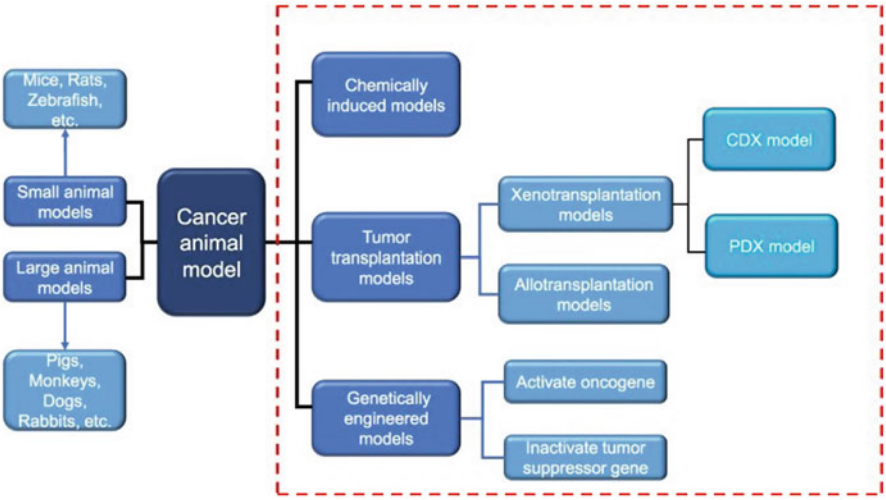

Fig. 1 Flowchart showing commonly used methods of classifying animal models in cancer research (Li et al. 2021)

have been found to be easily manipulated genetically, hence, allowing the natural mimicking of cancer cells that occurs in human tissues and body systems (Fig. 1).

Importance of Animal Models

Animal models being the most valuable and accessible tools for studying the biology and genetics of human cancer as studies have shown that they have helped tremendously to gain insight into the genetic mechanisms underlying transformation and progression of abnormal cells termed cancer. Animal models have not only reviewed the progression but have led scientist to the discovery of the molecular basis of how tumors are initiated, growth frequency, and most importantly their metastasis, which is a reoccurring problem to the treatment of cancer due to some abnormal cells ability to infect adjacent tissues and their subsequent proliferation on competent organs. Animal model has shown how some synthetic or natural occurring compounds can be utilized for anti-cancer drug discovery and testing (Pinho et al. 2012). Despite the ethical consideration surrounding animals, scientists have always seen animal models as power tool to studying and preempting cancer development in man. Many animal models are desirable for cancer research because of their anatomy, for example, the genetic makeup of mouse being cognate to that of human has greatly been explored by scientists. This suggests why mouse are the most preferred among many other animal models. Animal models such as mouse has helped study ways by which cancer cells trigger series of biological characteristics such as the occurrence, development, and metastasis of human cancer cells in vivo. We must bear in mind that these animals used as models are equally easy to be fed alongside

their ability to undergo gene modification and recombination (Mural et al. 2002). More importantly, Animal model serves as a good tool for anti-cancer research and valuable platform for drug discovery and verification. There are no perfect animal models for all varieties of human cancers; however, unlike rodents the genome of canines-dog families are also very similar to the human genetic makeup. Prominent examples include: wolves, foxes, jackals, and other members of the dog family – *Canidae* (Pinho et al. 2012).

The use of some canines as animal model for cancer research can be largely attributed to their ability to spontaneously form some cancers which are genetically and biochemically similar to human cancers in clinical, molecular, and histological features (Gardner et al. 2016). Schachtschneider et al. (2015), suggested they enhance the ability of researchers to perform porcrine-phenotype studies for many human diseases. In many other studies, observations have revealed human cancer genes present in Chimpanzee contain intact open reading frames and show a high degree of conservation between both species (Puente et al. 2006). Despite the physical outlook of man and Chimpanzee, detailed analysis, however, shows some differences in genes with special relevance to human cancer. The studies carried out by Puente et al. (2006), suggested small differences in cancer genes as those found in tumor suppressor genes, which might influence the differences in cancer susceptibility between humans and chimpanzee. Many animal models used in cancer research can be credited to fact that they possess genes similar to that of humans and more importantly, cancer initiation, infection, and progression are similar to that of man. One of many significant studies on cancer treatment using animal models have been exemplified by providing insight to xenotransplantation through critical study on the mechanisms underlying intrinsic resistance to colorectal cancer (CRC) to Vemurafenib – a synthetic drug used in the treatment of later stage melanoma. The success of this research has resulted into clinical trials where CRC patients were treated with a combination therapy targeting both $BRAF^{V600E}$ and EGFR. The study has also exposed the potency of using combined therapy for the treatment of cancer (Prahallad et al. 2012). It is important to note that, like humans' cancer tumors occur unpredictably also among animals in terms of timing, location, and number of lesions. Making animal models the best biological designs with the most appropriate set-up in the study of man's most deadly disease. Many other animal models apart from rodents, chimpanzee, and canines have also been explored.

Particularly, the Zebra Fish, another useful animal model, has enabled high through-put screening for drug discovery, drug validation, drug revalidation, and toxicity testing. Animal models have been useful tools in the area of toxicity testing also playing a vital role in pre-clinical trials where animal models are used in place of human testing. The process of developing a new and effective anti-cancer drugs are tedious, through animal models, focuses are made on preliminary drug screening, pharmacology, and pharmacokinetics. Animal models has help facilitate discovery of numerous new compounds with viable anti-cancer potentials with new mode of actions, they have also helped in having a better understanding on the pharmacodynamics of new drugs so as to predict their efficacy, potency, and toxicity level in humans. The recently developed clustered regularly interspaced short palindromic

repeats(CRISPR-Cas9)system for editing genome has also been applicable on animals models as well as cell lines and that of animal xenograft models which has facilitated recapitulation of human malignancies (Qin et al. 2015).

The most effective treatment for different cancer types and stages is heavily relied on preclinical research on animal models (Kersten et al. 2017). Xenotransplantation models have been useful in establishing novel combinatorial treatment strategies (Prahallad et al. 2012). Kang et al. (2003) proves xenograft studies has helped identify distinct gene expression signatures that mediate organ specific patterns of metastatic colonization, while Ghejar et al tells us those models show disseminated breast cancer cells reside adjacent to blood vessels, which tends to create a niche regulating dormancy of disseminated cancer cells. In the nearest future, application of patient derived tumor xenograft in animal models is expected to help personalize treatment using chemo- and targeted therapeutics.

Mouse Models in Cancer Research

Ovarian cancer is one of the most deadly cancer, ranked as the fifth most common cause of death among women in the USA. Epithelial ovarian cancer (EOC) is one of the most studies cancer known due to the fact that not too many animal models suits their progression, making ovarian cancer research using animal models very slow, unsuitable, and unreliable. Unlike many other cancer, EOC lacks clarity of study, and limiting information in terms of tumor origin stem. EOC has been more difficult to study because they are never known to have a well- defined disease flow chat and more also, researcher's inability to identify spectrum consisting the benign, invasiveness, and metastatic lesions. There exists the development of Syngeneic Mouse Models in Ovarian Cancer Research. In order to monitor the tumor metastasis or growth; mice with intact immune systems are used. The mice surface epithelial cells from C57BL/6 mice are transferred (ID8 cells), which is then injected back into C57BL/6 mice. Experimental designs using the Syngeneic mouse model has more accurately reflected the response of the immune system during tumor progression than the non-compromised models (Quinn et al. 2010).

Other application and advancements of mouse models in understanding cancer has been observed in the use of Genetically Engineered Mouse Models. Genetically Engineered Mouse Models (GEMMs) has given insight in the study of the primitive stages of some cancers that cannot be mimicked in xenografts – transplantation of living tissues or organ from a donor to the recipient. Different GEMMs gives room to evaluate how tumor arises from mutations at different anatomic locations as compared to human disease. GEMMs have helped in understanding why increased risk of developing ovarian cancer has been observed in women with familial BRCA1 mutations and develop cancer at a younger age than those with sporadic diseases (Walrath et al. 2010).

Mouse as an animal models is one of the most widely used model because of the availability of mouse sequence, easy of mouse re-engineering, well defined polymorphism information and well developed technologies to manipulate

mouse genome. Experimental mouse can now be easily assessed to provide information on specific gene and the role they perform in the progression of cancer cells while taking over expressed, under expressed and lost genes into considerations. In cancer treatment, RNA Interfering efficacy has been branded by its high effectiveness, potential and its ability of conveying silenced gene to the next generation. The induction of silencing in the advanced stages of growth, low cost compared to the other method of gene therapy and its high specificity when compared to other method of cancer therapy such as chemotherapy (Li et al. 2020).

Mouse Models are important tools for introduction and expression of genes in both cell culture and animal model system. The ability of replication-competent avian sarcoma-leukosis virus (RCAS) vectors to infect cells relies on the cell expressing the avian receptor *tv-a*has been introduced under the control of a cell lineage *tv-a* has been introduced under the control of a cell lineage specific promoter. These animals find great importance in experiments involving cell-lineage deneted infection by RCAS virus and for generating immune models of specific cancer. In order to maximally make use of the RCAS-tumor virus, a system as a high-throughput screening tool, it is necessary to efficiently and accurately clone genes into the RCAS vectors (Zhang et al. 2009). Several new mouse models have emerged for research in preclinical oncology, and great advancements has been recorded within the last decade. One novelty was the production of Non-germline genetically engineered mouse models (nGEMM), which exhibited great promise in producing transgenic mice at a low cost and with less time-consuming procedures (Lampreht Tratar et al. 2018).

Small Animal Models

Small animal models are commonly explored to examine the disease condition and viability of a disease. Small animal models are known to be quick to set-up, cost efficient, little or no complexity, and the results of such experiment are easily interpreted when compared to larger animal models. They are efficient screening protocol for novel drugs and cancer treatment development which then warrants further testing in large animal models before clinical trials. But important limitations in translational studies are identified as (i) the limited volume of bone and cartilage defects, (ii) the less thickness of the cartilage, and (iii) the high degree of flexion of those small animals and consequent partial weight-bearing condition, which are important drawbacks when compared with human conditions (da Silva Morais et al. 2018). Cancer care is predominantly depending on the success pre-clinical trials, which ascertain the safety and efficacy. However, animal models in medical research should be designed in such a way to mimic the disease environment; they must also be easily studied in vitro; they result must be reproducible and reliable, they must enable ease of treatment assessment (Schachtschneider et al. 2017).

Large Animal Models

Large animal models have very little example when compared to small animal models. It has been studied that small animal models offers great advantages compared to large animals, some of these advantages includes: well-studied genome, reduced genetic variation, fast generation time, ease of maintenance and handling, and cost effectiveness. Large animal models on the contrary offer a more anatomically similar experimental system where drug delivery and development to develop can be assessed. Dogs possess an experimental system allowing the examination of spontaneous development of tumors. Using these animals provides a comparative tumor study with human which is enabled due to the similar biological setup with human and biodiversity among dogs. Soft tissue sarcomas (STS), mammary, lung, oral carcinoma, as well as oral melanoma are common tumors associated with dogs. Dogs are the best of all the canines because of their similarities with human variants. Canines also provides special characteristics analogue to that observed in humans, this severs as an advantage to scientists to explore these models to recognize important genetic variation and specific gene mutations as observed in humans. Experimental system of canines has accelerated the rate of cancer drug discovery and developments (Schachtschneider et al. 2017).

Animal models have been used to study some cancer disease condition. Some the conditions cancer progression can be chemical induced, biologically induced while some of them can happen spontaneously. Animal models have been used to study transmission mechanisms of many viral induced cancers using mice. Example is found in the injection of cell free filtrate of leukemia-spleen homogenates into mice which then help to assess many cancer parameters such as inhibition potential, weight gain, enumerations of viable viral cells and elongation of survival time all which can also be monitored. The transmission of Rous sarcoma can be scientifically monitored through grafting of tumor fragments or injecting cell-free material from tumor homogenates into animal experimental systems such as mice (Kemp 2015). Some animal models with natural incidence of cancer have also been found to be a good experimental system playing some key roles in the study of some cancer cells. These models are explored because they closely mimic the clinical manifestation with great resemblance to human cancer both in progression and infection mechanisms. Although, some limitations arise with these type of animal models. (Alcoser and Hollingshead 2011).

Organ Site in Specific Animal Models

Some specific organ sites have been explored for screening and development of chemopreventive agents. In mammary cancer models, rats' models have been developed to serve as template for studying potential mammary cancer in methyl nitrosourea (MNU) induced mammary gland carcinogenesis. Practically, induced rats have been known to have high success rate in the study of animal tumors. In this model, female rat will be given single dose of intravenous injection of 50 mg MNU /kg per body weight, where

the pH is kept at 5.0. The chemotherapeutic agents will then be administered 5 days after the tumor induction. The result of such will give rise to tumor that are similar to well differentiated estrogen receptor human breast adenocarcinomas in terms of both gene expression and histology. It is important to note that this models are susceptible to manipulations which can modulate human estrogen receptor cancers (Russo et al. 1998). More than a decade ago, division of cancer prevention have reported with evidences on how animal models can be used to study aromatase inhibitors. Furthermore, mammary cancer models in rats have also been used to study hormonal impairment when chemo-preventive agents are being administered.

Mice has been used in the study of lung adenomas, especially those with protein error resulting from mutation. Mouse Lung Adenoma Model, female mice of about 5 to 6 weeks old will be administered with intraperitoneal (i.p.) dose of 100 mg B (a)P/kg body weight. The experimental animal will then be fed for more than 2 weeks to allow proper development of pulmonary tumors. From research, about 8 to 10 tumors develop per animals. After the onset of pulmonary tumors, chemo preventive drugs can then be administered through diet, gavage, and aerosol (Wattenberg et al. 2000).

Developing models for prostate cancer is not as easy unlike that of the colon, skin, breast and lungs. Prostate cancer prevention have been well studied using the Bosland models (Bosland et al. 1983). The Bosland models use MNU testosterone treated rats, following the treatments, the rat will develop many microscopic cancer in their dorsolateral prostate with high level of incidence. This model has allowed the evaluation and detection of the potency or activities of chemo-preventive drugs, such drugs includes: anti-androgen, prasterone, and retinoids (McCormick et al. 2007). Since prostate cancer has longer latency periods, the Bosland model is expensive, time consuming, and enquires a lot of test reagents. Aside from Bosland models, mouse prostate models have also been used to explore the activities of prostate chemotherapy. One of the widely used mouse prostate model is the transgenic adenocarcinoma of the mouse prostate (TRAMP) model which hires a probasin promoter; a second model uses C3(1)/T-antigen to mark the prostate. One distinctive features of these models is that the tumors develops fairly and rapidly unlike most human prostate tumors (Boocock et al. 2007).

Conclusion

Animal models are necessary for a better understanding of cancer progressions. The use of animal models in biomedical science for cancer research present a clearer knowledge of cancer initiation, progressions, and the development of novel therapeutics. Animal models have been utilized greatly to assess safeness, efficacy of chemical compound or natural products having anti-cancer potentials. Experimental setup provided by animal models gives a substitute means of determining the cause of onset of cancer and well-structured treatment. Animal models all enhance manipulation, induced mutations, and modelling in a manner which are all in possible to perform in patients.

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Stem Cell-Derived Exosomes: A Promising Therapeutic Role in Animal Models with Colorectal Cancer

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Abstract

Colorectal cancer (CRC), one of the most common afflicted cancers in both men and women worldwide, has caused a significant mortality rate. Distant metastases and recurrence are the two primary factors that contribute to patient death. Recent information has confirmed the cancer stem cells (CSCs) play a critical role in the development of cancer. CSCs are subpopulation of cancer cells with the ability to differentiate in several directions, proliferate indefinitely, and self-renew itself. CSCs play a critical role in the progress and recurrence of CRC. CSC-focused research has been conducted on a variety of malignancies, including CRC. This has led to the discovery and understanding of genes involved in the generation and maintenance of stem cells pluripotency as well as markers for CSCs, including those specifically investigated in CRC. The effectiveness of the current treatment against this malignancy, especially in instances of advanced CRC, is still lacking despite the adoption of new drugs and extensive therapies. Explicit understanding of mechanisms and factors that contribute to metastasis in CRC may be useful for identifying specific therapeutic targets to improve existing therapy. In recent decades, new treatment options for cancer have been emerged as a result of the increasing acceptance of the cancer stem cell theory. Exosomes are nanosized vesicles that are released by all cells, and they are now the focus of cancer therapy. Colonic mesenchymal stem cells (MSCs) experience morphological and functional changes as a consequence of CRC-derived exosomes, which aid in the growth of tumors and the progression of their malignant nature. Exosomes have been used as a delivery system for anticancer drugs in CRC. This review aims to summarize recent findings up to date several animal-based studies using exosomes derived from stem cells in CRC.

Keywords

Exosomes · Noncoding RNA · Stem cells · Angiogenesis · Colorectal cancer

Introduction

The colorectal cancer (CRC) is the third most commonly diagnosed malignancy in the worldwide; it is the second largest cause of cancer death (Navarro et al. 2017). At the time of diagnosis, a vast majority of people are in stage IV illness (i.e., advanced or metastatic tumors) and can only receive palliative care. The primary goals of CRC

screening are to remove adenomas and sessile-serrated lesions (SSL), as well as to identify early-stage CRC. According to one projection, approximately 147,950 new CRC cases would have been discovered in 2020, with 53,200 people dying from the disease (Siegel et al. 2020). Most CRCs progress through the adenoma-carcinoma cycle, which allows cancer prevention by removing precursor lesions and detecting CRC in its early, curable stages. Adenomatous polyps produce ~70% of spontaneous CRCs, and SSL causes the remaining 25–30% along the SSL-to-carcinoma pathway. Endoscopic polypectomy and surgery have lessened the mortality rates in CRC. These data suggest that the threat among younger generation is higher in the near future.

The cancer hallmarks shared by CRC and other cancers include their characteristic features of proliferation. As researchers investigated the origins and spread of these characteristics, or "hallmarks," it was evident that extracellular vesicles (EVs) play a major role in most cancers (Al-Sowayan et al. 2020). Cells produce spherical particles known as EVs and were identified in the late 1940s. Over 40 years later to the discovery of EVs, exosomes were identified originally as a distinct subtype of EVs.

The distinct cell population, in the 1990s, was first addressed as cancer stem cell (CSC) which is now recognized as a distinct population of cancer cells due to the increased interest and study (Lapidot et al. 1994). The CSC model, in conjunction with the clonal evolution model, is acknowledged widely as one of the most prominent cancer models. The clonal evolution model was first published in the 1970s, postulating that mutations in a specific set of somatic cell populations result in cancer. Moreover, one of the most interesting developments in the world of biomedicine has been the discovery of the therapeutic potential of stem cells. Decades of studies on stem cell biology have increased our understanding with regard to the molecular pathways that stem cells use to repair and regenerate tissues. Pluripotent and multipotent stem cells can restore tissue loss caused by degenerative disorders, traumas, or age. There is a significant proof that stem cells have a therapeutic impact via secreting soluble substances and producing exosomes (Lai et al. 2010).

Exosomes, which have shown considerable promise in a range of disorders, are also secreted in enormous quantities by stem cells. The interchange of biological materials via exosomes appears to be a key component in vesicle trafficking and signal transduction between normal and cancer cells. However, the role of exosomes generated by stem cells in tumor pathogenesis was unclear until recently (Wu et al. 2017).

Biogenesis of Exosomes

EVs are lipid bilayer-delimited particles that have been discovered as a new intercellular communication mediator. In the past decade, EVs have been divided into categories based on their size, content, and creation mechanism (Colombo et al. 2013). Apoptotic bodies (500 nm to 2 μm) are formed by bubbling of plasma membrane, microvesicles (MVs; 100–1000 nm) are formed by sprouting, and exosomes (30–150 nm) are released from the plasma membrane.

Exosomes are formed as a result of the endocytic process, including the formation of early secretory endosomes after cytoplasmic membrane invagination.

Subsequently, intraluminal vesicles are formed, leading to the formation of multivesicular bodies and the maturation of endosomes through acidification, with the ultimate release of exosomes. Endosomal sorting complex (ESCRT-0-III) has complexes that aid in EV biogenesis along with other related proteins (Colombo et al. 2013). These complexes along with other proteins aid in EV biogenesis, bud formation, and the release of EV from the cell. In addition, other external routes have been discovered to change the existing production (Dai et al. 2020).

Although the recent reports on ESCRT-independent pathway of exosomal biosynthesis have found to be associated with lipids and heat shock proteins sorting EV cellular material, both ESCRT-dependent and ESCRT-independent mechanisms are essential for its proper function (Wei et al. 2021).

Exosome Classification

Exosomes are often categorized based on their origin tissue or cell, but they can also be classified on the basis of biological concentration. The most frequent cell-specific exosomal proteins, such as adhesion molecules, integrins, MHC I and II, transferrin receptors, and other surface binding exosomes, along with non-specific protein exosomes, such as fusion and transportation proteins, heat shock proteins, and cytoskeleton proteins, contribute to the formation of multivesicular bodies and are found in the plasma membranes of the cell (Mashouri et al. 2019). These proteins are found mostly in the plasma membranes of the cell and are absent in the nucleus, mitochondria, or endoplasmic reticulum (Mashouri et al. 2019).

There are two types of exosomes: natural exosomes (derived from plants and animals) and synthetic exosomes. Exosomes in animals are further divided into normal exosomes and tumor exosomes or oncosomes, as exosomes are produced in both normal and pathological or tumor situations (Endzeliņš et al. 2017). Mesenchymal stem cells and immune cells release natural stem cells and play an important role in disease genesis as well as in tissue damage and repair, and they have intriguing therapeutic promise for cardiovascular disease (CVD) and a variety of neurological illnesses.

Normal exosomes can be discovered in biological fluids, such as saliva, plasma, urine, milk, bile, and ascites, and serve for therapeutic purposes (Cheng et al. 2018). Tumor exosomes not only are associated with tumor formation and metastasis but also help in the diagnosis of disease conditions, by acting as “diagnostic markers.”

Exosome Composition

Exosomes are lipid bilayer membranes produced by all cells, as reported initially in 1981 (Trams et al. 1981). Exosomes are produced by all cells detected in high concentrations in bodily fluids.

Exosomes contain proteins, RNAs, DNA, lipids, and other substances (Mathivanan et al. 2012). The “proteomic” investigations on dendritic exosomes

were undertaken in the late 1990s (Théry et al. 1999). Exosome molecular composition is influenced by donor cell types, epigenetic modifications, and diverse physiological and pathological environmental circumstances.

The most abundantly found exosomal components include lipid components, protein components, nucleic acids, and other substances that enable exosome stiffness, endocytosis, membrane trafficking, and signaling.

According to proteomic studies, exosomes play a range of tasks (Welton et al. 2010). Exosome protein components (CD9, CD81) that participate in cellular interactions on the exosome surface, exosome biogenesis components (e.g., ESCRT), and membrane fusion proteins all play key roles in human tissue healing (Qiu et al. 2019). mRNA, miRNA, transfer RNA, and ribosomal RNA transmit genetic information to the recipient cells, thereby influencing protein synthesis and biological activity (Konala et al. 2016). Unquestionably, miRNAs are crucial exosome contents due to their ability to trigger a wide range of activities in diverse groups.

Exosome Components

The most recent revision list shows that exosomes contain 9769 proteins (Xie et al. 2019). Exosome contents can be employed as prognostic indicators and/or as a grading system for the development of cancer. In tumor cells, exosomes also promote tumor development, metastasis, and angiogenesis, as well as mediating treatment resistance.

Exosomal Proteins

The EVs are composed of several components with exosomal protein as one of them. The EV protein of a signaling molecule family is known for their ubiquitination, but its ability to push proteins into exosomes is being debated till date (Moreno-Gonzalo et al. 2018).

Exosomal proteins are composed of a combination of membrane proteins, such as Ras-related protein GTPase (Rab), heat shock proteins (HSPs), and fusion proteins, that include four transmembrane cross-linked proteins, integrins, actin, and myosin for cytoskeletal development (Xu et al. 2018).

Moreover, CD9 facilitates the entry of the metalloproteinase CD10 into exosomes, whereas four transmembrane cross-linked proteins facilitate cargo access into exosomes. ALIX and TSG101 categorize these cargos further by, subsequently, arranging them on the plasma membrane as exosome components (Juan and Fürthauer 2018).

HSPs and intracellular proteins maintain appropriate protein folding and function, leading to anti-apoptotic activities (Taha et al. 2019). As tumor cells are under stress continually, such as hypoxia, acidosis, metabolic, and food shortage, Hsp90 production is high in many cancer cells. Hsp90 is implicated in the promotion of tumor development and progression as well as being linked to poor tumor prognosis. In a

recent study, one of the HSP was found to be responsible for exosomal formation and release. Exosomes containing Hsp90 would be unable to carry out important signal transduction between tumor and normal cells (Tang et al. 2019).

Noncoding Exosomal RNAs

MicroRNA

Exosomal miRNAs are noncoding RNAs that have been used as cancer markers during the development of cancer, tumor development, recurrence, and poor prognosis. Exosomal miRNAs in the plasma of lung cancer patients have been associated with poor prognosis (Endzeliņš et al. 2017). Plasma EVs were decreased considerably in a study on prostate cancer, where miR-1290 and miR-375 were shown to predict overall survival in prostate cancer patients with 10–80% mortality rate (Huang et al. 2015).

A study based on exosomes released by cancer cells and parental cells was identified and analyzed, revealing that six miRNAs were upregulated and five miRNAs were downregulated (Sun et al. 2017).

Exosomes from biological fluids can also be used as noninvasive new biomarkers. Exosome-derived miRNAs from biological fluids, including miR-21, miR-26, miR-122, and miR-150, are used as noninvasive indicators in cancer. Hu et al. (Puik et al. 2017) highlighted exosomal miRNAs as predictive indicators in alveolar cancer research, implying that exosomal miRNA might be used as a diagnostic biomarker for non-small cell lung cancer. The study on exosomal miR-126 also suggested its potential as a biomarker for lung cancer. Exosomal miRNA-23a levels have been shown to be elevated in the sera of lung cancer patients, and miRNA-23a levels are connected with proangiogenic activities positively, suggesting that miRNA-23a might be employed as a biomarker for lung cancer diagnosis. Exosomal miR- 222-3p might be used as a predictive biomarker for gemcitabine sensitivity, and exosomal miR-208a could be used as a predictive biomarker for radiation responses (Tang et al. 2016).

Exosome-bound miRNAs, such as miR-9, may further contribute to tumor development by enhancing tumor cell motility (Baroni et al. 2016). miRNA-mediated Ca^{2+} receptor instability led by tumor exosomes also results in tumor growth. miRNAs derived from mesenchymal stem cells (MSCs) are found to be transported directly to tumor cells, thereby promoting cancer progression and resistance to treatment (Roccaro et al. 2013).

lncRNA

Long noncoding RNAs (lncRNAs) are packed into exosomes selectively and work as the transmitter in cell signaling, regulating tumor development, invasion, and vasculature. The regulatory RNA packed into exosomes leads to cellular signaling

while also changing the tumor microenvironment (TME) (Hewson and Morris 2016). For example, in CRC, lncRNAs are upregulated leading to the epithelial-to-mesenchymal transition (EMT) progression followed by its angiogenesis and tumor resistance due to abnormal cancer signaling pathway mechanism.

Conigliaro et al. (Zhou et al. 2018) reported the enhanced vasculature in human umbilical vein endothelial cells (HUVECs) upon the action of lncRNA H19. Similarly, the role of lncRNA in tumor resistance has been reported in bladder cancer, wherein lncRNA was found to inhibit the effect of cisplatin due to abnormal cancer signaling pathway mechanism. Therefore, it serves as a possible target for bladder cancer resistance (Deng et al. 2016).

Circular RNA (circRNA)

Exosomal circRNAs are a class of RNAs present in all eukaryotes. They are produced mainly for alternative splicing (Chen et al. 2017). By reverse splicing, circRNAs that provide enzyme resistance and degrade nucleic acids are produced. These circRNAs compete with miRNA, preventing miRNA from binding to mRNA.

circRNAs play an essential biological role in cancer by acting as the gene and miRNA expression regulators and could have involvement in the cancer biological processes, such as carcinogenesis, growth, migration, and progression (Zhou et al. 2018). The expression of circRNAs was shown to be higher in plasma exosomes in one study, which was linked to increased endothelial permeability and fast tumor dissemination and metastasis. Furthermore, an increase in circDLEU2 increased leukemia cell proliferation in vitro, reduced cell apoptosis, and accelerated tumor development in vivo (Li et al. 2018).

In conclusion, due to their great stability, circRNAs may be identified noninvasively in physiological fluids. For example, circCNOT2 has been shown to be detectable in plasma, which can be used as a biomarker to select the best therapeutic method for nonsurgical progression monitoring (Smid et al. 2019).

Exosomal Source, Uptake, and Function

Endosome-derived vesicles, which have been proven to play a key role in cell-to-cell communication, are used to make exosomes. Previously, exosomes were assumed to be little rubbish sacs spat out by cells, and their true function was unclear. Recent research has indicated that exosomes, which operate as antigen-presenting vesicles, might boost immune responses and tumorigenesis (Muralikumar et al. 2021).

Mostly, exosomes are ingested by target cells by means of fusion, receptor-mediated endocytosis, and phagocytosis (Daßler-Plenker et al. 2020). According to a study (Daßler-Plenker et al. 2020), cells take up exosomes with smaller diameters. The kind and physiological state of the receiving cells, as well as the nature of the exosomes, impact exosome uptake. Exosome uptake is influenced by

the kind and physiological condition of the receiving cells as well as the nature of the exosomes.

Exosome-mediated intercellular communication has piqued researchers' curiosity in recent years. Exosome cargo is shared between exosomes and target cells in various ways, according to previous studies (Zhou et al. 2018; Daßler-Plenker et al. 2020). Exosomes can be taken up by a target cell by direct fusion with the plasma membrane, a receptor-ligand contact, or endocytosis by phagocytosis after they have been released. This process is aided by using various biological substances. Exosomal HSP-70 initiates a pathway that allows cardioprotective signals to be sent to the heart. T-cell molecules also regulate exosome uptake (Zhang et al. 2015). After being picked up by target cells, exosomes perform a crucial function in cells with intercellular communication and cargo transfer as key function. The role of exosomal uptake in the development and progression of hepatic diseases, cancer, and neurological diseases has already been reported. Exosomes have been linked to immune activation and tolerization, and research data suggested that exosomes may be used in immunotherapy. During a normal pregnancy, placental exosomes decrease immunity.

The role of exosomes in breast-feeding has been linked to immune activation and tolerization. Exosomes could also be used in immunotherapy, fetal immune development, and placental exosomes lower immunity during a normal pregnancy (De Toro et al. 2015). Furthermore, exosomes contain proteins that have the potential to be therapeutic or diagnostic in neurodegenerative disorders, cardiovascular diseases, and infection biology processes, by regulating the immune response to infectious diseases (De Toro et al. 2015). Exosomes from tumors influence the creation of new blood vessels, which aid in tumor angiogenesis, and also play a function in tumor cell proliferation. Exosomes control metastasis in cancer by stimulating the pre-metastatic niche (Miller and Grunewald 2015). Pathways involved in exosomal regulation also play an essential role in cancer cell drug resistance property (Miller and Grunewald 2015). Exosomes from varied origins may increase the immune response; however, some has a major impact on cancers' capacity to avoid immune monitoring (Miller and Grunewald 2015). There has been various research on exosome-based cancer detection and therapies, aside from their roles in cancer etiology. A previous research (De Toro et al. 2015) has investigated the roles of exosomes in addition to the ones described above; nevertheless, it is still unknown which precise class of chemicals present in exosomes that affect the target cells.

Exosome Isolation

A previous study has shown difficulties in the therapeutic use of exosomes due to the lack of a standard technique for extracting exosomes from other vesicles and identifying particular exosome subsets among diverse purification procedures (Gardiner et al. 2016). Exosomes can be found in both healthy and diseased individuals (Mimeault and Batra 2014). Exosomes are separated based on traditional separation methods based on centrifugal force, sedimentation, and filtration (Li et al. 2017).

Some developing and transdisciplinary methods for exosomal preparations have been used to improve the purity and efficiency of exosome separation. One of them is microfluidics-based exosome separation systems, which use the exosomes' physical and biological features at microscales. Innovative sorting processes, such as acoustic and electromagnetic manipulation, have been developed in addition to known approaches. Acquiring exosomes will be much easier with these separation platforms, and the isolation procedure will be shortened drastically.

Techniques based on active and passive microfluidics have been classified thoroughly, with the former relying significantly on external forces and the latter heavily on hydrodynamic and surface forces.

Exosome Preservation and Storage

In general, exosomes are kept and stored at a temperature of −29 °C. When stored at −80 °C or in liquid nitrogen with anti-freezing compounds, the formation of ice crystals inside exosomes has been avoided with increased storage life (Bahr et al. 2020). If exosomes are studied soon after isolation, the whole protein composition as well as a representative functional analysis will be retained. Exosome extraction from conditioned culture medium has been reported to be simpler, and the whole method appears to be easy; even so, due to their comparable size and the absence of cell-specific biomarkers, various kinds of EVs are co-isolated frequently.

Exosome Characterization

The surface of the exosomes is marked by various unique markers among the 9769 proteins discovered in exosomes from diverse sources. According to research, all MSC exosomes display the markers, CD55 and CD59 (Nakamura et al. 2015). These markers inhibit opsonin and coagulation factor activation, thereby stabilizing extracellular vesicle dispersion in physiological by using antibody-coated magnetic beads targeting the protein on the membrane surface (Zhu et al. 2020).

Stem Cells

Stem cells are precursor cells obtained from the inner cell mass of human embryo. Recently, stem cells have been shown to grow into cardiomyocytes as well (Kehat et al. 2001). Although it is possible to extract these cells, following cardioimplantation, due to ethical concern, immune rejection, and other challenges, its implementation is confined to cell culture experiments only (Zhang et al. 2002).

On the other hand, adult human stem cells (hematopoietic and mesenchymal) are present in mature tissues. The versatility of these cells creates other cell lineages unrelated to their initial organ of origin. As a result, these cells can be used to regenerate organs and repair cells in a range of species, including humans.

As far as mesenchymal cells are concerned, they can be derived from adults and can also be found in the human body fluids; therefore, there are no ethical concerns, but further study is needed (Zhang et al. 2002).

Aside from hematopoietic and mesenchymal stem cells, numerous other bone marrow-related cell types have been discovered to contribute to organ repair in myocardial models; hematopoietic stem cells (hemangioblasts) contribute to vascular structures; mesodermal progenitor cells are found in the mononuclear bone marrow cell fraction that matures to endothelial cells; and endothelial progenitor cells can transdifferentiate into stem cell factor and granulocyte colony-stimulating factor to stimulate primitive bone marrow cells, allowing them to migrate to infarct sites, replicate, differentiate, and contribute in heart repair (Orlic et al. 2001).

MSCs are found, most commonly, in bone marrow, cord blood, liver, dental pulp, and fatty tissues (Bieback and Klüter 2007). MSCs possess migratory and regenerative abilities; these abilities help in adhering to the in vitro conditions of the culture containers. MSCs secrete growth factors that have an autocrine and paracrine effect on the surrounding microenvironment, resulting in reduced inflammation, angiogenesis, tissue repair, and immune function suppression. The role of MSCs in regenerative medicine and cancer therapy responsible for the repair is a result of active bone marrow-derived native cells. Exosomal stem cells from MSCs cause tumorigenesis due to cell signaling. Even after all this time, the underlying mechanism of the MSC-tumor correlation remains unknown, but many studies state that interaction is due to MSC exosomes (Akyurekli et al. 2015). Therefore, exosomes are concluded to play an important role in the mechanism of signal transduction.

In addition to this, CSCs are found to be important regulators of cancer relapse due to the transport of genetic information between cells (Takebe et al. 2015). Considering the stem cell-like properties and their role in tumorigenesis, CSCs and CSC-derived exosomes serve to stimulate tumor signaling and cancer progression (Hannafon and Ding 2015).

Colorectal Cancer Stem Cells

CSCs are tumor cells that exhibit self-renewal, unrestricted proliferation and specialize in several orientations. CSCs make up a small percentage of cancer patients, yet they are linked to tumor metastasis, medication resistance, and recurrence after first therapy (Shen and Cao 2012). Traditional medicines and radiation may ease the symptoms of solid tumors, but they do not eliminate CSCs. Following therapy intervention, these CSCs may become latent and, eventually, become a cause of cancer recurrence.

The origin of CSCs is based on the formation of progenitor cells as a result of genetic alterations or genetic process dysregulation. Cells with epithelial features, such as EpCAM ^{high}/CD44^{+}, have been found in CRC and exhibit stem cell-like properties, suggesting that CSCs are formed from epithelial cells (Dalerba et al. 2007). With sufficient gene alterations, CRC CSCs are derived from developed intestinal epithelium causing tumorigenesis.

Colorectal CSCs, similar to other stem cells, exhibit self-renewal ability with dysregulated Wnt, Notch, and Hedgehog pathways, leading to tumorigenicity and drug resistance (Cleophas et al. 2017). CSCs from CRC have several characteristics similar to intestinal stem cells.

A set of surface indicators is used to identify the colorectal CSCs with CD44 as the most common CSC marker. Oct-4, CD51, CD24, CD26, and CD29 are among the more widespread CSC markers (Dalerba et al. 2007). These compounds are active physiologically in addition to being surface markers.

Stem Cell-Derived Exosomes

With a better knowledge of stem cell role in tissue regeneration, accumulating data suggests that exosomes generated by stem cells are responsible predominantly for the favorable effects. Owing to their capacity to transmit proteins, genetic information, and various chemicals to target cells, these 40–100 nm EVs from cells are regarded as significant participants in intercellular communication (Fig. 1). Exosomes provide researchers a fresh technique to enhance tissue regeneration, including bone repair (Hao et al. 2017).

Exosomes from MSCs Generated from Adult Bone Marrow

Adult stem cells known as mesenchymal stem cells (MSCs) can be found in body tissues and have immense potential in therapies. They are developed to treat several

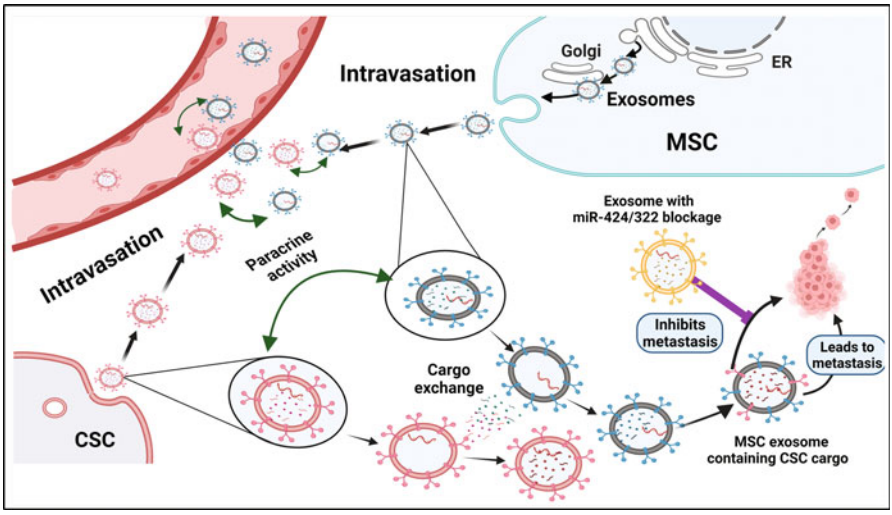

Fig. 1 Exosomes derived from stem cells and have multiple functional roles in cancer invasion, progression, and metastasis

diseases. MSCs produce EVs, which aid in tissue regeneration and immunomodulation (Setti et al. 2015), as well as paracrine chemicals, such as cytokines and growth hormones.

MSC Exosomes Generated from the Umbilical Cord

Umbilical cord (UC) exosomes are used mainly in healing wound. Increased angiogenesis via the Wnt/β-catenin pathway is thought to be a plausible mechanism for such beneficial regulation of these exosomes. Several investigations have indicated the advantage of studies in animal models in severe liver failure, heart attack, and autoimmune uveitis (Setti et al. 2015). Although UC exosomes are still in their early phase, their translational potential against a wide range of disorders is highlighted.

Exosomes Produced by MSCs Generated from Adipose Tissue

MSCs and exosomes produced from adipose tissue (AT) have been used mostly to treat skin ailments. Exosomes generated from AT have been used to treat several conditions, including nervous system disorders. The role of adipose tissue-derived exosomes in wound healing is due to the release of certain hormones and cytokines. AT MSC-derived exosomes have been shown, in studies, to reduce ovarian cancer cell proliferation and to be effective in restoring oxidative stress-induced apoptosis in cardiomyocytes (Liu et al. 2019).

Tumor and Stem Cell-Derived Exosomes

Natural MSC exosomes enhance tumor growth, according to a research review (Zhang and Yu 2019). On the other hand, MSC exosomes have been demonstrated to delay the onset of cancer and its development.

Angiogenesis

Angiogenesis that is abnormal or excessive during cancer development may aggravate the illness. MSC exosomes can improve cell-to-cell interaction and stimulate angiogenesis in the TME by improving the tubing capacity of endothelial cells (ECs). In addition, microRNAs play a major part in angiogenesis. miR-30b is purposed to promote directly the tube-like shape in vitro (Gong et al. 2017).

Growth of Tumors

Kalimuthu et al. (2016) demonstrated the apoptotic effect of MSC exosomes. Subsequently, Huang et al. (2020) verified that MSC-derived EV and exosomes

produced in vitro from bone marrow promote apoptosis and tumorigenesis in osteosarcoma.

Invasion and Metastasis

The whole tumor microenvironment, as well as the cancer cells themselves, influences tumor spread and invasion. Exosomes play an important function in the information transmission as part of the tumor microenvironment. In a study (Gu et al. 2016), it was reported that human cord exosomes obtained upon the activation of Akt pathway could aid in the proliferation and migration of GC. Another study (Sandiford et al. 2021) based on MSC exosomes demonstrated that exosomes from human bone marrow remain latent as CSCs for decades.

Role of Stem Cell-Derived Exosomes and CRC

CRC is the leading cause of mortality worldwide, characterized by the lymphocytic infiltration, which is caused by epithelial cells that form inside the gastrointestinal system's colon/rectum lining. The treatment of mutant RAS kinases, which are unresponsive usually to the existing therapy, is one of the most important unmet needs in CRC. Small interfering RNAs (siRNAs) targeting particular KRAS point mutations have antitumor effects in cancer and, probably, will be transformed into CRC models immediately (Banerjee et al. 2021).

Bone marrow-derived MSC (BM-MCSs) and exosomal miR-16-5p were explored by Yan et al. (Xu et al. 2019) in CRC, wherein exosomes generated from BM-MCSs that overexpress mir-16-5p were found to inhibit tumor development in vivo. In addition, exosomes produced from BM-MCSs inhibited CRC formation by upregulating mir-16-5p and downregulating ITGA2 (Xu et al. 2019). To guide the diagnosis and therapy of CRC, there is still a long way to go.

Stem Cell-Derived Exosome Role in Animal Models with Colorectal Cancer

Colorectal immunotherapy has been a regular treatment option for individuals with advanced illnesses when chemo/radiotherapy has reached its limit in recent years. DNA mismatch repair defects (dMMR) have been observed in CRCs previously, resulting in hypermethylation in the MLH1 gene promoter region (Kuismanen et al. 2000). This event resulted in an increase in high tumor mutational burden (TMB) and changed microsatellite sequences, resulting in high microsatellite instability status (MSI-H) in these malignancies. As a result, TMB and MSI status have been used to assess and select the right patients for immunotherapy. Significantly, researchers are looking at how EVs produced from immune cells during colorectal immunotherapy might influence the immune response and encourage immune

system reprogramming. Overall, EVs contribute to connecting the TME and local immune responses.

CRC development has been found to be aided by EVs generated by cancer cells, although the regulatory mechanism remains unclear. According to tumor-bearing nude mice trials, HCT116 cell-derived EVs transferred miR-25 and decreased SIRT6, thereby resulting in an increase in tumor development and metastasis, which play a role in CRC development and metastasis.

The injection of colon cancer EVs into the tail veins of NOD-SCID mice resulted in neoplastic transformation and metastases in the mice's lungs, proving the theory for the first time that malignant epithelial cancer characteristics may be transferred to distant target cells in vivo models. These models justify horizontal transmission of malignant features and support the idea that metastatic illness might be transmitted by circulating the genetic material. By inducing an anticancer immune response, modified EVs with miR-424/322 blockage reduced tumor start in a CRC animal model (Fig. 2). Understanding the biodistribution of EVs in vivo is one of the most important components of employing them as a therapeutic and/or a medication delivery method. The biodistribution pattern of EVs delivered intravenously to the animal was similar to that of previously published research using the same injection route (Choi et al. 2021).

Researchers employed various animal models to investigate the carcinogenic potential of the circulating tumor cells (CTCs). Morikawa et al. (1988) acquired metastatic cells from patients with colon carcinomas (CCs), in 1988, and converted them to cell culture. The separated cells were also implanted into several nude mice's

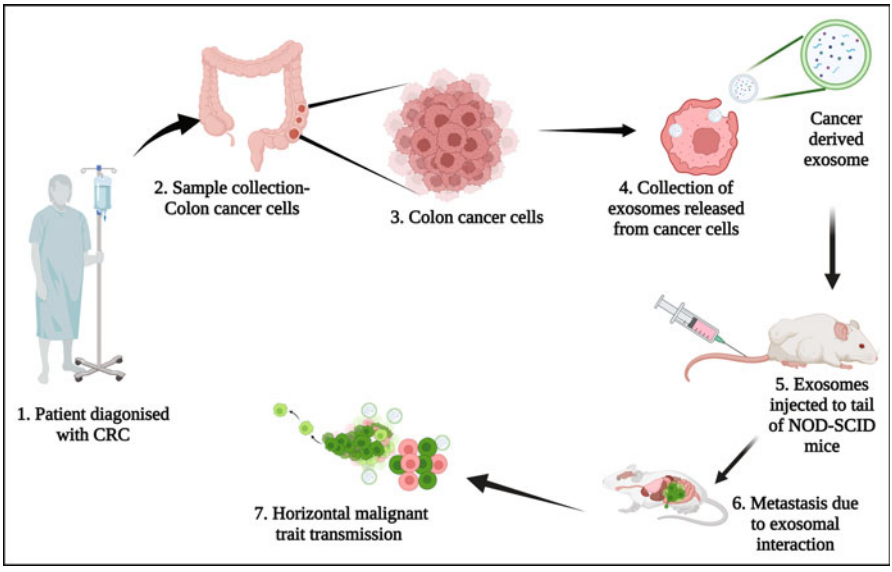

Fig. 2 Human CRC-derived exosomes are utilized for the development of animal model cancer metastasis

organs. After a few weeks, some of the animals had 1–5 liver tumor colonies in several organs, including the spleen and cecum. The nude mouse may be utilized to identify and grow metastatic cells from CCs, according to their preliminary findings.

Schülter et al. (2006) published a research work in 2006 and have demonstrated the unprecedented association between the production of colorectal metastases in certain organs and the relationship between CTCs and the endothelium of specific target organs' arteries. CTCs were detected within the pulmonary microcirculation, and cell adhesions were found regardless of size, according to their results. Regardless of metastatic capacity, all cell lines showed elevated adhesion rates, mostly in the liver and lungs and seldom in other organs. In another study, Kang et al. (Kang and Pantel 2013) offered genetically modified mouse models as alternatives to human cancer xenografts. These mice were used to assess the efficiency of investigational medications, including angiogenesis inhibitors and matrix metalloproteinase inhibitors. If the cells were tagged with molecular markers, the metastatic process in these models could be tracked. These animal models, as they are known, replicate the complicated condition of neoplasia in people in many ways. Local tumors were created by inserting human CRC cell lines orthotopically into the cecum of an immunodeficient mice, which resulted in distant metastases and CTCs with great repeatability. In their mouse model, CTCs did not overexpress CD47 or downregulate calreticulin. The generated tumors matched closely with their human counterparts and grew in immunocompetent hosts, according to the researchers. As a result, this mouse model resembled the equivalent human cancer.

Schölch et al. (Riau et al. 2019) published a study in 2016 and found a link between metastases and CTCs in an orthotopic mice model of CRC. They were able to extract and cultivate the resultant CTCs in vitro, and reinjected CTCs into mice showed their tumor-forming capacity. CTCs had lower levels of genes linked with cell–cell adhesion, indicating that they had a more metastatic activity than bulk tumor cells from hepatic metastases. DLG7 and BMI1 are stem cell markers that were elevated highly in CTC, leading to an accelerated tumor development and self-renewal potential.

The extraction of CTCs from orthotopic animal model led to establish tumorigenicity in cell line and animal model, followed by its qPCR expression investigation that had lower cell–to-cell adhesion. Furthermore, research was conducted to investigate the functions of MSC exosomes in the advancement of CRC, wherein a transforming growth factor-β1 (TGF-β1) antagonist was found to inhibit the functions of ITGA6. CRC cell invasion and EMT were reported to be reduced upon TGF-β1 inactivation. Tumor-derived exosomes promoted the differentiation of monocytes into M2-tumor-associated macrophages in animal models.

In another study (Huang and Feng 2017), CT26 tumor-derived exosomes were delivered into tumor-bearing BALB/c mice subcutaneously in an efficient manner, thereby inhibiting tumor development and increasing cytotoxic T-lymphocyte infiltration in the tumor tissue. The findings imply that antitumor responses were induced by a considerable reduction in the number of T-regulatory cells and upregulation of the interferon gene. The advantages of tumor-derived exosomes, such as their ability to act as antigen cargo and carry particular cancer antigens, have made these

nanosized particles a viable vaccination option in cancer immunotherapy. Future research should focus on a large population of tumor-bearing animal models. Upon hyperactivation of Wnt/β-catenin signaling in an animal investigation, exosomes derived from CRC cells were reported to enhance tumor growth and angiogenesis upon cell proliferation and migration (Huang and Feng 2017).

Cell culture and animal model studies based on exosomes have highlighted the role of exosomes to chemotherapy resistant-induced cell death. Furthermore, transferred exosomes also lead to the promotion of benign tumor to malignant cells as a result of cell-to-cell contact (Dallas et al. 2009).

Conclusion

Exosome cargoes have been associated with various melanoma activities, including colon cancer formation, metastasis, and treatment resistance. Proteins, microRNAs, and long noncoding RNAs all play key roles in these processes. Microenvironmental factors, such as hypoxia, have a significant impact on the characteristics of exosomes along with the cells they originated from. Exosomes generated from CRC cells, on the other hand, interfere with cancer chemoresistance. The specific processes of the exosomes are unknown due to their tiny size and varied population. The particular processes of exosome formation and their various effects on CRC cells are not known fully. As per the growing evidence, exosome influences the CRC tumor initiation, development, chemoresistance, and metastasis. Several studies and human bodily fluids have resulted in its use as a potent therapeutic.

Furthermore, exosomes and their contents are recognized as being particularly useful in the noninvasive diagnosis and treatment of CRC. In scientific and clinical investigations on CRC development and therapy, exosomes, particularly their payloads, play a range of roles. Furthermore, it is unclear if exosomal biomarkers can detect CRC earlier than other current signs. The use of exosome therapy in CRC requires further research and well-designed clinical investigations separately or combined with other techniques to eradicate CRC completely. They hold promise as a therapy option, especially for people suffering from advanced CRC.

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Experimental Model for Pancreatic Cancer and Its Therapeutic Implications in Clinical Research

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Abstract

This book chapter aims to provide an overview knowledge on different animal models that has wide range of research implications in pancreatic cancer studies. Being aware that the disease is a mute assassin and the symptoms are revealed only in the later stages makes the disease condition more vulnerable to patients and gives an alarming call for sooner effective therapeutic implications. Use of animal models plays an indispensable role in research. Notable physiological and anatomical resemblance of the animal models to human system makes it crucial for research studies. Though a number of anticancer agents are discovered, an optimal animal model is necessary for determining the mechanistic pathway through which the agent acts. This, in hand, makes understanding of various risk factors and different molecular pathways through which the cancer develops essential for determining the experimental animal model for the studies. This helps the investigator to choose an appropriate animal model to conduct their study. This chapter helps the investigators to get an overview on the so far used animal models in the pancreatic cancer studies. Also the benefits, strengths, and weakness of the animal models used in different subtypes of pancreatic cancer are discussed.

Keywords

Pancreatic cancer · Animal model · Clinical application · k-Ras signaling

Introduction

Pancreas is glandular organ situated in the abdomen, stretching from behind the stomach toward the spleen, weighing up to 100 g, 14–20 cm in length (Longnecker 2014), and divided into four parts namely, head, neck, body, and tail. Pancreas plays a dual role as an exocrine and an endocrine gland that is responsible for secretion of digestive juices and enzymes and secretion of hormones, respectively. Total 95% of pancreatic mass is part of exocrine pancreas leaving only 1–2% for the endocrine action (Longnecker 2014). The exocrine part is made of lobule-like structures made of acinar cells involved in production of zymogens aiding in digestion process. The endocrine part represented by cluster of cells called the pancreatic islets or islets of Langerhans is scattered over the entire pancreas. These islets are made of three types of cells namely alpha, beta, and delta cells which are responsible for secretion of hormones, glucagon, insulin, and somatostatin, respectively, and other hormones

namely vasoactive intestinal peptides, pancreatic poly peptides. Insulin is the major hormone secreted by the islet cells. The enterochromaffin cells are also found in the islets of pancreas.

Cancer is widely characterized as uncontrolled cell differentiation and cell growth. The basic reason for this uncontrolled cell growth is either activation of the oncogenes or inactivation of the tumor suppressor genes (Sarkar et al. 2013). Cancer development in pancreas is regarded as one of the fatal diseases due to escalated malignancy and poor diagnosis (Li et al. 2020). Pancreatic cancer is graded as the 14th most common cancer and the 7th highest cause of mortality due to cancer in the world (McGuigan et al. 2018). Data suggest that pancreatic cancer remains the most lethal malignancy recorded, due to its poor prognosis and increasing incidence/mortality ratio of about 94% (Bray et al. 2018). According to American Cancer Society, 2021, statistics on the frequency of pancreatic cancer among the people of United States shows 60,430 adults will be diagnosed with pancreatic cancer of which men account for 31,950 and women account for 28,480. It also states that it is the fourth leading cause of death based on cancer. The death rate for pancreatic disease is gradually increasing every year due to trouble in diagnosis of the disease. Estimation on number of deaths on pancreatic cancer this year is calculated as 48,220 of which men account for 25.270 and women account for 22,950. In 2018, it was noted that pancreatic cancer accounts for about 2.5% of total cancers (Bray et al. 2018). The incidence of pancreatic cancer is also related to geographical locations: The highest rate is reported in men in Central and Eastern Europe regions and for women in Western Europe regions (Bray et al. 2018). However, in African regions of Comoros and Sao Tome and Principe, there were nil cases reported on pancreatic cancer development in either of gender category (Bray et al. 2018). The incidence and mortality of pancreatic cancer have been depicted as close parallel lines, reveal its severity and fatal nature of disease condition (Oberstein and Olive 2013).

Pancreatic cancer is divided into two namely, exocrine pancreatic cancer and endocrine pancreatic cancer. Exocrine cancer is diagnosed most commonly (95%) while endocrine cancer is less common (5%) of total pancreatic cancer-reported cases (Rawla 2019). There are various subtypes of exocrine and endocrine cancers based on the cellular origin of the cancer. Pancreatic adenocarcinoma, including its variants, is the most common type of pancreatic cancer accounting for approximately 90% of the total diagnosed cases (Feldmann et al. 2007). This cancer originates at the head of the pancreas (~60–70%) and grows in the tail and body region (~15%). Specific symptoms are expressed only in final metastatic stages (Luchini et al. 2016). Pancreatic adenocarcinoma occurs in a gradual step-wise manner creating mutations in normal pancreatic mucosa. There are three well-recognized characteristic precursors for development of adenocarcinoma, namely pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN) (Esposito et al. 2014).

Pancreatic intraepithelial neoplasia is a noninvasive condition which is characterized by microscopic lesions not more than 0.5 cm in pancreatic ducts. It is also suggested that PanIN lesions may play a basal role in the development of the pancreatitis which can result in local injury which gradually leads to neoplasm

(Brune et al. 2006). Based on the morphological changes occurring in the tumor cells, PanIN is categorized into three stages (PanIN-1a/1b, PanIN-2, and PanIN-3), depicting the progression of the disease's severity (Hruban et al. 2001). A suggestive fact shows about 1.5% men and 1.3% women with PanIN are identified to develop pancreatic cancer from PanIN-3 within 11.3 to 12.3 years, respectively. At initial stage of PanIN, there is a notable mutation in the K-Ras oncogene along with shortening of telomere (Hruban et al. 2010), and as the malignancy progresses, there is marked inactivation of other tumor suppressor genes such as p16, CDNK27, p53, and SMAD4.

Intraductal papillary mucinous neoplasms are carcinomas mainly found in primary pancreatic duct or in one of the secondary ducts of the pancreas. The distinction between the presence of the carcinoma in the primary or secondary duct is essential as vigorousness of malignancy varies. Among total IPMN cases, about 70% of the tumor was found in resected pancreatic main duct and only 25% was found in secondary pancreatic ducts (Tanaka et al. 2006).

Mucinous cyst neoplasms are a rare type of premalignant pancreatic lesions (8%). Among resection of pancreatic cancer, mucinous cyst neoplasm accounts for about 25% and is more prevalent among women (Mohammed et al. 2014). The tail and body of the pancreas is affected (Din et al. 2020).

Pancreatic cancers are identified only in their later stages. The main rationale on lack of specific symptoms, and difficulty in diagnosis, is lack of advancements in providing screening tests and diagnostic tools for prompt identification of pancreatic cancer. But there are some general symptoms like jaundice, loss of weight, light colored stools, pain in abdomen, and tiredness (Siegel et al. 2018). These obstacles result in poor prognosis of pancreatic cancer. Patients with pancreatic cancer are found be associated with high glucose tolerance (Cersosimo et al. 1991).

Opportunistic Risk Factors

Risk factors associated with pancreatic cancer development include amendable risk factors (Fig. 1) and unamendable risk factors (Fig. 2).

Amendable Risk Factors

Smoking: It is one of the most importantly considered avoidable risk factor. About one-fourth of the diagnosed pancreatic cancer patients are found to be smokers and tobacco users (Pandol et al. 2012). The intensity of smoking, duration of smoking, and the age of the individual also have an impact in acquiring pancreatic cancer (Pandol et al. 2012). A review by Pandol et al. (2012), gives a brief summary on the possible roles and possible mechanistic pathways through which smoking can promote pancreatic cancer (Pandol et al. 2012).

Alcohol: Consumption of alcohol is associated with elevated risk of developing pancreatic cancer (McGuigan et al. 2018). A meta-analysis study reveals that

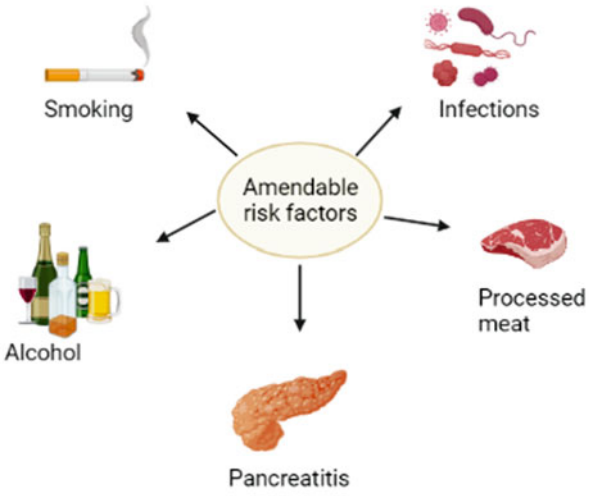

Fig. 1 Amendable risk factors of pancreatic cancer

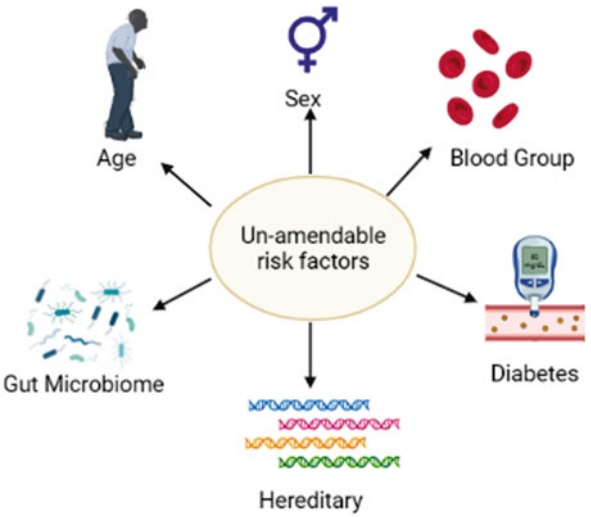

Fig. 2 Unamendable risk factors of pancreatic cancer

consumption of low and moderate levels of alcohol does not pose negative effects in the pancreas, whereas due to high levels of alcohol consumption the pancreas gets inflamed leading to pancreatitis (Wang et al. 2016), one of the chronic culprits in precipitating pancreatic cancer (Samokhvalov et al. 2015).

Chronic pancreatitis: Gradual increase of inflammatory condition identified by fibrosis in pancreatic tissue and loss of pancreatic cells such as acinar cells and pancreatic islets refers to pancreatitis. Pancreatic cancer development due to chronic

pancreatitis accounts for approximately 5% (Raimondi et al. 2010). The risk of developing pancreatic cancer from chronic pancreatitis is found to be 13 times higher than for normal individuals (Raimondi et al. 2010).

Infection: Studies on various infections which may be causative agent in developing pancreatic studies are carried out. Even though there are no sufficient studies made in order to strongly suggest the link between infections, by *Helicobacter pylori* (Guo et al. 2016) and Hepatitis C-infections, and increased risks of pancreatic cancer, there are noted positive results in association of these infections in developing pancreatic cancer (El-Serag et al. 2009).

Dietary factors: Dietary foods like processed meat and red meat are well known to have added preservatives, such as nitrate and N-nitroso groups, that are potent carcinogenic agents responsible for various cancer precipitations including pancreatic cancer (Lightsey 2012). Impact of various dietary products on pancreatic cancer accounts for about 30–50%, with suggesting evidences that certain specific food items have higher risk possibility resulting in disease condition (Midha et al. 2016).

Unamendable Risk Factors

Age: Pancreatic cancer is commonly referred to as an elderly disease. In general patients, those diagnosed with pancreatic cancer come under the age group more than 55 years, either in their seventh or eighth decade. Under rare conditions, patients around the age of 30 are reported.

Sex: Incidence of pancreatic cancer is high in male than in female. It was hypothesized that female reproductive organ factors may play an important role in decreasing the chances of getting pancreatic cancer. Thus, it was suggestive that either other environmental factors or the genetic factor plays an upper hand in high incidence of pancreatic cancer.

Blood group: Incidence of pancreatic cancer, especially pancreatic adenocarcinoma, is associated with different ABO blood grouping. An extensive study shows that the high risk of incidence of pancreatic cancer is associated with blood group A, blood group B, and blood group AB when compared to blood group O (Wolpin et al. 2009).

Gut Microbiota: Many studies were performed for investigating the role of gastro-intestinal microbial flora in developing pancreatic cancer. A review by McGuigan et al. (2018), elucidates that the alteration in the levels of microorganisms such as decreased levels of *Neisseria elongate* and *Streptococcus mitis* and increased levels of *Porphyromonas gingivalis* and *Granulicatella adiacens* plays a vital role in increasing the risk of pancreatic cancer (McGuigan et al. 2018).

Hereditary: It is noted that genetic susceptibility and previous family history also has incidence on increasing the risk of acquiring pancreatic cancer. Approximately on the whole, about 5–10% of the pancreatic cancer reported cases have given a statement indicating prior first-degree relatives diagnosed with this disease (Hruban et al. 2010). In general, if one first-generation individual is diagnosed with pancreatic cancer, the risk in acquiring pancreatic cancer is 9 times more than that of a normal

individual and 32 times more for family members where two or more first-generation relatives are diagnosed with pancreatic cancer (Becker et al. 2014).

Diabetes: A meta-analysis demonstration showed that there is two times increased probability of acquiring pancreatic cancer in patients with type-1 diabetes mellitus on comparison with non-type-1 diabetic patients (Stevens et al. 2007). Another meta-analysis on comparison of type-2 diabetic patients with non-type-2 diabetic patients showed the probability of acquiring pancreatic cancer was also highly similar to that of type-1 diabetic patients (Huxley et al. 2005).

Pathology of Pancreatic Cancer

Molecular Pathways in Pancreatic Cancer

Understanding the molecular pathways in which the disease progresses is essential for conceptual understanding of the aggressiveness of the disease, for designing a study on the disease progression, for choosing an optimal animal model, and also for developing drugs for prognosis. The hallmark-signaling pathways extensively studied in pancreatic cancer are Kristen rat sarcoma viral oncogene homolog (K-Ras), P16/cyclin-dependent kinase inhibitor 2A (CDKN2A), tumor protein P53 (P53), breast cancer 2 early onset (BRCA2), and SMAD family member 4 (SMAD4)/deleted in pancreatic carcinoma 4 (DPC4) (Jones et al. 2008). There are a number of growth factor molecules signaling receptors found on the cell surface of the cancer cells. They include the epidermal growth factor (EGF); fibroblast growth factor (FGF); insulin-like growth factor (IGF) and their receptors (EGFR), (FGFR), and (IGFR); platelet derived growth factor (PDGF); and vascular endothelial growth factor (VEGF) (Korc 1998).

K-Ras Oncogene

The activation of K-Ras oncogene is the initiative reaction that is commonly found in more than 90% of the pancreatic cases and is regarded as the earliest mutation observed in low-grade pancreatic cancer grading (Jones et al. 2008). The potential for a pancreatic cancer cell to proliferate and have sustained survival is attained by activation of K-Ras pathway. In general, continuous signaling process is required for sustained cancerous cell proliferation and survival; this applies for K-Ras signaling pathway also for pancreatic cancer proliferation (Collins et al. 2012). Ras belongs to G-protein super family group, regulated by guanine nucleotides, namely GDP (Guanosine di-phosphate) and GTP (Guanosine tri-phosphate). When Ras is bound to GDP, it is in inactive state, and when bound to GTP it is in active state (Polireddy and Chen 2016). Moreover, two main factors are found to regulate the active and inactive states of Ras, namely the Guanine Nucleotide Exchanger Factors (GEF) and the GTPase-activating proteins (GAP) (Polireddy and Chen 2016). GEF is involved in activation of the Ras signaling pathway by enhancing the exchange of

GDP to GTP, and the GAP is involved in halting the Ras signaling cascade by activating the GTPase enzyme activity that hydrolyzes GTP to GDP (Downward 2003). Thus, it is evident that any mutations in the GTPase enzyme system can result in alterations of Ras and its downstream effector pathways. The codon G12 and G13 are the important codons in K-Ras pathway; however, they are more susceptible for genetic mutations (Polireddy and Chen 2016). Mutation at this site can result in abolishment of GAP, thereby break off the GTPase-induced GTP hydrolysis. Subsequently, it prolongs the active state of Ras pathway assisting in pancreatic cell proliferation. Thus it can be understood that the abovementioned codons are specific regions to induce mutations in animals for pancreatic cancer-experimental model development. This mutation is adequate enough for inducing acinar cells pathogenesis leading to ductal metaplasia and pancreatic intraepithelial neoplasia (PanIN), progressing toward pancreatic ductal adenocarcinoma (PDAC) (Fig. 3). On activation of K-Ras-signaling cascade, the effector downstream signal cascades are as follows: canonical Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk), phosphoinositide 3-kinases (PI3Ks)/3-phosphoinositide-dependent protein kinase-1 (PDK-1)/Akt, Ral guanine nucleotide exchange factors (RalGEFs), and phospholipase Cε (Downward 2003).

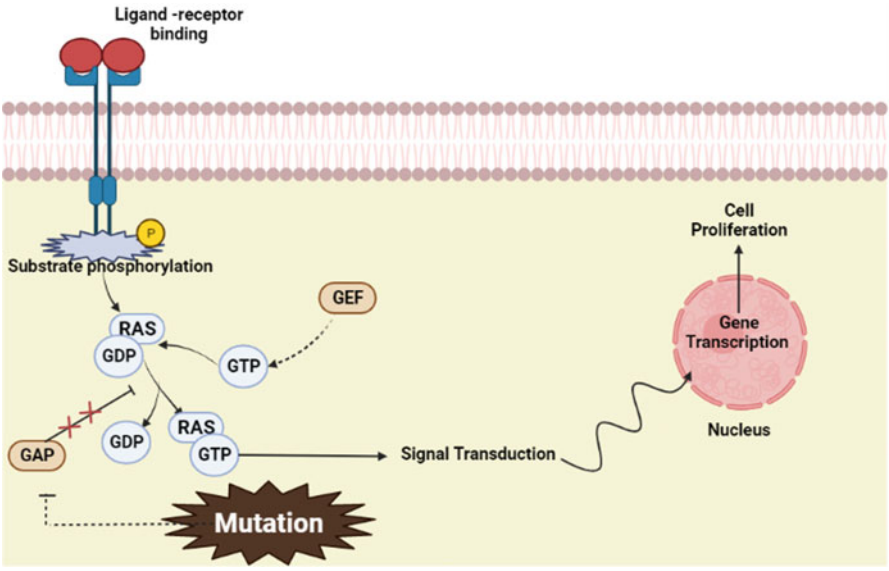

Fig. 3 K-Ras signaling pathway. On binding of ligand to the receptor, phosphorylation occurs which initiates Ras pathway. Ras bound with GDP (Guanosine di-phosphate) is in inactive state and on phosphorylation of GDP to GTP (Guanosine tri-phosphate) becomes active. This active Ras-GTP can lead to activation of a number of signaling cascades ultimately leading to cell-proliferation. GEF (Guanine Nucleotide Exchanger Factors) and GAP (GTPase-activating proteins) are the regulatory factors that are involved in exchanging of GDP with GTP and hydrolyzing Ras-GTP to Ras-GDP thereby inhibiting cell proliferation, respectively. On mutation, the activity of GAP is hindered and abolished and prolongs the active state of Ras

P16/CDKN2A Gene

P16/CDKN2A, Cyclin-dependent kinase inhibitor 2A, is a notable tumor suppressor gene that acts through the gene pathway, retinoblastoma (Ai et al. 2003). On oncogenic conditions, this gene is mainly targeted for inactivation of its action thereby providing a stable environment for the cancer cells to proliferate and grow. This gene is used as target for testing anticancer activity of various agents (Polireddy and Chen 2016). CDKN2A encodes for many proteins of which the two main proteins studied widely are p16(INK4A) and p14(ARF). These proteins are important tumor suppressor agents regulating normal cell division. During cell cycle, an orderly manner of cell division, CDK4 and CDK6 are involved in regulating normal cell division. When the cells are unable for further cell division, p16(INK4A) protein binds with CDK4 and CDK6 proteins and disables CDK4/6 ability to stimulate cell cycle. Thus, p16(INK4A) suppresses and regulates over cell division. Thus, in pancreatic cancer, suppression of p16(INK4A) gene is found to accelerate the pathogenesis and synergistically lead to development of PDAC (Pancreatic Ductal Adenocarcinoma) in K-Ras mutant (Aguirre et al. 2003). K-Ras activation also has negative impact on the CDKN2A mutation. Various mechanisms are noted to inactivate P16/CDKN2A, namely homozygous deletions, loss of heterozygosity, and epigenetic silencing by promoter methylation (Maitra et al. 2006). The p14 (ARF) protein is not directly involved in the tumor suppression, but it acts in an indirect way by protecting the p53 (an important tumor suppressor protein) protein which is involved in regulation of cell division and stimulating apoptotic pathway.

Tumor Protein P53 (p53)

The tumor protein p53 is a gene that encodes for a tumor suppressor protein p53. P53 is a potent tumor suppressor found in almost all the cells. They are located in the nucleus of the cells binding to the genetic material DNA. When there is damage in DNA to various external and internal factors, this protein, p53, examines DNA damage and interprets whether the DNA can be repaired or it must undergo apoptosis. Thus if the damage can be repaired, p53 activates genes responsible for the repair mechanism, and if the damage cannot be overcome then it activates the self-destructive pathway (apoptotic pathway). Thus to summarize with, p53 protein is found to act as a tumor suppressor mainly by preventing the division of cell containing mutated or damaged DNA. In PDAC condition, about 50–75% of P53 protein is inactivated through intragenic mutations combined with loss of the second allele (Redston et al. 1994).

SMAD4

SMAD is also called as the transforming growth factor Beta (TGF-β). This gene is involved in altering the environment around the cell in order to create an impact on the genetic material activity and protein production. Thus, on binding of TGF-β to

the cell surface receptor, there is an initiation of the downstream signaling cascade which involves in activation of SMAD proteins. These SMAD proteins then aggregate to form SMAD protein complex and migrate to the nucleus. After entering the nucleus, SMAD proteins then bind to specific sites at DNA and take control over the activity of genes involved in cell growth and cell division. In pancreatic cancer, the SMAD is inactivated and loses its ability to regulate genes responsible for cell division. It was noted that approximately 55% of the reported pancreatic cancer cases are denoted with SMAD4 inactivation either due to homozygous deletions or by intergenic mutations and loss of the second allele (Polireddy and Chen 2016). On loss of SMAD4, the pancreatic cancer cells enjoy supremacy over the growth rate by evading TGF-β growth-inhibitory signals (Siegel and Massagué 2003).

Epidermal Growth Factor (EGF)

EGF is an important group under growth factor family which is extensively studied for understanding the cell growth, cell development, and cell physiology and in human cancer studies (Wieduwilt and Moasser 2008). EGFR (Epidermal Growth Factor Receptor) is a transmembrane receptor belonging to the tyrosine group of receptors. It is made of an outer ectodermal part, a transmembrane part, and intracellular tyrosine kinase part with C-terminal tail (Wieduwilt and Moasser 2008). This receptor binds to specific receptors namely EGF and TGF-β (Polireddy and Chen 2016). On binding to the ligand, the receptor undergoes structural configurations such as dimer formation. This dimerization then activates the intracellular tyrosine residues and brings phosphorylation of C-terminal tail. The activated tyrosine kinase residues initiate a number of downstream-signaling cascade namely the Ras/MAPK, PI(3)K/Akt, PLCγ1/PKC, and STAT pathways (Wieduwilt and Moasser 2008). On pancreatic cancer condition, there is marked overexpression of EGFR, approximately diagnosed in about 90% of pancreatic tumor cases (Troiani et al. 2012) and recurrence of human pancreatic cancer (Tobita et al. 2003).

Insulin-Like Growth Factors (IGF)

IGF has a central role in governing the cell growth, cell development, and metabolism. IGF binds to three receptors namely the insulin receptor (IR), insulin-like growth factor-1, insulin-like growth factor-2, or mannose 6-phosphate receptor. IGF-1R specifically binds with ligands such as the IGF-1 and 2 and insulin receptor. IGF-1R is a tyrosine kinase receptor which is similar to insulin receptor. It is heterodimer glycoprotein made of two alpha and two beta subunits linked together with di-sulfide bonds, mainly controlling over apoptosis, cell growth, and differentiation. On binding to ligand, the tyrosine residue attached to the receptor is phosphorylated and activates downstream effector molecules. Based on the type of adaptor molecule activated, such as Insulin receptor substrates 1–4 (IRS) and Src homology collagen protein, the following cascade activation differs. For instance,

when IRS is activated, either PI3K/AKT pathway or Ras/Raf/MEK/Erk pathway is stimulated. Patients with PDAC showed elevated expression of IGF-1 and its receptor IGF1R indicating the association of high tumor grade and poor survival rate in patients (Valsecchi et al. 2012). An *in vitro* study on human pancreatic cell lines showed that additon of IGF-1 induced cancer cell growth but treatment with IGF-1 antibody attenuated the pancreatic cancer cell growth suggesting that IGF-1 plays a central role in the progression of pancreatic cancer (Bergmann et al. 1995).

Fibroblast Growth Factor (FGF)

FGF are transmembrane proteins that specifically bind their receptor FGFR (Fibroblast growth factor receptor). FGF proteins are signaling proteins produced by macrophages. They play a crucial role in maintenance of cell growth, proliferation, survival, migration, and differentiation. They are involved in repair and regeneration process of damaged tissues. Activation of FGF can recruit an adaptor molecule FGFR substrate-2 (FRS2) which is responsible for activation of various pathways like the RAS/MAP kinase pathway, PI3K/AKT pathway, and PLγ pathway, of which the RAS/MAP kinase pathway is the most predominant one. Increased mitogenesis and angiogenesis are found in pancreatic cancer cases which are mainly contributed due to increased expression of FGF and its ligands and their receptors (FGFR) (Nowak et al. 2005).

Vascular Endothelial Growth Factor (VEGF)

VEGF, also termed as vascular permeability factor (VPF), is an endothelial cell-specific mitogen (Ferrara et al. 1992) that binds to its specific receptors namely VEGFR-1 and VEGFR-2. There are many types of cells that produce VEGF namely tumor or cancer cells, macrophages, platelets, keratinocytes, and renal mesangial cells (Duffy et al. 2004). Thus targeting these factors has provided new strategies in cancer cells growth and treatment by mainly targeting the proangiogenic function by inhibition of the process neovascularization (Duffy et al. 2004). Being an angiogenic polypeptide, VEGF is actively involved in the promotion of endothelial cells proliferation and its survival by forming receptor-ligand complex (Olsson et al. 2006). Though pancreatic cancer is not highly vascular cancer, it is markedly observed that there is elevated mRNA expression of VEGF suggesting a positive correlation between the vascular density and the progression of the disease (Tsuzuki et al. 2001).

Animals Models in Pancreatic Cancer

Use of animals as an experimental model in research is a well-established implementation for scientific aspiration (Barré-Sinoussi and Montagutelli 2015). Due to similarities in the anatomical and physiological characteristics between mammals and humans, animal model is used to depict and understand various physiological mechanisms that

prompt the investigator to discover the key to unlock the complexity of the living system. Investigators examine organisms at dynamic levels, starting from the cells, molecules, tissue level, organ level, its physiological functions, and its molecular pathways. Use of animal models has helped a long way in conveying answers for a humpty number of scientific queries, hypothesis, etc. Animal models are an essential breakthrough for understanding the pathophysiology, detection of the biomarkers of a pathological condition, and an important step in the development of various drugs for amelioration of diseases (Saloman et al. 2019). Choosing an optimal animal model is a crucial step which depends on different aspects of the disease being studied.

Generally, various chemicals are used as models to induce carcinogenesis. If they are administered systemically, they are involved in tumor formation at multiple target organs and tissues. However recently, scientists have made a leap from following the traditional way of chemical-induced cancer model formation to proceeding with transplantation animal models due to significant benefits. Based on the site of tumor transplantation, the models are labeled as subcutaneous xenograft models and orthotropic xenographic models.

The earliest experimental pancreatic cancer induction model was made in 1941, in which 2-acetylaminofluorene (Carcinogen) was the inducer of hyperplastic foci, adenomas, and one acinar cell carcinoma in albino rat models (Wilson et al. 1941). Other pancreatic cancer model developments were held up until the twentieth century when there was an enforcement made by the governmental agencies to develop a pancreatic cancer model as there were increasing cases of pancreatic cancer in the United States (Standop et al. 2001).

Rats

A widely used classical model for pancreatic carcinogenesis is Azaserine treated of Wistar/Lewis rats. This model induces pancreatic cancer primarily through acinar cell damage gradually leading to carcinogenesis. Azaserine leads to tumor development gradually starting from cellular abnormalities (adenomas, nodules formation) to initiation of carcinogenesis and metastasis to the nearby organs (Rao 1987). Diet containing high fat content is considered as a major factor that is involved in initiation of pancreatic tumor-genesis. However, treatment with diet containing retinoids and calorie restriction has proved to downregulate the tumor formation (Longnecker 1990). A study by Longnecker (1990), reports that pancreases of rats are more vulnerable to hyperplasia and neoplasia not only to carcinogenic agents but also to noncarcinogenic agents (raw soya flour, cholecystokinin) (Longnecker 1990). A study showed that implantation of a polycyclic hydrocarbon 9,10-dimethyl-1,2-benzanthracene (DMBA) in pancreas of Sprague-Dawley rats precipitated adenocarcinoma by formation of spindle-shaped cell carcinomas and less differentiated adenocarcinomas of acinar cell types (Vesselinovitch et al. 1973). Using the same technique ductal cell proliferation, tubular adenocarcinomas, acinar cell carcinomas, fibrosarcomas, and invasive ductal adenocarcinomas were induced to study and investigate specific tumors (Pour and Wilson 1980).

Hamster

Use of hamster the *Mesocricetus auratus*, the Syrian golden hamster, as an experimental model was introduced in 1941 by Alder (Pour and Wilson 1980). However, use of hamster in cancer research was neglected for many years. But this species is found to be more useful as a pancreatic cancer model. Especially this species is found to show remarkable pancreatropic effects on induction with carcinogenic agents which was only less effective in other experimental animal models such as rats, mice, guinea pigs, and rabbits (Pour and Wilson 1980). This shows the vulnerability of hamster to carcinogens. Among various carcinogenic agents, BOP (N-nitroso-bis(2-oxo-propyl)amine) is the vigorous carcinogen which induced, specifically, pancreatic cancer (Pour and Wilson 1980) in shaved skin of hamster when exposed with few drops of the BOP. Most pancreatic cancer development is seen in the tail and body of the pancreas in hamster (Standop et al. 2001). As seen in pancreatic cancer patients (Cersosimo et al. 1991), hamsters also develop high glucose tolerance (Permert et al. 2001) during tumorogenesis (Ahrén and Andrén-Sandberg 1993). This fact strongly acknowledges that an irregular glucose metabolism was the primary cause for cancer. Similar to rats, hamsters also show pancreatic carcinogenesis on high fat diet consumption (Kazakoff et al. 1996).

Immune-Deficient Mice

Nude mice possess high advantage in serving cancer models. These mice are genetically mutated resulting in absence of thymus gland and therefore result in immune-deficit. These mice are athymic, thus resulting in immune-specific T-cell deficiency, and are hairless. Use of nude mice is considered to be important in investigating specific characteristic of pancreatic cancer starting from tumor growth to metastasis and also the efficacy of various anticancer drugs. Advantages in choosing nude mice in cancer studies are that it exhibits high efficiency in maintenance of the human cancers (Standop et al. 2001). Even studies on Severe Combine Immune deficiency (SCID) are done, in which neither T-cells nor B-cells are present and also studies on beige nude mice, mice absent with T-cells, B-cells, and natural killer cells (Shi and Xie 2000).

Establishment of Tumorogenesis in Animal Model

Subcutaneous Xenograft Models

In this model, human pancreatic cancer tissues or pancreatic cell lines are grafted under the skin, in subcutaneous region, in a nude mice model, or in severe immune-deficient mice (Saluja and Dudeja 2013). In this model, the tumor grows in the subcutaneous region, which is enriched with blood vessels providing an ideal environment for tumor growth. Usage of this model is advantageous in initiation

and monitoring of the pancreatic cancer. It is possible to continuously monitor the progression of cancer growth and measure of tumor size during the study period. When pancreatic cancer cell lines were used to graft, they undergo different mechanistic pathways to initiate the gene alterations responsible for tumor formation which shows variation from the mechanisms involved in normal tumor-genesis. Whereas the models with human pancreatic tissue graft under the skin showed a similar mechanism by which original pancreatic tumor progresses, this property increased the reliability on use of human pancreatic cancer tissue for inducing cancer. Thus in order to maintain the phenotype identical to the normal tumor progression in subcutaneous xenograft tumor model, it is essential to make use of human pancreatic cancer tissue to show identical histo-pathological patterns including the antigen expression (Capella et al. 1999). The biggest disadvantage in the usage of immune-compromised mice models is the inability to assess the effects of the immune system on the modulation in tumor growth when anticancer agents are administered. Regarding the future prospectus, this model using a human pancreatic cancer tissue can give rise to an array of new strategies in clinical applications to design treatment methodologies, develop new anticancer agents, and bring therapeutic invasions mechanistically for better outcomes.

Orthotopic Xenograft Models

In this model, pancreatic cancer tissue is grafted directly in the pancreas. This technique is laborious, complex, and challenging in many technical ways. On comparing with subcutaneous xenograft models, there are certain unique benefits achieved in this model. Some of the advantages are the exact characteristic of the cancer, and it can be studied extensively as the cancer grows in its normal ubiquitous environment thereby mimicking the original pancreatic cancer. Both pancreatic cancer cell lines and human pancreatic cancer tissue can be used for grafting showing distinct characteristics for both the types of grafting. Though this model is technically laborious and complex, about 60% of this model exhibited constructive local regional spread and metastasis of cancer (Loukopoulos et al. 2004). However, the disadvantages are quite similar to subcutaneous xenograft models. The disadvantages of orthotopic models are use of immune-compromised animal models, genetic variations in pancreatic cancer cell lines showed failure to mimic stromal cell reactions which were observed in humans diagnosed with pancreatic cancer.

Genetically Engineered Models of Pancreatic Cancer

A number of shortcomings in choosing the better experimental animal model led to invasion of genetic engineering which reproduced a plethora of genetically engineered animal models for pancreatic cancer (Frese and Tuveson 2007).

Genetic engineering has helped in targeting variety genes responsible for the cancer development. It made easier to examine the cellular targets in treating the cancer with various anticancer agents. The animal models are genetically engineered using transgenic, gene knock-in, and gene knock-out techniques in order to bypass specific genes into the model aided by retroviruses (Kong et al. 2020). An optimal model for depicting pancreatic cancer can be constructed using genetic engineering tools, by designing K-Ras-mutant animal model. The K-Ras mutant animal model is found to create intensive range of preneoplastic changes in pancreas (Hingorani et al. 2003). Further inactivation of p16/CDKN2A, TP53, and growth factors like DPC4/SMAD4 pathways can escalate a comprehensive spectrum responsible for pancreatic cancer development showing invasive, local, regional, and metastatic spread of the cancer. This model can be more reliable than the other models as in prediction of the treatment progress and efficacy of the anticancer drug administered (Fig. 4). Genetically engineered models are grouped under immune-competent animals which make them specifically useful in testing various new drugs and anticancer agents (Scarlett et al. 2016). Examples of some genetically engineered mouse models are KIC model (Pdx1-Cre, LSL-Kras^{G12D}, and Ink4a/Arf$^{lox/lox}$), KPC model (Pdx1-Cre, LSL-Kras^{G12D}, and LSL-Trp53$^{R172H/+)}$, KD model (Pdx1-Cre, LSL-Kras^{G12D}, and Smad4$^{lox/lox}$), and PDAC model by TGFBR2 knockout with Kras (Prf1a-Cre, LSL-Kras^{G12D}, and Tgfbr2$^{lox/lox}$) (Kong et al. 2020).

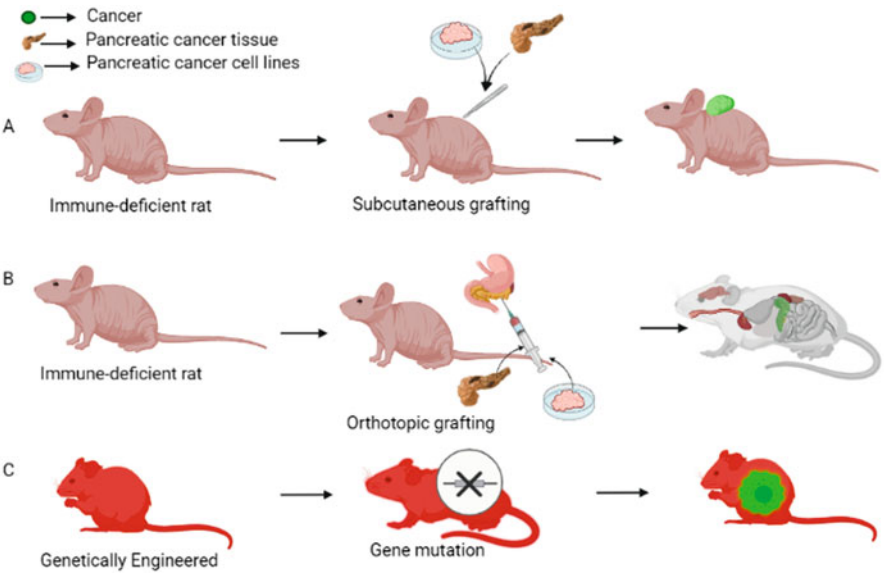

Fig. 4 Induction of cancer in animal models: (**a**) Depicts the subcutaneous xenograft model; (**b**) depicts the orthotopic xenograft model; and (**c**) depicts the genetically engineered model

Ideal Animal Model for Clinical Aspects

Selection of an ideal model for pancreatic cancer is crucial for understanding the complete pathogenesis and for testing various antineoplastic drugs for pancreatic cancer. Experimental animal models are a crucial channel that gives key takeaways in gaining in-depth knowledge and better understanding in the etiology of a pathological condition and to assess the potential risks associated with the disease condition and designing a better therapeutic agent. So in order to satisfy this need, the cancer induced in the animal model must be similar to the human cancer in morphology, physiology, and biochemical and clinical aspects (Standop et al. 2001). Though the experimental animals are induced with pancreatic cancer using various chemicals, the shortcoming associated with them is that the chemicals predominantly induce acinar cell tumors which are rare in humans. And usage of small animals to mimic any human disease can limit research possibilities (Standop et al. 2001). Rats are the widely used animal model in researches and in the case of pancreatic cancer have high possibility in developing acinar cell cancer (Pour and Wilson 1980). Nude mice (Immunodeficient mice) are considered as an "in vivo tumor bank" as they act as a reservoir in maintaining the human cancers providing a dynamic platform for tumor investigations and their mitigation. The Syrian golden hamster is also considered to be more beneficial in pancreatic tumors as they show unique similarities to the tumors in occurring in humans. In transplantation models, the most popular site for pancreatic cancer transplant is subcutaneous region (Saloman et al. 2019). Subcutaneous region is opted rather than orthotopic xenograft model in order to avoid the complexity in tumor induction and reducing the laborious work (Saloman et al. 2019). Subcutaneous grafting of the tumor cells also has its own advantages like easy visualization of the tumor growth, measurement of the tumor size, and effect of anticancer drug against the tumor. However, scientists are interested in orthotopic xenograft model, as they consider this model ideal for metastasis studies. Genetically modified animal models are considered as a worthy resource for preclinical testing of various novel anticancer therapies, which is advantageous over the use of other xenograft models (Scarlett et al. 2016). This model served for studying the noninvasive stage to invasive stage and metastasis (Scarlett et al. 2016). Though there are a number of advantages in using a genetically engineered model, the limitation of this model is that modification of all the cells may not be homologous to normal pancreatic tumor (Din et al. 2020). In xenograft models, the use of human cancer tissue fragments is preferred as the use of pancreatic cell lines does not completely follow the heterogeneity of the human pancreatic cancer. Knowing the fact that genetically engineered models are from immune-competent setup, they show promising role in the identification of new anticancer therapies (Scarlett et al. 2016). The major obstacle in inventing and designing new novel therapies for pancreatic cancer, specifically, is the immune-suppressive environment. The genetically modified group, in which the immune-suppressive environment is created, serves as a better tool to design new therapeutic tools (Scarlett et al. 2016).

Conclusion

Pancreatic cancer is like a ticking bomb which is explosive resulting in fatal outcomes mainly due to the asymptomatic nature of pancreatic cancer in initial stages. Pancreatic cancer incidence is increasing yearly. Though there are many innovations and advancements made in the identification of risk factors of pancreatic cancer, diagnostic tools, and methodologies to detect pancreatic cancer at an early stage, the mortality rate is still parallel to a number of new cases. There are many understanding made in the mechanistic pathways (K-Ras, inhibition of tumor suppressor genes, and growth factors) through which the pancreatic tumor forms. In order to achieve successful investigations, there is need to choose an animal model of optimal standard to provide sufficient reliable results.

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Human Embryonic Stem Cells as a Therapy for Alzheimer's Disease

40

Stephen Adeniyi Adefegha

Contents

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive decline in neurocognitive performance with symptoms such as neuroinflammation, decreased hippocampus volume, learning and memory issues, as well as problems with visuospatial functions. The mechanisms involved in the pathogenesis of AD include aggregation of the amyloid precursor protein, activation of cholinesterases, formation of senile plaques, hyperphosphorylation of tau protein, and activation of microglia. Several therapeutic strategies have been adopted toward the management of AD, especially stem cell technology. This chapter describes human embryonic stem cells as a viable therapy for the management of AD.

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Keywords

Human embroyonic stem cell · Alzheimer's disease · Senile plaques · Neuroinflammation · Activated microglia

Introduction

The global health burden of neurological disorders coupled with limitations in treatment and great progression rate of the disease makes it a global consideration. Dementia is a neurological disorder affecting over 50 million people with a future estimation set to 132 million by 2050 (Alzheimer's Association 2015, 2020). People with Alzheimer's disease have a wide range of difficulties, including cognitive decline, amnesia, and the inability to perform basic everyday tasks. It is estimated that mortality might occur anywhere between 5 and 12 years after the first signs of Alzheimer's disease (AD), one of the most prevalent and quickly advancing forms of dementia (Bruni et al. 2020). Even while research into Alzheimer's disease has made great strides, many aspects of the disease's multifaceted pathophysiology are still poorly understood. The presence of extracellular ß-amyloid deposits (also known as senile plaques) and an intracellular buildup of hyperphosphorylated tau (also known as neurofibrillary tangles) continues to be the primary focus in Alzheimer's disease diagnosis (Kent et al. 2020; Scearce-Levie et al. 2020).

Memantine, tacrine, galantamine, donepezil, and rivastigmine have all been approved by the FDA for clinical use in the treatment of Alzheimer's disease in the United States. Dysfunction in the cholinergic signaling pathway has also been implicated in the pathogenesis of AD as there tends to be a reduction in acetylcholine, an important neurotransmitter in the brain. The agents above have been effective in managing the symptoms associated with AD but with no effect on terminating the progression of the disease. The effectiveness of several stem cells transplantation as an alternative therapy for AD has been explored especially in animal models and some clinical trials resulting in cognitive functions and improvement with reference to hallmarks of AD (Shihabuddin and Aubert 2010).

Pathogenesis and Proteinopathy of Alzheimer's Disease

It has been shown that patients with Alzheimer's disease (A.D.) show progressive decline in neurocognitive performance with symptoms such as neuroinflammation, decreased hippocampus volume, and learning and memory issues as well as problems with visuospatial function. Neuronal synaptic synapses are lost in the basal forebrain and amygdala, as well as the hippocampus and the cortex, in Alzheimer's disease (A.D.). The accumulation of harmful proteins such as amyloid-(A) plaques and neurofibrillary tangles is also observed (Delbeuck et al. 2003; Popovic and Brundin 2006). Hyperphosphorylated tau protein and A-42 are two neurotoxic proteins that accumulate excessively, produce neurotoxicity and synaptic failure, making A.D. a

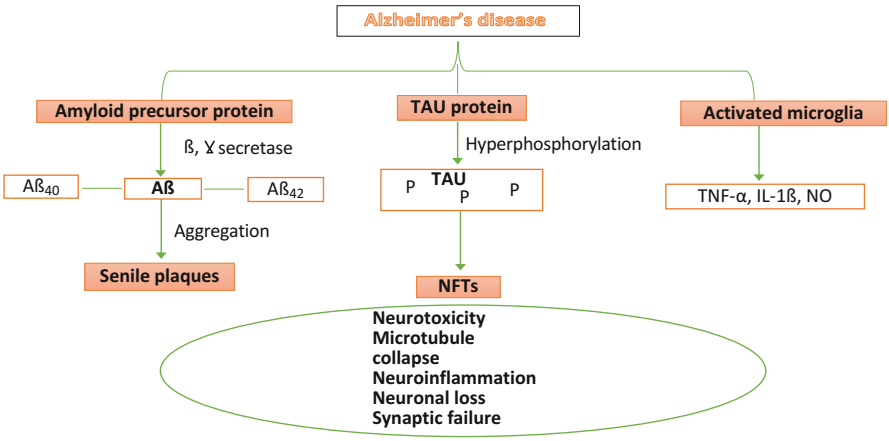

Fig. 1 Mechanisms of neuronal damage leading to Alzheimer's disease

proteinopathy, according to this study. Helping to maintain the stability of microtubules and facilitating axonal transport, Tau is an essential component of neurons. Because of this, microtubules began to break down and clump together, forming neurofibrillary tangles (Khan and Bloom 2016; Bali et al. 2017). There are extracellular deposits of beta amyloid (A) that are evident as amyloid plaques in the AD brain because the amyloidogenic pathway is triggered by the misfolded precursor protein amyloid (APP), which is an abnormally folded protein (Querfurth and La Ferla 2010). Neurotoxicity and subsequent neuroapoptosis in the CNS are caused by the abnormal amyloidogenic pathway, which results in the accumulation of the predominating A-40 (90 percent of A.D) and the fibrillogenic A-42 (10 percent of AD) (Perneczky and Alexopoulos 2014; Bali et al. 2017; Pallas and Camins 2006; Hardy 2009). Activated microglia release cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and nitric oxide (NO), which may aggravate neuroinflammation when combined with amyloid plaques (Walker and Lue 2005) (Fig. 1).

Stem Cell and Its Potency

Stem cells are unspecialized cells that can self-renew and divide indefinitely, allowing them to develop into any type of cell in an organism. All the tissues and organs of the body are constructed from stem cells that develop differentiating into various organs during embryonic development (providing a renewal capacity in most organs). One of the characteristics of stem cells is their ability to develop into many cell types. Stem cells that are totipotent and pluripotent have the greatest potential for differentiation and are derived from the pre-embryonic phases of human development. Totipotency allows cells to form both embryo and extraembryonic structures as seen in zygote (fertilized egg) while pluripotency allows formation of all germ layers but not extraembryonic structures, such as the placenta. Zygotes give

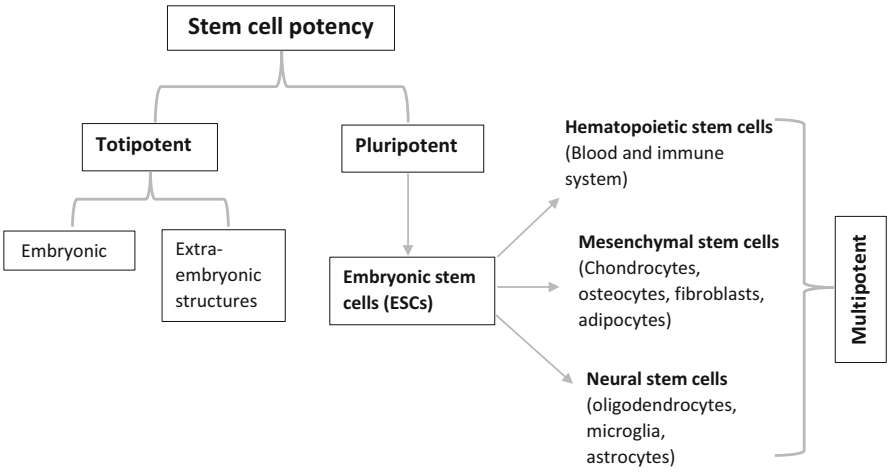

Fig. 2 The stem cell potency

rise to blastomeres while a cluster of blastomeres forms blastocyst which has an inner cell mass of embryonic stem cells (ESCs) that are pluripotent. Embryonic stem cells can differentiate into multipotent stem cells (hematopoietic, mesenchymal, neural stem cells) that are committed to give rise to some but not all cells of a given organ (Fig. 2).

It is possible to develop various brain and nervous system cells from hematopoietic stem cells (blood-forming) and neural stem cells (nerve-forming) (Berger et al. 2020; Si et al. 2020). Mesenchymal stem cells have immunomodulatory properties (Song et al. 2020) that can develop into chondrocytes, osteoblasts, fibroblasts, and adipocytes (Song et al., Ankrum et al. 2014; Si et al. 2019). If allowed to mature, multipotent stem cells can differentiate into oligo- or unipotent progenitors, each with a distinct lineage (Fig. 2).

Stem Cells in Alzheimer's Disease

Advances in research on management of A.D have been able explore several pharmacological agents that manage the symptoms but not the progression of the disease. More than 60 percent of people using these drugs become tolerant to them and develop adverse effects (Kumar et al. 2015) since they are neuromodulatory agents that only treat symptoms and do not impact the primary pathological aspects of Alzheimer's disease (AD) (Zetterberg and Bendlin 2021). For example, drugs that target amyloid- (Cummings, et al. 2017), acetylcholinesterase (AChE), antioxidants (Zandi, et al. 2004), and cholinergic and glutamatergic neurotransmission (van Marum 2008) are examples of these well-known agents. Deficiency of A-42 plaques in the brain affects stem cell proliferation and survival of neurons and glia, and hence, A.D. might be considered a stem cell disease with complex multifactorial brain disorders.

A successful therapy targeted toward A.D should be able to repair, replace, and prevent further progression. The goals of stem cell therapy are to slow the progression of Alzheimer's disease (AD), remove the source of the problem, permit the localization of therapeutic cells to defective brain regions, stimulate tissue repair and maintenance, and subsequently recover cognitive function. A variety of mechanisms, including hippocampus neurogenesis, paracrine effects, antiamyloidogenic potentials, and anti-inflammatory activity, contribute to the therapeutic potential envisaged from stem cell transplantation (Fouad 2019).

ESCs, MSCs, NSCs, and iPSCs are some of the most commonly used stem cells in AD research (Yang et al. 2013; Chen et al. 2014; Penney et al. 2020). It is possible for mesenchymal stem cells to develop into mesodermal cells such as bone, fat, and cartilage (Caplan 2017). In addition to their immunomodulatory and paracrine properties, MSCs do not require genetic matching and can be obtained from bone marrow, fat, and umbilical cord. A possible drawback of using MSCs from these sources in therapy is that they are less likely to target the brain and nervous system, which have been shown to be particularly vulnerable in the year A.D. In the central and peripheral nervous systems, NSCs release neurotrophic factors and can develop into specific neuronal lineages, such as neurons and supportive glial cells, in order to promote neurogenesis (Kim et al. 2006). NSCs are difficult to harvest from adult brains because of their deep location and difficulty in accessing them during the fetal period of development. NSCs from aborted fetuses pose an ethical and immunorejection concern since they come from fetuses that were terminated for medical or nonmedical reasons (Hoornaert et al. 2017). Skin or blood cells can be used to make stem cells, which are known as induced pluripotent stem cells, because they have already undergone a significant amount of differentiation (IPSCs). The creation of iPSC-derived brain cells that properly match the development and maturation of diverse brain cell types is difficult, however. Regular genomic change tests and the use of delivery systems that do not integrate must be taken into account in the event of IPS cell genome instability (Kwon et al. 2017; Zhang et al. 2018).

Human Embryonic Stem Cells

There are numerous ways in which regenerative medicine and disease modeling can benefit from using human embryonic stem cells, which can differentiate into a variety of cell types and have a high capacity for self-renewal (Klimasnkaya et al. 2014; Zhu and Huangfu 2013). Extrinsic growth factors and intrinsic transcription factors help HESCs maintain their pluripotency and self-renewal potential (Xu et al. 2008; Liber et al. 2010). Research on human embryonic stem cells has shown numerous immunogenic rejection possibilities and the potential for uncontrolled cell development with the creation of teratomas (tumors). Cell transplantation therapy has unwanted side effects (Wessel Schmidt 2007; Acharya et al. 2009; Fong et al. 2010; Ratajczak et al. 2014; Chen et al. 2014). HESCs have been reprogrammed into functional human neural stem cells (hNSCs) that can target the brain by further dividing into various neural cell types, including neurons, astrocytes, oligodendrocytes, and spinal motor

neurons, without the risk of tumor formation. This method has been used to overcome this obstacle (Araki et al. 2013; Reubinoff et al. 2001; Adib et al. 2015; Lee et al. 2007; Reubinoff et al.). These stem cells can be used to generate dopaminergic and cholinergic neurons, which can be used to repair damaged brain tissue and improve memory in animal models (Moghadam et al. 2009; Goings et al. 2004; and Arvidsson et al. 2002). NSCs produced from ESCs have been found to proliferate faster and differentiate more efficiently than somatic NSCs (Colombo et al. 2006).

There are basically two ways of using ESCs as a therapy in neurodegenerative disease. First, signals that mimic an appropriate in vivo environment can be used to produce the desired neural cells in vitro before transplant or otherwise transplanting the ESC-derived NSCs and relying upon the host environment to provide the appropriate signals. Culturing ESCs in vitro under standard and controlled conditions can direct predifferentiation into viable and pure populations of neural cells (Shihabuddin and Aubert 2010). In order to exclude nonneural cells during ESC neural development, embryoid bodies (EBs) are often formed and then grown in a serum-free selective medium. Since the bulk of the EBs-derived cells do not survive in this serum-free, selective medium, cell numbers in the cultured pool are drastically reduced. Neuronal progenitor cells (NPCs) are generated from ESCs into specific neurons in vivo when they are implanted into specific brain areas. In vivo investigations (Cai and Rao 2007) have shown that the risk of tumor formation and immunogenic reactivity can be minimized with these ESC-derived NPC and neuron transplantation approaches (Kim et al. 2015).

There are a number of significant components and signaling pathways that are responsible for mediating the process by which ESCs are differentiated into neurons in a controlled manner in vitro. This process is analogous to the neural induction that takes place throughout the development of an embryo. Inducers and pathways include the -catenin and wnt signaling pathway (Cajánek et al. 2009; Tonge and Andrews 2010), the retinoic acid pathway (Shan et al. 2008; Theus et al. 2006), the Notch pathway (Lowell et al. 2006; Das et al. 2010), and the transforming growth factor/bone morphogenetic protein (TGF/BMP).

Human Embryonic Stem Cells: A Prospect in Targeting AD

Human embryonic stem cells (hESCs) with their self-renewing and differentiating ability into NSCs have significant potentials for use in AD therapies (Wang et al. 2006; Israel et al. 2012). ESCs and brain-derived NSCs have been used in stem cell research in AD (Liu et al. 2020). Targeting embryonic stem cells to the brain improves its microenvironment and facilitates the survival and activation of endogenous stem cells of the brain (Philips and Robberecht 2011). More so, they stimulate connections between neurons and improve cognitive functions and metabolic activities in the brain (Blurton-Jones et al. 2014). ESC-derived NSCs transplanted into the brain of Alzheimer's disease model in rodents caused a reduction in cholinergic deficits and short-term memory disruption, synapse formation, and improved cognitive functions (Moghadam et al. 2009; Acharya et al. 2009). To restore cognitive

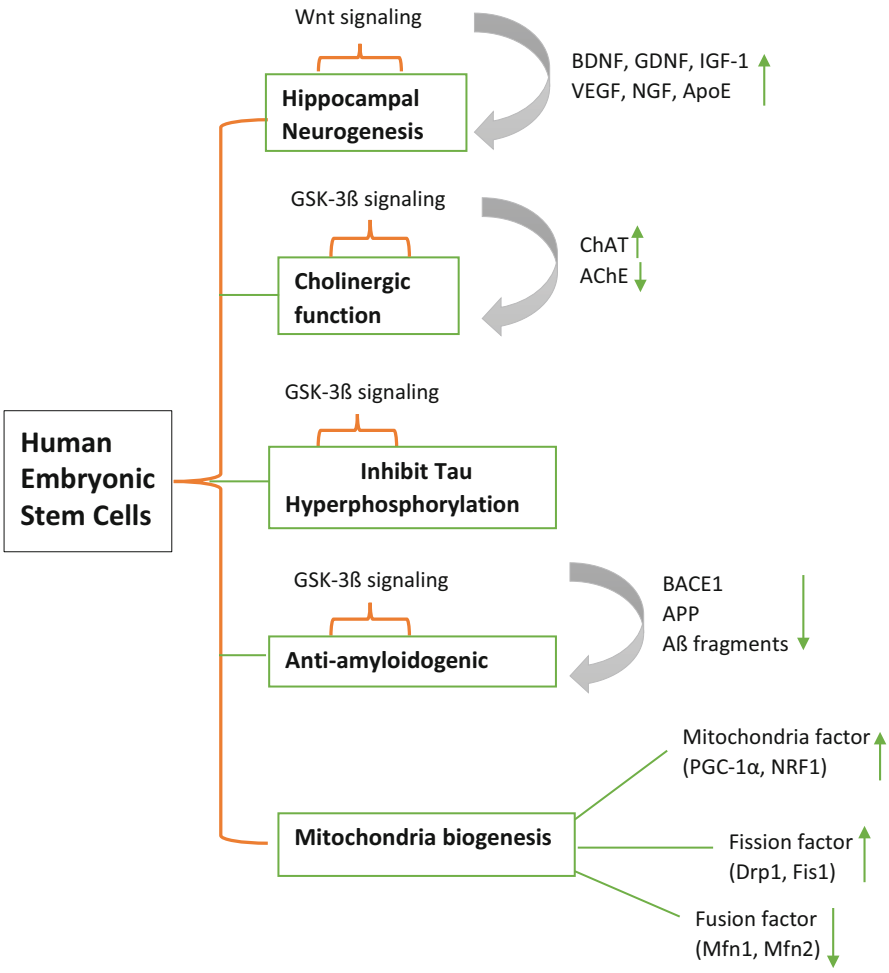

Fig. 3 Mechanisms targeting AD in human embryonic stem cells

functions in the brain of AD patients, human embryonic stem cells must induce neurogenesis, improve cholinergic function, reverse hyperphosphorylation of Tau protein, be antiamyloidogenic, stimulate paracrine factor secretion, and enhance mitochondria biogenesis, among others (Fouad 2019) (Fig. 3).

Induction of Hippocampal Neurogenesis

In learning and memory, neurogenesis in adult hippocampus (in the dentate gyrus) is key; facilitating this process may be counteractive against AD and its numerous symptoms. Approximately 800 neurons are estimated daily to be produced in

adulthood in a disease-free state with a decline to around 100 neurons and decrease in rate of hippocampal neurogenesis in aging. Consequent to this, there is need for hippocampal neurogenesis to increase in double-folds in AD due to an aberrant loss of hippocampal neurons. Neurogenesis, or the regeneration of neurons, aids in the maintenance of neurocognitive function by transforming neural stem cells into functioning neurons that can integrate into the host's preexisting neural circuit (dentate granule cells, or DGCs) (Ming and Song 2005; Jin and Galvan 2007). Cognitive decline can be caused by an imbalance between neuroregeneration and neurodegeneration, resulting in defective neurogenesis (Haughey et al. 2002).

Alzheimer's disease treatment relies heavily on human embryonic stem cells' neurogenic potential and the mechanism through which they regulate endogenous neurogenesis (Zhang and Jiang 2015; Fang et al. 2018). Upregulation of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) is possible by stem cell therapy. Apolipoprotein E (ApoE) and epigenetic regulators are examples of effector molecules, as are transcription factors like insulin growth factor-1 (IGF-1), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) (Gadadhar et al. 2011; Horgusluoglu et al. 2017; Blurton-Jones et al. 2009; Kim et al. 2012; Klinge et al. 2011; Jin et al. 2002; Garcia et al. 2014). Mutations in the human ApoE gene may contribute to the onset of Alzheimer's disease. GABAergic transmission on immature adult-born DGCs is affected by these mutations, which in turn inhibits adult-born DGC maturation in both morphology and function. Oh et al. (2015) used a mouse model to show that hESC-derived NSCs can stimulate endogenous neurogenesis in the dentate gyrus through the Wnt signaling pathway. Specifically, this was discovered in the dentate gyrus.

Secreting Paracrine Factors: Role of Brain-Derived Neurotrophic Factor (BDNF)

Paracrine chemicals released by the brain's neurons promote cell survival, increase synaptic connections, and enhance memory and learning. For neuroprotection, neuronal survival, and synaptic plasticity, hESC transplants can trigger a paracrine impact through the release of BDNF from the cells (Ager et al. 2015; Blurton-Jones et al. 2009; Yan et al. 2014). BDNF has been connected to cAMP response element-binding protein (CREB), a DNA-binding protein that acts as a transcription factor and regulatory molecule for BDNF. Long-term and short-term memory formation and retention are enhanced by the presence of BDNF and CREB in close proximity, which has been shown to regulate the production of the BDNF gene (Song et al. 2015; Dominguez et al. 2016; Lee et al. 2013; Suzuki et al. 2011). In Alzheimer's disease, researchers believe that BDNF expression and release are compromised (Fumagalli et al. 2006). The circulating concentration changes predispose to AD, and its expression decreases in the hippocampus, temporal and frontal cortices of people with AD (Peng et al. 2005; Fahnestock 2011).

Restoring Cholinergic Functions

The neurocognitive deterioration seen in Alzheimer's disease (AD) patients has been related to the degeneration of BFCN cholinergic neurons and the resulting cholinergic dysfunction. Neurons from human embryonic stem cells can mend and replace neurons that have been damaged in the host's nervous system (Telias and Ben-Yosef 2015). Predifferentiation into neural stem cells of embryonic stem cells (ESCs) and transplantation of these cells improves memory function in people with AD (Moghadam et al. 2009). Transplantation of human ESCs into the vitreous and hippocampus tissues led to the stable formation of functioning cholinergic neuronal populations, as well as their differentiation into BFCNs, according to the study of Bissonnette et al. (Yue and Jing 2015). Choline acetyltransferase (ChAT) neurotransmitters are more abundant in the forebrain when hESCs differentiate into neurons that look like choline acetyltransferase (ChAT) neurons (Li et al. 2008). Increased Trk-dependent Akt/Gsk3 signaling from stem cells promotes the production of cholinergic neurons (Li et al. 2016).

Anti-hyperphosphorylation of Tau

Tau is a protein largely found in neurons' axons, where it helps keep microtubules in the axons more stable. It plays a role in the maturation of neurons and the maintenance of cytoarchitecture during various physiological processes (such as development, hibernation, and hypothermia). Kinases like GSK-3 (Glycogen Synthese Kinase 3 Beta) phosphorylate these proteins during these processes. Overphosphorylation of tau protein is noticeable in neurons of AD patients due to an imbalance in kinase/phosphatase system resulting in abnormalities found in this diseased state. This abnormal hyperphosphorylated tau accumulates in brain cells to form misfolded protein structures called neurofibrillary tangles (NFTs). hESCs could inhibit abnormal phosphorylation of tau through the GSK3β signaling pathway (Lee et al. 2015) and thereby increase stability of microtubules and restore cognition (Li et al. 2016).

Antiamyloidogenic Potential

There is a deposition of amyloid (Aβ) plaques in the brain of AD patients which is neurotoxic and causes loss of neurons, synapses, and neural impairment (Walsh and Selkoe 2004). Accumulation of this neurotoxic plaques has been linked to irregular activity of β-site Amyloid Precursor Protein-Cleaving Enzyme 1 (BACE1) which cleaves Aβ precursor (APP), a membrane spanning protein at its β-secretase site. This enzymatic reaction gives rise to amyloid beta (Aβ) fragments (Aβ40 and Aβ42), a soluble precursor protein released outside the cell and thus considered the rate-limiting step of Aβ production. Aβ is presumed the culprit in the pathogenesis of AD, and its accumulation in the brain causes formation of senile plaques resulting in neuronal

damage or death. Stem cells have been observed to facilitate clearance of Aβ and reduce its toxic reaction thus enhancing cognitive recovery (Bae et al. 2013; Choi et al. 2014). More specifically, NSCs have the ability to express metalloproteinase 9 (MMP9), an Aβ peptide-degrading enzyme (Miller et al. 2003). Additionally, stem cells can modulate the expression of BACE1 through Akt/GSK3β signaling and consequently alter APP processing to improve cognitive function (Li et al. 2016).

Impairment in the export of Aβ through the blood-brain barrier to the periphery is considered a factor responsible for accumulation of Aβ and consequent plaque formation in AD. Pericytes are cells in the central nervous systems (CNS) that maintain the blood-brain barrier and regulate entry of immune cells. Thus, hESCs transplantation can be effective in inhibiting pericyte loss to enhance transport of amyloid-β across the blood-brain barrier and promote its phagocytosis (Li et al. 2016, 2021).

More so, the expression of blood-brain barrier endothelial cell receptors have been observed to change with the development of AD with a decrease in the expression of efflux receptors and increase in the expression of influx receptors (Silverberg et al. 2010). hESCs therapy can help stimulate the expression of this receptor to increase the expression of efflux receptor to remove Aβ fragments while decreasing the expression of influx receptors.

Mitochondrial Biogenesis

Mitochondria generate energy required by the human body for vital functions and are thus termed the power house of the cell. Neurotransmission, membrane potential generation along the axon, neurogenesis, neural proliferation, neural differentiation, and dendritic remodeling are dependent on the presence of ATP and buffer Ca2+ -ion concentration produced by the mitochondria of the nerve cells especially at the synapses (Gazit et al. 2016; Cheng et al. 2010). Oxidative stress which is an imbalance between the concentrations of ROS generated and the presence of radical scavengers (the antioxidant system) has been observed in neurodegeneration. As electron is transported during oxidative phosphorylation in the mitochondria, there is possibility of high generation of ROS which becomes detrimental when not efficiently scavenged. The brain, especially the hippocampus and the cortex, is highly susceptible to oxidative stress due to high dependence on mitochondrial energy production, high energy consumption, and high content of polyunsaturated fats most vulnerable to oxidative alterations (Cobley et al. 2018). Abnormalities in mitochondrial function in AD include mitochondrial biogenesis, shape and number, oxidative phosphorylation, Ca2+ buffering, and ROS generation and mutation of mitochondrial DNA, as well as mitochondrial transportation along the neural axon (Cai and Tammineni 2017; Chen and Yan 2010). It has been discovered that PGC-1, a protein that regulates mitochondrial biogenesis, is downregulated in Alzheimer's disease together with its target gene (NRF-1) (Rohas et al. 2007; Qin et al. 2009). Fission and fusion regulate the morphology and structure of mitochondria, which are often

thread-like or tubular in shape (Mishra and Chan 2016) and are therefore critical for neuronal survival (Lu et al. 2009).

This improved cognitive function by increasing the expression of mitochondrial fission factors (Drp1 and Fis1) and increasing the number of mitochondria in an AD transgenic mice model by reducing Mfn1 and Mfn2 expression by neural stem cells in the model (Zhang et al. 2015).

Signaling Pathways Mediating hESCs Function

Wnt Signaling

Wnt signaling and its components are involved in hippocampal neurogenesis and can serve as signals from the microenvironment to support differentiation and maturation of neurons into functional dentate granule cells and contribute to hippocampal function (Toda and Gage 2018). The components of Wnt signaling that are key players in these processes are the Wnt ligand, Frizzled receptors (FZD) and coreceptors (LRP5), Dvl, GSK3β, β-catenin, and Wnt antagonist (Dkk1,). The Wnt/−catenin signaling pathway is activated by the Wnt glycoprotein (Jackstadt et al. 2020; Serafino et al. 2020) and Frizzled receptors (seven transmembrane helices) (Gordon and Nusse 2006). After binding to the frizzled receptor's N-terminus extracellular cysteine-rich domain (CRD) and its coreceptor (LRP5), the Wnt signaling pathway activates, resulting in the transcription of neurogenesis-related genes. The recruitment and subsequent phosphorylation of Disheveled (Dvl) is facilitated by the C-terminus of FZD, which mediates the contact between FZD and Dvl (Zeng et al. 2005; Bilic et al. 2007). The phosphorylation of -catenin by GSK3 (Perugorria et al. 2019) and subsequent ubiquitination and destruction of -catenin are prevented by these interactions. When TCF/LEF binds to –catenin, it stabilizes and accumulates in the cytoplasm, where it translocates into the nucleus to form a transcriptional complex and regulate the expression of target genes (Nusse and Clevers 2017; Rossini et al. 2013). Enhancing hippocampus neurogenesis requires GSK-3 inhibition in the Wnt signaling pathway (Varela-Nallar et al. 2015; Zeng et al. 2019). Gene Cyclin D1 is one of the downstream genes targeted by Wnt/-catenin signaling; they increase neuronal differentiation by activating TCF/Lef-binding DNA-binding transcription factors such as NeuroD1 to promote hippocampal neurogenesis (Kuwabara et al. 2009).

There are endogenous inhibitors of the Wnt signaling that prevents the downstream expression of target genes important for neurogenesis. Secreted frizzle-related proteins (sFRPs) (mostly sFRP3) bind directly to Wnt ligand and prevent their interaction with FZD receptor while Dickkopf 1 binds to LRP5 (Wnt coreceptor) to interfere in its interaction with FZD/Wnt complex (Clevers and Nusse 2012). Preventing these interactions due to upregulation of sFRPs and Dkk1 consequently leads to phosphorylation of β-catenin and its degradation (Cotrim et al. 2013). These Wnt antagonists sFRPs and Dkk1 have been shown to be a regulatory mechanism for hippocampal neurogenesis (Jang et al. 2013; Seib et al. 2013). In brains of AD patients after

postmortem, an increase in Dkk1 levels was observed (Caricasole et al. 2004) while knockout of sFRP3 gene in mice has been shown to increase proliferation of NSC confirming their involvement in neurogenesis.

Wnt/-catenin signaling is downregulated in the AD brain, and the Wnt coreceptor LRP6 is dysregulated and malfunctioning. Upregulation of DKK1 and sFRPs expression, activation of GSK3, and degradation of -catenin are all seen (Jia et al. 2019). By stabilizing -catenin in the cytoplasm for translocation into the nucleus to modulate expression of target genes involved in neurogenesis, hESC transplantation can promote neurogenesis via the Wnt signaling pathway. As a result, GSK3 and Wnt antagonists (Dkki and sFRPs) must be inhibited.

As a serine/threonine kinase, GSK-3 (GSK-3 and GSK-3) has been found to phosphorylate and inhibit glycogen synthase activity (Frame and Cohen 2001). Alzheimer's disease (AD) pathogenesis is linked to the phosphorylation of GSK-3 at its Ser 21 in GSK-3 and Ser 9 in GSK-3, which inhibits its activity and prevents the downstream phosphorylation and inhibition of target genes (A peptides and tau proteins) that can disrupt neurogenesis, neuronal survival, and synaptogenesis. A protein complex interaction, such as the Wnt pathway, can also influence GSK-3 activity (Fig. 4). Stem cells have been revealed to restore cholinergic functions, prevent tau hyperphosphorylation and prevent amyloid plaque formation via the GSK-3β signaling pathway (Li et al. 2016), and thus GSK-3β is revealed to increase during aging (Lee et al. 2006) (Fig. 4). In cholinergic neurons, activation of GSK-3β causes a reduction in acetylcholine levels due to a decrease in the activity of choline acetyltransferase (ChAT) that mediates acetylcholine synthesis and possibly increase in activity of acetylcholine esterase that hydrolyzes acetylcholine and stops its neurotransmission function (Xiong et al. 2019; Zhao et al. 2013; Wang et al. 2017). Furthermore, inhibition of GSK-3β prevents phosphorylation and subsequent inactivation of pyruvate dehydrogenase (PDH) which catalyzes the conversion of pyruvate to acetyl CoA and ultimately prevents the reduction in acetylcholine in cholinergic neurons (Hoshi et al. 1996) (Fig. 4).

Wnt pathway activation phosphorylates and inhibits GSK3, which hinders the processing of APP by reducing transcription of BACE1 (app cleaving enzyme) and also affects tau hyperphosphorylation (Parr et al. 2015; Tapia-Rojas et al. 2016; Hernandez et al. 2013). Wnt pathway is also essential for the creation, integrity, and function of the blood-brain barrier (BBB), preventing impairment in A export through the BBB that can lead to accumulation of A and plaque formation (Engelhardt and Liebner 2014; Liebner et al. 2018).

Brain-derived neurotrophic factor (BDNF) is widely expressed in the central nervous system and is involved in neuron formation and survival, axon and dendritic growth and guidance, synaptic plasticity, long-term potentiation, and neurotransmitter release (Tapia-Arancibia et al. 2008). In the hippocampus, cortex, and basal forebrain, these activities are critical for learning, memory, and cognitive functions (Bekinschtein et al. 2008). Memory enhancement is linked to increased expression of BDNF and its receptor, tyrosine kinase-coupled receptor (TrkB), but changes in their expression can lead to cognitive impairment (Song et al. 2015).

In order for BDNF to be expressed and released, CREB activation and binding to the cAMP response element (CRE) in the promoter region of BDNF are necessary

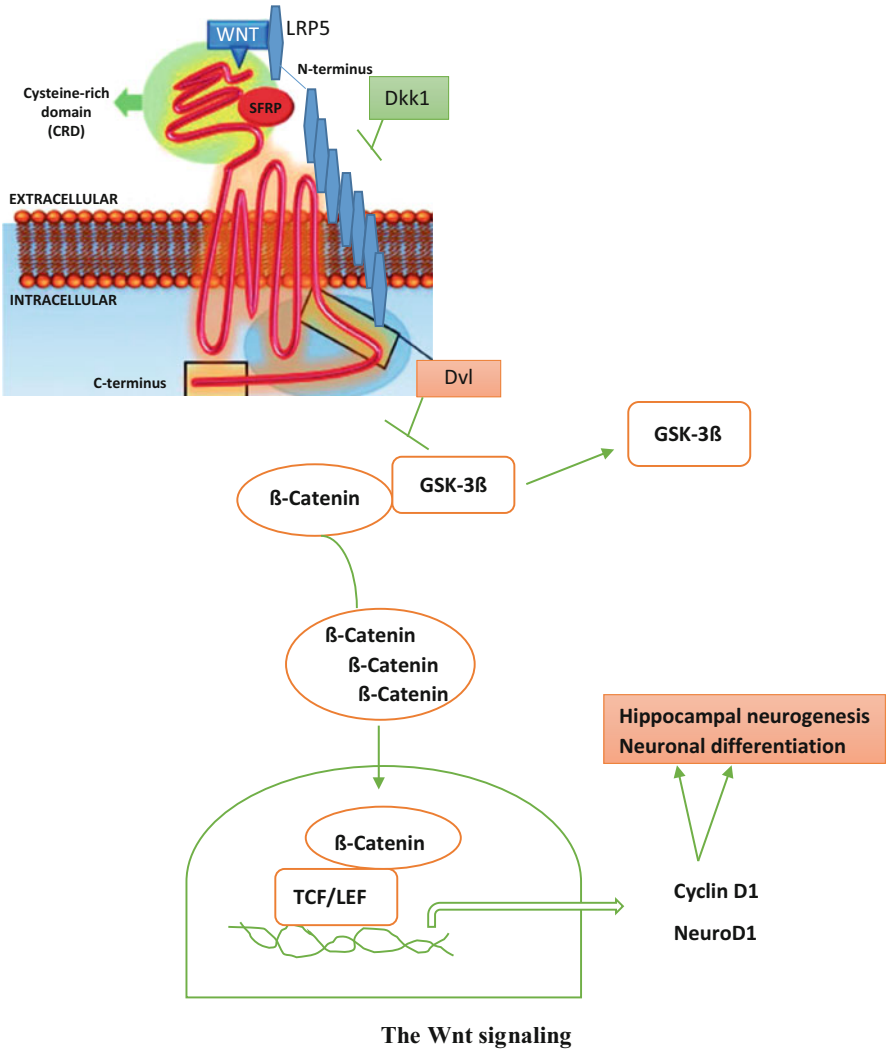

Fig. 4 The involvement of the GSK-3β pathway

(Yoo et al. 2017). This boosts the transcriptional activation of BDNF by activating CREB at its serine-133 residue, which is phosphorylated by PKA, PI3K/AKT, and mitogen-activated protein kinase 2 (Rosa and Fahnestock 2015; Alberini 2009). The glycogen synthase kinase-3 (GSK3) inactivates CREB by phosphorylating its serine -129 (Fig. 5). CREB is activated by phosphorylation of cyclic AMP (cAMP)-dependent kinase, which initiates the CREB signaling cascade and BDNF transcriptional activation (Won and Silva 2008; Cohen and Greenberg 2008). Following the activation of adenylate cyclase by neurotransmitter stimulation of G-protein-coupled

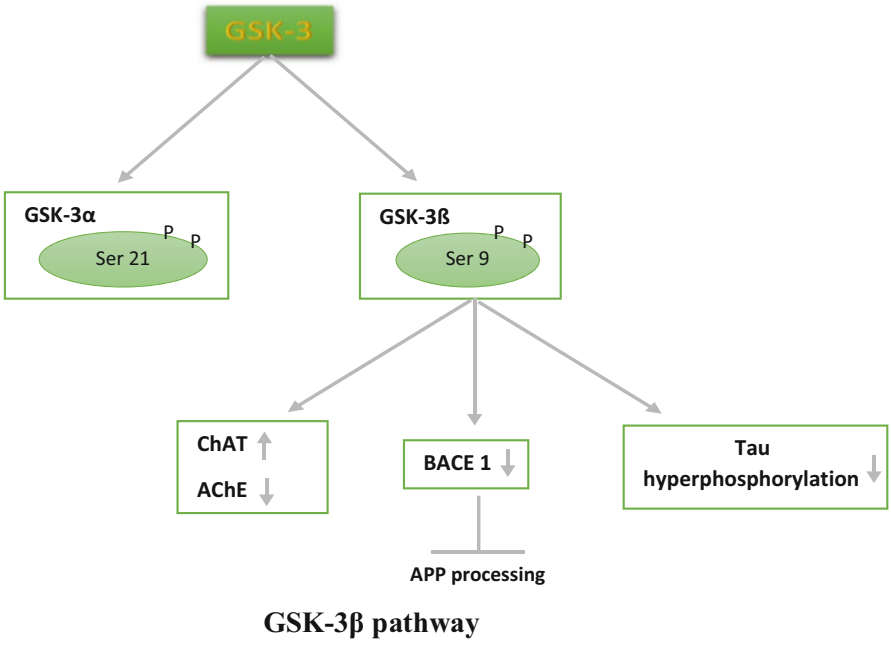

Fig. 5 The BDNF/CREB pathway

receptors (GPCR), the amount of cAMP rises. As a result of cAMP release, protein kinase A is activated by dissociating its catalytic component from its regulatory subunits (Fig. 5). PKA's catalytic subunits enter the nucleus and phosphorylate CREB at Ser133 in its kinase-inducible domain (KID), causing nuclear localization and activation of the protein (Guo et al. 2017). The ubiquitin-proteasome system (UPS) regulates PKA activity by degrading its regulatory subunits. Apart from phosphorylation, CREB-binding protein (CBP) is a coactivator of CREB and is essential for gene transcription activation by binding to promoter regions of genes implicated in learning and memory (Ravnskjaer et al. 2007). In a rat model of Alzheimer's disease, increased CBP has been shown to improve cognitive impairment. The transcription of BDNF is initiated by the activation of CREB and the recruitment of CREB-binding protein (CBP) (Dyson and Wright 2016) (Fig. 5).

Inhibition of PKA/CREB-mediated gene expression reduces the effect of BDNF in neurons and is impaired in AD's brain (Xue et al. 2016; Rosa and Fahnestock 2015). Aβ toxicity downregulates CREB-mediated transcription of BDNF by lowering the level of cAMP and subsequent phosphorylation of cAMP response element-binding protein (CREB) protein (España et al. 2010; Pugazhenthi et al. 2011). Thus, hESC transplantation can be channeled toward the cAMP/PKA/CREB pathway or used to deliver exogenous BDNF to the brain (Fig. 6).

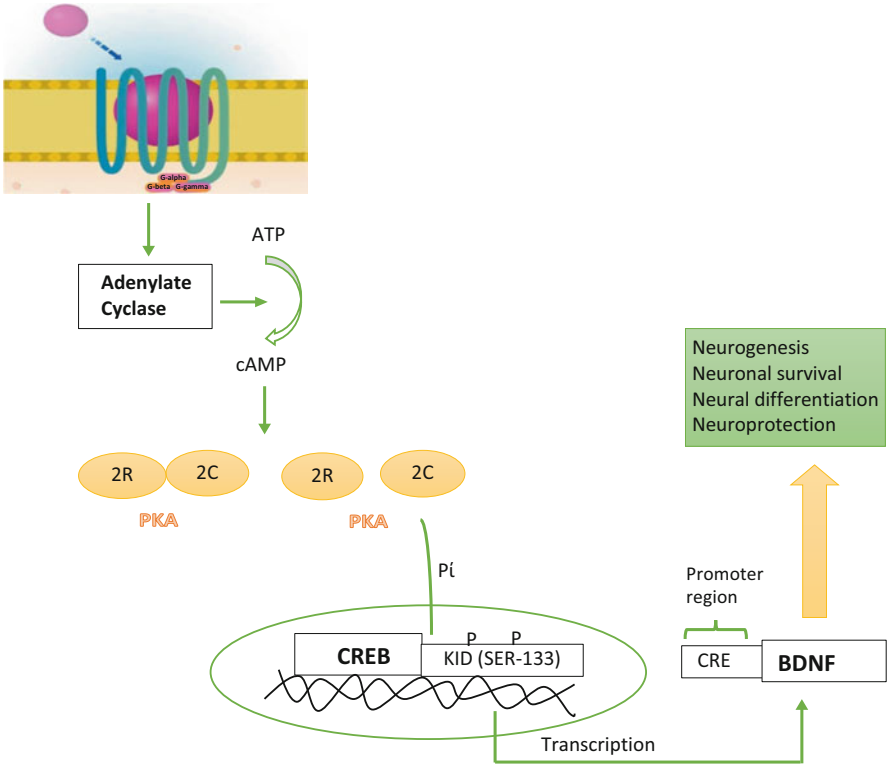

Fig. 6 The BDNF/CREB pathway

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Triple-Negative Breast Cancer (TNBC): Clinical Features and Therapeutic Targets

41

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Abstract

Triple-Negative Breast Cancer (TNBC) is one of the main antagonistic and dangerous subtypes of breast cancer, well-known for the deficiency of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER-2). It has a poor diagnosis when judged against further subtypes of breast cancer. The lack of a logical classification scheme for TNBC impacts both existing and new therapy options. TNBC remains an unmet clinical challenge due to its aggressiveness and the lack of targeted treatments. It has ductal histology, a high rate of propagation, and mitotic activity. Chemotherapy is considered more sensitive for TNBC. It is connected with an advanced hazard of distant revival, a higher rate of visceral and central nerve metastases, a shorter time to recurrence, and a worse prognosis after reappearance than hormone receptor-positive subtypes, indicating a more aggressive clinical course. This chapter provided an overview of pathogenesis, clinical features, and pathways associated with TNBC. This chapter highlights current TNBC therapy options, both standard and experimental.

Keywords

Triple Negative Breast cancer (TNBC) · Clinical features · Molecular pathways · Drug targets · Chemotherapy · Medications

Introduction

Globally, cancer is the second foremost cause of fatality. An estimated about ten million deaths, 70% of deaths due to cancer in 2019, is considered one of the world's burdens. Cancer can accumulate in any part of the body when the cell grows abnormally, and it starts to spread all over the body (organ or tissue), which paves the way to metastasis. In men, liver, stomach, prostate, lung, and colorectal cancers are commonly affected, while women's cervical, thyroid, and breast are mainly affected. Breast cancer is one of the deadliest ones among women, even in men but not that aggressive; it is a heterogeneous disease with different subtypes with diverse medication responses and medical outcomes. Based on the genes expressed in cancer, four vital molecular subtypes of Breast Cancer comprise Luminal-A, Luminal-B, Triple-negative/basal-like, and HER2 (Boyle 2012). And

also the fifth one is Normal-like which closely resembles Luminal A. Study reported by the American Cancer Society, about 73% are luminal A, 11% are luminal B, 12% are triple-negative, and 4% of HER2 were reported in the USA. Estrogen, progesterone, and HER2 receptors are widely expected in breast cancer, and these receptors are the key to the development of medications and diagnosis options. Regrettably, these receptors are absent and lead to the most violent form of BC called Triple-negative breast cancer (TNBC); the name itself resembles; it tests negative for the three essential receptors.

Lacking these receptors makes it more difficult for the common treatments such as hormone therapy, and the drug candidates that target estrogen, progesterone, and HER-2 are still ineffective. TNBC is more common among women with the BRCA1 mutation and African-American women younger than 40. It is entirely different from other forms of cancer; they can grow and spread very fast with minimal diagnosis and medication due to the lack of possible drug targets. According to epidemiologic studies such as the Carolina Breast Cancer Study, basal-like tumors were more expected to develop in women with early menarche. Higher parity, younger age at full-term pregnancy, shorter duration of breastfeeding, higher body mass index, and higher waist to hip ratio, particularly in premenopausal patients (Carey et al. 2006). TNBC has a poor prognosis, high metastasis is prone to recurrence further, and they have only inadequate treatment opportunities compared to other types of breast cancer. The primary cause for this is that ER, PR, and HER2 expression is all negative, making specialist endocrine and targeted treatment ineffective. Finally, chemotherapy has been noted as the best management producer for TNBC. A growing body of research suggests that employing neoadjuvant chemotherapy regimens in the treatment of TNBC resulted in a considerably higher pathological remission rate than hormone receptor-positive breast cancer and may improve TNBC patients' prognosis. Combination regimens based on taxanes, anthracyclines, cyclophosphamide, cisplatin, and fluorouracil are recommended by the National Comprehensive Cancer Network. As a result, selecting appropriate chemotherapy medications and optimizing chemotherapy regimens are critical for a successful treatment conclusion.

Biological Warnings of TNBC

American Cancer Society (ACS) reported a study in which the signs and symptoms of TNBC are much more similar to other types of Breast cancer. Most commonly, a mass or lump indicates the initial indication of TNBC; they are painful and swelling all over the parts of the breast, and it may be even without a lump. Other probable symptoms include:

- Breast skin dimpling.
- Pain in nipple or breast.
- Inward of the nipple, sometimes discharging occurs other than milk.

- Swelling in lymph nodes.
- Itchiness, irritation, or hardness.

Epidemiology

TNBC accounts for 170,000 of the global breast cancer burden (Sorlie et al. 2006). Using tools like EMBASE, Scopus, Medline, & Web of Science databases, investigations testified on the TNBC prevalence in India between January 1, 1999, and December 31, 2015 (Sandhu et al. 2016). The Carolina Breast Cancer Study (1993–1996) was a population-based case-control study that included an oversample of premenopausal and African-American women. They looked at the occurrence of subtypes of BC within menopausal subsets and racial and found links to the tumor's size, auxiliary nodal status, nuclear pleomorphism, mitotic index, combined grade, p53 mutation, and genes associated with breast cancer. TNBC was found in both obese and non-obese African-American women analyzed previously and afterwards at the age of 50 (31% vs 29%); 29% vs 31% in obese or non-obese. TNBC seems to be more prevalent in black women than in white women. Approximately 375,761 invasive BC were identified (counting non-Hispanic white women are 276,938 and non-Hispanic black women are 21,681). Non-Hispanic white women had a greater lifetime incidence rate than non-Hispanic black women and Hispanics. However, the occurrence was highest among non-Hispanic black women aged 44 years, who also had advanced rates of stage 3 and 4 illness and an elevated frequency of TNBC across all age categories (Amirikia et al. 2011) (Fig. 1).

Diagnosis and Treatment

There are fewer therapy choices for triple-negative breast cancer than for other kinds of invasive BC. This is because the tumor cells lack estrogen and progesterone receptors and enough HER2 protein to effectively develop hormone treatment or targeted medicines. Patients often need a lump removed (a lumpectomy) or the whole breast initially (a mastectomy). Then there are chemotherapy therapies targeting cancer cells that can't be seen, such as those still in the breast or have spread to other body regions (Fig. 2).

Lumpectomy

The malignant tumor and a rim of surrounding tissue are removed during a lumpectomy, often known as breast-conserving surgery. For persons with cancer in only one breast area and a tumor less than 4 cm in size, a lumpectomy followed by radiotherapy is likely to be just as beneficial as a mastectomy. In rare situations, women who have been analyzed with early-period BC in one breast may elect to

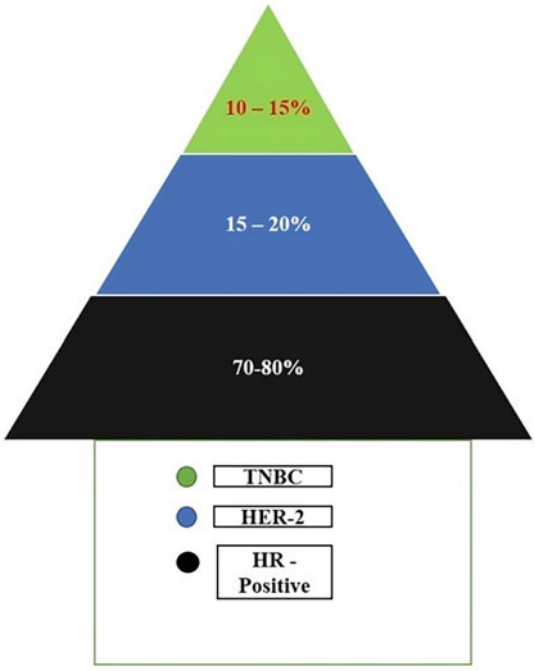

Fig. 1 Epidemiology of TNBC subtypes

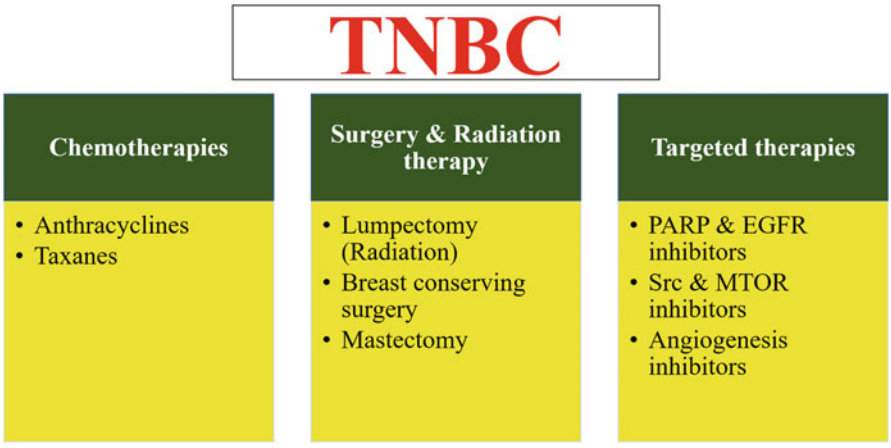

Fig. 2 TNBC treatment approaches

have that breast and the other healthy breast removed – a procedure known as a double mastectomy. Removing the other healthy breast is known as contralateral prophylactic mastectomy.

Mastectomy

The surgeon will remove the breast and any neighboring lymph nodes during a mastectomy to determine whether the tumor has spread. Few women prefer to have a reconstruction of the breast done simultaneously as their hysterectomy.

Radiation

After radiation therapy, Lumpectomies are carried out. This is when your breast is exposed to high-energy radiation to eliminate any leftover cancer cells. It takes roughly 20 min per day on average. Most ladies attend 4–5 days per week for 6 weeks. Radiation therapy will do this.

Chemotherapy

TNBC has a high level of biological aggression. However, it has been evidenced that they react better to chemotherapy than other kinds of breast cancer with a poor prognosis. This is attributable to a shorter disease-free time in adjuvant and neoadjuvant settings and a more aggressive course in metastatic settings (Ismail-Khan and Bui 2010). Chemotherapy is an effective systemic treatment because it can reach and eliminate cancer cells throughout a patient's body. Chemo medicines go through the patient's bloodstream to do this. Chemotherapy medications used to treat triple-negative breast cancer can be administered in various methods, including instilled into a vein through an intravenous (IV) drip, injected into a vein or muscle using a needle, taken orally as a tablet or capsule, and swallowed like a liquid. Pointing to DNA repair complexes, P53, cell proliferation, and targeted therapy are some of the therapeutic methods for TNBC management (Berrada et al. 2010). Platinum compounds have been used to treat breast cancer since the early 1970s, with over 200 medical examinations in breast cancer patients. Early applications of platinum therapies were investigated in advanced breast cancer patients who provided an outstanding, notable result in both single-agent and amalgamation with other drugs.

Platinum agents were not widely used, possibly because other medications in development at the time, such as taxanes, had a higher therapeutic index. Single-agent administration of cisplatin resulted in objective response rates ranging from 42% to 54% in small studies. Still, the rate of a comeback was poorer in women who had already undergone chemotherapy for metastatic illness. The response rate plummeted to 0–9% when cisplatin was given after other therapies. Notably, cisplatin was employed in these investigations independent of ER, PR, or HER2 status. Numerous mixture regimens were also examined, especially cisplatin combined with taxanes. However, there seemed to be little need to preserve these mixtures once taxanes were shown to be exceedingly active and far less toxic (Crown 2001).

Cytotoxic Treatment

The mainstay of treatment for treatable and progressive TNBC is cytotoxic chemotherapy. Anti-tubulins, anthracyclines, alkylating agents, time tabolites, and blocks of platinum are among the agents that have action in contained and progressive disease. Standard adjuvant and neoadjuvant regimens commonly include an anthracycline and an alkylating drug, given concurrently or successively before or after a taxane (docetaxel) (docetaxel or paclitaxel). When utilized in the neoadjuvant situation, these provide the highest pCR rates, but they produce the lowest reappearance rates in the adjuvant setting (Andreopoulou et al. 2017). Recent clinical trials suggest that adding more chemotherapeutic drugs to this arsenal could yield additional benefits. Utilizing the neoadjuvant platform may speed up new developments in TNBC, which currently has no targeted choices for different adjuvant treatments beyond chemotherapy.

Therapeutic Targets: At the Level of Gene

Micro RNAs (mi RNAs or mi Rs) are non-coding RNAs with 19 to 25 nucleotides that control gene expression by targeting mRNAs based on their sequence. RT-PCR examined the 49 primary TNBC cases and discovered that miR-21, miR-210, and miR-221 were considerably over articulated in the TNBC, while miR-10b, miR-145, miR-205, and miR-122a were suggestively under-expressed. The molecular evidence supports the concept that miR-221/222 plays a part in basal-like BC's aggressive clinical behavior. The level of miR-221 expression was substantially linked to HR status. HR-negative patients tended to have more significant plasma miR-221 levels (Ding et al. 2019). As a result, miRNA silencing treatments may be a helpful technique in miRNA therapeutics when combined with anti-tumor medications and chemotherapy treatments. Peptide Nucleic Acid (PNA) may be a DNA analog in which N-(2-aminoethyl) glycine units replace the sugar-phosphate spine. Even though it may be a hypothesis that miRNA-targeted compounds based on PNA can effectively treat human disorders, it is still trusted that this may be connected to significant patients based on clinical trial information (Fig. 3).

Immunotherapy

In early-stage TNBC, immune checkpoint TILs are common, relate to higher pathological complete response (pCR) to neoadjuvant chemotherapy, and are prognostic of disease-free survival (DFS) and overall survival (OS) (24–26). The expression of immune-regulatory checkpoints in the tumor microenvironment is an adaptive strategy for tumor resistance to invading lymphocytes (Keenan and Tolaney 2020). In preclinical and early clinical investigations, various intratumoral immune modulators and targeted medicines have been employed to enhance the response to PD-1/PD-L1 inhibition. Chemotherapeutic drugs can increase the action of

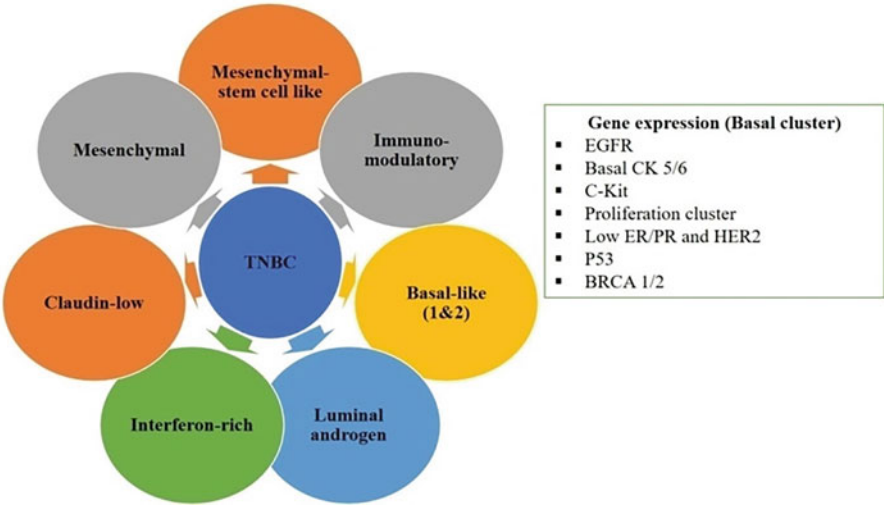

Fig. 3 The molecular taxonomy of TNBC.

immunotherapy, such as immune checkpoint inhibitors, by stimulating/releasing antigens, resulting in the encouragement of immunogenic cell death. Past the metastatic setting and indeed within the first-line setting, clinical trials analyzing to utilize of safe checkpoint inhibitors are presently underway, either as a single treatment or in different combinations with other therapies (Mediratta et al. 2020).

Treatment with Neoadjuvant

The results of studies examining the benefits of neoadjuvant checkpoint inhibitor therapy have been inconsistent. In the KEYNOTE-173 Phase 1b (NCT02622074) and I-SPY2 Phase II (NCT01042379) studies, neoadjuvant chemotherapy with pembrolizumab revealed tolerable tolerability and hopeful anti-cancer efficacy in patients with initial stage TNBC. The KEYNOTE-522 3rdPhasetrial (NCT03036488) looked into neoadjuvant chemotherapy with or without surgery, pembrolizumab, and adjuvant pembrolizumab or placebo (Lee et al. 2020). The PCR rate of the neoadjuvant combination was substantially more significant than that of the placebo-chemotherapy group (65 vs 51%). It's worth noting that the PCR benefit was similar (15%) in both the PD-L-positive and -negative subgroups, implying that neoadjuvant pembrolizumab may help patients independently of their PD-L1 levels. This contrasts with the progressive scenario, where atezolizumab is only beneficial to PD-L1-positive individuals. The toxicity profiles for each treatment were similar (78 vs 73%), with similar frequencies of grade 3 treatment-related AEs (78 vs 73%). Sacituzumab govitecan (IMMU-132) is an anti-Trop-2-SN-38 antibody-drug conjugate (ADC) that was approved by the FDA as an advance treatment in February 2016 for treating TNBC patients who had had a minimum of two therapies for the metastatic illness (Weiss et al. 2019). In 60 quantifiable patients with metastatic TNBC who had ordinary a middle of

5 (extend 2–12) earlier lines of treatment, and stage II patient's inquiries about proceeds to appear as a good middle survival advantage.

Breastfeeding and Triple-Negative Breast Cancer

Breastfeeding has been considered a conceivable exclusion in the quest for danger issues for this subtype with a bad prognosis (Anstey et al. 2017). The AMBER Consortium's findings bolster the validity of the earlier conclusions. Although Palmer et al. did not give information on breastfeeding length, they did indicate an odds ratio of 0.81 when likening the danger of ER-negative and, more particularly, TNBC in parous women who had ever or never nursed. When considering racial/ethnic inequalities in this exposure, the link between breastfeeding and TNBC risk is particularly fascinating. Preceding experimental studies of BC risk have found that African American case and control patients have a lower prevalence of nursing history than their non-Hispanic white counterparts. Beyond nursing, further research is needed to determine the more comprehensive environmental, biological, and cultural elements that subsidize triple-negative breast cancer incidence and disparities.

Given that most African American ladies are parous, age-specific rate rates of TNBC in African American women are 50% to 100% higher than in non-Hispanic white women; a 66% influence portion is probable to have significant public wellbeing implications. There are many ins and outs to heartening women to breastfeed and promoting workplace and healthcare communications to help new mothers breastfeed more successfully. Reducing inequities in TNBC occurrence may provide further impetus (Ambrosone and Higgins 2021).

Advanced Medications

Pembrolizumab was tested in a mixture with the investigator's choice of chemotherapy as a first-line medication choice for locally inoperable, recurrent, or metastatic TNBC against placebo plus chemotherapy (KEYNOTE-355 Phase III trial, NCT02819518) (Kwapisz 2021). In patients whose tumors communicated PD-L1 (CPS 10), a significant PFS advantage with the pembrolizumab-chemo combination was distinguished (9.7 months vs 5.6 months for chemotherapy alone) (34). The trial's other primary goal, overall survival, is now being evaluated (Fig. 4).

Molecular Pathways of TNBC

Different molecular pathways are engaged in TNBC, and many studies are focused on the pathways to identify the biological drug targets and drug therapies. Although a pair of medicine is presently present in process scientific trials, the biology in the back of TNBC continues to be largely unknown. It is understood that the TNBC represents awesome heterogeneity which complicates scientific remedy strategies

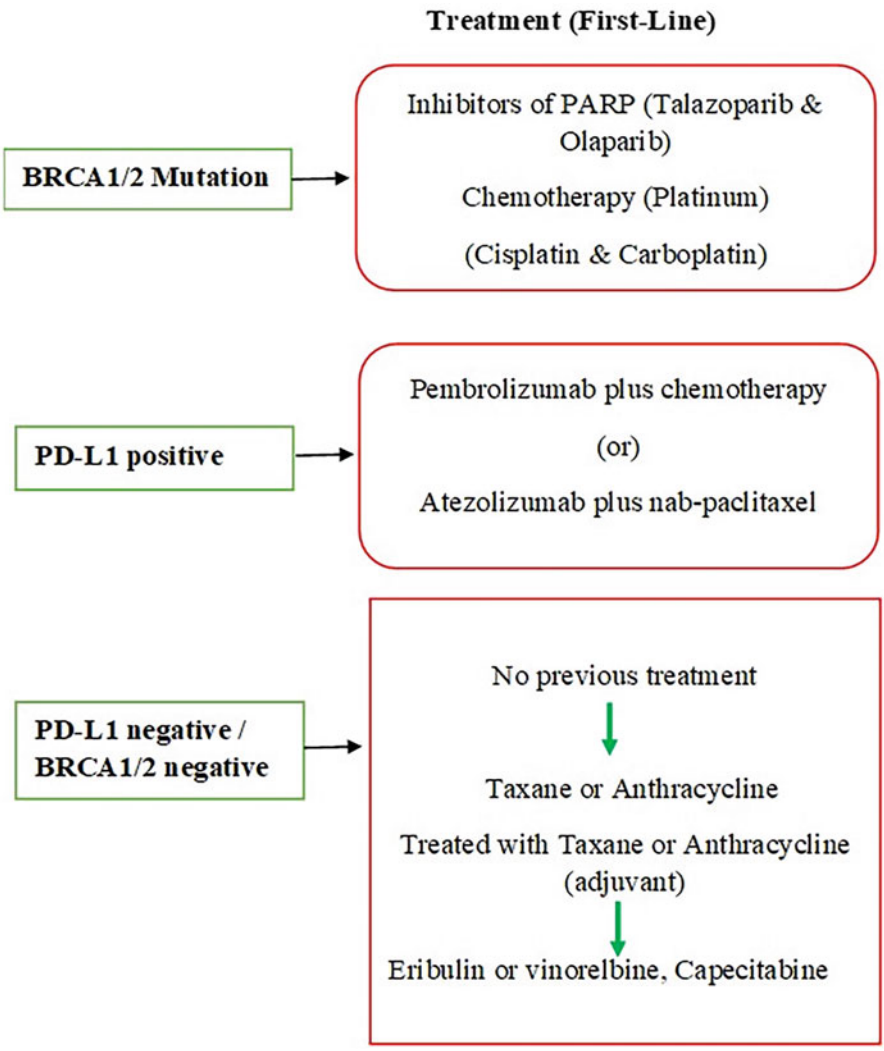

Fig. 4 First-line management for TNBC

(Nedeljkovic and Damjanovic 2019). A further class of TNBC can also additionally assist in reaching higher scientific final results (Fig. 5).

Ras/MAPK Pathway

This pathway is widely involved in the promoting proliferation of cells, angiogenesis, and differentiation of cells. N – Ras, K – Ras, and H – Ras are the different members of the Ras family, which are activated to diffuse the growth signaling of the cell membrane to the nucleus through a sequence of phosphorylated proteins that includes

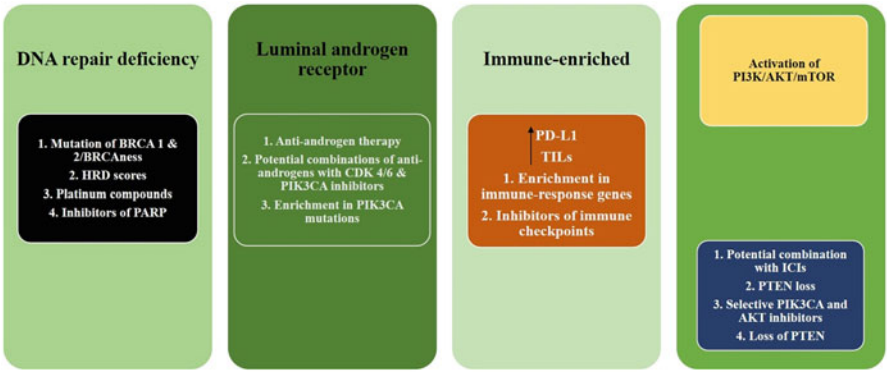

Fig. 5 The molecular pathways of TNBC

extracellular signal-regulated kinase, Raf, and MAPK kinase 1 (Giltnane and Balko 2014). At the same time, the frequency of mutations withinside the Ras/MAPK signaling pathway is <2% in TNBC, reproduction variety versions of specific genes from the Ras/MAPK pathway were tested to be related to TNBC (Loi et al. 2016).

PI3K/AKT/mTOR Pathway

RTKS activates the PI3K family members, stimulating PI3KS FOSFORYLATE phospylatebynositol 4,5 diphosphate (PIP2) at phosphatidylinositol-3,4,5triphosphate (PIP3), causing a downstream of Akt phosphorylation. The poor diagnosis of TNBC patients is associated with the hyperactivated AKT and mTOR; supported success in diagnosing experiments, twin inhibition of those molecules could represent a promising strategy for TNBC medications (Cossu-Rocca et al. 2015).

Models of TNBC

Syngeneic models, environmentally induced tumor models, genetically engineered mouse models (GEMMs), transgenic models (MMTV-PyMT model), and cell line-derived GEMMs are mouse models. In contrast, in vivo models for human CTC shedding and biology need either breast cancer-derived cell lines, or xenografts (PDXs) derived from patients' xenografts (Arroyo-Crespo et al. 2019).

Transgenic Mouse Models (MMTV-PyMT)

The MMTV-PyMT (mouse mammary tumor virus-polyoma middle tumor antigen) breast cancer mouse model is well established and extensively researched. MMTV-PyMT mice progress spontaneous mammary tumors that match the development and human breast cancer morphology meticulously. While MMTV-PyMT primary

tumors have been demonstrated to express HER2, levels are low compared to human HER2+ cell lines with augmented HER2 (Attalla et al. 2021). MMTV-PyMT tumors were associated with ER-negative "luminal" human breast cancers, a gene moniker comparable to the luminal-AR (LAR) TNBC subtype defined by high AR countenance and the molecular apocrine ER/PR negative, but AR+ tumors reported preceding molecular subtyping.

Patient-Derived Xenografts (PDXs)

PDX is made by implanting primary human tumor cells or tumor tissue fragments into immunocompromised mice. Even though most models are developed in mice without a functioning human immunization, they are regarded as quite relevant to clinical, particularly when orthotopically implanted (e.g., human breast tumor tissue from the operating room implanted into the mammary fat pads of mice). PDOX, which are orthotopically implanted PDX models, have been shown to review key genomic, histological, proteomic, and transcriptomic features of the tumors from which they were originated; and it is the best models of metastatic disease in human and anti-cancer therapy response, and it is more aggressive phenotypes in TNBC PDX models (Turner et al. 2020).

Genetically Engineered Mouse Models (GEMMs)

GEMMs provide a solid foundation for testing ideas about tumor formation, progression, interaction with the microenvironment, and therapy response. Multiple GEMMs have been produced with mutant alleles that are conditional, typically inducible, and constitutively active. However, because similar genetic changes such as TP53 deletion occur in several human breast cancer subtypes, it's not always clear which human subtype a given GEMM most closely resembles (Park et al. 2018). The gene expression profiles of more than 27 distinct mouse GEMM of mammary carcinomas to several human data sets were examined to find the models that most closely resemble human disease subgroups. Genetically engineered rats, on the other hand, can be helpful models for studying the involvement of the Ras, BRCA1, and BRCA2 genes in the evolution of malignant breast cancers. Growth factors and receptors can be used in genetically engineered mouse models of TNBC cancers. GEMMs offer an alternate platform that solves these flaws. When combined with CDXs and PDXs, it provides good preclinical validation to help bring drug candidates into the clinic and identify which patient subgroups could benefit from targeted therapy.

Conclusion

TNBC appeals to collective attention because of its exclusive clinical pathology and molecular features. Chemotherapy remains the limited effective systemic medication for patients with TNBC. This chapter widely shows that characterization of TNBC

with biological signature and diverse changes in many cancer pathways and the medication options are discussed. The information moreover outlines that TNBC speaks to a heterogeneous group of breast cancers. The unique built-up classification of TNBC as basal-like cancer ought to be changed, with a requirement for an advance examination and the creation of extra, progressed, exceedingly particular biomarkers for this sort of cancer.

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Therapeutic Molecular Targets of Cancer and Animal Models: Adequacy and Drawbacks

42

Lucian Hritcu and Oana Cioanca

Contents

Abstract

Cancer has become a serious public health problem worldwide. The selection of applicable experimental animal models is essential for the transfer of basic scientific knowledge about cancer in treatment interventions in humans. In vivo analysis is essential for the life science, especially for the development of diagnostic drugs. Mouse-supported cancer models are known to be widely used in cancer research. Mouse models have transformed the ability to study in vivo gene and protein activities and gain a deeper knowledge of their molecular pathways and processes. Grafts, including chemically or genetically modified mouse tumors, are the most used rodent cancer models.

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This chapter describes the most common cancer animal models and therapeutic molecular targets focusing their compatibility and shortcomings.

Keywords

Cancer · Animal models · Therapeutic molecular targets · Adequacy · Drawbacks

Introduction

All phases lead in the multistage development of cancer, including preservation of proliferation initialization, prevention of growth inhibitors, replication immortality, resistance to cell death, tumor-promoting inflammation, induction of angiogenesis, activation of infiltration and metastasis, genomic instability and mutation, immunity prevention of destruction, and thus destruction of cellular energy (Hanahan and Weinberg 2011; Cekanova and Rathore 2014). Cancer is the second leading cause of death in the world. Overall, cancer is becoming more and more common. In 2014, approximately 1,665,540 people were cancer patients in the USA of which 585,720 died as a result of cancer (Siegel et al. 2013). In Europe, 1,267,000 cancer deaths are expected in 2021, with age-standardized rates of 130.4/100,000 males(−6.6% since 2015) and 81.0/100,000 women(−4.5%). Lung cancer had the highest rate of rise among cancer categories, increasing by +6.5% to 14.5/100,000 in 2021.

To increase cancer detection and early treatment efficiency, specific tumor molecular targets must be discovered. As a result, it is highly desirable to use and develop relevant cancer models in vitro and in vivo (Cekanova and Rathore 2014) (Table 1).

Cancer models, whether created naturally or artificially, have properties like human tumors. Cells in reducing 2D cultures change cell shape, disrupt signaling

Table 1 Cancer models: advantages and drawbacks

Cancer model	Advantages	Drawbacks
In vitro model	Easily implemented, low-cost assay (van Staveren et al. 2009) Highly controlled circumstances (Ayuso et al. 2021) Homogeneity (Burdall et al. 2003) Identification of molecular pathways (Riedel et al. 2008) Reproducibility (Hirsch and Schildknecht 2019)	Inability to simulate the variety of human cancer cells (Sajjad et al. 2021) Low physiological relevance (Howells et al. 2007) Disrupt signaling pathways (Sever and Brugge 2015)
In vivo models	Easy to establish (Gargiulo 2018) Recapitulate human tumor history (Sajjad et al. 2021) Use the same microenvironment as human cancer (Li et al. 2021) Facilitate understanding of the genetic basis of cancer (Balmain et al. 2003)	Costly and time consuming (Lokman et al. 2012) Requires immunodeficient host (Okada et al. 2019) Long tumor latency (Gómez-Cuadrado et al. 2017)

pathways, and fail resistance mechanisms in in vitro cancer models (Ben-David et al. 2018). As a result, many pharmaceutical agents that showed therapeutic promise in vitro have been shown to be ineffective in clinical trials (Morgan et al. 2018). Despite the development of increasingly clinically relevant 3D patient–derived organoid models, they are not exact genetic representations of their tumors of origin (Roerink et al. 2018).

In vitro cancer models have a few advantages, including highly regulated conditions, homogeneity, identification of molecular pathways, and reproducibility.

In vivo models are also insufficient (Clarke and Fisher 2020). For example, genetically modified mouse models and patient-derived xenografts show genomic changes at the tumor and host levels that are more physiologically relevant than in vitro models (Kersten et al. 2017). In addition, metabolism, physiology, cellular interaction, the microenvironment, and lack of a functional immune system distinguish the xenograft environment from the patient's original environment (Villacorta-Martin et al. 2017).

This chapter presented the advantages and drawbacks of animal cancer models, as well as the therapeutic targets that are relevant in both human and animal carcinogenesis.

Rodent Models in Oncology

Animal models of cancer offer a unique way to determine what causes cancer and how to treat it, making them a valuable resource for cancer researchers. The complexity of modeling cancer in mice has increased to the point that scientists can now observe and control a complex disease process in a way that humans cannot. We only now know the full potential of mouse cancer models and what new approaches are needed to maximize the value of this investment for cancer patients due to the model design constrains and technological advances, as well as the significant underutilization of existing models (Frese and Tuveson 2007).

Xenograft Mouse Model

The human tumor xenograft is widely utilized models. Interrupting genes crucial in immune cell development, survival, and function have resulted in several immunodeficient mouse breeds. The capacity to develop these immunodeficient animals is crucial for creating humanized mice that can be utilized to examine the progression of human cancer (Tian et al. 2020). Immunodeficient mice were created to inhibit discarding of human cancer cells by immune system (Shultz et al. 2012). T and B mouse lymphocytes deficits is linked to the forkhead box N1 (*Foxn1*) gene deletion (Fogh et al. 1977), recombination activating gene 1 (*Rag1*) (Mombaerts et al. 1992), recombination activating gene 2 (*Rag2*) (Shinkai 1992), protein kinase, and DNA-activated and catalytic polypeptide (*Prkdc*) genes (Bosma et al. 1983). Deletion of the interleukin 2 receptor subunit gamma (*IL2rg*) (Ito et al. 2002) or

2-microglobulin (*B2m*) (Christianson et al. 1997) genes cause deficiency or disfunction of mouse NK cells, but nonobese diabetic (NOD) mouse background (Takenaka et al. 2007) or knock-in human (Herndler-Brandstetter et al. 2017) or NOD (Yamauchi et al. 2013) *Sirpa* genes prevent macrophages phagocytosis. The most often used immunodeficient mouse strains NOD/*Prkdc*^{scid} (NOD/SCID), NOD/SCID IL2rg$^{-/-}$ (NSG or NOG), and Balb/c Rag1$^{-/-}$ IL2rg$^{-/-}$ (BRG) were used in human oncology studies, employing a combination of these genetic approaches (Shultz et al. 2012).

The athymic "nude mouse" will readily receive the xenograft (Richmond and Su 2008). Human cell line xenotransplantation is a tractable, quite simple controllable, and experimentally nimble methodology. Hajitou et al. (2008) developed a primary tumor from a soft tissue sarcoma cell line in the rat hind limbs. Consequently, the use of xenograft models obtained in a cell line reduces the influence of unrelated cells in the study of a single factor (Wykosky et al. 2015), improves the use of experimental cellular operations in the study of signaling pathways and tumor-related molecular mechanisms (Kung et al. 2014), developed animal models of acute myeloid leukemia to mimic the progression of fluid tumors (Saland et al. 2015), and assisted in the study of metastatic mechanisms by intravenous or intraperitoneal injection (Santel et al. 2010). Recently, Woodfield et al. (2017) described the development of a new cell line based on a mouse model of an orthotopic xenograft that recapitulates human hepatoblastoma. The human hepatoblastoma cell line is commercially available for in vivo preclinical studies. However, HepG2 tumor strongly matches the human hepatoblastoma. Also, Larsson et al. (2018) reported a cell xenograft mouse model of pediatric glioma stem cells that were very similar to the patient's clinical course. According to the authors, this model can be used to measure the patient's response to preclinical research.

Because they alleviate some of the issues that arise when employing cell cultures, patient-acquired xenografts (PDXs) are one of the most often utilized models of human cancers in mice (Siolas and Hannon 2013). In the PDX, human-derived tumor cells are transferred to immunocompromised mice (athymic nude mice, severe combined immunodeficiency [SCID] mice, or other immunocompromised mice) under the skin or the type of the organ from which the tumor originates (Tanaka et al. 2021). According to Xu et al. (2018), PDXs could be considered the best model for researching tumor heterogeneity and are presently the efficient toll for evaluating cancer-related processes. The three most utilized PDX mice were nude, nonobese diabetic (NOD)/SCID, and NOD SCID (NSG) mice. Nude mice were athymic, meaning they have a congenital deficiency of T and normal B cells, as well as increased natural killer (NK) cell activity (Pelleitier and Montplaisir 1975). C.B.-17-SCID mice were crossed with NOD mice to form NOD/SCID mice. T and B cells were deficient in SCID and NOD/SCID mice from birth.

The use of human tumor xenografts to test drug treatment responses has several important advantages: (1) the complexity of genetic and epigenetic abnormalities inherent in the human tumor population is reflected in the human tumor tissue being used for transplantation; (2) human tumor xenografts are a first step in developing personalized molecular treatment methods for clinical application; (3) findings from

a human tumor biopsy can be available in a matter of weeks to provide an indication of treatment response; (4) a single tumor biopsy can be used to test different therapies; (5) before patients are treated with potentially ineffectual treatments, tissue microarray and gene microarray data can be easily retrieved from human biopsies and xenograft tissue for complex screening; (6) orthotopic xenografts can be successfully implanted in the natural organ milieu in order to assess the tumor's impact on its microenvironment using tissue-specific modulations; and last but not least (7) xenografts generated from nonobese diabetic/severely compromised immunodeficient (NOD/SCID) mice have been "humanized" by injection of blood or bone marrow cells to fully restore the tumor's immune response (Richmond and Su 2008). There are some important limitations to monitoring and/or predicting a cancer treatment response using a mouse xenograft model: (1) an orthotopic tumor model is time consuming, costly, and technically difficult to create; (2) lymphocyte-mediated responses to tumors are lost in athymic nude or SCID mice, whereas nude mice lose some T cell responses, and SCID mice lose both T cell and B cell responses; and (3) lymphocyte-mediated responses to tumors are lost in athymic nude or SCID mice, with nude mice lose some T cell responses and SCID mice lose both T cell and B cell responses (Richmond and Su 2008).

Chemically Induced Rodent Model

The use of DNA-damaging chemicals, which lead to several correction mechanisms that occasionally lead to DNA sequencing errors, is a method of generating DNA changes. Chemical carcinogens alter the genetic and epigenetic structure of susceptible cells and give them a selective growth advantage; these cells may become cloned, genomically unstable and possibly malignant. The first stage of carcinogenesis is the formation of tumors, which occurs when normal cells are exposed to chemical or physical carcinogens (Fig. 1).

Some chemical carcinogens, such as (1) N-nitroso compounds, (2) heterocyclic amines, (3) polycyclic aromatic hydrocarbons, (4) food additives, (5) polychlorinated biphenyls, (6) antineoplastic agents, (7) naturally occurring compounds, and

Fig. 1 Chemical carcinogenesis

(8) synthetic compounds, have been identified as being used to induce carcinogenic lesions like human malignancy.

N-nitroso compounds are carcinogenic to various animal organs and can cause cancer in about 40 different animals, including higher primates (Bogovski and Bogovski 1981). Among them, N-nitrosodiethylamine (DENA) is the most commonly used cause of liver cancer in mice (DA COSTA et al. 2014). DENA is a member of the N-nitroso family of compounds, which are known to be highly carcinogenic and known in food, beverages, cosmetics and personal care items, tobacco, and other substances (Rath and Canaes 2009). About 300 N-nitroso compounds were examined for carcinogenicity; 90% of them induce cancer in 40 animal species, and in a variety of organs (Stuff et al. 2009). In rats and mice, chemical carcinogens such 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) have been shown to belong to the heterocyclic amine (HCA) family and cause breast carcinomas (Machida and Imai 2021). PhIP is the most prelevant HCA subtype, having board carcinogenicity as an initiator (including DNA damaging and mutagenesis) and estrogenic activity as a promoter. PhIP exposure has been associated to breast (Naiki-Ito et al. 2007), colorectum (Tang et al. 2011), and prostate cancer in rats (Tang et al. 2013).

7,12-Dimethylbenzanthracene (DMBA) belonging to the polycyclic aromatic hydrocarbons (PHA) has been documented to induce mammary tumorigenesis in rats (Yang et al. 2021).

Mice exposed to food supplement potassium bromate ($KBrO_3$) was found to be involved in intestinal carcinogenesis (Yokoo et al. 2016).

Polychlorinated biphenyl quinone exposure induced breast cancer in rats as reported by Qin et al. (2022).

Carcinogenesis has been linked to interaction with organic substances. Aflatoxin B1 (AFB1) is the most powerful carcinogen among others known to produce hepatocellular carcinoma in rats (Kittichaiworakul et al. 2021). Exposure to asbestos fiber is central to mesothelial carcinogenesis in rats as described by Okazaki et al. (2020). Also, aristolochic acid is considered to be responsible for carcinogenicity in rats associated with upper urothelial cancer (Bárta et al. 2021).

Synthetic carcinogen has been demonstrated to induce carcinogenetic effects in animals. 1,2-dimethylhydrazine dihydrochloride (BMH) exposure induced colon carcinoma in rats (Zhang et al. 2021). Azoxymethane (AOM) is a potent cancer inducer widely used in rats for colon cancer as reported by Zhou et al. (2021). Klaus et al. (2017) suggested that short-term exposure to low doses of methylazoxymethanol acetate (MAMAc) increase the number of time-dependent DNA adducts in the Eker rat kidney, including extremely stable and promutagenic O^6-methylguanine (O^6MG) adducts. Hepatic damage including hepatocarcinoma has been attributed to phenobarbital (Pathak et al. 2020).

Chemically created rodent cancer models can help researchers for better understanding of the cancer complexity, but this method is difficult, time consuming, and requires high-throughput sequences to detect mutations.

Genetically Engineered Mouse Model

The heterogeneity and diversity of human tumors can be captured using genetically engineered mouse (GEM) cancer models (DuPage and Jacks 2013).

However, faster progress in transferring studies from a mouse cancer model to the clinic is hampered by the limitations of these models, which better represent the complexity of human malignancies. Previously, immunodeficient mice with allogeneic and xenograft tumors have been used, subcutaneously or orthotopically transplanted. Due to their wide availability and low cost, these models are widely used in various studies. Another form of rodent model used in cancer research is transgenic mice, in which oncogenes can be produced constitutively or conditionally.

Pronuclear injections, which involve the direct implantation of DNA into fertilized zygote, can also yield GEMs (Cekanova and Rathore 2014). Overexpression of endogenous or foreign genes can be examined via pronuclear injections. This method is used to quantify the impact of gene in this mechanism because oncogene expression is one of the most important phases in the process carcinogenesis.

Unlike pronuclear injection, which involves random integration of exogenous transgenes, gene targeting involves precise changes to endogenous genomic regions. This method can be used to induce genetic alterations like deletions, mutations, inversions, and translocations into embryonic cell lines derived from early stage mouse embryos (Martic-Kehl 2016).

The main disadvantage of GEM is that it only targets just few copies of the gene. The vast heterogeneity of human tumor cells cannot be explained in this way. Creating a GEM costs time and resources and it usually begins during the precertification phase. Animal tumor development is unpredictable and slow. They have different biochemistry, physiology, and anatomy than humans. The importance of GEMs for human cancer has yet to be determined.

While proponents of GEMs argue that the problem is not in the models themselves, but in the experimental setting that has not been developed to transfer successful GEM research to human cancer. Therefore, it is very important to develop criteria for evaluating the value of a particular GEM for a specific experimental setup. It includes pathological evaluation, disease progression, tumor microenvironment, molecular signaling pathways, and environment variables (Sajjad et al. 2021).

Therapeutic Molecular Targets of Cancer

Anticancer drugs with improved molecular targeting have been created by researchers. Cancer cells have genetically altered cellular targets that are critical for tumor formation and survival.

Therapeutic Molecular Target for EGFR-Mutated Non-Small Cell Lung Cancer

The advent of molecular targeted therapy, particularly the development of small molecule EGFR inhibitors, has recently opened up new therapeutic options for non-small cell lung cancer (NSCLC), the most common lung tumor histology (Haber et al. 2005). NSCLC is the most common type of lung cancer, accounting for more than two-thirds of cases (Majeed et al. 2021), with the majority of patients (84%) currently diagnosed with advanced disease. The identification of targeted mutations (EGFR, ALK, PI3K/AKT/mTOR, RASMAPK, RET, MET, BRAF, and NTRK/ROS1) in patients with advanced NSCLC altered the therapeutic paradigm (Yuan et al. 2019). Deletion of exon 19 (EX19del) and point mutations of exon 21 (L858R) in the EGFR gene have been identified as carcinogenic regulators in nearly 20% of patients with lung cancer (Majeed et al. 2021).

For the moment, the development of the third generation of EGFR inhibitors is ongoing. Lazertinib (YH25448) targets EGFR mutations, including T790M, with rash, pruritus, and paresthesia as side effects (Ahn et al. 2019). Olmutinib was tested in patients with EGFR mutant NSCLC who had failed EGFR tyrosine kinase inhibitors (TKIs) and had proven T790M mutation in an open label, worldwide phase 2 research (Park et al. 2021).

The fourth generation of EGFR TKI, including EAI045 and BLU945, has been studied in order to solve C797S, the most common pathway of target resistance to osimertinib (Schalm et al. 2020).

Therapeutic Molecular Target for FLT3-ITD Mutation in Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a kind of hematological cancer marked by clonal proliferation of improperly differentiated myeloid progenitor cells (blasts) (Döhner et al. 2015). AML is a complex disease caused by multiple genetic mutations, but two identical genetic events are necessary to induce leukemia. Class I mutations that activate signaling pathways lead to proliferation and class II mutations that alter transcription factors involve bone marrow differentiation (Steffen et al. 2005). Among the FTL3 inhibitors, midostaurin and gilteritinib have been described for use in the treatment of AML with FTL3 mutations. Midostaurin was first discovered to inhibit protein kinase C (PKC) (Caravatti et al. 1994), but has now been identified to have activity against a variety of other kinases, including KIT, PDGFR, VEGF, CDK1, FLT3, and others.

Therapeutic Molecular Target for VEGF and mTOR Pathway in Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the seventh most common cancer in men and the ninth most common cancer in women, accounting for around 2–3% of all cancers in

adults (Ke and Shen 2017). The targeted therapies for advanced RCC are classified into two groups depending on their methods of action, however their activities overlap significantly (Heng and Bukowski 2008). Bevacizumab, sunitinib, and sorafenib have some direct anticancer efficacy by blocking the vascular endothelial growth factor (VEGF) system, which is a key modulator of tumor angiogenesis. Temisirolimus and everolimus decrease mammalian target of rapamycin (mTOR) kinase signaling in tumor cells, resulting cell cycle arrest, enhanced apoptosis, and angiogenesis suppression. Several intrinsic factors in tumors, such as hypoxic cytokines, oncogenes, and inactivation of tumor suppressor genes, upregulate the VEGF and mTOR pathways (Meric-Bernstam and Gonzalez-Angulo 2009). Both routes promote tumor angiogenesis, although their specific inhibitors have been found to be ineffective (Porta and Szczylik 2009).

Conclusions

The preclinical rat cancer models described in this chapter that can be utilized for diagnosis, treatment, or prognosis. Due to their extremely high accuracy, molecularly controlled anticancer drugs are effective in treating cancer. Newly targeted immunotherapeutic agents are common in most subtypes of cancer. Cancer cells have genetically engineered self-signaling targets that are important for tumor growth, survival, and growth. Targeted treatment is a practical option for treating patients with various types of cancer.

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Therapeutic Approaches and Models in Retinoblastoma

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Contents

Abstract

In cancer research, animal models constitute an essential aspect of preclinical investigations. In various human malignancies, such as ovarian, breast, colon, liver cancers, and tumors in the eye, nonhuman tumor models have aided in determining the path of carcinogenesis and estimating diagnostic and treatments. In vitro investigations have limitations since they are done outside of the human body's complicated milieu. The complete scope of pathophysiological alterations

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that occur in neoplasms can be revealed by probing the cellular processes of tumor growth within the complexity of an organism. This chapter intends to construct prototypes that parodist all aspects of retinoblastoma, including its genesis, development, and metastasis, using molecular and imaging technology breakthroughs. In retinoblastoma research, combining xenograft and genetic models has and will support the way for a deeper understanding of retinoblastoma tumor biology and devising and testing viable diagnostic and therapy methods.

Keywords

Retinoblastoma · Animal Models · pRB · Tumor · Retinal cells · Pediatric ocular malignant

Introduction

The most common malignant tumor of the eye in children is retinoblastoma, which affects one out of every 15,000–20,000 live births. The tumor is caused by the inactivation of the retinoblastoma (Rb) gene at both alleles, leading to abnormal pRB protein production. The tumor suppressor gene pRB regulates terminal differentiation, DNA replication, and cell cycle progression (Chang and Ying-Ping 2013). When pRB activity is lost in retinal progenitor cells, the cell cycle regulation is disrupted, and the proliferation of cells is uncontrolled. Whether it is random or hereditary, retinoblastoma can appear unilaterally or bilaterally. Understanding genetic inheritance and advances in diagnostic technologies has revealed the way for the first effective genetic diagnosis (preimplantation), allowing for early detection and genetic prediction. According to Xu et al. (2004), it is feasible to test embryos for mutation of RB1, transplant a good embryo after in vitro fertilization, and have a successful gestation and delivery. Furthermore, early detection and treatment of family members at risk of developing retinoblastoma is possible because of accurate RB1 mutation detection (Tomar et al. 2017) (Figs. 1 and 2).

Alfred Knudson (1971) anticipated the tendency of retinoblastoma following a statistical examination of incidence in the early 1970s. In 1987, the retinoblastoma gene was discovered, confirming his theory. The bilateral variant is usually inherited, but the unilateral form is not. The tendency to tumor development in the hereditary type is inherited from a parent who carries one mutant allele of the RB1 gene. When a child inherits one copy of a damaged gene from their parents, they are 1000 times more likely to lose a second copy than if the mutation occurs spontaneously. Because RB1mutant copy is present in all cells and RB1 mutation of the second allele can happen in numerous cells of the retina, this variant is more likely to be multifocal. Patients with RB1 deficiency in nonretinal cells are more likely to develop secondary malignant neoplasms such as osteosarcomas. Unilateral retinoblastoma is a kind of retinoblastoma that develops when both copies of the RB1 gene are somatically altered or deleted in the developing retina (Diana et al. 2017). Tumorigenesis is a multistep process in which cells multiply indefinitely, resist apoptosis, generate

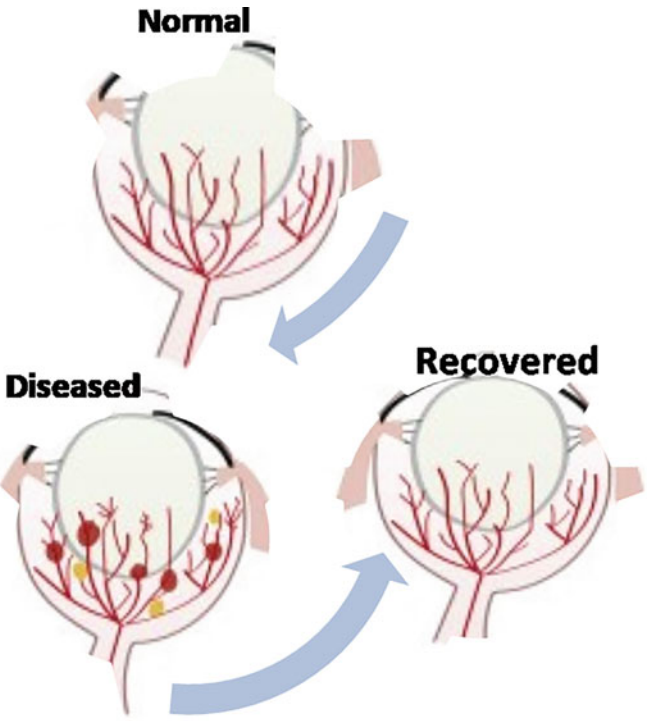
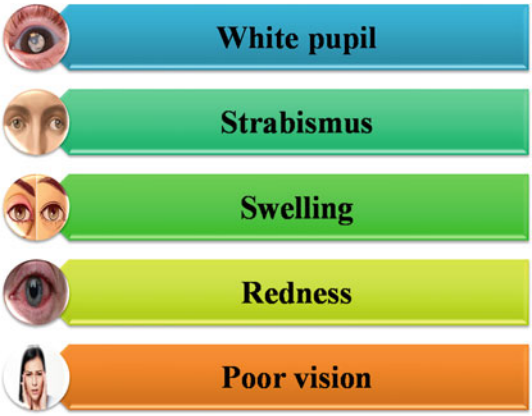


Fig. 1 Depicts diseased and treated eyeballs from retinoblastoma

Fig. 2 Represents symptoms of retinoblastoma



blood vessel formation, escape growth suppressors, activate metastasis, escape immune damage, and destabilize cellular energetics. These cancerous cells have a strong predisposition for causing genetic instability and inducing an inflammatory response. Retinoblastoma is caused by the mutation of the RB1 gene, which results

in the retinoma and numerous genomic variability. In retinas with nonproliferative areas, numerous copies of genes on chromosome 1 at the arm, such as MDM4 and KIF14, were found. Various copies of oncogenes such as E2F3, KIF14, MYCN, and DEK are seen in retinoblastomas with enhanced mitotic activity and the tumor suppressor gene deletion CDH11 (Brigitte et al. 2015). The exact sequence of events that promote retinoblastoma carcinogenesis is unclear, and tools like whole-genome sequencing of malignancies might help researchers figure out how benign retinoma becomes malignant retinoblastoma.

The three domains that make up the human pRB protein are the A/B region or "pocket" domain (RBP), the C-terminal domain (RBC), and the N-terminal domain (RBN). RBP's binding cleft of Leu-X-Cys-X-Glu (LxCxE) allows it to interact with numerous proteins, including oncoproteins and transcription factors (Guzman et al. 2020). Most research has focused on the link between pRB and the E2F transcription factor family. The structure of pRB and post-translational alterations that influence pRB's activity in various developmental stages impact how it interacts with other proteins. The pocket structure of the RBN domain can interact with the RB protein physically. The RBC domain is vital for the pRB- DP/ E2F complex to interact. The three pRB domains (pRB- DP/ E2F) are linked by linker sequences, allowing the protein to be flexible. Post-translational modifications to these linker regions, most notably CDK-dependent phosphorylation, influence pRB's communication with other proteins and functional actions. The phosphorylation of pRB causes pRB protein to be inactivated, resulting in derepression of transcriptional and cell cycle progression. There are 14 phosphorylation sites, 2 acetylation sites, and 6 methylation sites found in human pRB. Further, the pRB protein was SUMOylated and ubiquitinated. These post-translational modifications can promote or prevent other alterations that govern pRB activities, including its capacity to recruit chromatin-remodeling factors (Burke et al. 2012).

On human chromosome 13, a mutation in the RB gene causes retinoblastoma, a type of intraocular cancer that affects children. The tumor suppressor characteristics of the RB gene are well recognized. When cells are in the S phase, active RB products regulate cell division by impairing the function of E2F (a critical nuclear transcription factor). pRb is a tumor suppressor gene that is active when it is hypophosphorylated. The hypophosphorylated form of pRB binds with E2F in the early G1 phase, suppressing transcriptional activity; however, the hyper-phosphorylated form of pRB releases E2F in the late G1 phase, permitting transcription of genes essential for progression to the S phases (Mirzaei et al. 2016). The mutant RB gene product cannot govern cell proliferation, survival, or differentiation because it is often phosphorylated in malignant cells. The importance of miRNAs in tumor suppressor networks, such as the RB gene, is well established.

According to Nemour's study, the RB gene is specifically targeted by miRNAs in cancer cells. MiR-449, for example, inhibits RB phosphorylation in prostate cancer cells, limiting cell proliferation. RB1 is a tumor-suppressor gene because it inhibits apoptosis. RB-induced apoptosis in lung cancer cells can be prevented by increased expression of miR-17-92. Mir-17-92 is carcinogenic in a wide variety of tumors. The RB1 gene is also targeted by MiR-192, which slows lung cancer cell growth and

accelerates cell death. Nonsmall cell lung cancers also reduce or eliminate the expression of miR-34a and miR-15a/16, which act in concert to slow down the progression of the cell cycle. There is no p53 mutation in retinal tumors, even though pRB and p53 pathways are generally inactive in most human tumors. There are no p53 mutations in RB tumors, and therefore the p53 pathway is inactivated. There are genetic mutations in the RB1 and P53 pathways, and the genes that regulate them are affected by these mutations. After RB1 ablation, MDM2, p53, MDMX, and Arf are activated to monitor tumors. According to recent research, miR-24-mediated Arf suppression may be able to stop p53 tumor surveillance activation in retinoblastoma when RB1 is lost. This is because miR-24 suppresses Arf.

Mir-34a is a cell proliferation inhibitor that promotes the expression of p53 while inhibiting the transcription factor E2F, conferring on its tumor suppressor activity. MiR-34a inhibits p53-induced apoptosis, senescence, and cell cycle arrest by downregulating the expression of numerous oncogenes, including CD 44 and SIRT1, CD44, the antiapoptotic factor BCL2, and MYC. The modest functional significance of miR-34a in the p53 pathway is unknown in RB tumors. The study of miRNAs in RB tumors is expected to shed light on the processes underlying cancer growth and tumorigenesis (Golabchi et al. 2017). Numerous studies have established a link between miRNA expression and RB. Numerous miRNAs were found to be overexpressed in RB tumors compared to normal tissues, including miR-513-1, 513-2, 518c, 198, 492, 498, 503, 29-2, 320, and let-7e. In RB tumor tissues, oncomiRs such as miR-19b, 18a, 198, and 106a were upregulated, whereas tumor suppressor miRNAs such as the let-7 family, miR 15a, 16-1, and 34a were downregulated. Ectopic expression of Let-7 inhibits proliferation and carcinogenesis by downregulating oncogenes such as HMGA2, Ras, and Myc. Ras.

The goal of treatment in retinoblastoma is to remove the tumor while minimizing collateral damage to surrounding tissues. The primary treatment goals are to prevent metastasis, lower the risk of long-term secondary malignancies (e.g., osteosarcoma, soft-tissue sarcoma), spare the eye, and preserve vision (Ancona et al. 2020). To optimize treatment outcomes, pediatric oncologists, ocular oncologists, radiation oncologists, and geneticists must collaborate when caring for a child with retinoblastoma. The stage of the tumor, its laterality, the number of tumor foci (unifocal, multifocal, or bilateral), the location and size of tumors within the eye, the presence of vitreous seeding, the child's age and health, and the family's preferences all play a role in determining treatment. The type of tumor affects treatment options (Fig. 3).

Intravenous: Systemic chemotherapy is typically administered via an intravenous catheter and consists of a two, three, or four-drug regimen. The most frequently used mediators are DNA crosslinking agents (carboplatin, cisplatin), DNA topoisomerase 2 inhibitors (etoposide, topotecan, and teniposide), and Vinca alkaloids. The most frequently used regimen is vincristine/etoposide/carboplatin.

Intra-arterial: Melphalan was discovered to be beneficial in the late 1980s for the in vitro treatment of retinoblastoma. Yamane et al. (2004) began treating patients with intracarotid artery melphalan, developing a safe and active procedure.

Intravitreal: Laser treatment, standard chemo reduction therapy, and intravitreal chemotherapy (IAC) are often used to treat retinoblastoma tumor seeds in the

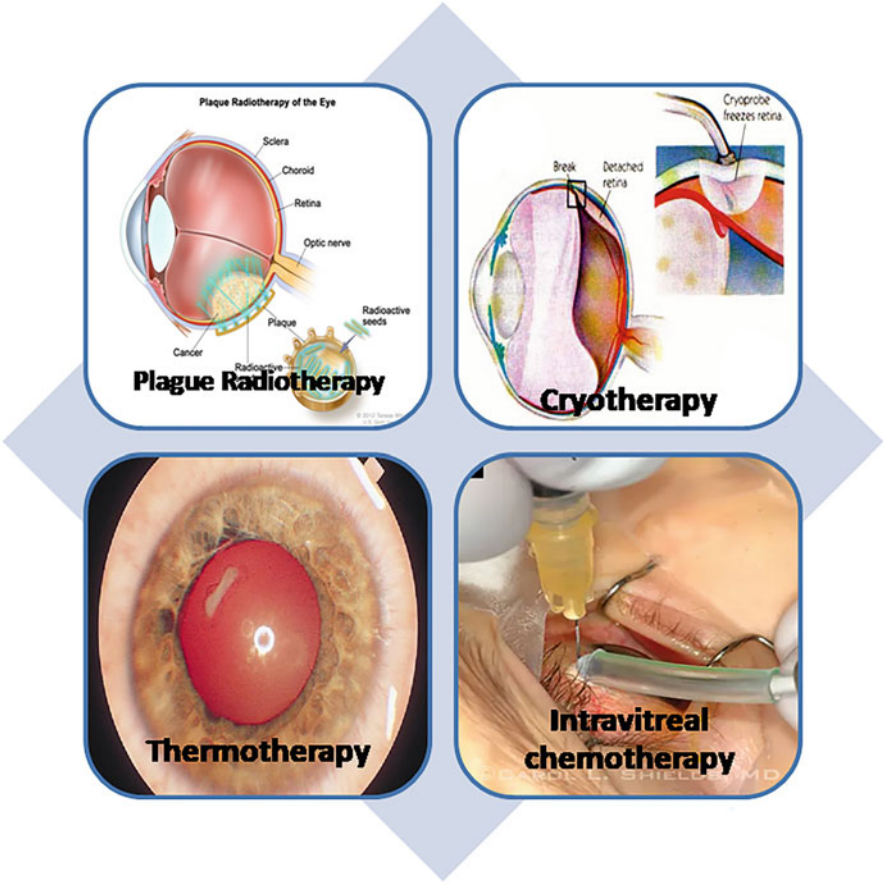


Fig. 3 displays treatment options for retinoblastoma

vitreous and subretinal regions. Treatment of glassy seeds is complicated due to the lack of vascularization in the vitreous. Alternatively, an intravitreal technique can be used, in which the healing agent is injected into the eye's vitreous cavity through the pars plana under aseptic conditions (Kaneko and Suzuki 2003).

Periocular: Chemicals used to treat cancer can be injected into the eye under the conjunctiva or subretinally. Because the sclera has a lot of surface area and is very good at letting small molecules through, periocular injection allows drugs to be delivered across the sclera without risking puncturing the globe. Because periocular chemotherapy quickly reaches vitreous levels, it can reach them 6–10 times faster than intravenous treatment (Mendelsohn et al. 1998). Most of the time, chemotherapeutic drugs have been used to treat tumor recurrence and control retinoblastoma as an add-on to systemic chemotherapy. Animals are used in research on retinoblastoma.

It has been practically impossible to determine the etiology of retinoblastoma cases that deserve nucleation in human samples because of viable tissues' late appearance and scarcity. As a result, animal models are vital in researching retinoblastoma formation and tumors. Since the tumor appears to form spontaneously only in humans, creating an animal model that resembles retinoblastoma in terms of appearance and function.

Enucleation is still the recommended therapeutic choice for individuals with advanced subtype group E retinoblastoma with buphthalmos, neovascular glaucoma, aqueous seeding, and transscleral extension. With little globe tension, the eye is gently withdrawn from the orbit to limit the progression of tumors into the orbit. A large portion of the optic nerve is removed for histological examination. After enucleation, an orbital implant is inserted to provide sufficient volume in the socket and allow the four rectus muscles to combine for movement. When the procedure is completed, there should be fresh tissue taken from the eye for genetic testing.

Aside from the eyes that have not responded to previous treatments, those with an optic nerve resection margin tumor and extraocular retinoblastoma that has progressed through the sclera, orbit, or intracranially can all benefit from external beam radiation (American Society of Clinical Oncology 2011).

The extraocular disease is uncommon in modern countries, although it is more common in people who have been neglected or treated incorrectly. Tumors can infiltrate the orbital soft tissue around the eye through emissary capillaries in the sclera. Approximately 60–85% of patients with orbital retinoblastoma can be cured with systemic chemotherapy and external beam radiation (40–45 Gy). Treatment may spread to the optic nerve, brain, and meninges if more spinal fluid is needed. There is a low cure rate for recurrences in the central nervous system, with vincristine, cyclophosphamide, doxorubicin, platinum, and epipodophyllotoxin-based medicines the most effective treatments. No preclinical or clinical evidence supports the use of intrathecal chemotherapy. The skull and spine may be irradiated with 25–35 Gy. By hematogenous transfer, retinoblastoma may spread to the bone marrow, bones, lungs, or liver. Cancer patients with metastatic disease can benefit from multiple-agent, high-dose chemotherapy and autologous stem cell transplantation.

For the treatment of retinoblastoma, topotecan, a topoisomerase inhibitor, can either be used alone or in combination with other drugs. Experiments in a transgenic mouse model of retinal cancer showed that sustained-release topotecan reduced tumor burden (Eleanor et al. 2016). Retinoblastoma tumor volume can be reduced by injecting topotecan in a fibrin sealant around the eye. Topotecan can reduce retinoblastoma tumor burden whether supplied systemically, by intravitreal injections, or via IAC.

Retinoblastoma treatment advances are aimed at creating an ideal system that is both affordable and simple to use by ophthalmologists or ocular oncologists – even in developing countries – as well as that is near effective to the primary tumor and the tumor seeds, has few side effects, and can achieve a long-term treatment effect without spreading tumor cells. Many retinoblastoma patients require anesthesia for intraocular treatment, which necessitates prolonged drug-release strategies.

Retinoblastoma has no feasible molecularly targeted therapy; even though RB1 was the first tumor suppressor gene to be found and cloned secondary mutations, differential expression of other cancer-related genes than RB1 and doubts regarding retinoblastoma's biological genesis might all be to blame, according to some theories (Zhang et al. 2011). To treat retinoblastoma, small molecules including MDMX–p53 response (nutlin-3a) inhibitors, histone deacetylase (HDAC) inhibitors, and spleen-tyrosine-kinase (SYK) inhibitors have been discovered and developed. These medicines have greatly improved our knowledge of tumor biology.

A fatal toxin that kills tumor cells is produced by injecting a bacterial or viral gene into tumor cells. This is known as suicide gene therapy. To combat vitreous seeds, phase 1 studies used intravitreal injections of an adenovirus vector containing HSVtk, followed by ganciclovir treatment. Retinoblastoma gene therapy will likely be utilized as a complement to existing treatments for refractory vitreous seeds, not a first-line treatment, despite these fascinating findings.

Recent advances in nanotechnology-based cancer treatment delivery methods have made it possible to deliver drugs to particular locations with greater precision while also increasing their bioavailability. These include biodegradable liposomes, polymer polyesters, gold nanoparticles, mesoporous silica, and dendrimers (Patra et al. 2018). Long-term drug release is possible with these particles, which might increase the therapeutic efficacy of their medications. Research on nanoparticle-based solutions in the treatment of retinoblastoma showed increased drug delivery rates and longer intravitreal half-lives for chemotherapy medicines in this cancer.

Because the vitreous and subretinal tissues are not penetrated during suprachoroidal injection with microneedles, this method of administering medication to the choroid and retina has a minimal risk of extraocular tumoral dissemination. A rabbit model showed that bevacizumab injected into the suprachoroidal space was safe and effective when administered to the tissues of the posterior segment (Ifat et al. 2021). There are 32-gauge microneedles with a short slope and 0.8–1.0 mm in length that enter the supraciliary space through the sclera. A small needle injects chemicals into the supraciliary space, where they spread into the suprachoroidal area.

Several hereditary retinoblastoma animal models with moderate to high similarity to the human tumor type have been produced in the last three decades. p107 has been linked to the retinal development of mice, which necessitates the deletion of both genes to generate retinal cancer.

The first and most comprehensively studied transgenic models were created (Windle et al. 1990). LHb promoter-driven expression of the oncogenic SV40 early region in anterior pituitary gonadotrope cells is achieved in this paradigm. PP2A phosphatase is linked to the pRB family of oncoproteins, as are p53 and pp2A. Both Large-T and Small-T oncogenes in the SV40 early region are thought to induce transformation. This method successfully induced retinoblastoma in mice expressing T-Ag/t-Ag from the IRBP promoter. Models of human retinal gliomas were created, but the cells' origins remained a mystery until recently.

Researchers have used gene knockout approaches to generate chimeric mice with Rb/cells solely in the retina to construct Rb animal models. These chimeras did not

develop retinoblastoma despite the Rb/cells' contribution to the growing central nervous system. Ectopic mitoses and significant cellular degeneration were found in the developing retina of chimeric Rb/embryos. Retinoblastoma might be prevented in mice lacking the Rb gene by adding p107, according to Robanus-Maandaget et al. (1998). The protein p107 and RB1 are incredibly similar in sequence and biological properties. While the p107 gene was downregulated in human postnatal retinas and explant cultures lacking Rb, evidence suggests that p107 replaces Rb in mice.

Retinoblastoma xenograft models have made it possible to rapidly study cell lines and tumor cells in immune-compromised experimental mice. When McFall et al. (1977) injected 107 cells from the Rb cell lines Y79 and WERIRb into rabbits with weakened immune systems, they saw tumor development in the eye's anterior chamber. Slit-lamp examination of the tumors revealed the highest growth between days 17 and 22 following injection. Angiogenesis and edema in the eye were also observed in the histological sections. A rabbit model of retinoblastoma was created by implanting 106 WERI-Rb cells into the subretinal area (Shin and Hans 2011). Tumor development was evident with fundoscopy during the first week after injection, with vascularization beginning in the fifth week and tumor growth continuing until the eighth week, according to their testimony. Histological sections confirmed the presence of an intraocular tumor in the subretinal area near the optic nerve and vitreous cavity lesions, but no evidence of metastasis was seen. Retinoblastoma-like live tumor cells were also detected surrounding blood arteries and necrotic regions. This model has a critical flaw: Tumors develop in the subretinal region instead of the retina.

Conclusion

Research into retinoblastoma has risen in the last three decades due to the discovery of cancer-like experimental models. The development of Rb malignancies and the course of carcinogenesis has benefited from our growing knowledge of the role of the Rb gene in tumor suppression. Clonal proliferation, blood-to-eye transmission, intraocular spread, and exophytic spread would be present in an ideal retinoblastoma animal model. For reliable diagnostic techniques based on marker research and medication effectiveness evaluation, and prognostic signal prediction, the creation of these animal models is essential.

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Transmissible Animal Tumors as Models for Cancer Research

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Contents

Abstract

Transmissible and transplantable animal models of tumors were applied by many investigators to scrutinize the mechanisms involved during the progress of cancer to metastasis and to assess anticancer effects of the agents under investigations. Transmissible tumors have been stated in three wild species; these are devil facial tumors in Tasmanian devils, canine transmissible venereal tumors in dogs, and

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leukemia in soft-shelled clams. Otherwise, tumors can be transferred in humans through pregnancy, organ transplantation, and even accidentally from patient to surgeon. Nowadays, human tumor xenografting into mice has been broadly applied in cancer biology. Human-derived tumor models have been established by many publications to study tumor biology and drug therapeutic effects and resistance. This chapter gives an updated overview about the types of transmissible and transplantable animal models of tumors and their possible applicability to improve understanding the mechanisms and treatment of cancers as well as to identify therapeutic targets.

Keywords

Transmissible cancer · Types · Properties · Possible applicability in research

Introduction

Cancer develops when cells exhibit mutations that direct them towards programs of sustained and stimulated cell division. The process of natural selections promotes the most prolific subclones that frequently steer the tumor towards more aggressive phenotypes. Cancer is elective and often leads to death of its host; therefore, it is an ultimately self-destructive entity and of short life (Murchison 2008).

According to previous studies, many different cell types are strongly associated with the heterogeneous cancer microenvironment (Junttila and Sauvage 2013) and exhibit complex tumor-microenvironment interactions (DelNero et al. 2013). The major constituents of the tumor microenvironment are the extracellular matrix (ECM), interstitial cells, growth factors, and cytokines (Quail and Joyce 2013). The ECM is mostly composed of proteoglycan, glycosaminoglycan, and collagen (Novikova et al. 2017). Endothelial cells found in blood vessels, lymphocytes, immune cells, and fibroblasts are good examples of interstitial cells that are defined as cells, which lie in the spaces between the functional cells of a tissue or an organ (Quail and Joyce 2013). Hypoxia (Milane et al. 2011), inadequate nutrition (Xu et al. 2012), and low pH are all characteristics of the tumor microenvironment (Webb et al. 2011), and it uses biochemical cues like growth factors and cytokines to regulate the connections between tumor cells and interstitial cells. Integrin governs the interactions between cancer cells and ECM, creating a feedback loop to the cancer (Desgrosellier and Cheresh 2010). Integrin interacts directly with ECM constituents to create the traction required for cancer cells' invasions (Winkler et al. 2020). Integrin breakdown in organs, tissues, and basement membranes is a key histological indicator of invasive carcinoma progression. Integrin also affects the adhesion-dependent regulation of cancer cell multiplication by regulating ECM remodeling and altering the localizations and activities of proteases (Desgrosellier and Cheresh 2010). The ECM is a major component of tumor that plays multiple extremely important roles, viz., mechanical support, microenvironment modulation, and being a source of signaling molecules (Huang et al. 2021). The ECM and interstitial cells

may have important roles in hosting tumor cells and the development of cancer cells transmitted via physical transfer or through experimental injection or inoculation (Baghban et al. 2020). In this regard, many types of transmissible and transplantable animal tumor models have been developed and reported by several previous publications (O'Neill 2011; Lui et al. 2022).

Therefore, this chapter aims to provide an overview of the types of transmissible cancers and their applications as transmissible or transplantable animal tumor models for assessing new anticancer drugs or for the study of mechanisms of action.

Transmissible Cancers

Transmissible tumors diverge from the traditional cancer paradigm in the sense that they can be transmitted and proliferate throughout a host population as allografts (Murchison 2008). Transmissible tumors have only been found in three wild species: devil facial tumor (DFT) in Tasmanian devils (Wilcox 2021), canine transmissible venereal tumor (CTVT) in dogs (Das and Das 2000), and leukemia-like disease called disseminated neoplasia in in different species of marine bivalves, such as clams, mussels, and cockles (Metzger et al. 2015; Hammel et al. 2022). Furthermore, tumors have been transferred in humans through gestation (Tolar and Neglia 2003), organ transplantation (Gandhi and Strong 2007), or even accidentally (for example, from patients to surgeons) (Welsh 2011). Due to the similarities between transmissions among individuals and tumor metastasis within patients (Ujvari et al. 2016), the study of transmissible tumors is useful to study cancer biology in a broader sense.

Tumor cell passage between invertebrate and vertebrate hosts requires an environment and behaviors that facilitate tumor cell transfer, such as tumor tissue characteristics that elicit the massive expulsion of malignant cancer cells, promote tumor cell adaptability, which permits survival and development in an another host, and a host tissue, or a host tissue (Ujvari et al. 2016).

Canine Transmissible Venereal Tumor (CTVT)

CTVT is a malignancy that is spread via physical transfers of live tumor cells through injured skin and/or mucous tissues (Küçükbekir et al. 2021). CTVT is the superlative of old known somatic cell lines, and it originates from myeloid cells (Mukaratirwa and Gruys 2003). Dogs' external genitalia are where CTVT neoplasms are most frequently seen (Mukaratirwa and Gruys 2003; Küçükbekir et al. 2021). Cancer cells exchanged between canines during mating may spread if the penile and vaginal mucosa suffer significant abrasions and bleeding (Transmissible Cancer Group 2022). CTVT results in tumors, which are usually associated with the external genitalia of both males and females of dogs. The tumor tissue or cells can be transmitted to naive animals through contact with wounded skin or mucosa when the

host tumor cells lose their ability to present MHC classes I and II molecules (Murgia et al. 2006).

The transplantation of CTVT cells into mice indicates that this tumor could only be transmitted to healthy animals with the same MHC or into immunocompromised receivers because CTVT cells initiate immune responses in healthy recipients (Murgia et al. 2006). The transplantation theory and the genesis of CTVT led these studies. The transplantation theory is based on the discovery that experimental tumor transplanting can only occur utilizing vital cancer cells that have survived (Park et al. 2006). Other studies have found that CTVT cells can be produced from lymphohistiocytic cell mutations caused by chemicals, viruses, or radiation, and that these tumor clones can then be spread through allogeneic transplantation (Murgia et al. 2006).

CTVT tumors that have been experimentally transplanted have three unique growth stages: the progressive, stable, and regressive stages (Chu et al. 2001; Liao et al. 2003; Frampton et al. 2018). CCL5 (C-C motif chemokine ligand 5) was identified as a possible driver of CTVT regression (Frampton et al. 2018). After experimental transfer by 10–20 days, tumor is frequently palpable. In this regard, the starting progressive phase that normally continues for few weeks is marked by fast tumor volume growth with a doubling period of 4–7 days and an estimated 50% cell loss (Chu et al. 2001). Tumor development is substantially slower in the next stable phase, with a doubling duration of 20 days and a determined cell loss of 80–90% (Mukaratirwa et al. 2006). Phase of up to 80% of CTVT tumors go through a returning to an earlier phase after the stable period, which can continue weeks, months, or eternally (Chu et al. 2001; Liao et al. 2003). Tumors of a size 100 cm^3 can be entirely removed during the regressive phase, which normally lasts 2–12 weeks. Percent 1–20 of implanted tumors start a second period of rapid development that leads to metastasis, instead of regressing (Chu et al. 2001; Murchison 2008). Spontaneous tumor regression can occur, probably as a result of immune system response (Liao et al. 2003).

Ahrar et al. produced a canine lung tumor model in 2002 by inoculating CTVT fragments intra-arterial or percutaneously, which was later used in research on percutaneous radiofrequency ablations (Ahrar et al. 2003). Metastatic lung cancer is well documented that it gets its blood supply from the bronchial and the pulmonary artery; central tumors have a significant bronchial circulation, while peripheral tumors have a strong pulmonary circulation (Milne and Zerhouni 1987). In addition, Sun et al. (2021) developed an animal model based on Ahrar et al.'s (2002) model and succeeded in delivering blood to a large animal lung tumor model. All experimental dogs were effectively injected, resulting in 10–20 mm patchy, irregular, well-defined hyperdensities in the inoculated lung areas by the 6th week of inoculation. Solitary pulmonary nodules with a mean maximum diameter of 1.716 ± 0.102 cm were found at the infected sites in all dogs, and they expanded rapidly reaching a mean maximum diameter of 2.392 ± 0.076 cm at the 8th week and 2.734 ± 0.138 cm at the 10th week. In addition, at the 10th week, many tiny distal subpleural nodules with a diameter of less than 1.0 cm were also discovered.

CTVT can provide useful tumor animal models for future research into their human cancer-like characteristics, including any possible cancer stem-cell (CSC) component (O'Neill 2011).

Devil Facial Tumor Disease (DFTD)

The DFTD is a transmissible Schwann's cell malignancy (Murchison et al. 2010; Wilcox 2021), which has decimated the Tasmanian devil (*Sarcophilus harrisii*) populations (Hawkins et al. 2006; Woods et al. 2020). The cancer cells in this disease model are formed from Schwann's cells and are disseminated among devils through biting, which is a common habit during mating seasons (Woods et al. 2020). Since its discovery in 1996 in Tasmania's far northeast, the disease has spread across the majority of the devils' natural territory. More than 80% of the devil population has been estimated to have died as a result of DFTD (Hawkins et al. 2006). In DFTD, the "infectious" agent is the cancer cell, which is delivered as an allograft through bites (Hawkins et al. 2006). Allogeneic skin graft rejection is efficient in Tasmanian devils (Kreiss et al. 2011), and they can develop both cytotoxic and antibody responses against xenogeneic cancer cells (Brown et al. 2011). Primary DFTs are more common around the neck and face (Loh et al. 2006). The tumors ulcerate, become friable, and metastasize as the disease progresses, and the affected devils often die within 6 months following the onset of lesions (Pyecroft et al. 2007). DFTD, like CTVT, can provide useful animal cancer models for future research into their human counterparts, including any CSC component (O'Neill 2011).

Devil facial tumor 1 (DFT1) is a cancer clone that poses a threat to Tasmanian devils. Kwon et al. (2020) investigated the genomes of 648 DFT1 tumors collected between 2003 and 2018 across the clinical spectrum. They discovered that DFT1 is a distinct stable lineage whose genome exemplifies how cancer cells adapt to a variety of microenvironments and survive in a parasitic niche.

Clam Leukemia (CL)

Although disseminated, hematopoietic, or hemic neoplasia (HN) has been noted in a number of bivalves, it was only recently recognized that this malignant clonal cell line may be transferred horizontally in soft-shell clams (*Mya arenaria*) (Metzger et al. 2015). CL is defined by abnormal hemolymph cell proliferation, lack of phagocytic capacities, expression of a new surface antigen, and TP53 tumor suppressor protein cytoplasmic sequestration (Walker et al. 2011). CL was initially identified in the 1970s and has since spread over North America's eastern coast, decimating soft-shell clam communities (Metzger et al. 2015). In marine bivalves, leukemia-like transmissible cancer HN has demonstrated capability to infect individuals from various species (Garcia-Souto et al. 2022).

Transplantable and Transmissible Tumors in Animal Models

Cancer cells or tissues are implanted in mice or rats to create transplantable tumors. There are two types of implantations, the first of which is heterotropic transplantation that includes the transplantation of tumor cells at a site different from the original site; for example, carcinoma implanted subcutaneously or intraperitoneally. Such method usually involves the intraperitoneal (i.p.) or subcutaneous (s.c.) route of inoculation, and the tumor develops into ascites or a solid tumor, respectively. Another type of transplantable tumor is the orthotopic model, wherein cancer cells are transplanted to the anatomic site from which a tumor is derived (e.g., transplantation of lung cancer cells in the lung). This approach results in tumor models that have characteristics similar to human cancer, including responsiveness to chemotherapy, gene expression, and metastatic potential. The orthotopic transplantation (OI) of tumor cells can be accomplished in two ways: (i) direct injection of cancer cells or (ii) surgical OI (SOI), which involves implantations of complete fragments of the tumor cells orthotopically following surgical excision. The use of SOI improves the tumor model's repeatability and metastatic potential, and these transplantable models can be divided into two groups based on the cancer's origin and the host employed (Zhao et al. 2012).

Syngeneic models involve implanting cancer cells into inbred individuals of animals with a similar genetic background to the derived cells, such as the L1210 leukemic cell lines taken from the DBA/2 mouse and produced in the same species. Such models are initially generated after a carcinogen is administered in animals from which stable cell lines are then generated. These cell lines can be transplanted into mice with the same genetic background as the malignancies they were produced from. The benefit of syngeneic models is that the tumor microenvironment and the host stem from the same species of origin in transplanted tissues. When considering the close relationship between the tumor and the host, this is very beneficial to success. Nonetheless, because these models are primarily developed from homozygous inbred mice and so lack the genetic complexity of human tumors, they lack a number of critical molecular hallmarks of human cancers. Furthermore, they may not have the same constellation of mutations as human patients due to species-specific changes in oncogenesis (e.g., differences in xenobiotic metabolism) (Voskoglou-Nomikos et al. 2003). For tumor animal models that are more closely related to human pathology, transplantable tumor of human origins should be applied. The transplantations of these human tumors in mice could lead to severe immune graft rejection by immunocompetent cells in the host. For this plan, athymic (nude) mice with deficits in their immune responses have been developed to efficiently accept such foreign transplanted material. Before the emergence of athymic mice, the animals were immunocompromised by irradiation, thymectomy, or steroids to be ready for xenotransplantation (Khleif and Curt 2000). The first nude mouse was derived instinctively in closed colonies of albino mice in the laboratory in Ruchill Hospital, Glasgow, Scotland, and was revealed by Isaacson and Cattanach as lacking fur (Rygaard Jand Povlsen 1969). A mutant gene (nu, for nude) is located as an autosomal recessive gene on chromosome 11 of this mouse, and it is responsible

for the absence of hair, short lifespan, slow growth, and limited fertility. Mice with the homozygous nu/nu mutation do not have a thymus, whereas heterozygous nu/1 mice have. The *nu/nu* athymic mice have a modest number of T cells that come from a heterozygous mother immunologically. The functions of B cells, on the other hand, are normal, and natural killer cell activity is increased. The successful xenografting of human tumors into nude mice and the preservation of tumor histological and biological identity over several passages in vivo changed many aspects of cancer research. Tumor cell lines could be transplanted into nude mice by a variety of methods, including intraperitoneal and intravenous injections. When nude mice are implanted with human cancer cells, the kinetics of the cells change. More often than not, the doubling period is shorter than that of the original tumor, which continues to shrink with time. Regardless, several human tumor xenografts retain their original morphological and molecular characteristics. As a result, most anticancer drug development methodologies rely on xenografted human malignancies (Guillen et al. 2022).

Preclinical animal tumor models are the most informative way to collect clinically relevant data on the mechanism of action and therapeutic efficacy of novel anticancer drugs. The development of cancer in specific tissues targeted by oncogenes of transgenic mice (Palmiter and Brinster 1985), the evaluation of new cytotoxic agents via measurements of the inhibition of the tumor diameter during the growth of primary tumors implanted s.c. into mice (Wilmanns et al. 1993), or the activation of host immune cells against metastatic diseases found in the lungs of mice after the removal of their primary renal adenocarcinoma by nephrectomy are required for successful animal models (Dinney et al. 1992). Each of these models has distinct advantages and limitations that are defined by the experimental questions that are asked by the research investigator about the biology and therapy of cancer.

Immunodeficient animals have been used as an important tool in modeling human glioblastoma for many years. Glioblastoma is most typically propagated and tested in such animals at the subcutaneous flank region (heterotopic), while orthotopic (intracranial) xenograft models have found growing usage recently. For both orthotopic and heterotopic research, allograft and xenograft tumors are often produced from persistent human glioblastoma cell line. Additionally, invasively orthotopic xenografts have been developed from surgical tissues that were originally cultured as tissue spheroids (Mahesparan et al. 2003). Invasively intracranial tumors have been formed from heterotopic xenografts grown by the direct transplantation of surgical resection specimens and then supported by repeated passaging in nude mice flanks (Taillandier et al. 2003). In allograft and xenograft models, several human and mouse cell lines have been used. Tateishi et al. (2015) investigated the susceptibility of IDH1-mutant tumors to NAD + depletion using the severe combined immunodeficient (SCID) mouse. In a 2013 study, Ashizawa et al. looked at how the STAT3 inhibitor STX-0119 affected the growth of cancer stem-like cells produced from recurrent glioblastoma in NOD-SCID mice and NOD/Shi-scid IL2rγ^{null} mice. Other mice strains, viz., athymic nude (Nu/Nu) mice (Wykosky et al. 2015), CD1 nude mice (Mercurio et al. 2016), and athymic nude Foxn1-nu mice have also been previously applied (Szabo et al. 2016).

Since the 1970s, several animal models for brain metastasis xenotransplantation have been created utilizing cancer cell lines from various cancers, including renal cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, breast carcinoma, and melanoma. After intravenous or intracoronary administration at various take rates, brain metastases were observed in nude mice (Fidler et al. 2002). MDA-MB-231, one of the most frequently utilized cell lines, was obtained from a pleural effusion of a human breast carcinoma in 1973 at the MD Anderson Cancer Center (Higuchi et al. 1980). The same facility also developed a number of other cancer cell lines, such as MDA-MB-157 (Young et al. 1974) and MDA-MB-435 (Zhang et al. 1991). Then, Lorger and Felding-Habermann demonstrated that extravasation across the blood-brain barrier (BBB) in mice between days 3 and 5 after ICA injections could result in the infection of several firefly luciferase-tagged breast cancer cell lines (MDA-MB-231BR, MDA-MB-435, 4 T1, MDA-MB-231, and MCF-7). Astrocytic and microglial responses to tumor cells captured in the brain microvasculatures were immediately apparent (Lorger and Felding-Habermann 2010). Heyn and associates demonstrated in 2006 using magnetic resonance imaging of mouse brains that a single tumor cell might enter the brain (Heyn et al. 2006). Additionally, after administering MDA-MB-231 cells to nude mice, researchers found that matrix metalloproteinase 2 may play a role in the spread of breast cancer to the brain (Tester et al. 2004).

A different research examined the effects of farnesoid X receptor (FXR) activation (FXR overexpressions and the agonist GW4064) and inactivation (FXR siRNA and the antagonist guggulsterone) on the growth of colon cancer cells using nude mice. Additionally, FXR overexpression in stably transfected human colon cancer cells decreased cell proliferation and inhibited the development of human colon cancer xenografts in nude mice (60% decrease in tumor volume and 50% decrease in tumor weight) (Peng et al. 2012).

Male Wistar rats were fed a diet containing 0.1% N-ethyl-N-hydroxyethylnitrosamine (EHEN) for 2 weeks before receiving daily s.c. injections of beta-cyclodextrin at a concentration of 450 mg/kg body weight for a week. Tumors were excised from these animals and three times administered either intraperitoneally (i.p.) or subcutaneously (s.c.) to newborn Wistar rats (4 weeks old). Tumors appeared where the inoculations were made as a result of the s.c. injection. The i.p. mode of inoculation caused tumors to grow more quickly than the s.c. route, and half of the i.p.-injected animals had invasion into their lungs, intestine, peritoneum, spleen, liver, and stomach. Only 2 of the 12 i.p.-injected rats developed lung metastasis. Alveolar or papillary patterns resulted from the transplantable tumor cells (Hiasa et al. 1984). Chemical induction of renal cell carcinoma by ferric nitrilotriacetate (Fe-NTA) was also applied to induce the development of cancer cells that were subjected to implantation in the syngeneic models (Sobczuk et al. 2020). In the same line, Kobayashi et al. (2010) presented a situation in which immunocompetent August Copenhagen Irish (ACI) rats were exposed to Fe-NTA and produced rat RCCs (renal cancer cells). Tumor cells (1×10^6) were also injected into the subcutaneous tissue of the animals' abdomens. The tumors that had grown a week later exhibited moderately differentiated basophilic cell carcinoma. An

orthotopic animal model showed metastatic progression to the lung 2 weeks after a 1×10^6 inoculation into the left renal subcapsular region. Modified cells expressing luciferase developed metastatic malignancy in the lungs in a same amount of time after implantation (Sobczuk et al. 2020).

In 2010, Lu et al. found that the γ-tocopherol-rich combinations of tocopherols had chemopreventive effects in carcinogen-induced A/J mice and H1299 human lung cancer cell xenograft tumors. Otherwise, after receiving an injection of SK-Hep-1 cells into their tail vein, athymic nude mice were administered lycopene or β-carotene twice weekly for 12 weeks (Huang et al. 2008). Additionally, Schleicher et al. (1988) found that retinoids had an antimetastatic impact in HM1-F5 (a malignant hamster melanoma cell line) transplanted into athymic mice. These retinoids include 2-hydroxyethyl retinamide, N-[4-hydroxyphenyl] all-trans-retinamide, and 13-cis-RA.

Prostate cancer cell lines could be used to develop tumors in rats. These cells can be taken from chemically produced or spontaneous rat prostate malignancies and transplanted into rats to establish xenograft models, or from chemically induced or spontaneous rat prostate tumors and implanted in rats to construct syngeneic models. (Tumati et al. 2013; Neubauer et al. 1986; Kawai et al. 2005; Pollard et al. 2010). These prostate models are orthotopic if prostate tumor cells are inserted into the prostate, the tumor's primary place of origin (McCullough et al. 2014). However, these prostate models become heterotopic when the prostate tumor cells are injected in different locations, such as through subcutaneous implantation (Pollard et al. 2010) or in the subcutis of the dorsal surface of the rat tail (Neubauer et al. 1986). As a syngeneic model, the PAIII cancer cell line is produced from an autochthonous prostate tumor in Lobund-Wistar (LW) rats. The androgen-intensive cancer cell line, generated from spontaneous prostate carcinomas in LW rats, may generate large primary tumors when subcutaneously implanted (Pollard et al. 2010). Harvey Pollard et al. (2010) utilized this model to research the function of vitamin C in carcinogenesis and discovered that pharmacological doses of this molecule may prevent the growth and spread of tumors. Rat prostate cancer cell line (PLS10) was generated from testosterone- and P-dimethylaminobenzaldehyde-induced carcinomas in the dorsal prostate of male Fischer 344 (F344) rats, and may be injected into additional animals (Kawai et al. 2005). In order to investigate the hyperthermic impacts of magnetic particles on prostate cancer, the PSL10 cancer cell line cells were injected into the flank of F344 rats as a syngeneic heterotopic model; the authors of such research revealed that hyperthermia is an effective treatment for prostate cancers (Kawai et al. 2005). Rat xenograft prostate cancer models are infrequently employed in experimental investigations owing to the limited number of immune-deficient rat strains that are accessible for application. Andresen et al. (2003) tested the capacity of the immune-deficient male homozygous Sprague-Dawley rats to develop bone metastases from the human prostate cancer cell line CRW22. In order to evaluate the outcomes of high-doses of image-guided radiation exposure coexistent with biological agents' treatment, advanced orthotopic prostate cancer models of human prostate cancer (PC3) transplanted into the prostates of nude or Copenhagen rats were employed (Tumati et al. 2013).

The Copenhagen rat Dunning R3327 adenocarcinoma provided the majority of the in vivo rat tumor lines for evaluating the effects of new medications for treating hormone-dependent prostate cancers. Male Copenhagen rats with spontaneous dorsal prostatic adenocarcinomas were used to produce prostatic tumor cell lines in 1963. Thereafter, multiple sublines were developed from Dunning R3327, such as AT-4-R3327-5, AT-1, MAT-Ly-Lu, and PAP (Tennant et al. 2000). Each subline contains distinct characteristics and phenotypes that resemble various aspects of human prostate malignancies, including as slow-growing androgen-responsive tumors (Lamb and Zhang 2005). In vivo transmission of these cell lines is possible without losing their tumorigenicity (Tennant et al. 2000). This rodent's tumors are slow-growing, nonmetastatic, androgen-responsive, and they preserve the histological structure and biochemical characteristics of the rat's dorsal prostate gland, making it a good choice as a model for cancer research in general and chemoprevention research in particular (Tennant et al. 2000).

Sugase et al. (2020) discovered a noninvasive technique by injecting metastatic uveal melanoma cells into the spleen of a mouse model of hepatic metastatic xenograft. In contrast to the hepatic injection of tumor cells, the splenic injection led to the spread of hepatic tumors across the liver. Based on their findings, the researchers came to the conclusion that this new orthotopic liver metastatic mice model would prove valuable in preclinical drug screening and the investigation of liver metastasis pathways.

More recently, Guillen et al. (2022) have developed a human breast cancer-derived (PDX) xenograft mice model for breast cancer and organoid grown from PDX tumors in culture to study drug response and resistance, tumor heterogeneity and development, and model metastatic diseases. PDX models epitomize human tumors with high fidelity and exhibit treatment responses that are consistence with human responses (Woo et al. 2021). They can be used to investigate tumor heterogeneity in response to treatment, patterns of cancer evolutionary dynamics throughout tumor progression and under therapeutic pressure, and mechanisms of treatment resistance (Woo et al. 2021; Guillen et al. 2022).

Conclusion and Future Perspectives

Many of the current comprehension and therapeutic approaches were first assessed and confirmed using animal models of cancers including transmissible and transplantable animal models of tumors. More progress in adding information about the molecular mechanisms implicated in the development of cancer and cancer metastasis using transmissible and transplantable animal models of tumors may lead to further discoveries of novel therapeutic targets. Human xenograft tumors in animal models have advantages of being able to exhibit human cell specific biology in vivo. Many aspects of the orthotopic tumor model, in which mouse and human cancer cells are implanted into the same organ of origin, include a relevant site for host interactions, the establishment of metastasis, site-specific therapeutic dependency,

and organ-specific gene expression. Thus, orthotopic implantation remains an affordable method to study therapeutic strategies and identify molecular targets associated with progression and therapy of cancer.

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Part III

Animal Models for Cancer Drug Discovery: Recent Advances

RNA Interference in Experimental Animal Models: Its Application in Cancer Research and Therapy

45

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Contents

Abstract

Cancer, is a major health problem in the world, that has to be faced with new technologies and methods. Chemotherapy is still being widely used treatment option for cancer along with radiotherapy but is associated with many side effects. A large amount of expenditure is being spent on cancer research, yet effective treatment strategies need to be developed. RNA interference is seen as a promising and highly potential strategy in cancer therapy due to high specificity and economic advantage. It has been used in the generation of many animal models,

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especially mice and rats. Many preclinical studies have been performed using RNAi and are found successful in minimizing the tumor growth. Due to various complexities associated with the delivery of RNAi, it has still not emerged as a commercially available treatment option. In this review, various aspects related to RNAi, cancer, and animal models have been discussed. Additionally, several genes which have been targeted in the preclinical trials, parameters which have to be considered while designing RNAi drugs, and various aspects that can be explored further in RNAi technology to successfully emerge as a commercial treatment option have also been discussed.

Keywords

Cancer · Animal models · RNA interference (RNAi) · Short hairpin RNA (shRNA) · Small interfering RNA (siRNA)

Introduction

Cancer is one of the most dreadful diseases and a major challenge in public health worldwide. It is a genetic disease in which the cell death mechanism is deregulated, thus leading to abnormal cell proliferation. Cancer cells differ from the normal cells due to abnormal activation of genes, their overexpression, mutation in proto-oncogenes or deletion of tumor suppressor genes.

Conventional treatments for cancer include surgical resection, chemotherapy (anti-cancer drugs), radiotherapy, and their combination, which though have improved the patient survival in many different cancers, but still are associated with many side effects. Moreover, they have chances of recurrence and pose economic challenges. With the identification of many new molecules involved in various pathways and techniques which enable stable delivery of drugs to the specific cells in cancer tissue, a large field of research remains open.

Since the early twentieth century, chemicals have been used to treat cancer. Surgery and radiation therapy are also widely used but are not effective for metastatic stage, due to which chemotherapy has an advantage over them. So, many a times, a combination of these is used (DeVita and Chu 2008). Chemotherapy, although has been a standardized treatment, exerts various ill effects in multiple organ systems. These after-effects along with worse quality of life endure a research interest towards new strategies which are at least equally effective as chemotherapy but have lesser side effects, economical, and can be tolerated by the patients in a better manner (Gegechkori et al. 2017).

RNA Interference

RNA interference (RNAi) is a mechanism of gene silencing which has the ability to target various genes related to cancer and is a member of non-coding RNAs.

Although these do not go through translation for making up of proteins, it does not mean that they have no function at all. In Eukaryotes, RNAi is a post-transcriptional gene silencing (PTGS) regulatory mechanism which causes silencing of specific genes. Macromolecules which are distinctive components of RNA silencing include Dicers, argonaute proteins (AGOs), and duplex-derived RNAs.

Small interfering RNAs (siRNA)and microRNAs (miRNAs) are two major categories of small RNAs that regulate several genes. They also play an important role in the defense of genome from invasive nucleic acids. These small RNAs are 20–23 nt long, which provide protection against exogenous and endogenous genes, like that of bacteria and transposons, respectively (Carthew and Sontheimer 2009).

The miRNAs are regulators of several genes, while siRNAs on the other hand protect the integrity of genome (Carthew and Sontheimer 2009). siRNA can target the expression of genes in unstable manner and for shorter period in addition to inducing various responses of innate immunity such as that of interferon. Short hairpin RNA (shRNA) technique was developed in order to achieve the silencing of gene through transfection into genome.

Cancer and RNAi

A huge amount of attention is being recently given to cancer treatment using RNA molecules. RNAi is an economical and fast strategy (Mahmoodi et al. 2019). Although there are other methods of gene silencing such as CRISPR/CAS9 and TALEN, due to high potential, precise mechanism, high specificity, and lesser side effects, RNAi is usually favored (Mansoori et al. 2014).

The targeting of specific cells is an important aspect of cancer therapy. Chemotherapy has various toxic effects and affect normal cells. RNAi can be an important mechanism for targeting cancer cells without affecting the normal cells, thereby causing comparatively lesser side effects. Genes responsible for activation of oncogenes can be targeted. When the cells withmutated tumor suppressor gene is replaced with wild-type tumor suppressor gene, it may cause suppression of tumor formation by restoring cells to normal cell phenotype. Also, cells where abnormal activation of oncogenes has occurred, it can be suppressed by antisense RNA. RNAi can enable targeting of many different targets which are usually difficult to treat with conventional therapies.

RNAi is still being explored as a powerful means to treat cancer. There are a large number of studies, both in vivo and in vitro, which suggest that RNAi can be used for treating disease involving protein overexpression (Dykxhoorn and Lieberman 2006). It is efficient and has a huge potential in cancer treatment. Various genes involved in tumor formation along with a cascade of genes involved in cellular pathways promoting tumor progression can be silenced. Moreover, personalized medicine for the patients can be developed which can control the tumor progression (Bora et al. 2012). Due to less side effects and comparatively more specificity, RNAi outweighs other therapies (Rao et al. 2009).

Gene Silencing Through siRNA and shRNA

RNAi is induced by perfectly base paired double stranded RNA taken up in cytoplasm. The production of siRNA takes place in two stages called starting and effecting. In the former stage, a 200–500 bp long double stranded RNA is cleaved, resulting in mature smaller 21–23 bp siRNA molecules having a 3′ overhang and an enzyme called Dicer helps in the cleavage (Agrawal et al. 2003).

In the Dicer protein, an important role of excising siRNAs from the ends is played by PAZ and RNase III. PAZ domain, to which a ribonuclease called Argonaut is shared, binds to the ends of the RNA having 2 nt long 3′ overhangs. One strand of the RNA duplex is cleaved by RNase III active sites, which leads to the generation of new ends with 3′ overhangs leaving a 5′ monophosphate. Some organisms such as mammals consist only one Dicer protein that causes the biogenesis of both miRNAs and siRNAs, whereas other organisms have multiple Dicer proteins. For example, in *Drosophila melanogaster,* Dicer-1 and Dicer-2 are required for miRNA and siRNA biogenesis, respectively (Tomari and Zamore 2005).

Argonaute is a bilobed protein in which PAZ domain is located in one lobe and the PIWI domain in the other lobe. The PAZ domain binds to the 3′ end of the RNA which causes strand binding. The PIWI domain has an RNase H fold that sometimes can help in the cleavage of the target after base-pairing (Parker et al. 2004).

In organisms such as drosophila, the trimer consisting of Dicer, transactivation responsive RNA-binding proteins (TRBP), and Argonaute 2 (Ago2) form risk loading complex, which binds to double stranded RNA, dices it into siRNA, then delivers it in Argonaute and generates functional RNA-induced silencing complex (RISC) by discarding the passenger strand. In mouse, it has been demonstrated that dicer is not critical for loading of RISC, as it has been shown that null allele of dicer can assemble siRNAs into functional RISC (Carthew and Sontheimer 2009). This signifies that siRNA can be synthetically induced in RISC and the processing due to Dicer may be avoided.

The two strands of the duplex are named guide strand and passenger strand. In case of shRNA, the synthesis takes place in nucleus of the cells and then it gets into the cytoplasm for further processing. In the effecting stage of siRNA production, the two strands are subjected to separation due to Helicase activity. The guide strand is taken into the RISC, while on the other hand, endonucleases act on the passenger strand (Agrawal et al. 2003). During the strand selection, in which guide strand is selected for RISC complex, there is no presence of target mRNA. The RISC is preformed and is already programmed to cleave and identify a specific sequence. Rather, the selection of the strand depends on the thermodynamic stability (Tomari and Zamore 2005).

When the RISC complex carrying the guide strand is directed to the target mRNA, it leads to mRNA degradation due to the presence of Argonaute. The decrease in the target gene expression is called post transcriptional gene silencing. PIWI domain in the Argonaute protein is the inducer of this degradation. The cleavage occurs through breaking of phosphodiester bonds in the target. This leads

to the formation of products having a 5′monophosphate and 3′ hydroxyl ends (Agrawal et al. 2003). Single stranded siRNA can be loaded directly in Argonaute proteins but siRISC assembly pathways are necessary when the siRNA is double stranded after the action of Dicer (Rivas et al. 2005).

The degradation process is then completed by exonucleases in the cells. After cleavage, the target is freed from the siRNA and RISC is then able to cleave more targets. On the other hand, when they get connected to the homogeneous strand and then the formation of double stranded RNA takes place with the help of RNA polymerase, a continuation of interference pathway can occur (Carthew and Sontheimer 2009).

Gene Silencing Through MicroRNAs

miRNAs have been shown to be strongly associated with cancer. The miRNA transcription takes place with the help of RNA Polymerase II. In animals, the transcription units can lead to formation of one or more miRNA. They may also form a protein and an miRNA. The miRNA genes can produce multiple products because of the retainment of nucleotide changes, and not due to gene duplication (Bartel 2004; Lu et al. 2008). This resultant nucleotide sequence may form a new product when it is expressed. Thus, the primary miRNA transcript (Pri-miRNA), consisting of a stem loop structure with about 33 bp in length with a terminal loop and flanking regions, is formed. The excision of the stem loop structure from the transcript occurs in the nucleus, which leads to the formation of a product called pre-miRNA (Bartel 2004). In animals, this cleavage is done by Drosha which is dependent on DGCR8 for appropriate positioning of its catalytic site at specific distance from the junction between stem and flank (Carthew and Sontheimer 2009). The pre-miRNA is transported into the cytoplasm where cleavage of the terminal loop occurs with the help of Dicer enzyme that leads to the formation of mature miRNA/miRNA duplex of around 22 bp length. Dicer gives more preference to the terminal region for cleavage. The PAZ domain of Dicer determines the 3′ end cleavage site. The miRNA is then assembled into RISC to which only one strand remains attached.

The regulation of mRNAs takes place through miRISC where miRNA acts as an adapter. In animals, the binding sites for miRNA on mRNA are located in the 3′ UTR. Also, for the regulation of mRNAs without binding sites for miRNA, an RNA recognition factor is bound to miRISC which represses the mRNA. After binding, this can lead to either cleavage of the mRNA molecule, or may restrain its translation. This isdependent on the complementarity of miRNA and mRNA. Cleavage of mRNA takes place in case of perfect complementarity while translation gets repressed due to some central mismatches. Arguments have been made about translational repression to be the default mechanism, independent on the complementary (Carthew and Sontheimer 2009). A diagrammatic representation of RNAi mechanism is portrayed in Fig. 1.

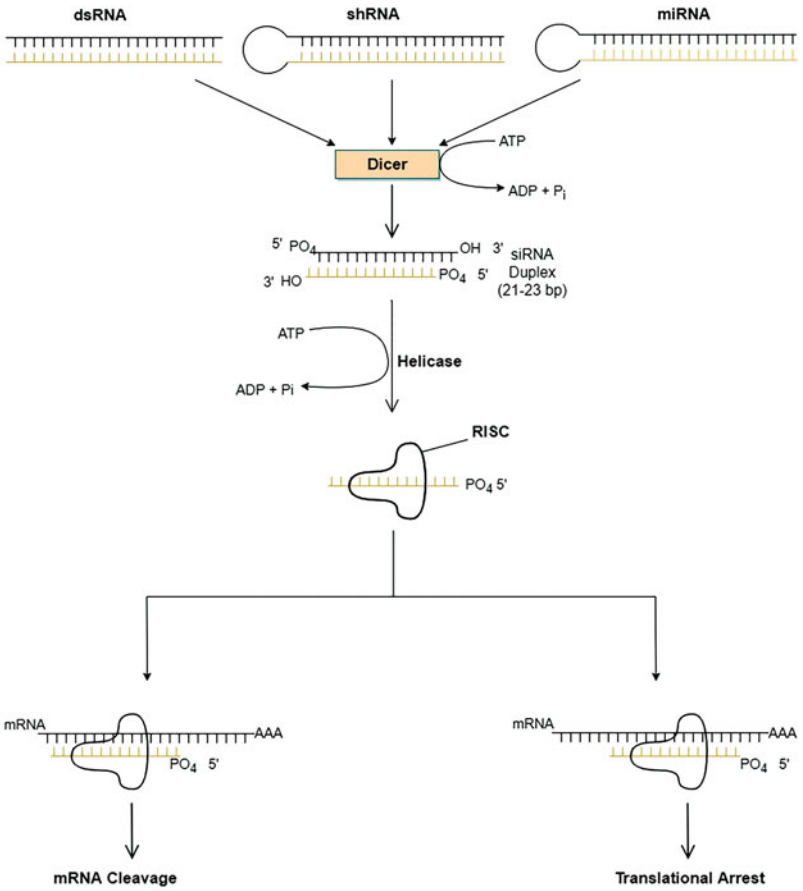

Fig. 1 Diagrammatic flowchart of RNAi mechanism. Double stranded DNA as in case of virus, shRNA carried in a delivery vehicle and miRNA which are endogenous care converted into siRNA with the help of Dicer enzyme. The duplex product has 5′ phosphate group as well as 3′ overhang. The helicase enzyme in the RISC assembly unwinds the RNA duplex with the help of ATP. The RISC associated with the anti-sense RNA then looks for the complementary mRNA in the mRNA pool in the cytoplasm resulting in either targeted mRNA cleavage or translational arrest

Animal Models

Cancer research is limited due to the difficulty to access lesions in various stages in cancer development. The interactions between various cell types are not fully recapitulated during cell proliferation and differentiation. Furthermore, during in vitro culture, phenotypic and genotypic changes occur and emphasize the importance to study tumor biology from initiation to metastatic growth (Winslow and Jacks 2015).

The gene expression can be knocked down with the help of RNAi in animals. This can form cheaper option in order to generate knock out or knock down animals. The gene silencing achieved through siRNA has been observed to be stable and can be inherited to the next generation. A large diversity of cells can be targeted using siRNA from blastocysts to adult animals. Thus, this strategy has important advantage for therapeutic applications.

A number of organisms have contributed to the knowledge and understanding of cancer biology at molecular and cellular level. Research on animals such as rats, mouse, chickens, and rabbits have contributed a lot in the cancer biology before the development of genetically engineered mouse. Although discoveries in RNAi have been made in organism such as Drosophila, more experiments regarding cancer research have been done in mouse and rat models. Mice have a shorter generation time, close genetic relation with the humans, and are smaller in size due to which they are preferred than other animal models (Winslow and Jacks 2015).

Various Models Used

Spontaneous Model: These animals are not genetically engineered, and they would help to determine the effect of a particular mutation. The animal is analyzed to see the tumor development with age. The growth of the tumors is unspecified, can develop in any organ and their genetic basis of unknown.

Carcinogen Induced: In these models, the animal is treated with carcinogens (physical or chemical). This leads to the formation of spontaneous tumors with unknown genetic mutation.

Xenograft Models: In these models, the animals are injected with human cancer cells. These cells can be genetically engineering in the lab, and then injected. The animals used are immunocompromised (lacking a thymus) and large number of cancer cells are injected to develop the tumors.

Orthotopic Models: The mice are injected with mice or human tumor cells and these can be engineered genetically. The cells can be injected into the tissue of origin. But, a large number of cells are required for the tumor induction.

Genes to Target

RNAi-based gene silencing can be used to silence a wide array of genes like oncogenes, tumor suppressor genes, genes that promote apoptosis, inhibit of cancer-promoting miRNAs, etc. Also, genes involved in angiogenesis and cell cycle progression can be targeted. It has been seen that RNAi when used with a chemotherapeutic drug is better in suppressing cancer progression than individual chemotherapy. Also, silencing of gene at the transcriptional level is also possible as siRNA can induce heterochromatin formation and can form DNA methylation.

Several genes are involved in various stages of cancer. Genes such as β-Catenin, WT1, RhoA, and RhoC are involved in mitosis and thus increase the cell

proliferation whereas IGF-1R is involved in the regulation of cancer cell differentiation. Genes namely Neuropilin-2 (NRP-2), EGFR, VEGF, and VEGF R2 are involved in angiogenesis. RhoA and RhoC are also involved in inhibition of apoptotic pathways in addition to EphA2, Cyclin D1, and Survivin and thus increase the cell survival. Moreover, RhoA and RhoC can regulate the cell motility, invasion, and metastasis. EphA2 increases the adhesion to extracellular matrix. Survivin inhibits apoptosis through intrinsic and extrinsic pathways and can increase the chemoresistance causing tumor recurrence. Livin promotes cancer progression through inhibiting both extrinsic and intrinsic pathway of apoptosis.

A list of various cancer-related genes that have been targeted using siRNA and shRNA in various mouse and rat models is in given in Table 1.

Delivery of RNAi In Vivo

In RNAi technology, the delivery of siRNA to specific tissues is a great challenge. This is because of the size and negative charge of the siRNA which causes difficulty in crossing the plasma membrane. Most of the cells find it difficult to internalize the siRNA. After delivery into the cells, the siRNA should escape from the endosomal and lysosomal degradation, which is also a challenge (Zhang et al. 2020b). Therefore, developing methods which can significantly overcome these hurdles are important. As a result, new methods of delivering siRNA into the cell are necessarily important.

An effective delivery vehicle is needed for the protection of the naked or chemically modified siRNA from degradation and thus enables it to cross the target cell membrane and release from the endosome (Karim et al. 2018).

The delivery vehicles are of two categories: viral and nonviral vectors.

The viral vectors include adenovirus, retrovirus, and adeno-associated virus (AAV). About 69% of gene therapies include viral vectors. The genetically engineered viral vectors have increased specificity, decreased pathogenicity, unassisted cell entry, and enhanced gene expression efficiency. However, these viral vectors can induce innate immunity response and carcinogenesis, and are more expensive which account for their disadvantages. This opens the way for requirement of developing nonviral vectors (Karim et al. 2018).

Nonviral vectors are comparatively less expensive, biologically compatible with high specificity. They are further categorized into organic and inorganic carriers. The former is divided into three types based on their composition: cationic lipids, cationic polymers, and lipid-polymer hybrids. The decreased rate of endosomal escape, retarded release of siRNA and toxicity decrease the success rate of organic carrier based approach. Inorganic nanocarriers are therefore the focus for delivery of siRNA. They are biocompatible and flexible enough to be functionalized with different ligands and surface coating materials. They are also compatible with known therapeutic agents. Some examples of inorganic nanoparticles include gold nanoparticles, magnetic nanoparticles, and pH-sensitive carbonate apatite nanoparticles (Karim et al. 2018). Various formulations used for transfection of siRNA

Table 1 Genes targeted using siRNA and shRNA in various animal models and their outcomes

Genes silenced	Animal model used	Cells injected	Outcome of the preclinical study	Reference
17β-HSD1	Nude female BALB/c mice	T47D-WT cells or T47D-17β-HSD1 cell	Decease in proliferation of tumor cells	(Li et al. 2019)
3WJ-EpCAM-D5D	Female homozygous nude mice (NU/J	4T1	Decrease in tumor size and lung nodules when a combination of siRNA along with dihomo-γ-linolenic acid (DGLA) was induced.	(Shah et al. 2020)
AQP3	BALB/c nude mice	XWLC-05	Human lung cancer invasiveness and proliferation were suppressed	(Xiong et al. 2017)
Bag-1	Balb/C nude mice	LoVo	Tumor was inhibited by 69%	(Huang et al. 2016)
Bcl-2	Balb/c mice	PC-3	Tumor was decreased by 65%	(Sonoke et al. 2008)
Bcl-2 (shRNA)	Balb/C mice	GBC-SD	Volume of tumor decreased by 50%	(Geng et al. 2013)
Bcl-xL	Male BALB/c nude mice	PC-3	Efficiency of siRNA delivery was about 62% when atelocollagen is used as a vehicle	(Takei 2019)
CatK	Male SCID mice	C4-2B	Prostate cancer tumor bone metastasis was inhibited in mouse.	(Liang et al. 2019)
CCND1 (shRNA)	BALB/c nude mice	SKOV3	Increase in sensitivity to Olaparib treatment through RAD51 downregulation by CCND knockdown	(Zhong et al. 2019)
CD47	C57B2/6 mice	B16F10	Tumor was inhibited by 90%	(Wang et al. 2013)
CDC42	C57BL/6 mice	LLC1	Size of the tumor and proteins related to metastasis were reduced	(Zhang et al. 2020a)
Cyclin B1	NMRI nude female mice	B16-F10	Reduction of tumor size by 44%	(Kedinger et al. 2013)
CYP1A1	BALB/c nude mice	A549	Significant inhibition of tumor growth	(Zhang et al. 2019a)
EIF5A2 (shRNA)	Nude mice	PC-3 M IE8 cells	Inhibition of tumor growth as well as metastasis	(Zhong et al. 2020)
EphA2	Balb/C mouse	SGC 7901	Tumor growth was inhibited by 43%	(Yuan et al. 2012)

(continued)

Table 1 (continued)

Genes silenced	Animal model used	Cells injected	Outcome of the preclinical study	Reference
IGF-1R	Male nude mice	A549	Tumor volume was decreased by 60%	(Dong et al. 2007)
LINC01234 and SHMT2 (shRNA)	BALB/c nude mice	Lovo and HCT119 cells were injected prior to shRNA intake	Decrease in the tumor growth and invasion	(Lin et al. 2019)
Livin	Nude mice	LiBr	Size of the tumor was decreased by 64%	(Wang et al. 2017)
MDM-2	Athymic mouse model	H2009 cells, NSCLC cells	Growth of tumor reduced by 67%	(Yu et al. 2013)
MDM-2, c-myc, and VEGF	Female C57B216 mice	B16-F10	Tumor load was reduced by 20–30%	(Li et al. 2008)
NRP-2	Male athymic nude mice	HTC-116	Reduction in tumor volume by 91.3%	(Gray et al. 2008)
PD-L1	SCID-hu mice	PaTu8988	Reduction in tumor growth and lung metastasis, and increase in survival time	(Wang et al. 2019)
PRDM14	Female BALB/c nude mice	MDA-MB-231 and HCC1937	A reduction in breast tumor formation was seen. Lung or liver metastasis did not develop even after 45 days	(Taniguchi and Imai 2019)
RANBP9 (RAN binding protein 9) (shRNA)	Male BALB/c mice	HCT116 and HT29 cells	RANBP9 knockdown promoted colon tumorigenesis in mice	(Qin et al. 2019)
RhoA	Female nude mice	MDA-231-MB, human breast cancer cells	Tumor volume was reduced by 85%	(Pillé et al. 2005)
RhoA & RhoC (shRNA)	Male Balb/C mice	HCT-116, human colorectal carcinoma cell line	Tumor volume was decreased by 37%	(Wang et al. 2010)
RhoC	Balb/C nude mice	SUM149, human inflammatory breast cancer cell line	Tumor volume was reduced by 35%	(Xu et al. 2017)
Ribonucleotide reductase M2 (RRM2)	Female BALB/c nude mice	Human ovarian cancer cell line SKOV3	Tumor volume was decreased	(Xue et al. 2019)

SAP102	Female SPF rats	Walker 256 breast cancer cells	Intrathecal injection resulted in lessened the pain with respect to cancer induced bone pain (CIBP) which indicated a potential treatment	(He et al. 2020)
Survivin	Balb/C mice	4 T1 cells	Tumor volume was reduced by 66%	(Fan et al. 2017)
Survivin	Female Balb/C mice	4T1 cells, mouse breast cancer cells	Reduction in tumor volume by 55%	(Sun et al. 2016)
Survivin	NMRI nude female mice	B16-F10 cells, murine melanoma cell lines	Reduction in tumor volume by 50%	(Kedinger et al. 2013)
Survivin siRNA	Female BALB/c mice	4T1	Codelivery of DOX and survivin siRNA in mice by PEI modified SFNPs decreased the proliferation of breast cancer cells and also induced apoptosis	(Norouzi et al. 2021)
Neuromedin U receptor 2 (NMUR2)	Spague-Dawley rats	Walker 256	Bone cancer pain was reduced through inhibition of PI3K/AKT and PKC/ERK pathways	(Peng et al. 2019)
TRIM24	Nude mice	PC-3	Decrease in tumor growth and bone loss was observed	(Shi et al. 2019)
TROP2 (shRNA)	BALB/c nude mice	GBC cells	There was a significant decrease in tumor weight	(Li et al. 2017)
TRPV1 (shRNA)	Sprague–Dawley rats	Walker 256	Successful for treatment of bone cancer pain	(Zhang et al. 2019b)
VEGF	Female nude mice	PC-3	Inhibition of tumor growth by intratumoral and intravenous administration	(Kim et al. 2008)
VEGF R2	Female nude mice	N2A	Reduction in tumor volume by 90%	(Schiffelers et al. 2004)
VEGF-C	Balb/C mice	4T1 cells	Tumor volume was reduced by 28%	(Liu et al. 2016)
VEGF-C	Balb/C mice	A549	Tumor volume was reduced by 48%	(Feng et al. 2011)
WT1 (shRNA)	Balb/C nude mice	A549, H1299, and H1650 cells	Tumor volume was decreased by 69–76%	(Xu et al. 2013)

(continued)

Table 1 (continued)

Genes silenced	Animal model used	Cells injected	Outcome of the preclinical study	Reference
WT1 (shRNA)	Female C57BL/6 mice	B16F10	Weight of the tumor was reduced by 34% and survival rate was improved by 62.5%	(Saavedra-Alonso et al. 2016)
XIST (shRNA)	Female nude mice	A549	Decrease in pulmonary metastasis	(Li et al. 2018)
β-Catenin (ShRNA)	Male athymic nude mice	AGS cells	Reduction in tumor volume by 75%	(Chang et al. 2017)

include microemulsions, nanogels, liposomes, and polymers. Modifications are done to improve the cellular uptake such as PEGylation, binding of moieties such as antibodies and ligands for active targeting, and cell-penetrating peptides (Karim et al. 2018).

Various methods of delivery using aptamers, liposomes, dendrimers, antibodies, exosomes, and polymers have been studied. There has been a tremendous advancement in the optimum delivery with the use of aptamers. The aptamers are nucleic acid sequences that are synthetically designed to bind to a cell-specific antigen. The aptamer designed to bind to prostrate specific membrane antigen (PSMA) efficiently delivered the siRNA and silenced the oncogenes in prostate cancer cells, resulting in the reduction of tumors in the mouse xenograft models (Ren and Zhang 2014).

A method involving protamine-antibody fusion protein is also utilized in which the antibody binds to a cell-specific protein. Breast cancer cells expressing ErbB2 was successfully targeted through the protein-specific antibody using non-viral vectors (Li et al. 2001).

Stable nucleic acid lipid particles (SNALPS)are also used for targeted delivery of siRNA. It was reported that siRNAs encapsulated in SNALPs cause about 90% target silencing with no toxicity (Zimmermann et al. 2006). miR-34a mimics encapsulated in SNALPS and delivered efficiently, decreased the tumor cell growth in vitro and in vivo (Di Martino et al. 2014).

Chemical modifications performed during siRNA designing namely introducing 3′ end phosphorothioate linkages and modifying 2′ ribose position in the strand prevent exosomal degradation of siRNA. Treatment of 2′-OMe-phosphorodithioate-modified siRNAs in ovarian cancer mouse model showed antitumor activity when combined with paclitaxel. Moreover, silencing of GRAMD1B protein that is involved in chemoresistance, using of siRNA led to synergistic anti-cancer effect in combinational treatment with Paclitaxel (Wu et al. 2014). Magnetic nanoparticles are also being used in drug delivery which can release drugs in the target tissue by using an external magnetic field.

As we discussed earlier, viruses are also used to deliver RNA molecules in the animal models such as AAV, herpes virus, lentivirus, etc. AAV does not induce an inflammatory response in the host and can efficiently express shRNA or artificial miRNA in different cells for a longer period. Due to this, AAV is mostly favoured and is comparatively safer. Cell survival genes are regulated by androgen receptor (AR) and administration of AAV-ARHP8 viruses resulted in apoptosis and reduction in tumor growth in nude mouse xenografts (Sun et al. 2010). Furthermore, the administration of AAV containing the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene under the control of human telomerase reverse transcriptase (hTERT) led to apoptosis of tumor cells and suppression of tumor growth in hepatocellular carcinoma xenograft mouse model (Wang et al. 2008).

A brief overview regarding various methods of delivery of RNAi molecules are shown in Fig. 2.

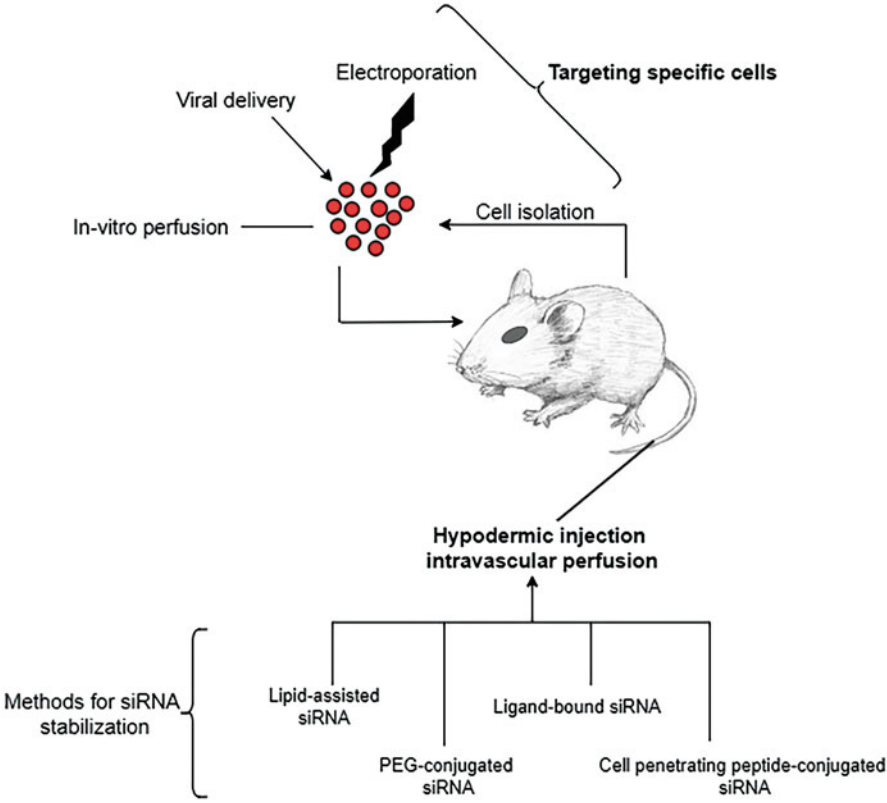

Fig. 2 In vivo delivery of RNAi molecules. The siRNA molecules can be injected directly into the model organism. Various siRNA stabilization methods are used. Tumors can be induced in the mouse by isolating the cells and silencing its specific genes through viral delivery and then injecting the cells back into the animal model.

RNAi and Cytotoxicity

An ideal RNAi therapy would silence the target genes without affecting the other cells with no side effects. However, RNAi drugs in reality have off target effects and an immune response. When a gene having a sequence similar to that of target gene is silenced or repressed, it is called off target gene. These have to be understood and solved properly for proper application of RNAi. The off-target effects can be minimized by using ligands capable of detecting the cell-specific receptors and antibodies having high affinity and selectivity Also, proper concentration of siRNA should be used, as the target genes can be silenced at low optimal concentration.

Conclusions and Future Scope

In this book chapter, the mechanism of RNA interference, its need in cancer research and animal models that have been developed using RNAi technology were discussed. RNAi has a great potential and is one of the most promising solutions to cancer. It has several benefits over other methods and can be used in a wide range of tumors such as renal carcinoma, breast cancer, and melanoma. Since it targets only specific genes, it has lesser side effects and is highly effective. New techniques are now available to do efficient transduction.

Limitation occurs due to degradation of RNA when it is being carried in blood-stream as well as during cellular uptake. Due to the delivery problem, it is confined to local administration. For some specific cells, RNAi delivery remains problematic due to various complexities of the tumor microenvironment and extracellular matrix through which RNA has to pass. Various signaling pathways associated with cancer, cross talk, and feedback loops cause complications to the gene therapy using RNAi. However, for many patients who develop drug resistance, gene therapy targeting specific genes is recommended. Therefore, RNAi has a greater application in the field of cancer research and therapeutics.

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Association of Animal Models in the Field of Translational Medicine: Prediction and Validation

46

Debora Bizzaro

Contents

Abstract

During clinical development, the success rates for drugs remain low despite large investments. A prominent reason for the poor rate of translation from bench to bedside is generally assumed to be the failure of preclinical animal models to predict clinical efficacy and safety. This failure could be held to problems of internal validity (e.g., poor study design, lack of measures to control biases) and external validity (e.g., poor reproducibility of a research finding, translational failure) in preclinical animal studies. To analyze the significance of animal research impartially, we must warrant that (1) the experiments are conducted and reported according to best scientific practices; (2) the selection of animal models is made with a clear and thorough translational rationale behind it. Once these criteria are met, the true significance of the use of animal models in drug development can be ultimately attested.

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Preclinical animal models · Internal validity · External validity · Experimental bias

Introduction

During clinical development, the success rates for drugs remain low despite large investments. During the last decade, thanks to innovative molecular biology approaches, a lot of possible new drug targets were identified and a large number of promising drug candidates directing these potential new targets have entered clinical development. However, this was associated to the decline in the success rates for drugs due to the absence of efficacy in humans during clinical trials (De Martini 2020; Hwang et al. 2016). On average, 17 years pass before effective research-based findings are applied into practice, and even then only an estimated 14% of those findings result in health care delivery changes (Olson and Oudshoorn 2020).

A complex multifactorial process is at the base of the poor rates of successful translation from bench to bedside and includes, as one of the most important components, the failure of preclinical animal models in predicting clinical efficacy and safety (Ferreira et al. 2020).

More strict success criteria could be useful to decrease the failure rates during the preclinical studies, especially during target validation for a potential new drug. Efforts to explain these failures have focused on the internal and external validity of preclinical animal models (Henderson et al. 2013; Ferreira et al. 2020).

So far, the internal validation is the most considered and analyzed aspect of the problem. It included inadequacy in study design such as low power, inappropriate endpoints and inaccuracies in study conduction, analysis, and reporting. These inaccuracies lead to unreliable data which ultimately means unnecessary suffering for the animals and potential risks for human clinical trial participants (Ioannidis 2017).

On the contrary, there has been relatively little discussion of the other key factor influencing translation and assessing the reliability of the research, namely the external validity. It is defined as the degree to which research results derived in one experiment setting, species, or strains can be consistently applied to other different settings, species, or strains (Pound and Ritskes-Hoitinga 2018). Of course, in the field of preclinical animal research, external validity is of the utmost importance.

When adequately designed and conducted, preclinical animal studies with adequate internal and external validation are essential in the discovery and development of new drugs. The current chapter highlights some strategies that researchers should take into account to improve the translational potential of the preclinical animal models.

Validity of Preclinical Animal Research

Internal Validity

Internal validity refers to the basic principles of study design, such as conduction, analysis, and results reporting. Frequently, preclinical animal studies suffer from serious problems of internal validity, in particular low power, inappropriate endpoints, and lack of measures to avoid bias such as randomization or blinding (Denayer et al. 2014).

Recently, Jankovic et al. found that only 7% of the published studies they analyzed from journals with relatively high impact factors retained strong internal validity, despite all having undergone a rigorous review process. They highlighted two major flaws in internal validity: lack of randomization and the use of pseudoreplication (repeating an experiment using the same animal) that can lead to misleading data and overestimation of the sample size (Jankovic et al. 2019). Similarly, other research found that only 14% of the papers reported blinding in animal selection and results in evaluation to avoid bias (Kilkenny et al. 2009). Moreover, only 3% of all studies reported sample size and statistical power calculation and more less studies defined a primary outcome variable (Macleod 2011).

Therefore, to avoid biased conclusions and production of false positive or negative results, the preclinical studies with animal models should, at least, contain the appropriate control groups, be repeated in independent sets of animals with adequate statistical power to guarantee significant results, and apply treatment randomization, as well as a blinded outcome assessment (Pound and Ritskes-Hoitinga 2018; Schmidt-Pogoda et al. 2020).

External Validity

The external validity extends behind the specific setting of experiment and is referred to as the grade of generalization of study results, i.e., how replicable they are in other environmental conditions, experimental setting, study populations, and even in other strains or species of animals, including humans (Pound and Ritskes-Hoitinga 2018). Poor external validity may implicate: i) poor reproducibility of a research finding (e.g., the same experiment in a different laboratory by a different investigator produces different results); ii) translational failure (e.g., an effective treatment in an animal model has not therapeutic effect in a human clinical trial).

McKinney and Bunney were the first to recommend criteria on external validity of animal models in 1969, mainly focusing on affective disorders (McKinney and Bunney 1969). In 1984, these external validations were simplified to three criteria: predictive, face, and construct validity (Willner 1984). These are the most widely accepted criteria for model validation, although others were proposed (Denayer et al. 2014; Ferreira et al. 2020).

Predictive validity is defined as the determination of how preclinical animal models are effective in predicting currently unknown aspects of the human disease or the clinical efficacy of a drug.

Face validity refers to the likeness in pathobiology, symptoms, and signs among animal models and human diseases. In many cases, the pathobiology underlying the symptoms of the disease is poorly understood, so the assessment of facial validity is often hampered.

Construct validity is defined as how the method of induction of the disease phenotype in animals replicates the currently known disease etiology in humans (similarity in the biological dysfunction).

By definition, a model cannot be a perfect reproduction of the human disease. Consequently, all the three criteria cannot be met by only one model; for example, a model might have sound predictive validity but totally lack face validity, or vice versa. A combination of diverse animal models could be surely more similar to the clinical situation than a single complex model.

The three criteria offer a general external validation and there is controversy in their ranked importance, mostly due to the discrepancy in their definition. Generally, their importance should be based on the purpose of the model. Indeed, according to the aim of the animal model, the criteria to be respected may change. For example, face validity may be more important in animal models for pathobiology studies, whereas in preclinical drug discovery, predictive validity tends to hold the most weight. Understanding which validity a model can and cannot provide is fundamental for accurate preclinical assessment of novel therapeutic agents (Denayer et al. 2014).

Association of Internal and External Validity

Despite it is a common perception that internal validityis actually a prerequisite of externalvalidity (i.e., by resolving the problems of internal validity the clinical translation would be more successful), the available evidence does not support this sight. Indeed, it is important to highlight that preclinical animal studies need to be both internally and externally validated if they have to be translated into benefits for humans (van der Worp et al. 2010). Both internal and external validity are critical, yet researchers often encounter a trade-off between them, such that strengthening the features of one type of validity weakens the other. Some of the strategies used to increase internal validity could together decrease external validity. For example, using homogeneous study populations to standardize experiments and to maximize test sensitivity inexorably prejudice the external validity of the findings, resulting in poor reproducibility (van der Worp et al. 2010). Preclinical studies are usually performed in a fairly homogeneous approach (e.g., mice of the same sex, age, and genetic background). Despite this may ease the use of as a small number of animals as possible to obtain a statistically significant result, it does not really represent the real human condition of a pool of individuals from various genetic and environmental backgrounds. Results more applicable in spite of the animal's (or human's)

characteristics would be reached by combining a heterogeneous population of subjects with the right analytical techniques (Pound and Ritskes-Hoitinga 2018).

How to Refine the Preclinical Animal Models

Taking into account both internal and external validity, numerous aspects should be weighed in performing preclinical studies.

I. Selection and attrition bias: refer to the biased distribution of animals to treatment and control groups and can be prevented by randomization. Randomization is always required even if homogeneous population (such as same sex and/or age, inbred mice kept under identical housing conditions) was used, since individual differences still prevail. Since selection bias may occur either consciously or subconsciously, operator-independent methods may be preferable (e.g., random number generators). Selection biases may also occur if animals' inclusion or exclusion criteria are weakly defined. Complications that require exclusion of animals (e.g., reaching of humane endpoints or occurring of complications unrelated to the experimental treatment that make the outcome analysis worthless) are an intrinsic risk in preclinical animal studies. To avoid this kind of bias, all animal inclusion and exclusion criteria should be clearly predefined, and the operator accountable of these steps should be unaware of the treatment allocation (van der Worp et al. 2010). The risk, if these criteria are not well specified, is the unequal distribution of withdraws among treatment groups, defined as attrition bias.
II. Performance and detection biases: the first occurs when there is a systematic difference in the animal care and/or experimental procedures (apart from the treatment under investigation) between the treatment groups. Detection bias occurs when the outcomes are determined differently in animals of distinct treatment groups. Both these biases may occur consciously or subconsciously, therefore the best approach to exclude them is blinding. In contrast to randomization, blinding is not always achievable and it is essential that authors explicitly report the blinding status of the staff involved in experimental steps that may affect the outcome of the study (Denayer et al. 2014).
III. Sample size and power analysis: according to one of the 3R principles (reduction) the researcher should minimize the number of animals utilized in biomedical experimentations. However, this should be well-adjusted with the statistical power essential to obtain relevant data (Button et al. 2013). When possible, sample size calculation and power analysis should be carried out, specifying the desired statistical power, the level of statistical significance, and the minimal effect size considered to be relevant.
IV. Reproducibility of results: generally, experimental set-ups could be highly standardized in a single lab, while minimal differences in environment (such as staff, noise) or experimental procedures (e.g., a xenograft model with a

diverse cell line) in another laboratory may harvest important differences in results avoiding their generalization potential in a wider context (Richter 2017).

V. Treatment time course: treatment of animal models is often started very shortly after or even before the disease onset. In this condition, the treatment is prophylactic, evidently in contrast to the human "real-life" condition in which the treatment is usually therapeutic, therefore started only after the clear manifestation of the symptoms and diagnosis. As a consequence, in an animal model the potential pharmacological effect may be wrongly overestimated (Malfait and Little 2015).

VI. Animal species and strain: The selection of species and strain of animals for a particular model should be carefully performed. Primarily, the animal target should be sensible to the active principle of the drug to be tested. In addition, the health status, age, and gender of the animals should be matched as strongly as possible to the "real-life" clinical condition. Instead, the animals used in preclinical research be likely more young and healthy, while numerous human diseases develop in older age and in association with other co-morbidities (Malfait and Little 2015). Furthermore, many animal models do not have the complexity necessary to precisely reproduce human conditions. Clearly, in such cases the findings from animal studies can give misleading results and are improbable to be appropriate for human patients.

VII. Reporting: together with the problem of insufficient reporting of experimental procedures that limits the reproducibility of the same experiment, a further obstacle is that experiments with positive and statistically significant results are more likely to be disseminated to the scientific community than negative ones. This is due to selective analysis and selective outcome reporting. Selective analysis occurs when numerous statistical analyses are performed but the Authors present only the one with the most statistically significant result; selective outcome reporting occurs when numerous result variables are analyzed but only the ones that are significantly influenced by the treatment are reported (Tsilidis et al. 2013). To avoid these potential biases, primary and secondary outcome variables as well as the statistical approaches to testing for treatment effects should be defined before the onset of the study.

VIII. Efficacy and safety assessment: efficacy is generally analyzed in preclinical disease models treated with a therapeutic dose of the drug but without examination of side effects, while safety is assessed in healthy animals to whom was administrated the drug at high dose. A safety margin is then defined by comparing the effective doses to that outlined in the safety assessment. However, this margin might be overestimated. Indeed, healthy and young animals used for safety analysis could develop less potential side effects as compared to diseased and more frail subjects. On the other hand, estimating the efficacy without considering the side effect could make it impossible to administer corresponding doses in a clinical setting. A possible solution would be to use diseased animals in parallel to standard healthy animals for safety testing of the new drugs.

Conclusion

Animal models are an essential aspect of any drug development experiment. However, inaccuracies in experimental design, conduction, and publication (whether conscious or not) persist to afflict research based on animal models. Facing these problems and underlying causes is an essential step in the direction of successful improvement of experimental design and conduct. Researchers, but also reviewers, and journal editors should not only support such methods of refinement but rigorously implement them. Otherwise, the reliability and ethical justification of animal research may be permanently damaged.

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Detection of Tumors Through Fluorescence Conjugated Dye in Animal Model

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Anjoy Majhi, Sandip Paul, and Pinki Saha Sardar

Contents

Abstract

The animal experiment serves as a vital link between the cell and clinical experiments. Animals have similar anatomy, physiology, and genetics to humans, and the progression of animal diseases is similar to that of humans; hence studying the animal model is significant. The mouse genome is quite similar to the human genome and can imitate a variety of biological features. Fluorescence imaging has received a lot of attention in the last two decades as a method of detecting a variety of cancers. Fluorescence imaging has the ability to provide high-resolution, high-contrast images to doctors, allowing them to more effec-

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tively diagnose and treat cancer patients. In the field of healthcare, molecular imaging plays an essential role in the diagnosis of disorders inside the body and provides accurate results.

Keywords

Fluorescent probes · Dyes · Fluorescence imaging · Bioluminescence imaging · Animal model · Cancer research

Introduction

Cancer is one of the primary or second leading causes of death (13% of all deaths) throughout the world (Trabulo et al. 2011), specifically among individuals under the age of 70 in 91 of these nations, according to the World Health Organization (WHO) in 2015 (Bray et al. 2018). The uncontrolled growth of abnormal cells, the preliminary symptom of the disease, can be treated more effectively in earlier detection of malignant cancer cells. The nanoparticle technologies, one of the most thriving methods for the detection of cancer cell inside the body, could be perceived by imaging technique (Jin et al. 2010). Fluorescent probes are extremely proficient of showing biosensing and bioimaging because of their excellent solubility, high specificity, simple method for preparation, and high fluorescence intensity (Paul et al. 2019). At present the investigations on design, synthesis, and development of fluorescent probes have great interest in biological and clinical research because of the direct visualization and dynamic information obtained by the process (Paul et al. 2019). Fluorescent probes can bind specifically with the receptor present in the cell, resulting to sharp change in wavelength of the emitted light along with the fluorescence intensity.

Fluorescence imaging technique received an enormous attention in the last two decades to detect the wide range of malignant cells. Fluorescence imaging technique with its high prospectus offers clinicians to identify and treat cancer patients more effectively with the help of high-resolution, high-contrast images. Early identification and treatment are obligatory for eradicating cancer in a patient, and fluorescence imaging technique for this has the capability to detect non-advanced, even precancerous tumors that would have been missed by imaging using white light or radiation.

Molecular imaging technique plays an important role in the field of healthcare to make a diagnosis of diseases inside the body with the proper outcome. Magnetic resonance imaging (MRI), ultrasound, tomography, gamma scintigraphy, optical imaging, etc. are the various forms of imaging techniques to consider for the interest of current research (Jenkins et al. 2016). Computed tomography is another method for the detection of an affected cell, but it has some drawbacks such as high cost of such imaging technique and needs to expose the tissue to radiation. Optical imaging is also known to be a very sensitive and cost-effective process for the detection of the cells (Janib et al. 2010). The setbacks for optical imaging are that the images obtained are generally low in resolution and have a limited tissue penetration

(Janib et al. 2010) to focus in the UV-Vis regime. Tissues exhibiting the reduced absorption to light in the far-red (i.e., 775 nm–925 nm) out to the near-infrared (NIR) range (Chance et al. 1998) facilitate this review to examine the recent development of molecular imaging technique where fluorophores emit in those regions. NIR fluorophores gained immense interest in medical imaging process and overcome some drawbacks, such as background noise and able to image deeper in the human body (Frangioni 2003). There are four drawbacks of small molecule fluorophores such as low quantum efficiency, limited aqueous solubility, low signal-to-noise ratios, and short in vivo circulation lifetimes. These drawbacks could be minimized or nullified by encapsulating the fluorophore into a nanoparticle or making it to attach on the surface of the nanoparticles. Over the years, fluorescent nanocarriers such as dendrimers (Lallana et al. 2012), surface cross-linked micelles (Torchilin 2007), biodegradable polymeric nanoparticles (Yap et al. 2009), and other carriers have been developed. Among all these techniques, platforms based on polymeric materials, especially biodegradable nanoparticles, gained enormous consideration due to the flexibility offered by macromolecular synthesis methods, improved drug solubility, high drug loading capacities, and their ease of multi-functionalization (Pokorski et al. 2011).

Animal models are the experimental objects of animal and related materials used in the medical research to replicate the cells in the human body. Cancer is the center of awareness for people all around the world, making it a hotspot for medical studies. After cardiovascular disease, it is the second most common cause of morbidity and mortality. Simultaneously, attempts are taken for a considerable increase of animal models to develop and be employed in cancer research. Chemical induction, xeno-transplantation, gene programming, and other methods of creating cancer animal models are more and more diversified as research moves on (Li et al. 2021). Since, it can retain the milieu of the originating tumor as well as the basic properties of cells, the patient-derived xenotransplantation (PDX) model has been a research hotspot in recent years (Li et al. 2021). Animal models are able to be used for investigating the biochemical and physiological processes that lead to the occurrence and progression of cancer in objects, as well as to screen cancer medications and test gene therapy (Li et al. 2021).

Preclinical animal models, a stage between cell culture and human clinical trials, are useful tools for improvising the treatments for malignant diseases. Mouse and, more recently, zebrafish models are among the various animal models commonly utilized in cancer research. Indeed, most cellular pathways are largely conserved in humans, mice, and zebrafish, making these models particularly interesting. Animal models are frequently generating new ideas for cancer research as a great tool for understanding the pathophysiology of human diseases and researching on prevention of disease and principles of the treatment. However, there are some disparities or inconsistency observed between animal models and humans in terms of physiology, genetics, and immunity. As a result, an animal model that serves as better mirror human biological traits and can replace humans in preclinical research is urgently needed in today's scientific development. With the advancement and refinement of gene-editing technologies in recent years, it has been possible to create a wide range of

cancer animal models, which have considerably aided cancer research. Researchers are engaged in developing a model that more closely resembles the human genome that will be more beneficial for the cutting-edge research (Davis et al. 2018).

Cancer Research on Animal Model

The experiment on animal model is a very important bridge between the cell experiment and clinical experiment. Animals have similar anatomy, physiology, and heredity to that of human, and recent growth of animal diseases is somewhat similar to that of human thereby enhancing the study and research exploiting the animal model. Animal models are useful in cancer research since they help us not only to understand the genetic foundation of cancer, the role of certain genes, gene mutations in cancer formation, and their progression but also the design and deployment of anticancer drugs (Schachtschneider et al. 2017). As research on precision medicine and tailored medicine progress, researchers are working to develop standardized and personalized tumor models that are increasingly similar to human tumors (Xu et al. 2019). Cancer animal models are created using a variety of animal kinds and construction methods, and the effectiveness of each animal model in tumor research reflects its own characteristics. The mouse genome is highly similar to the human genome, and it can mimic a variety of biological characteristics in vivo. It also includes the existence, development, and metastasis of human cancer cells and benefits of easy feeding, low cost, and gene-editing methods (Li et al. 2021). It is a great platform for drug discovery and testing, as well as a good tool for cancer research. The four most common methods for producing animal cancer models are chemically induced models, cell line-derived xenograft (CDX) models, patient-derived xenograft (PDX) models, and genetically modified mouse (GEMM) models (Mendes et al. 2020). This model is based on a tumor model that created is by chemical carcinogens, and it has the advantage of imitating the onset of human cancer directly at the outset of the carcinogenic process (Liu et al. 2015). In this method around 30–50 weeks are required to form the tumor after using carcinogens, which is one of the disadvantages of this method (Minicis et al. 2013). The patient-derived xenograft (PDX) model is a mouse model that can be generated by implanting tumor tissue samples from cancer patients directly into mice, which closely resembles the histology and genetics of the tumor (Hidalgo et al. 2014). Many antineoplastic medications have shown to be effective in preclinical animal models but unable to play an important role in cancer patients. An animal model must be developed that not only replicates the tumor microenvironment but also has a "humanized" immune system. Human hematopoietic cells, lymphocytes, or organs are implanted into immunodeficient mice to replicate the human immune system in the humanized mouse model (Shultz et al. 2012).

The zebrafish cancer model is the most popular among the vertebrate model, and the genomes of zebrafish are homologous and similar to those of humans, making them a useful model to study effectively the formation of cancer cell (Teittinen et al. 2012). The zebrafish model has certain distinct advantages in cancer research as

compared to the most regularly used mouse models (Veinotte et al. 2014) such as (1) compact in size, inexpensive in cost, and quick to reproduce; (2) it can be monitored and track the multiplication, dissemination, and metastasis of cancer cells in real time using transparent embryos; and (3) both transgenic and immunodeficient zebrafish (White et al. 2008) can stay translucent after reaching adulthood. Transgenic, genome-editing, xenotransplantation, drug-induced toxic damage, and other methods have been used to create a variety of zebrafish cancer models.

The most common disease at present in women is the suffering from breast cancer, and it is becoming more common as people become older (Ullah 2019). The mouse has long been used to examine the breast cancer cells. It has a number of drawbacks, including a long cycle, a high cost, and a complicated operation, to name a few (McCarthy et al. 2007). The advancement of research on the zebrafish model is being used as the experimental subject in an increasing number of breast cancer investigations. The effect of BMP antagonists like GREMLIN-1 (GREM1) on breast cancer cell invasion and exudation using xenograft zebrafish breast cancer (co-) injection models has also been investigated (Ren et al. 2019). According to this model, GREM1 promotes fibrosis, intravasation, and extravasation in breast cancer-associated fibroblasts (CAF). Furanodiene and 5-FU (5-fluorouracil) are used to treat zebrafish as a part of the breast cancer xenotransplantation paradigm (Zhu et al. 2019). The results demonstrated that the two medications had a clear synergistic anticancer impact as compared to the findings of furadiene alone.

Lung cancer has the greatest morbidity and fatality rates than any kind of cancer. To enrich the detection and treatment on lung cancer, researchers need to learn more about the disease's pathophysiology and underlying molecular mechanisms. Implanting LINC00152 knockout lung cancer cells into zebrafish produced a xenotransplantation model. The silencing of LINC00152 inhibited the proliferation and spread of lung cancer cells as compared to the control group using stereoscopic microscopy (Shen et al. 2020). The zebrafish model can be used to assess the efficacy and safety of anti-lung cancer drugs for more effective cancer treatment. Zebrafish lung cancer model is used to test the effect of DFIQ (a novel quinoline derivative) on non-small cell lung cancer (NSCLC) in vivo (Huang et al. 2020). It was discovered that DFIQ might inhibit cancer cells to some extent by monitoring cell proliferation, migration, and apoptosis. With the in-depth examination of the zebrafish tumor model, the current research would provide a new path for the molecular research mechanism of many cancers.

With the advancement on cancer research, more animals are being employed to create animal models. Small animal models, viz., mice, rats, zebrafish, fruit flies, and others, are currently the most widely used for the experiment in cancer research. Mice and zebrafish are the most commonly used among them. Small animal models provide numerous advantages, including high reproductive ability, low cost, and ease of upkeep, to name a few. Small animals, on the other hand, have the difficulty to operate usually due to their small size and limited blood supply, and also on small animals, surgery and radiography are difficult to accomplish (Mei et al. 2010). The introduction of large animal cancer models such as dogs, nonhuman primates, tree shrews, and pigs has opened up new avenues for research.

Canine genomes are more comparable to human genomes than rodent genomes (Pinho et al. 2012). Canines can develop tumors that have clinical, molecular, and histological characteristics that are similar to human tumors (Gardner et al. 2016). Unregulated genes implicated in human breast cancer were also found in canine breast cancer when researchers looked at gene expression in human and dog breast cancer and normal breast samples (Uva et al. 2009). Phosphatase and tensin homolog (PTEN) gene has also loss of expression in both canine breast cancer and human breast cancer (Ressel et al. 2009). Nonhuman primates are closely related to humans and share many characteristics with them, including physiology, metabolism, immunity, genetics, and many others, making them a good model for the studies on cancer. Chimps and humans, practically all human cancer genes, are well conserved (Puente et al. 2006). The use of nonhuman primates has been limited due to their high breeding and feeding costs, complex experimental procedures, and also due to ethical concerns (Pouladi et al. 2013). The tree shrew is a brand-new experimental creature; its entire genome is quite close to that of primates, and its physiology, biochemistry, tissue architecture, and immunology are all human alike (Fan et al. 2013). *Lentivirus* is used to create a tree shrew pancreatic cancer model and RNA sequencing to evaluate the gene expression profile (Tu et al. 2019). A tree shrew breast cancer model using lentivirus expressing the PyMT gene and chemotherapy drugs commonly used in human breast cancer (cisplatin and tobramycin) (Ge et al. 2016) have been discovered to significantly inhibit tree shrew breast tumors. The pig genome is highly homologous to the human genome and has highly conserved epigenetic regulation (Schachtschneider et al. 2015). Pigs are great animal models for cancer research because their anatomical, physiological, and genetic properties are remarkably comparable to those of human. Diethylnitrosamine (DEN) is used to produce hepatocellular cancer in pigs and discover that partial hepatic embolism could aid in the model's creation (Mitchell et al. 2016). Gene-edited pigs have also been used as a novel method to study cancer-related genes. Gene-edited pigs expressing Cas9 under Cre enzyme induction and a lung cancer pig model were created by activating one oncogene (KRAS) and five tumor suppressor genes at the same time (TP53, PTEN, APC, BRCA1, and BRCA2) (Wang et al. 2017). Most importantly, several studies have shown that the pharmacokinetics of young pigs may be predicted in children (Roth et al. 2013), paving the way for the development of anticancer medicines using piglet models.

Cancer Imaging and Targeting with Near-Infrared Dyes

Fluorescence imaging in the near-infrared (NIR) region is the topic of interest in noninvasive molecular imaging. NIR dye conjugation with cancer-targeted biological and chemical substances to both covalent and non-covalent molecules emerged as a feasible strategy for providing real-time data on biomarkers and molecular activity connected to cancer. Some nonconjugated multifunctional NIR dyes, in particular, have shown the way for extremely specific and sensitive cancer imaging by demonstrating outstanding and selective accumulation in vivo in tissues, organs,

or tumors. The question is that the additional multifunctional NIR dyes will emerge not just for imaging applications but also for target-specific diagnosis and even therapy of diseases, as more effort is put into overcoming the disadvantages of current NIR dyes and combining multi-modalities.

Because of the low tissue autofluorescence and high tissue penetration depth in the infrared spectral window, the development of multifunctional drugs for simultaneous tumor-targeting NIR fluorescence imaging is expected to have a significant impact on customized oncology in the future. Establishment of stable, highly selective, and sensitive molecular probes is critical for molecular imaging of cancer in the NIR region. Indocyanine green, the most extensively employed organic dye, has shown promising result as nontargeting agent for optical imaging in the clinical research. Significant progress has recently been achieved in the development of new dyes based on NIR that can act as tumor targets. Traditional NIR organic dyes having a number of limitations, viz., poor hydrophilicity and photostability, low quantum yield, insufficient biological system stability, low detection sensitivity, and so on, were solved using current design methodologies. Tumor-targeted imaging is possible when these NIR dyes or NIR dye-encapsulated nanoparticles are combined with tumor-specific ligands. The ligands are generally used small molecules, peptides, proteins, and antibodies. The multifunctional NIR dyes that preferentially accumulate in tumor cells display a unique optical and pharmaceutical properties.

Newly Developed NIR Dyes for Cancer Imaging

In biological applications, organic dyes that are active in the near-infrared region achieved a lot of attention. The traditional dyes have also several limitations like NIR organic dyes; only a few NIR dyes are easily available. In recent years, NIR dyes (such as cyanine dyes, squaraine, phthalocyanines, porphyrin derivatives, and BODIPY (borondipyrromethane) analogs) are fashioned for substantial development, with enhanced chemical and photostability, high fluorescence intensity, and long fluorescent life. To avoid aggregation in biological systems and tissues, several dyes have been modified to be more water-soluble. Small chemical compounds having two aromatic nitrogen-containing heterocycles joined by a polymethine bridge are known as cyanine dyes (also known as polymethine cyanine dyes). The visible cyanines monomethine and trimethine (Cy3) absorb light, but adding one vinylene moiety (CH=CH) to the chromophore causes a bathochromic shift of about 100 nm (Mishra et al. 2000). Cyanine dyes (such as Cy5, Cy5.5, Cy7, and their variants) are the most widely used NIR fluorescent dyes, with high molar absorption coefficients and fluorescence quantum yield values (Ballou et al. 2005). FDA has approved of indocyanine green (ICG) for assessing blood flow and clearance for nearly 50 years. In aqueous solution, many cyanine dye showed poor photostability, low quantum yield, high plasma protein binding rate, undesired aggregation, and mild fluorescence (Haughland 2002). Significant development of cyanine dyes was observed for overcoming these constraints. According to studies, a stiff cyclohexenyl substitution at the center of the polymethine linker significantly improved the

photostability and fluorescence quantum yield of cyanine dyes (Patonay et al. 1991). Electron withdrawing ability of 3H-indolenine and the photostability of newly produced 3Hindocyanine dyes are reported (Chen et al. 2006). They also discovered that substituting an electron-donor group for the central chlorine atom in the cyclohexene ring can improve the photostability of the dyes (Song et al. 2004). Other studies have found that when cyanine dyes bind to nucleic acids or proteins, the fluorescence efficiency is considerably improved due to the rigidization of the fluorophores (Yarmoluk et al. 1999). Recently, several NIR cyanine dyes with a significant Stokes shift, intense fluorescence, and improved water solubility are produced by adding carboxylic or sulfonic acid groups (Peng et al. 2005). After conjugation with tumor-targeting ligands, zwitterionic heptamethine indocyanine dye with superior in vivo properties is also reported (Gragg 2010).

Squaraine dyes have an oxocyclobutenolate core with aromatic or heterocyclic components on both ends (Volkova et al. 2007). Squaraines have outstanding physical and chemical features, including intense absorption bands, a high molar absorption coefficient, and good photoconductivity (Keller et al. 2006). However, increasing the water solubility of conventional squaraines reminds a significant issue due to their massive and planar hydrophobic conjugated structures. In aqueous solution, only a few NIR squaraines emit at wavelengths greater than 800 nm (Saxena et al. 2003). KSQ-4-H is a squaraine containing four water-solubilizing sulfonate moieties in a squaraine framework, and it demonstrated that it entirely dissolved in PBS buffer, having a band at 775 nm in the visible range of the absorption spectra (Umezawa et al. 2008). This dye is thought to have a lot of potential in terms of protein identification and in vivo imaging. Bis-squaraine dyes are achieved by conjugating two squaraines with a monothiophene or pyrene unit, and it can join to protein bovine serum albumin (BSA) as probes with non-covalent labeling to increase near-infrared fluorescence band (Nakazumi et al. 2005). A squaraine has two rotaxanes (SRs), one with four tri(ethyleneoxy) chains on the squaraine to improve water solubility and the other with an encapsulating macrocycle to promote photostability. This squaraine has a better stability in a variety of solvents, including water and protein, viz., serum albumins, indicating that it might be used to build a variety of highly stable near-IR imaging probes.

Recent Approaches to Create Multifunctional NIR Dyes with Cancer-Targeting Properties

The selective and sensitive detection of the cancer cell could be perceived by a suitable material having appreciable cancer-targeting capabilities. Chemically or biologically conjugated cancer-specific ligands (such as chemical compounds, peptides, proteins, antibodies, aptamers, and so on) can accurately recognize the biomarkers associated with cancer cells and is the most typical technique for a chemical to gain active targeting capabilities. Combining imaging agents with or without target ligands is another possibility in nanoparticles (or polymers), which can act as carriers for delivering agents to tumors via a specific receptor-mediated targeting

pathway or the tumor microvasculature's enhanced permeability and retention (EPR) effect (Maeda et al. 2009). Another target-activatable strategy has been developed: instead of targeting specific cell surface receptors, imaging agents that undergo a chemical reaction when they interact with their intended target convert the probe from a non- or weakly fluorescent condition to one that have noticeable quantum yield values (Maeda et al. 2009; Luo et al. 2011).

Cancer Imaging and Targeting with NIR Dyes Coupled with Target Molecules

Conjugation of the fluorochrome with a ligand that binds to specific biological targets is a common and straightforward way to increase the accumulation of contrast agents at cancer sites. Non-bound probes are removed from circulation, while bound probes bind to the targets and are maintained at the target location. When cancer ligands are conjugated with NIR dyes with greater signal-to-background ratios, conjugates can be employed as cancer-specific imaging probes. The most common organic NIR dyes used for in vivo NIR cancer imaging are polymethines, and dye-ligand conjugates can be made in a variety of ways. Furthermore, some compounds may have poor optical stability and other flaws that are only relevant to cancer imaging.

Nanoparticles with NIR Dye Embedded in Them for Cancer Targeting and Imaging

The functional nanomaterials have recently been produced by a novel method, for increasing the sensitivity and specificity of molecular imaging detection. Nanomaterial-based molecular probes, or nanoprobes, help to increase the permeability and retention (EPR) in the tumor microvasculature and can be used to target tumors. With the rapid advancement of nanotechnologies, the development of dye-encapsulated nanoparticles has exploded to overcome the limits of free dyes. Organic dyes that are nano-encapsulated emit more intense fluorescence and have significantly greater photostability than primary dyes due to the protective nanomatrix and amplification of the fluorescent signal. The nanoparticles having enormous surface area extend the in vivo circulation period of NIR dyes, allowing for long-term repeated imaging. Targeting, imaging, and therapeutic components are delivered by nanometer-sized particles and have been the subject of extensive research in order to construct several modalities and are not possible with individual components. In order to make cancer imaging and treatment, low-density lipoprotein (LDL) and a variety of dye-encapsulated nanoparticles made from silica have been created and tested. These nanoparticles provide a good framework for developing multifunctional probes for cancer imaging and treatment; clinical application is still in its early stages.

Based on the findings of the preceding investigations, the target-activatable method has lately emerged as an appealing modality that has piqued people's curiosity. Nontargeting probes cannot be triggered or granted fluorescence, preventing nontargeting site interference and allowing for targeting imaging.

Enzyme-activatable probes frequently contain more than two chromophores, which are linked together by a particular peptide linker in close proximity. Because of the quenching effect caused by proximity or resonance energy transfer, the probes are mostly black and emit little or no fluorescence. The polymer probes are activated to release the fluorophores after internalization by tumor cells and breakdown of the peptide linker by specific enzymes overexpressed in tumor cells, leading in the restoration of fluorescence emission. As the probes without activation cannot create fluorescence, the background signal from nontargeting molecules is often low. Even in vivo, at sub-nanomole levels in deep tissues, the imaging and detection sensitivity is quite strong.

pH-sensitive probes are a type of probe that responds quickly to changes in pH. The method using pH-activatable probes is based on the presence of unique chromophores, which are very sensitive to protonation and deprotonation (Wang et al. 2010). At the very least, the chromophores have a hydrogen-ion (H+) receptor unit and have varied pKa values as a result of the regular release or restoration of H+ with varying pH values in solution.

NIR Dyes Are Multifunctional and Native for Cancer Detection and Imaging

Despite the fact that the chemical conjugation procedures described above have had some success, there is still much dispute about their delivery and specificity. Furthermore, chemical conjugation could affect the specificity, affinity, and dispersion of these medicines in cells and tissues, allowing them to be targeted only at specific cancer cell types. Furthermore, in order to diagnose solid tumors, to overcome the limitations of a longer plasma half-life, excessive background fluorescence, and poor transport, NIR dyes must be coupled with macromolecular ligands. As a result, there is an imperative need to investigate these issues and formulate novel or alternative solutions. Generating NIR dyes with inherent cancer-targeting characteristics that do not require chemical conjugation is a sensible and desirable strategy for avoiding these limitations. Without chemical attachment of tumor-target ligands or target activation, in vivo imaging of NIR heptamethine indocyanine dyes and porphyrin derivatives with preferential tumor accumulation has recently been discovered. These multifunctional NIR dyes extend the current approach and illustrate the promising role in cancer-targeted imaging.

The majority of cyanine dyes, on the other hand, are tumor nontargeting medications with low intrinsic tumor selectivity. A couple of new heptamethine dyes that can target tumors while also allowing for NIR imaging have recently been published, displaying considerable advantages over traditional fluorescent dyes used in research and clinical practice. Two prototype dyes, for example, may reach the NIR area at roughly 780 nm and can be easily identified by an NIR fluorescent-detecting

technology that is used without substantial autofluorescence interference. These multifunctional dyes have a low molecular weight (always less than 1000 Da) and are biomacromolecule like, making them ideal for imaging. They also have exceptional pharmacological and dynamic properties. These dyes are also quite durable in serum, and when non-covalently bound to proteins like albumin, they show a significant increase in fluorescence, which improves their optical intensity. Some dyes have proved to have distinct tumor-targeting properties. These dyes preferentially aggregate in tumor cells after systemic delivery via intravenous or intraperitoneal routes, while unbound dyes are promptly removed from circulation and the hosts' interstitial fluids, resulting in a good signal-to-background ratio. Surprisingly, these absorption and retention mechanisms of colors in cancer cells are similar *across* cancer cell types and animals. They can be taken up by a wide range of human tumor cells as well as animal cancers. When compared to healthy tissue, these multifunctional heptamethine dyes have a signal-to-noise ratio of more than 20, whereas more than 2.5 is considered significant tumor accumulation in other studies, signaling huge promise in tumor-targeted imaging (Shi et al. 2010). Important features of these dyes are their photostability after formalin fixation, which allows for the development of sensitive methods for detecting target areas, such as malignancies in surgical specimens, as well as additional histological detection and evaluation. Human prostate cancer cells were mixed with human blood cells; NIR dye imaging, for example, could clearly identify cancer cells, and these dyes are so sensitive that they can detect as few as 10 cancer cells per milliliter in whole blood.

Hydrophobic dyes accumulate in tumor cells by a variety of ways, and it is unclear why these dyes have such a unique ability to target and retain in tumor cells. The increased negative inner transmembrane potentials of mitochondria in tumor cells have been well documented, and in response to these greater negative inner transmembrane potentials, several delocalized lipophilic cations particularly target cancer cell mitochondria. Heptamethine dyes are lipophilic cationic chemicals in nature, and they preferentially accumulate in the mitochondria of live tumor cells that play important role for the preferential accumulation of these dyes along with other intrinsic biological pathways. These characteristics open up the prospect of establishing a sensitive method for monitoring tumor cell death after treatment. Inhibiting organic anion transporting polypeptides (OATPs), which are well-known and effective, dramatically reduces the accumulation of these dyes, which suggests that the process of dye accumulation in tumor cells could be linked to the differential expression of transporters in tumor cells compared to normal cells (Luo et al. 2011).

Bioluminescence Imaging (BLI) Technique

In vivo bioluminescence imaging, or BLI, is a promising current and future molecular imaging technique. It allows imaging of internally generated light in living small animals that is linked to certain physiological and/or pathological cellular processes. This noninvasive technique allows for the quantification of the geographic and temporal advancement of the process of interest in the same animal, as well as the identification of animal-to-animal differences (Signore et al. 2010).

Bioluminescence imaging uses luciferase reporter genes as internal light sources in living animals. Animals are usually designed to produce the luciferase gene in a specific tissue and/or biological process utilizing tissue-specific promoters. If the promoter's activity is only dependent on a single protein, this method enables for in vivo monitoring of that protein's transcription activity. Another technique is to clone the luciferase cDNA into the gene's locus whose expression could be tracked over time. In contrast to the previous strategy, the expression of the protein of interest is visualized in this case, not its activity. Both of these methods allow for noninvasive real-time imaging of a variety of biological processes. The mouse model has been the most often utilized to research BLI cellular mechanisms up to now, although zebrafish models for BLI have recently been characterized.

The most common BLI reporter gene is firefly (*Photinus pyralis*) luciferase, a heat-unstable enzyme with a half-life of about 2 hours that can be used to analyze biological process dynamics. Animals do not manufacture the luciferin substrate for the light-producing process; hence there is essentially little background in the animal tissues, resulting in an excellent signal-to-noise ratio. Luciferin is rapidly distributed throughout the animal after intraperitoneal (i.p.) injection and passes through blood-tissue barriers, including the placenta (Lipshutz et al. 2001). Similarly, to mice, luciferin can be injected intraperitoneally or simply dissolved in aquarium water in zebrafish models, allowing the fish to swim. Many different luciferases, in addition to the firefly, are available for in vivo BLI. The sea pansy *Renilla reniformis*, the click beetle *Pyrophorus plagiophthalamus*, the marine copepod *Gaussia princeps*, and the recently produced deep-sea shrimp-derived NanoLuc are among the most extensively used.

Conclusion

The animal experiment is a very important bridge between the cell experiment and clinical experiment. Animals have similar anatomy, physiology, and heredity to that of human, and development of animal diseases is similar to that of human, so it is important to study the animal model. Fluorescence imaging as a noninvasive imaging technique has the potential to offer clinicians with high-resolution, high-contrast images, allowing them to identify and treat cancer patients more effectively. Molecular imaging plays an important role in the field of healthcare for the diagnosis of diseases inside the body, and it gives appropriate results. Magnetic resonance imaging (MRI), ultrasound, tomography, gamma scintigraphy, optical imaging, near-infrared (NIR) fluorescence imaging, bioluminescence imaging, etc. are the various forms of imaging techniques.

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Recent Advances of Metal-Based Anticancer Agents and Their In Vivo Potential Against Various Types of Malignancies

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Priyatosh Nath, Abhijit Datta, and Suman Adhikari

Contents

Abstract

Metal-based complexes have tremendous capacity to interact with biological molecules and modify their actions. Due to their ability to modify enzymatic action, metabolism and molecular signaling; metal-based scaffolds exert many pharmacological activities. Several research works have described that metal complexes, besides their extensively studied cytotoxic antineoplastic impacts, further reverse tumor immune escape besides directly facilitating the immune cell function, leading to superior antitumor effects. Till date a huge number of metal complexes were designed, characterized, and synthesized as well as tested for their anticancer activity with both in vitro and in vivo system. While a massive range of metallodrugs have been developed as well as illustrated as favorable and effective in vitro dynamic anticancer agents, few have revealed effectiveness in in vivo models. Many of the metallodrugs also entered into clinical trials and received FDI approval for their use in treating cancer patients. The in vivo

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experiment assessing the anticancer potential of cancer drug results plays a crucial role in decision-making to permit a drug for clinical trials. The latest discoveries in the field of anticancer metal-based complexes of Pt, Au, Cu, Ru, Pd, and Ir were reported in this work. The in vivo antitumor potency of various metallodrugs and their mechanisms of action were discussed. The authors have further enlightened the importance of in vivo experiments which may be helpful in directing the future research with metal-based scaffolds.

Keywords

Cancer chemotherapy · Metal complexes · Anticancer drugs · In vivo experiment · Clinical trial

Abbreviations

BRD4	bromodomain-containing protein 4
CD4	a glycoprotein marker present on the surface of helper T lymphocyte
CD45	a common glycoprotein marker present on the surface of immunologically active white blood cells
CD8	a glycoprotein marker present on the surface of cytotoxic T lymphocyte
c-Myc	a transcription factor
COX-2	cyclooxygenase 2
CTL	cytotoxic T lymphocytes
CTR	copper transporter proteins, a type of protein channel which helps in active transport of molecules in and out of the cell
DNA	deoxyribonucleic acid
ECF	extracellular fluid, the fluid present outside the cell, e.g., plasma and tissue fluid
HMG	high-mobility group protein
hMSH2	human mutS homolog 2 protein
hUBF	human upstream binding factor
ICF	intracellular fluid, the fluid present within the plasma membrane of a cell
MMP-9	matrix metallopeptidase 9, an enzyme protein involved in the breakdown of extracellular matrix
MOFs	metal organic framework
NK	natural killer cell, an innate immune cell that exerts anticancer and antiviral immunity
NKT	natural killer T cell
OCT	organic cation transporters, a type of protein channel which is associated with the transport of organic cations in and out of the cells
PD-L1	programmed cell death protein 1, an inhibitor of immune response
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase

TBP	TATA binding protein
TDO	tryptophan 2,3-dioxygenase
γ-H2AX	phosphorylated form of H2A histone family member X, it is a sensitive molecular marker of DNA damage and repair

Introduction

Cancer has turned into a major general health concern impacting human life throughout the world. As per the WHO, cancer can be caused by a combination of internal genetic factors and many external or environmental factors like exposure to radiation (ultraviolet and ionizing radiation) and carcinogenic chemicals (tobacco smoke components, aflatoxins, arsenic compounds, pesticides, etc.) as well as biological carcinogens (infections from certain viruses, bacteria, etc.). The number of cancer cases is rising day by day and needs more potent therapeutics to keep in check (WHO/Europe 2020; Blackadar 2016).

Medicinal inorganic chemistry has uncovered new opportunities for the design and development of therapeutic medicines that aren't possible with organic chemicals (Orvig and Abrams 1999; Thompson and Orvig 2006). The medicinal chemist can leverage a wide range of reactivities due to the extensive array of coordination number of metals and structural geometries, accessible redox states, and thermodynamic as well as kinetic features, besides intrinsic characteristics of the cationic metal ion along with ligand itself. Based on the metal chosen, its oxidation state, and coordinating ligands, as well as the coordination arrangement, metallodrugs have the potential to have a distinctive mode of pharmacological action. Study of the activity and interactions among metal derivatives with DNA as well as proteins gave rise to substantial breakthroughs in biochemical processes and advance metallodrug development (Barry and Sadler 2013; Bugarcic et al. 2012). Substantial attempts have been done for the synthesis of transition metal antitumor compounds in the aftermath of the successful application of cisplatin (Fig. 1) some decades back (Yang et al. 2016; Liu et al. 2017; Kang et al. 2017). Ruthenium-based metallodrugs such as imidazolium-trans-DMSO-imidazole-tetrachlororuthenate (NAMI-A), imidazolium trans-[tetra-chlorobis(1H–indazole)-ruthenate(III)] (KP-1019) (Leijen et al. 2015; Hartinger et al. 2008), NKP-1339 (a sodium counterpart of KP1019) (Fig. 2) (Trondl et al. 2014), and titanocene dichloride (Kröger et al. 2000) are examples of a few metal-based complexes that have been taken in clinical trials as a result of pioneering research in the sector. Redox-active mono(thiosemicarbazone) copper compounds (Jansson et al. 2010; Kowol et al. 2012; Lovejoy et al. 2011); auranofin (a gold(I) derivative) (Fig. 2) that inhibits replication of DNA and RNA, in addition to synthesis of protein (Mirabelli et al. 1985); and osmium(II) arene compounds which aim at the mitochondria in addition to inducing cell apoptosis (van Rijt et al. 2014) are some other metal-based compounds with anti-malignancy potency. Transition metal-based complexes have alluring characteristics which organize them to be viable antitumor agent alternatives to organic molecules. As a result, there is an ongoing requisite for developing novel metal-based derivatives which could one day be converted into anticancer

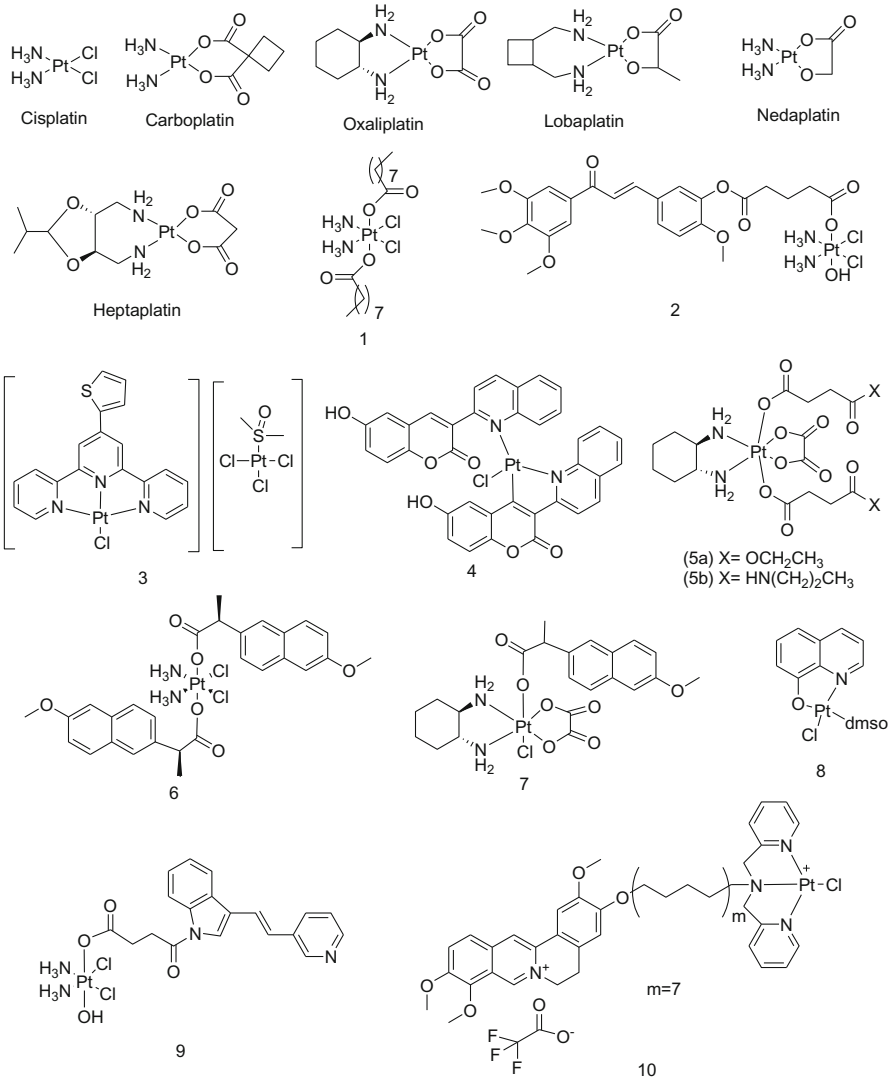

Fig. 1 Platinum-based anticancer drugs

medications, particularly those with fewer complicacies and/or are operational in case of chemoresistant malignancies.

Several traditional antitumor medications are encapsulated in liposomes along with easy delivery of active drug molecules to the cell; a lot of them have been permitted for clinical usage or are going through clinical trials. Additionally, nanoparticle albumin-bound (nab) paclitaxel (Abraxane in the USA and Japan) has achieved approval in recent times for treating metastatic cancer of the breast. The drug delivery system of anticancer medications was developed depending on the

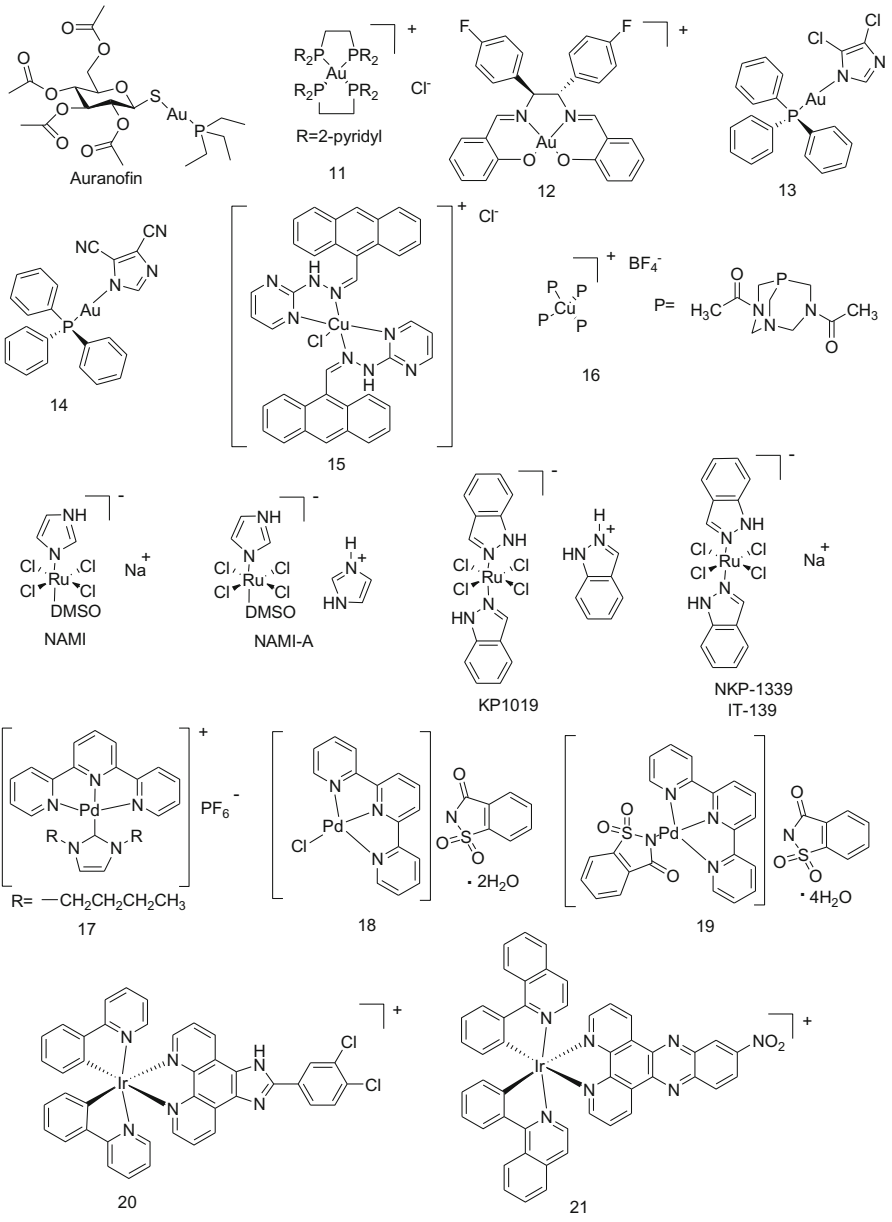

Fig. 2 Non-platinum-based anticancer drugs

perception of achievement of a better clinical action besides its tolerability. Yet, these agents are frequently discontinued owing to harsh aftereffects which include organ failure due to toxicity and disturbed homeostasis.

In the last few years, some of the researches have emphasized the first in vivo advantages of nano-MOFs for treating diverse ailments in animal models. In the future years, sensational developments in relation to the preclinical in vivo assessment of MOFs (Chen et al. 2017; Simon-Yarza et al. 2017; Zhang et al. 2017a; Hartlieb et al. 2017; Yang et al. 2017; Sene et al. 2017; Zhang et al. 2017b; Gao et al. 2017; Adhikari et al. 2019) can be assumed. Nevertheless, these in vitro test results are distant from reproducing in an in vivo condition. Hence, it is essential to highlight that the absence of in vitro cytotoxicity does not express safety as well as the biocompatibility of the tested molecule. Generally, toxicity problems should be undertaken basing on a further rationality of safe-by-design methodology while taking into consideration about the nano-MOFs for biomedical operations. Even though there exists a vast quantity of research in the literature of metal-based complexes with promising anticancer activity in vitro affecting cancer growth and angiogenesis to metastasis and also enhancing anticancer immunity, only a few have been evaluated in animal models for their in vivo efficacy against carcinogenesis and cancer. Many of these have yet to receive approval for human use. Antitumor metal compounds must therefore be tested in in vivo models before being tested in humans. In this chapter the authors hope to highlight some instances of recent metal-based compounds that demonstrated antineoplastic action in vivo. This study is not anticipated to be extensive, but rather to call attention to the diversity of significant metal complexes having in vivo action in order to spur future research into metal complexes as anticancer therapeutic candidates.

In Vivo Anticancer Activities of Platinum Complexes

Metal complexes designed on platinum are notable owing to their medicinal applications in the treatment of different types of malignancies. Data reveals that almost 50% of all malignancy patients get a platinum drug for chemotherapeutic treatment solely or as a combined therapy with other chemo drugs (Galanski et al. 2005). Utilization of platinum for the treatment of cancerous tissues dates back to the 1960s when Barnett Rosenberg discovered the antineoplastic activity of cisplatin (Rosenberg et al. 1969). Since then many platinum-based metal complexes were synthesized as promising agents for cancer treatment (Kelland 2007); some of them are cisplatin, oxaliplatin, lobaplatin, carboplatin, nedaplatin, heptaplatin, etc. (Fig. 1). Platinum therapeutics in a very short period of time earned a huge clinical success in cancer treatment, but slowly some disadvantages and deleterious side effects also started to came into notice. Patients receiving treatment using platinum-based drugs begin to show toxicity symptoms and reported many adverse health complications like emesis, renal toxicity, alopecia, ototoxicity, fatigue, peripheral neuropathy, myelosuppression, etc. (Oun et al. 2018). Another major update came as drug ineffectivity among some of cancer cells or resistance, where the prevailing drugs failed to kill the malignant cells because of certain somatic evolution. Cancerous cells which are resistant to cisplatin show reduced cellular absorption of drug, drug efflux, enhanced detoxification, increased DNA repair, and inhibition of

apoptosis (Florea and Büsselberg 2011). As an effort to defeat the general toxicity and resistance, a large quantity of platinum-based compounds has been synthesized applying the traditional structure-activity relationships (SARs) as well as verified for their pharmacological activities. The simple hypothesis behind this is the concept that a simple difference in structure will result in some altered functional activities (Goodsell 2006). The evaluation process includes testing drugs in vitro in cancerous cell lines, then in in vivo animal model, and finally for clinical trials. Here the in vivo anticancer activities of the most recently synthesized platinum complexes will be discussed. Platinum-based compounds are acknowledged to cause apoptosis of malignant cells through mitochondrial dysfunction in in vivo models of Lewis lung carcinoma (LLC), human non-small-cell lung carcinoma (NCIH460) xenograft, subcutaneous A549 xenograft, and HeLa xenograft (Novohradsky et al. 2017; Huang et al. 2020; Qin et al. 2019a; Qin et al. 2019b). Brabec and associates designed a number of Pt(IV) compounds to investigate why octanoato (OA) axial ligands improved the cytotoxicity of platinum(IV) compounds more than branching isomers like VPA (34). Pt(IV)diOA (**1**) (Fig. 1) overcomes cisplatin resistance, is considerably extra effective than the branched Pt(IV) valproato isomer, and has promising anticancer activity in vivo. Pt(IV)diOA's efficacy is due to a number of causes, including increased cellular accumulation, which correlates with increased toxicity and DNA platination. Pt(IV)diOA causes DNA hypermethylation besides dysfunction of mitochondria, lowering membrane potential in malignant tissues at levels far lesser in comparison with the IC_{50} value of free ligand OA, implying that Pt and OA ligand work together. Exceptional anticancer influences of Pt(IV)diOA is due to increased cellular absorption that allows high quantities of cisplatin as well as OA to be accumulated in cells at the same time. A number of tubulin-targeting platinum(IV) compounds including chalcones have displayed extensive antitumor action against a variety of cancerous cells (Huang et al. 2020). Compound **2** (Fig. 1) had the maximum effective antitumor effects against tested cancerous cell lines, having IC_{50} 0.190.37 μM, and in vivo data showed that complex **2** could considerably reduce tumor growth in the A549 xenograft model, with no obvious negative consequence. Compound **2** invoked apoptosis which was related with a breakdown of the MMP and a rise in reactive oxygen species within the cell, along with modifying the expression of apoptosis-associated proteins (e.g., Bax and Bcl-2) in cellular studies. Three new binuclear platinum(II) complexes, comprising the terpyridine derivatives, were designed as well as characterized by X-ray diffraction study and showed a marked antiproliferative activity against human TSK-OV-3 (ovarian cancer cells), A549 (lung carcinoma cancer cells), NCI-H460 (non-small-cell lung cancer cells), and human liver Hl-7702 normal cells comparable to that of positive control drug cisplatin (Qin et al. 2019a). The binuclear Pt-tpbtpy (**3**) (Fig. 1) was reported to dramatically suppress tumor growth in an NCI-H460 xenograft model when supplied at 10.0 mg kg^{-1} every two days (inhibition of tumor growth rate (IR) ¼ 70.1%, $p < 0.05$). The potential of organoplatinum(II) compounds incorporating quinoline-coumarin scaffolds against cisplatin-resistant human lung adenocarcinoma (A549/DDP) and cervical carcinoma (HeLa) cell line was designed, synthesized, and examined by Qin and co-workers (Qin et al. 2019b). In comparison

with *cis*-Pt(DMSO)$_2$Cl$_2$ and the starting quinoline-coumarin ligands, all of the novel complexes demonstrated increased anticancer activity on A549/DDP and HeLa cells. After treating for 21 days, mononuclear green luminescent Pt compound **4** (Fig. 1) (2.0 mg/kg every two days) showed promising antitumor activity in vivo.

Treatment with platinum-based compounds also induces antitumor immune response and increases the count of immune cells (macrophage, dendritic cells, helper T cells, cytotoxic T cells, and NK cells) in in vivo mouse model bearing L1210 leukemia, CT26-induced solid tumor, Lewis lung carcinoma, and breast cancer cell line (MDA-MB-231) xenograft (Göschl et al. 2017; Arsenijevic et al. 2017; Jin et al. 2020). A series of four platinum(IV) compounds derived from oxaliplatin was reported by Go¨schl et al. and their capability of interacting with DNA explored in the absence as well as presence of the reducing agent ascorbic acid (Göschl et al. 2017). Their cytotoxicity was assessed on many colon cancer cell lines (HCT116, HCT15, and the oxaliplatin-resistant subline HCT116oxR) in hypoxic in addition to normoxic conditions showing that **5a** and **5b** (Fig. 1), having axial (4-propylamino)-4-oxobutanoato ligands, had significant cytotoxic activity. The in vivo experiments in L1210 leukemia-bearing mice revealed that **5a** and **5b** compounds can considerably extend the average life span of mice. To examine the activity in solid tumors, CT26 tumors in immune-deficient SCID/BALB/c and immune-competent BALB/c mice were given treatment of **5b**. Only immune-competent mice responded to treatment, suggesting that activity shows intensive dependence on its immunity. Several nitrogen donor ligands have been coordinated to the $PtCl_4$ scaffold pointing at the widening of action spectrum of newly synthesized platinum(IV) and dinuclear platinum(II) compounds (Arsenijevic et al. 2017). Among the tested compounds [($PtCl_4$(en)] (en = ethylenediamine)] cisplatin exhibited the maximum cytotoxicity against malignant cells of human and murine lung through induction of apoptosis in lung carcinoma cells. In [$PtCl_4$(en)]-treated mice, no sign of induced toxicity of [$PtCl_4$(en)] was found, viz., water consumption, changes in food, or loss of body weight, as well as nephrotoxicity and hepatotoxicity. In tumor-bearing mice, [$PtCl_4$(en)] increased the count of CD45+ leukocytes in the lung tissues, including CD11c + dendritic cells, F4/80+ macrophages, CD8+ cytotoxic T cells (CTLs), and CD4+ helper cells, and cytotoxic NK, NKT, and CTLs in the spleens, resulting in reducing cancerous lesions in the lungs, signifying its potentiality of inducing an antiproliferative immune response in animal models. Guo, Wang, and colleagues reported on a Pt(IV) compound (**6**) (Fig. 1) comprising cisplatin and the axial naproxen ligands that outperformed cisplatin and NPX in terms of cytotoxicity, apoptosis, antimigratory, and anti-inflammatory activities (Jin et al. 2020). In vivo investigations in Blac/C nude mice with MDA-MB-231 tumors revealed a significant anticancer efficacy (tumor growth suppression of around 46%) and negligible toxic consequence. It was shown that complex **6** does not get reduced into Pt(II) moieties or interact with DNA. Complex **6** downregulates the expression of COX-2 and PD-L1, suppresses the release of prostaglandin, and lowers the BRD4 protein expression and the phosphorylation of extracellular signal regulated kinases 1/2 (Erk1/2) while preventing c-Myc oncogene. Furthermore, complex **6** forms a chimera adduct with nuclear DNA, causing DNA damage and upregulating γ-H2AX.

Recently Chen and colleagues successfully accomplished designing, synthesizing, and testing the antiproliferative activities of a series of naproxen-incorporated platinum(IV) compounds in vivo as well as in vitro (Chen et al. 2020). The most promising compound of the series, naproxen platinum(IV) hybrid oxaliplatin derivative **7** (Fig. 1), displayed a significant potency of overcoming drug resistance and improving malignant tumor selectivity in comparison with hybrid cisplatin analogs. Furthermore, the toxicity of compound **7** was found less than cisplatin and oxaliplatin. Naproxen platinum(IV) hybrid oxaliplatin derivative **7** results in the inhibition of COX-2 and alleviates tumor-associated inflammation and marked inhibition of MMP-9 in CT-26-induced homograft tumor-bearing BALB/c mice. It has been noticed that naproxen-incorporated Pt(IV) moiety enhances its accumulation in tumor cells substantially (Chen et al. 2020). Platinum-based compounds also show in vivo antitumor activity against osteosarcoma, HepG2 (hepatocellular carcinoma cell line) xenograft, and HeLa tumor xenograft by increasing the production of ROS, and the inhibition of tryptophan 2,3-dioxygenase (TDO), as well as of telomerase, has been disclosed (Ruiz et al. 2019; Hua et al. 2019a, b; Qin et al. 2019c). Two platinum(II) compounds [$Pt(Cl)_2$(quinoline)(dmso)] and [PtCl(8-O-quinoline)(dmso)] (**8**) (Fig. 1) of quinoline derivatives have been developed, and structural characterization was done (Ruiz et al. 2019). The cytotoxicities of the two mononuclear platinum(II) derivatives were examined for bone cancer in human. [PtCl(8-O-quinoline)(dmso)] (**8**) caused a reduction of the viability of the cell of multicellular spheroids and decreased the volume of malignant tumor on athymic nude mice N:NIH(S) $Fox1^{nu}$ without persuading any complexity. Gou and associates reported a range of platinum(IV) compounds with an immune checkpoint TDO inhibitor (Hua et al. 2019a, b). Besides having noticeable cytotoxicity on a variety of tested cancerous cell lines, they also exhibited improved antitumor immune response. Complex **9** (Fig. 1), in particular, was 35 times more potent in comparison with cisplatin in TDO-overexpressed HepG-2 cancerous cells. More crucially, in vivo experiments showed that it might enhance cisplatin's anticancer efficacy while suppressing TDO's expression as a powerful immune modulator. Luminescent compound **10** (Fig. 1) having a jatrorrhizine scaffold demonstrated a significant anticancer action in addition to lesser toxic consequences in comparison with cisplatin (Qin et al. 2019c). By targeting p53 and telomerase, it showed great selectivity against HeLa cells ($IC_{50} = 1.00 \pm 0.17$ nM). Moreover, even at a low dosage of 1.00 ± 0.17 nM, it caused mitochondrial and DNA damage, as well as a significant rate of apoptosis. For the development of therapeutically important platinum complexes, it is important to understand the factors that lead to organ toxicity and lack of clinical success. It will help in designing novel anticancer platinum-based complex.

Mechanism of Action of Platinum Complexes

The anticancer mechanisms of platinum complexes are widely studied (Dasari and Tchounwou 2014). Here the authors will discuss the mode of action of cisplatin, as a model platinum complex. The extended research carried out globally has revealed a

clear picture of cisplatin's cytotoxicity against cancer cells. Cisplatin-induced cytotoxicity varies among cancer types in a dose-dependent way. Inside the cell, cisplatin binds with the DNA which hinders DNA replication and transcription mechanism and induces cellular apoptosis by activating various signaling pathways like death receptor signaling and mitochondria-mediated death signaling (Florea and Büsselberg 2011). Some of the major points in this whole process include cellular uptake of platinum complexes, aquation/activation within intracellular environment, access to the cell nucleus and platination of DNA, and finally affecting the cell signaling necessary for cell survival and apoptosis (Fig. 3).

Cisplatin is administered in patients intravenously into blood. Owing to its strong reactivity with sulfur of the amino acid cysteine, it binds with the plasma proteins as well as rapidly diffuses into the tissues. The binding of cisplatin with plasma proteins leads to inaction of a lion portion of cisplatin molecules, and only a little quantity enters the cell after crossing various obstacles. The uptake of cellular internalization of

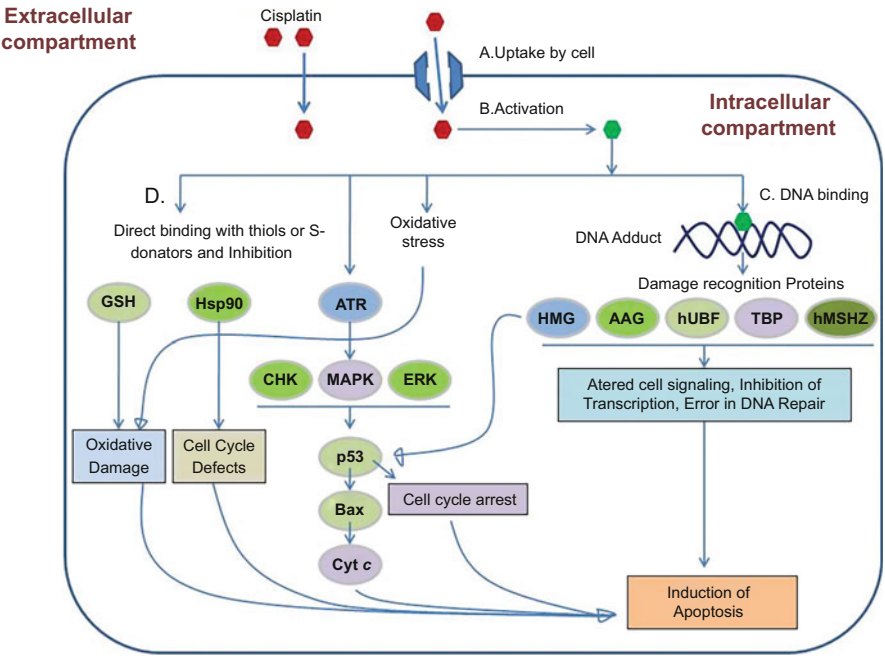

Fig. 3 A brief mechanism of anticancer activity shown by cisplatin. (**a**) Uptake by cell: entry of cisplatin occurs by passive diffusion and by active transport. (**b**) Activation: a series of aquation reaction causes substitution of chloride by water molecules and activates cisplatin. (**c**) DNA binding: active cisplatin interacts with N7 of adenine and guanine nucleotide in the major groove of DNA to form DNA-protein as well as DNA-DNA interstrand-to-intrastrand cross-links. Various damage recognition proteins next identify and interact with those adducts and bring about changes in cell signaling regulating cell cycle, controlling cell survival and apoptosis, inhibiting transcription, and hampering DNA repair. Additionally, cisplatin also inhibits GSH, which is an important antioxidant enzyme, thus raising oxidative stress

the neutral cisplatin molecule is mainly achieved by passive diffusion and additionally by facilitated or energy-driven active transport process. Passive diffusion depends on the SAR profile of the synthesized complex. In recent years the role of copper transporter proteins (CTR1 and CTR2) and organic cation transporters (OCT1 and OCT2) in active transport of platinum complexes was discovered (Howell et al. 2010; Zhang et al. 2006).

Once inside the cell, the low chloride ion concentration (≈2–30 mM) in the intracellular fluid (ICF) compared with the chloride ion content (≈100 mM) of the extracellular fluid (ECF)/plasma helps in the substitution of chlorides in cisplatin with water molecules. The aquation reactions lead to the formation of activated mono- and diaquo-cisplatin which are very much reactive to nucleophilic center (Fuertes et al. 2003). The activated cisplatin bind with DNA and form adducts. Cisplatin and other platinum compounds primarily interact with N7 of adenine and guanine nucleotide in the major groove of DNA to form DNA-protein as well as DNA-DNA interstrand-to-intrastrand cross-links (Dasari and Tchounwou 2014). Intrastrand cross-links are the main type of adducts with about 60–65% 1,2-d (GpG) cross-links and 20–25% d(ApG) cross-links (Fuertes et al. 2003). The 1,2-intrastrand adducts formed are reported to play a key role in cisplatin's anticancer activity. Two important observations in this regard are the specific recognition of 1,2-intrastrand adducts by HMG (high-mobility group) proteins and the least efficient nucleotide excision repair for this type of adducts which leads to DNA damage (Fuertes et al. 2003). Proceeding into cisplatin's mechanism of action, the abovementioned structural distortions in DNA double helix caused by platinum binding are identified by the mismatch repair protein hMSH2 or hMutSα component of mismatch repair complex and many damage recognition proteins including nonhistone chromosomal high-mobility group 1 and 2 (HMG 1 & 2), TATA binding protein (TBP), etc. and subsequently transduce DNA destruction signals along the downstream effector controlling cytotoxic pathways (Siddik 2003). It has been reported that binding of HMG proteins with cisplatin-DNA adducts causes inhibition of cellular functions vital for survival (Fuertes et al. 2003). Also, exposure to cisplatin was found to cause overexpression of HMG 1 which interacts with DNA adducts promoting cytotoxicity and hampering DNA repair (Siddik 2003). Similarly, the binding of hUBF, TBP, 3-methyladenine DNA glycosylase (AAG), etc. sequesters their natural activity and prevents them from participating in transcription (Jordan and Carmo-Fonseca 2000; Khan et al. 2019). These recognition proteins in this way initiate many such events effecting normal functioning of the cell. The cisplatin-induced DNA damage activates a number of pathways culminating in cell cycle and the activation of p53 and directs cells toward apoptosis (Jordan and Carmo-Fonseca 2000). Though activation of p53 is the consequence of DNA damage, multiple other factors are also involved into this. HMG 1 and 2 facilitate the binding of p53 with DNA and cause its activation (Jayaraman et al. 1998). Cisplatin is reported to cause activation of ATR (ATM and Rad-3-related protein). ATR is a kinase working upstream to the p53 gene and controls its transcriptional activity. Activated ATR causes activation of p53 by phosphorylating serine-15 residues (Damia et al. 2001). ATR targets many other downstream targets like

CHK1 kinase and MAPK (mitogen-activated protein kinases) and causes their activation which is responsible for the phosphorylation of p53 at serine 15 and 20, threonine 81 residue, etc. (Siddik 2003). MAPKs are very important signaling molecules involved in most of the cellular procedures like cell proliferation, survival, and differentiation as well as apoptosis. Cisplatin treatment activates ERK1/2 which facilitates the phosphorylation and activation of p53 (Persons et al. 2000). Transactivation of p53 activates a number of genes associated with cell cycle, repair, and apoptosis (Hafner et al. 2019). The fate between survival and death is decided by the severity of DNA damage. If the DNA is damaged beyond a critical threshold level, the cellular repair capacity is overwhelmed, and apoptosis is induced. Cisplatin induces Bax, increases the ratio of Bax/Bcl-2, and translocates pro-apoptotic Bax from the liquid phase of the cytoplasm to the mitochondria initiating a cascade of events including discharge of cytochrome-*c* and activation of caspases which results in apoptosis (Siddik 2003). Cisplatin also induces apoptosis in cancer cells through death ligand Fas/FasL-mediated activation of caspases 8 and 3, which is also expedited by p53 (Siddik 2003). Apart from damaging DNA, cisplatin induces oxidative stress in the cancer cells leading to apoptosis (Brozovic et al. 2010). As mentioned earlier, cisplatin has a strong reactivity against thiols and S-donor biomolecules; glutathione is one of the major targets of cisplatin adversely affecting the antioxidant activities of cell (Fuertes et al. 2003). Cisplatin also inhibits Hsp90 (heat-shock protein 90) which has a role in cell cycle regulation (Fuertes et al. 2003). Other targets of cisplatin includes telomerase enzyme important for maintenance of telomere ends. Inhibition of telomerase by cisplatin therefore fails to preserve the telomere during successive cell division leading to cell death (Fuertes et al. 2003).

In Vivo Anticancer Activities of Gold Complexes

Gold is another exciting metal, getting attention from researchers for the design and development of antineoplastic drugs. Gold(III) or gold(I) complexes have gained a huge consideration in the realm of metallodrug chemistry because of their advantageous antiproliferative and antitumor effects. Gold(III) compounds, which are isoelectronic to Pt(II) compounds, are regarded to have great antineoplastic potency; besides, their stability in biological contexts can be increased by introducing multidentate ligands. Furthermore, gold(III) compounds exert their influence not through damaging DNA as is widely assumed, but by suppressing intracellular protein function and/or affecting normal mitochondrial function. The chemistry of gold biological system in support of its anticancer activity is reviewed (Zou et al. 2015). Anticancer activities of gold-based compounds are due to their ability to target enzymes including thioredoxin reductase, glutathione reductase, and cysteine protease (Zou et al. 2015). Gold(III) porphyrins, gold(III) corrole, gold(III) complexes with tridentate c-deprotonated ligands and multidentate N-donor ligands, gold(III) dithiocarbamate complexes, gold(I) complexes with thiolate and phosphine, gold(I)-NHC derivatives, gold(I) alkynyl derivatives, and gold(I) thiourea compounds are reported for their anticancer activities. In recent years, many gold-based compounds were synthesized, and their in vivo anticancer activity was tested.

In vivo treatment with gold compounds inhibits tumor growth of tumor xenograft induced by injecting HeLa (cervical carcinoma), T24 (urinary bladder carcinoma), HTC-116-luc2 (colon carcinoma), and PC3 (prostate cancer) cell lines (Srinivasa Reddy et al. 2020; Khan et al. 2019; García-Moreno et al. 2015; García-Moreno et al. 2016; Walther et al. 2020). In bortezomib-sensitive or bortezomib-resistant myeloma cells, bis-chelated tetrahedral gold(I) phosphine derivative **11** (Fig. 2) was recently studied as a thioredoxin reductase (TrxR) inhibitor (Sze et al. 2020). Gold (I) phosphine complex **11** was tested against human RPMI8226 xenograft in NOD/SCID mice and was found to downregulate the oncogene MYC and significantly inhibit myeloma growth in vivo. The complex inhibited cell proliferation and apoptosis by inducing ROS production. Gold(III) Schiff base complex **12** (Fig. 2) with potent anticancer action linked to ER stress induction was also reported (Bian et al. 2020). The compound reacts rapidly with thiols such as NAC and glutathione, signifying that it can be reduced quickly to gold(I) in the cellular environment. Gold (III) Schiff base complex **12** minimizes the CCl_4 toxicity in the liver, improves damage by downregulating the TrxR enzyme, and also reduces inflammation preventing chronic liver cancer. Gold(I) complexes **13** and **14** (Fig. 2) containing the N-Au-P or P-Au-Cl backbones and having azolate/phosphane moieties were tested for their anticancer activities (Gambini et al. 2018). Treatment of A17 tumor-bearing syngenic mice with gold complexes (**13** and **14**) causes downregulation of STAT3 and Cox-2, both known for their role in progression and growth of cancer. Though a huge numbers of gold compounds have been developed as well as assessed for their action, very few were approved for clinical trials. In the future researchers must focus more on in vivo studies to identify novel mechanisms, routes, and delivery vehicles of novel gold complexes.

In Vivo Anticancer Activities of Copper Complexes

Copper is an important metal micronutrient required for various physiological functions in the human body. Copper acts as catalytic cofactor of many enzymes and participates in oxidation-reduction reactions. Some of the critical enzymes which require copper for their functional activities are superoxide dismutase (SOD), cytochrome-*c* oxidase, lysyl oxidase, and tyrosinase (Denoyer et al. 2018). The study of coordination complexes of important metals was one technique used in the search for novel metal-based anticancer compounds. This study was originally conducted with the assumption that living organisms already have metabolic routes for certain metals, resulting in minimum side effects. Copper compounds' mode of action isn't thoroughly understood, and it could include a variety of chemical reactions. Copper has two oxidation states, (+I) and (+II), resulting in a versatile redox chemistry that could result in the generation of ROS. Because cancer cells are more sensitive to oxidative stress than normal cells, ROS generation is thought to be a rather selective process. Copper compounds are capable of overcoming acquired cisplatin resistance, besides having a larger spectrum of activity and reduced toxicity. The uses of copper complexes as cancer therapeutics are due to their involvement in enzyme function, maintenance of extracellular matrix, and metastasis. Tetrathiomolybdate, clioquinol, disulfiram, and

elesclomol are some of the important copper complexes widely tested for their anticancer activities through clinical trials in cancer patients (Denoyer et al. 2018). Copper complexes also target topoisomerase I and II function, disturb DNA topology, and cause double-strand break leading to induction of apoptosis (Molinaro et al. 2020). In vivo treatment in various cancer models with copper complexes was found to hinder the growth of tumor through ROS-mediated mitochondrial apoptosis activating Bcl-2 proteins and caspases 3 and 7 (Mahendiran et al. 2017; Liu et al. 2020). Distorted octahedral copper(II) derivative of the type $[Cu(ttpy)_2]Cl_2$ [ttpy = bis(4′-(4-tolyl)-2,2′:6′,2″-terpyridine] has been synthesized and characterized by X-ray analysis (67). C–H•••π intermolecular and intramolecular contacts stabilize crystal packing in solid-state structure. Copper(II) complex revealed elevated cytotoxicity in comparison with the anticancer drug cisplatin against EAC tumor cell line. Cu(II) complex accumulates in the cell nucleus and brings many functional changes, according to cellular uptake experiments. In female Swiss albino mice, in vivo treatment of copper (II) complex effectively suppresses tumor growth in EAC cells. Condensation of 9-anthraldehyde with 2-hydrazinopyrimidine led to the isolation of pyrimidine anthrahydrazone-derived Schiff bases (Liu et al. 2020). Against the human normal liver cell line HL-7702, complex **15** (Fig. 2) was extremely cytotoxic. The five-coordinated pyramidal Cu(II) complex **15** also exhibited a significant reduction in tumor growth in the T24 tumor xenograft mice model, without causing any weight loss.

Treatment of Lewis lung carcinoma (LLC) and malignant glioma implanted in mice with copper complexes shows growth inhibitory and antiangiogenic activities as they target the EGFR/Src/VEGF signaling pathway (Gandin et al. 2015; Li et al. 2015). The homoleptic, tetrahedral Cu(I) complexes derived from phosphine ligands exhibited astonishing antiangiogenic activities (Gandin et al. 2015). Complex **16** (Fig. 2) caused a considerable decrease in tumor mass without significant loss of body weight, indicating that it had minimum side effects than cisplatin. Bis(diethylthiocarbamoyl) disulfide (disulfiram) was shown to have cytotoxic effects in a human cancer cell lines, and because of its metal-chelating characteristics and capacity of inactivating Cu/Zn superoxide dismutase as well as matrix metalloproteinases, disulfiram has been reported to hinder angiogenesis. Li et al. investigated the antiangiogenic properties of disulfiram/Cu to see if copper can improve disulfiram's angiogenesis suppression (Li et al. 2015). It was found that in the Matrigel plug assay, the rat aortic ring assay, and the glioblastoma xenograft model, Cu/disulfiram also prevented the formation of new microvessels. Moreover, copper targets the EGFR/Src/VEGF signaling path, which increases disulfiram's antiangiogenic action in endothelial cells. Based on their activity to affect multiple signaling pathways, copper complexes have promising future possibilities with anticancer therapy.

Antitumor Activities of Ru, Pd, and Ir Derivatives

The antiproliferative activities of Ru, Pd, and Ir compounds are well evaluated. All of them are reported to exhibit anticancer potential, enhancing production of ROS, arresting cell cycle at various phases, and inducing apoptosis, TrxR inhibition, etc. (Lee et al. 2020; Liu and Sadler 2014; Scattolin et al. 2021; Cheng and Qi 2017).

Initially, ruthenium metallodrugs were developed to mimic the activity of platinum anticancer drugs. Early amine- and chloride-containing ruthenium compounds {*fac*-$[Ru^{III}Cl_3(NH_3)_3]$} reflected this, and DNA was long thought to be the main target (Durig et al. 1976). During the last decade, however, this viewpoint has shifted dramatically, and it is now experimentally clear that ruthenium-based anticancer drugs have a significant prospect as cytostatic as well as cytotoxic medication with unique modes of action. Tetrachloridobis(indazole)ruthenium(III) (KP1019, NKP-1339, IT-139) and tetrachloride dimethyl sulfoxide (imidazole)ruthenium(III) (NAMI, NAMI-A) (Fig. 2) scaffolds were discovered and developed into two therapeutically developed Ru(III) complexes. IT-139 (previously called NKP-1339) (Fig. 2) is the sole Ru antitumor drug currently under clinical trial, with promising phase I results suggesting unique anticancer efficacy (Fuereder and Berger 2017). Ruthenium complexes inhibit in vivo growth of Ehrlich ascites carcinoma (EAC), HeLa (cervical carcinoma) cell xenografted tumor growth, CT26 cell-induced colon cancer xenograft, etc. The anticancer mechanism involves cell cycle arrest, telomerase activity inhibition, and mitochondrial dysfunction, along with inducing apoptosis (Elsayed et al. 2020; Meng et al. 2019; Tamasi et al. 2017; Mendes et al. 2017).

Since 1980, palladium(II) compounds have been explored as anticancer drugs (Alam and Huq 2016). TOOKAD®, a palladium compound prepared for vascular-targeted photodynamic (VTP) treatment, has been acknowledged for clinical application for prostate cancer patients. Palladium complexes are viable substitute to traditional Pt(II) complexes, exhibiting cytotoxicity as well as improved selective nature for malignant cell lines, although palladium complexes have some disadvantages like high lability, poor solubility, and a rapid ligand exchange rate than corresponding Pt complexes. Many anticancer palladium complexes show in vivo activities against xenografted tumors through mitochondrial dysfunction and suppression of endothelial growth factor receptor (EGFR) signaling, by DNA damage and cleavage of poly-(ADP-ribose) polymerase (PARP), caspase 3 activation, etc. (Fong et al. 2016; Ari et al. 2014; Ikitimur-Armutak et al. 2016). Palladium(II)-NHC derivatives are usually reactive to substitution and reduction, and their pharmacological properties are rarely investigated. Fong and associates reported a novel series of palladium(II) compounds which reveal stability when physiological thiols are present (Fong et al. 2016). Cyclometalated palladium(II) complex **17** (Fig. 2) demonstrated promising anticancer activities toward cancer cell lines but exhibited lesser cytotoxicity for normal human fibroblast cells. Pd(II) complexes prevent tumor growth significantly in a nude mouse model, according to in vivo anticancer studies. Ari and colleagues reported a palladium(II) derivative [PdCl(terpy)](sac).$2H_2O$] (**18**) (Fig. 2) containing saccharinate and terpyridine derivative (terpy) which was found effective against breast cancer cells in addition to mice models (Ari et al. 2014). This same compound was found to cause a considerable interruption in the growth of Ehrlich ascites tumor cells cultured in Balb-c mice, similar to paclitaxel and to a greater extent more superior than cisplatin (Ikitimur-Armutak et al. 2016). Treatment of C57BL/6 mice bearing LLC cell injected tumor with palladium (II) complex **19** (Fig. 2) harvested from saccharinate and terpyridine ligands causes a significant inhibition of tumor growth and exerts antiangiogenic activity which prevents the formation of new blood vessels and thus obstructs the supply of

nutrients to the tumor mass and inhibition of metastasis (Cetin et al. 2017). The designed Pd(II) complex **19** was found to be more effective than cisplatin at shrinking tumor size and had fewer hepatotoxic side effects.

Iridium-based compounds have recently attracted a lot of attention due to their potent anticancer characteristics, diverse photophysical properties, and low side effects. Iridium complexes have a greater variety of molecular structures than platinum complexes due to the easily achievable structural modification in the peripheral ligands, allowing for a wider range of pharmacological activities. The iridium complexes because of their unique spectroscopic features can be used for simultaneous imaging applications, which is a clear advantage for employing them as anticancer drugs. The wide and controllable emission spectrum of iridium compounds allows for the development of dual-functional anticancer medicines as well as multicolor luminescent probes (Ma et al. 2019). Iridium complexes inhibit in vivo growth of various forms of tumor xenografted in athymic nude mice (Bai et al. 2020; Zhang et al. 2019; Gu et al. 2021). A number of Ir(III) compounds of 2-phenylpyridine and phenanthroline derivatives have been synthesized and fully characterized (Bai et al. 2020). Ir(III) compounds as well as the corresponding liposomes that encapsulated iridium(III) complexes were tested for their in vivo antitumor activities. When compared to corresponding iridium(III) complex **20** (Fig. 2), liposome-encapsulated iridium(III) complex (**Ir-20-Lipo**) effectively prevents tumor growth (57.45%). Mixed ligand Ir (III) complex **21** (Fig. 2) derived from 11-nitrodipyrido[3,2-a:2′,3′−c]phenazine and 1-phenylisoquinoline ligand systems significantly inhibited the migration of malignant cells and inhibited their growth at the G0/G1 phase (Zhang et al. 2019). Gu et al. designed, synthesized, and characterized a novel Ir(III) complex fabricated from 1-phenylisoquinoline and 12-(1,4-dihydroquinoxalin-6-yl)-4,5,9,14-tetraazabenzo[b] triphenylene (Gu et al. 2021). The synthesized iridium complex has moderate-to-low cytotoxicity against the tested cancer cells, whereas corresponding liposome-encapsulated iridium(III) complex has exceptional antitumor activity on the same malignant cells. Moreover, liposome-encapsulated iridium(III) complex suppressed tumor growth in xenografted nude mice in vivo, with a percentage of tumor growth suppression of 75.70%. In addition to the synthesis of novel metal-based complex, it is important to evaluate their biological system activity. The study of physicochemical interactions within the biological system, stability, bioavailability, and binding ability with cancer-specific receptors may help in identifying future molecules with novel anticancer properties.

Compound	In vivo activity	Mechanism of action	Reference
Pt(IV)diOA	Inhibits the growth of xenografted Lewis lung carcinoma (LLC) in mouse	Enhanced platination and hypermethylation of DNA, reduction of mitochondrial membrane potential, and induction of apoptosis	(Novohradsky et al. 2017)

(continued)

Compound	In vivo activity	Mechanism of action	Reference
cis,cis,trans-[Pt $(NH_3)_2Cl_2$((E)-2-methoxy-5-(3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-en-1-yl)phenyl glutarate)(OH)]	Inhibition of tumor growth in a lung carcinoma A549 mouse xenograft model	Induction of apoptosis through mitochondrial-dependent pathway	(Huang et al. 2020)
[Pt(tpbtpy)Cl] [Pt $(DMSO)Cl_3$] (tpbtpy-Pt)	Inhibition of tumor growth in a non-small-cell lung cancer NCIH460 xenograft mice model by 70.1% ($p < 0.05$) at a dose of 10.0 mg/kg body weight	Induces apoptosis in cancer cells through mitochondrial dysfunction and inhibition of telomerase enzyme	(Qin et al. 2019a)
Organoplatinum (II) complexes incorporating quinoline-coumarin derivatives (H-QC1-H-QC11): $[Pt^{II}(QC4)(H\text{-}QC4)Cl]\cdot CH_3OH$	Inhibits HeLa xenograft tumor growth in mouse model. The tumor volume reduces by 42.7% after quinoline-coumarin complex treatment	Induction of mitochondria-dependent apoptosis	(Qin et al. 2019b)
Oxaliplatin-derived platinum (IV) complexes	Inhibits cancer progression and increases life span in L1210 cell-induced leukemia and CT 26-induced solid tumor mice model	Induces anticancer immunity and also apoptosis in cancer cells	(Göschl et al. 2017)
Platinum(IV) complex $[PtCl_4(en)]$ (en = ethylenediamine)	Reduces metastasis LLC1 cell in mouse model	Enhances antitumor immunity by increased occurrence of CD45+ leukocytes, comprising F4/80+ macrophages, CD11c + dendritic cells, CD4+ helper and CD8+ cytotoxic T cells (CTLs) in the lungs, cytotoxic NK, NKT, and CTLs	(Arsenijevic et al. 2017)
Platinum(IV) complex with naproxen (NPX) as axial ligand(s)	Display potent antitumor activity in mice model bearing MDA-MB231 tumor	Enhanced antitumor immunity by downregulating PDL1. Also downregulates Cox-2 which is useful in checking metastasis	(Jin et al. 2020)

(continued)

Compound	In vivo activity	Mechanism of action	Reference
Naproxen platinum (IV) hybrid	Inhibit tumor growth in a CT26 homograft mouse model	Inhibits COX-2 and MMP-9 which lowers tumor-associated inflammation and reduces tumor progression	(Chen et al. 2020)
Quinoline-based platinum complexes: [PtCl(8-O-quinoline)(dmso)]	Inhibition of growth of neoplasm in an osteosarcoma MG-63 mouse xenograft model	Increased production of ROS and induction of apoptosis	(Ruiz et al. 2019)
Oxoplatin monoconjugate of and (E)-4-oxo-4-(3-(2-(pyridin-3-yl)vinyl)-1H-indol-1-yl)butanoic acid	Inhibition of growth of neoplasm in a hepatocellular carcinoma HepG2 mouse xenograft model	Inhibit tryptophan 2,3-dioxygenase	(Hua et al. 2019a)
Pt(II) complexes containing jatrorrhizine derivative ligands: $[Pt(Jat)_2Cl]Cl$	Inhibition of growth of neoplasm in a cervical carcinoma HeLa cell xenograft mice model by 48.8%	The mechanisms include platinum-prompted apoptosis caused by telomerase inhibition, dysfunction of mitochondria, damage of DNA besides sub-G1 phase arrest in HeLa cells	(Qin et al. 2019c)
Gold(III) compounds comprising ligand cyclometalated triphenylphosphine sulfide: $[Au(\kappa^2\text{-}S_2CNEt_2)\{\kappa^2\text{–}2\text{-}C_6H_4P(S)Ph_2\}]PF_6$	Inhibition of growth of neoplasm in a cervical carcinoma HeLa cell xenograft mice model	Apoptosis induced via increased production of ROS	(Srinivasa Reddy et al. 2020)
Gold(III) isoquinoline derivatives: $[Au(L^1)Cl_2]$ and $[Au(L^2)Cl_2]$	Inhibit tumor growth in a urinary bladder carcinoma T24 cell xenograft mouse model	Arrests cell cycle at S-phase by upregulation of p53, p27, and p21 and downregulation of cyclin A and cyclin E. causes depolarization of the mitochondrial membrane potential, ROS generation, and induction of apoptosis	(Khan et al. 2019)

(continued)

Compound	In vivo activity	Mechanism of action	Reference
Alkyne gold(I) derivatives:	Inhibition of growth of neoplasm in HTC-116-luc2 colon cancer cell xenograft in nude mice	Arrest cell cycle in the S phase and induce apoptosis	(García-Moreno et al. 2016)
1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene gold (I) dimethylamino dithiocarbamate and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl-1-thiolate derivative	Inhibit tumor growth in a prostate cancer PC3 cell xenograft in mouse	Inhibits proliferation of tumor cells as indicated by reduced Ki-67 expression	(Walther et al. 2020)
A tetrahedral bis-chelated gold(I) phosphine derivative	Inhibit the growth of human myeloma RPMI8226 xenograft in immune-compromised NOD/SCID mouse	Mechanism includes downregulation of MYC oncogene, which is known to drive myeloma progression	(Sze et al. 2020)
Gold(III) Schiff base complexes: 2,2′-((1E,10 E)-(((1S,2S)-1,2-bis (4-fluorophenyl)ethane-1,2diyl) bis(azanylylidene)) bis(methanylylidene)) diphenol	Reduce damage and prevent liver cancer caused by the carcinogen CCl4 administration in ICR mice	Downregulation of TrxR expression	(Bian et al. 2020)
(4,5-Dichloro-1H-imidazolate-1-yl)-(triphenylphosphane)-gold (I) and (4,5-dicyano-1H-imidazolate-1-yl)-(triphenylphosphane)-gold(I)	Inhibits the growth of A17 tumors transplanted in mice	Downregulation of STAT-3 and Cox-2	(Gambini et al. 2018)
bis(4′-(4-tolyl)-2,2′:6′,2″-terpyridine)copper (II) derivative $[Cu(ttpy)_2]Cl_2$	Inhibits the growth of Ehrlich ascites carcinoma (EAC) tumor in mice	The compound encourages EAC cell apoptosis via a ROS-facilitated mitochondrial path	(Mahendiran et al. 2017)
9-PMAH-Cu and 9-FPMAH-Cu	Inhibits the growth of tumor in mouse models bearing T24 cell xenograft	Induction of apoptosis through increased production of ROS and mitochondrial dysfunction	(Liu et al. 2020)
$Cu(P)_4]BF_4$	Inhibit the intramuscular growth of syngenic Lewis lung carcinoma (LLC) in C57BL mice	Antiangiogenic	(Gandin et al. 2015)

(continued)

Compound	In vivo activity	Mechanism of action	Reference
DSF/Cu	Inhibit the growth of malignant glioma U87 xenograft tumor in mice	Reduced expression of VEGF	(Li et al. 2015)
$RuCl_3$(DMSO)	Inhibits the growth of EAC (Ehrlich ascites carcinoma) in a mouse model	Cell cycle halt in the G2/M phase along with apoptosis	(Elsayed et al. 2020)
Ru(η6-p-cymene)Cl-(L1)	Inhibits the tumor growth of cervical call HeLa xenograft in a mouse model	Telomerase inhibition and mitochondrial dysfunction-induced apoptosis	(Meng et al. 2019)
fac-[$Ru^{II}(CO)_3Cl_2$(N_ 3 -DMBI)]	Inhibit the growth of CT26 colon cancer xenograft in mice	Apoptosis	(Tamasi et al. 2017)
Organometallic ruthenium(II)-cyclopentadienyl complexes	Inhibition of growth of triple-negative breast cancer (TNBC) orthotopic tumors induced by injecting MDAMB231 cell line in mouse	Induces necrosis in cancerous cells. Also inhibits cancer metastasis	(Mendes et al. 2017)
[Pd(C^N^N)(N,N′-nBu_2 NHC)]	Inhibition of growth of NCI-H460 cancer cell xenograft in nude mice	Mitochondrial dysfunction and inhibition of EGFR signaling pathway	(Fong et al. 2016)
[PdCl(terpy)](sac)·$2H_2O$]	Inhibits the growth of EAC cell-induced solid carcinoma in BALB/c mice	Activation of caspase 3 and cleavage of PARP	(Ari et al. 2014)
PdCl(terpy)](sac)·$2H_2O$] (sac = saccharinate and terpy = 2,2′:6′,2″-terpyridine)	Inhibition of the growth of Ehrlich ascites carcinoma (EAC) in Balb-c mice	Increased expression of p53, PCNA	(Ikitimur-Armutak et al. 2016)
Palladium(II) (Pd)-saccharinate complex having terpyridine	Significant reduction of LLC-induced tumor volume in mice	Enhanced anticancer immunity and prevention of angiogenesis	(Cetin et al. 2017)
Ir$(ppy)_2$(DCPIP)](PF_6)	Inhibits the growth of tumor in mouse models bearing A549 cell xenograft	Induction of apoptosis through ROS-mediated lysosome-mitochondria dysfunction	(Bai et al. 2020)

(continued)

Compound	In vivo activity	Mechanism of action	Reference
$[Ir(piq)_2(adppz)](PF_6)$	Inhibition of tumor growth of gastric cancer SGC-7901 cell xenograft into nude mice	Apoptosis induction through ROS-mediated mitochondrial pathway	(Zhang et al. 2019)
$[Ir(piq)_2(DQTT)](PF_6)$	Inhibit tumor growth of SGC-7901 cell xenograft in mouse by 75.80%	Induction of apoptosis through increased intracellular ROS production and mitochondrial dysfunction	(Gu et al. 2021)

Conclusion

From the above discussion, it can be concluded that the metal-based complexes are rich in active anticancer properties against diverse types of cancers. The metal complexes are found to inhibit tumor growth by arresting cell cycle, mitochondrial dysfunction, enhanced production of ROS, DNA binding, modulating enzyme activities, and inhibiting angiogenesis, metastasis, etc. in animal models. In vivo experiment results are indispensible for a proposed therapeutic molecule in considering it for therapeutic trials. In an in vivo experiment, the direct administration of a drug in the body of an animal model more or less replicates the condition of actual drug-disease interaction in a patient body. This type of experiment not only gives results oriented to the therapeutic potential of a proposed molecule but also determines whether it has any toxicity to the bodily organs and systems. Most of the time, the synthesized or nature extracted molecules which gives interesting results in in vitro setup fail to reproduce the same in in vivo conditions. Within the biological system, most of them show side effects which encroach into organ toxicity leading to functional failure and even death. When a molecule is tested in an in vivo setup, three types of results can be seen: (i) not at all reproduce the vitro results and toxic; (ii) replicates a part of in vitro results and toxic; and (iii) replicates a part of in vitro results and nontoxic to minimum toxicity. The last criteria mentioned are ideal for continuation with therapeutic trials. Despite these promising prospects, the translation of in vitro investigations into in vivo models is still a work in progress. It is well acknowledged that only in vitro investigation approach is insufficient for determining clinical activity, especially as pharmacokinetics has a significant influence on pharmacodynamic action. In order to ensure that the medication doses limit target and cancer cell growth, data from in vivo model systems is vital. Furthermore, the tumor models in the few in vivo publications were uncharacterized (syngenic/xenograft models) and hence neither clinically significant nor molecularly described. In vivo experiment must generate data on possible therapeutic activity, mechanism of action, and systemic toxicity if any drug molecule has to qualify as a potential

drug candidate. As in vivo experiments are expensive and need animal sacrifice, therefore it is more ethical to collect all possible types of data and report them scientifically.

As a crucial step for human testing, authors urge researchers to do more pharmacokinetic and pharmacodynamic investigations of their novel metal-based compounds in the future. The majority of the instances discussed in this study were confined to proving anticancer efficacy in vivo besides basic cytotoxicity testing. Knowing a medication's pharmacokinetic and pharmacodynamic characteristics, on the other hand, is critical for developing dose administration regimens and lowering toxicity effects as well as improving targeted drug delivery. Further comprehensive realization of the action mechanism of a few complexes can further possibly support to enhance the effectiveness and selective nature of the complexes via sensible design of drug. Depending on repeated efforts that research workers all over the world have devoted in representing the in vivo antitumor activity of metallodrugs, it can be predicted that this is just a matter of time before novel metal-based compounds having a higher efficiency besides lesser toxic nature will be approved as anticancer drugs.

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Mouse as a Preclinical Model for Studying Small Noncoding RNAs Involved in Colorectal Cancer

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Contents

Abstract

Colorectal cancer (CRC) is the third most diagnosed cancer and occupies the second position in death among other cancer deaths in both sexes due to unmet

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screening programs and therapeutic strategies. Small noncoding RNAs such as microRNA (miRNA), PIWI-interacting RNA (piRNA), silencing RNA (siRNA), small nucleolar RNA (snoRNA), and tRNA-derived fragments (tRFs) play a critical role in colorectal carcinogenesis, and few of these sncRNAs could be used as a diagnostic, prognostic, predictive, and therapeutic biomarker of CRC. Among these sncRNAs, miRNA is extensively studied in CRC in vitro and in vivo experiment. To accurately elucidate the role of these different sncRNAs in CRC, the mouse model plays a spearheaded role among other animal models. Generally, immunocompromised mice are used to generate different xenograft mice models like cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) models. Genetically engineered mouse models are widely used to create knock-in and knockout transgenic mice for studying oncogene and tumor suppressor genes involved in CRC. Now, Cre-LoxP system and cluster regularly interspaced palindromic sequence (CRISPR)-based genome editing technology have revolutionized the field of mouse cancer models and have had a more immediate impact on the development of more effective systems about different human cancers. In the future, various types of mouse models could be constructed by using this xenograft and genome editing technology which is more suitable and can throw more light on discovering the role of small RNA as a biomarker of CRC.

Keywords

Small noncoding RNA and their types · Small RNA associated with CRC · Chemical induced mouse models · Peritoneum models · CDX mouse model · PDX mouse models · Transgenic mouse models, issues, and future perspective

Introduction

Colorectal cancer (CRC) is one of the world's leading cancers, and it is considered an enormous executioner of lung, prostate, and breast cancer. However, more extraordinary advancements have been made to understand the pathogenesis and colorectal carcinogenesis and formulate new treatment approaches against CRC, such as endoscopic and surgical excision, radiotherapy, immunotherapy, palliative chemotherapy, targeted therapy, and extensive surgery local ablative therapies. Still, CRC is the third most commonly diagnosed cancer in recent years in malignancy and the second most deadly cancer among other global cancers due to the unmet screening programs, therapeutic strategy, and increasing incidence rates (Xi and Xu et al. 2021). In 2016, there were an expected 95,270 new cases of colon cancer and 39,220 new cases of rectal cancer. An estimated 49,190 people would die of colon and rectal cancers in the same year (Benson et al. 2017). In 2022, according to the American cancer society, the total number of newly diagnosed CRC cases is 1,51,030 in both sexes, and the estimated death by CRC is 52,580. It has been calculated that the incidence rate of CRC is declining by about 2%/year of the people

aged 50 years and above while increasing by about 1.5%/year of the people below 50 years of age (Siegel et al. 2022). Currently, research is ongoing to understand what type of genetic and epigenetic changes happen in the protein-coding region of the genes involved in the initiation and progression of CRC. To understand these genetic changes and epigenetic changes and to discover new therapeutic targets, various types of small noncoding RNAs (sncRNAs) have come into focus in scientific research. Uncovering the role of sncRNAs such as miRNA, siRNA, piRNA, snoRNA, and tRFs involved in the progression of colorectal carcinogenesis is very important. To stout these sncRNAs, researchers have chosen the preferable animal, like a mouse, as a preclinical study tool for understanding the molecular carcinogenesis of different human cancer. The mouse model has formed a link between in vitro experiment (cell experiment) and clinical study (Zhang et al. 2011). The mouse is widely used in cancer research due to some similarities to human beings in their anatomy, physiology, heredity, genetic features, and occurrence and development of some diseases and cancer (Li et al. 2021). Various mouse models have already been developed to generate a particular mouse strain using genetically engineered technology and the transplantation or grafting process. A transgenic knock-in and knockout mouse model is used to study a particular gene of interest. Grafting tissue or cells from patients into a mouse is used to develop a xenograft model that can be used to study sncRNAs to know their regulatory function under colorectal carcinogenic conditions. These models provide a foundational tool for discovering new anticancer drugs and therapies against CRC (Tian et al. 2020). The sncRNAs are polymeric RNA molecules with less than 200 nucleotides formed from the nonprotein-coding region of mRNA. The cellular system contains various types of small RNA, which can be used as potent diagnostic, prognostic, and therapeutic biomarkers for CRC, such as miRNA, siRNA, piRNA, tRF, sno RNA, etc. (Chen et al. 2019b). miRNAs can regulate the expression of the various target genes that participate in the prevalence and development of colorectal carcinogenesis (Schee et al. 2013). In colon cancer, miRNA is currently being employed as a novel serum/plasma-based biomarker (Vychytilova-Faltejskova et al. 2016).

Research is going on in genetics to detect the role of other small RNA in the development of CRC and their potential role as a biomarker. piRNA is a noncoding single-stranded RNA involved in the male germline development of drosophila. piRNA is also known as cancer-specific signatures and is abnormally expressed in human cancer. piRNA is involved in CRC by doing different epigenetic modifications to activate various oncogene (OG) and inhibit various tumor suppressor genes (TSG). Emerging evidence has demonstrated that piRNA could be used to diagnose and treat various cancers as well as CRC also (Liu et al. 2019). siRNA is used to treat various cancer by targeting oncogenes and genes involved in angiogenesis, metastasis, survival, antiapoptosis, and resistance to chemotherapy. Nanoparticle and liposome-based siRNA delivery are widely used to treat CRC (Huang et al. 2008). A lot of research has been done by taking snoRNAs in cancer, and researchers have found its deregulated role in cell proliferation, tumorigenesis, and metastasis of CRC. It can be used as an important prognostic biomarker and to discover new therapeutic targets in CRC (Huang et al. 2020).

Small RNA and Their Types

Small RNAs are actively transcribed but lost their protein-coding ability. They follow these three mechanisms mainly for the regulation of gene expressions, such as posttranscriptional gene silencing (PTGS), RNA activation (RNAa), and chromatin-dependent gene silencing (CDGS). These sncRNAs are involved in various cellular processes such as cell proliferation, differentiation and migration, apoptosis, and metabolism and are also involved in the pathogenesis of cancer, neurodegenerative disease, renal disease, diabetes, hepatic disease, infective disease, etc. (Zhang et al. 2009). Deployment of small RNA-involving pathways for spatio-temporal law of the transcriptome has fashioned the evolution of eukaryotic genomes and contributed to evaluate the complexity of multicellular organisms. There are different types of small RNAs involved in CRC development.

microRNA (miRNA)

miRNAs are a kind of noncoding RNA (ncRNA) that regulates the expression of target genes by complementary base pairing with the target gene and play a crucial role in the prevalence and development of cancers. miRNAs synthesize in the cell, but its action is limited to that cell and can also transport via exosome to another organ. Recent data suggest that miRNAs are aberrantly expressed in many human cancers, and they may play significant roles in carcinogenesis (Schee et al. 2013). Thousands of miRNAs have been discovered, and many have been used for cancer diagnosis and treatment purposes. It is apparent that miRNAs play a noteworthy part in host-pathogen interaction, apoptosis, and tumorigenesis (Kusenda et al. 2006). There are still several challenges to be resolved before establishing miRNAs as clinical diagnostic and prognostic tools for colon cancer. Because the amount of free-circulating RNAs in serum varies from individual to individual, this biasness could not be introduced into the results (Vychytilova-Faltejskova et al. 2016).

Piwi-Interacting RNA (piRNA)

piRNA is a mysterious class of noncoding single-stranded RNA with 26–31 nucleotides. They are involved in male germline development, heterochromatin modification, silencing the selfish elements of DNA, and maintaining the integrity of germline DNA. They protect the genome from the expression of various transposable elements by silencing it and help in spermatogenesis and germ stem cell maintenance. They are very stable, can be detected in body fluids, and readily cross cell membranes. piRNAs come into focus because they can show epigenetic regulations on genes in the nucleus and cytoplasm (Huang et al. 2010). piRNA makes a complex with piwi protein, which plays an essential role in pathogenesis and cancer development, and mutation of this protein in mice can cause defects in gametogenesis (Kuramochi-Miyagawa et al. 2004). Roughly 20,000 piRNA

qualities have been recognized inside the human genome. These brief RNAs were initially considered key utilitarian controllers for germline support and transposon quieting. Rising information uncovers that the aberrant expression of piRNAs could be a unique and particular focus to know how it regulates the development of colon cancer by modulating various molecular mechanisms. Because of their modified expression in cancer patients, it is essential to relate with clinical results and highlights their critical role in the progression of infection (Huang et al. 2010).

Small Interfering RNA (siRNA)

siRNA is a double-stranded small noncoding RNA consisting of 20–25 nucleotides involved in posttranscriptional gene silencing by perfect base pairing with the target gene. siRNA has now been in focus because of its specific and efficient gene-silencing mechanism. Based on this, siRNA-based therapy has been building up and has an excellent guarantee for treating numerous infections, neurodegenerative disorders, cardiovascular disorders, and different types of cancer. Nanoparticles and liposomes are majorly used to deliver siRNA into cells to control the oncogenes involved in angiogenesis, metastasis, survival, antiapoptosis, and resistance to chemotherapy. siRNA can be presented into the cells by utilizing either chemically synthesized siRNA oligonucleotides (oligosiRNA) or vector-based siRNA, which permits long, enduring, and more steady quality quieting (Alshaer et al. 2021).

Transfer RNA-Derived Fragments (tRFs)

tRFs are an essential class of noncoding RNAs made up of 14–32 nucleotides and derived from mature and precursor tRNA by site-specific endolytic cleavage. Till now, 4 types of tRFs have now been discovered. When enzymatic cleavage happens on the D loop of 5′ ends and TψC loop of 3′ ends of mature tRNA, it forms tRF-5 and tRF-3, respectively. tRF-1 and internal tRF(i-tRF) are derived from premature tRNA and the internal region of mature tRNA, respectively. tRF-3 and tRF-1 have been first confirmed to play a role in gene silencing in the HCT116 colon cancer cell line (Yu et al. 2020). Similar to miRNA, it plays a crucial role in developing various types of malignancies in humans by regulating cell proliferation, progression, differentiation, invasiveness, and metastasis property of the cells. It also develops metabolic disorders, neurological disorders, virus infections, immune disorders, inflammation, etc. Various high-throughput sequencing technologies have gathered evidences that tRFs are dysregulated and aberrantly expressed in various cancers (Xiong et al. 2019). One study using small RNA sequencing has shown that tRFs are differentially expressed in colon cancer. tRFs make a complex argonaute (AGO) and regulate mRNA expression (Haussecker et al. 2010). In mouse embryonic fibroblasts, 5′ and 3′ CCA tRFs have been shown to bind AGO2 to regulate cell functions (Keam and Hutvagner et al. 2015).

Small Nucleolar RNA (snoRNA)

snoRNA is also one type of small noncoding RNA, transcribed autonomously with the help of RNA polymerase II enzyme and involved in posttranscriptional modification of ribosomal RNA(rRNA), mRNA editing, tRNA genome imprinting, telomere maintenance, and alternative splicing of rRNA. It has two classes: One is C/D box and the other H/ACA box snoRNA, known as SNORD and SNORA, respectively, responsible for ribose methylation and pseudouridylation of rRNA (Liang et al. 2019). Rising evidences have reported that aberrant expression of snoRNAs played an essential role in various human malignancies, including colorectal cancer (CRC). It has been found that the expression of SNORA42 and SNORA71 are high in CRC, which has been proven by using small RNA sequencing technologies (Zhang et al. 2020).

Small RNA Associated with CRC

Small RNAs are differentially expressed in CRC and have already been discussed earlier. Various types of miRNAs are involved in CRC; some are downregulated, and some are up-upregulated, known as a tumor suppressor and oncomicroRNA, respectively. To study the role of these miRNAs in the molecular pathogenesis of human CRC, only animal model can exactly recapitulate human CRC and generate more meaningful insight into the miRNAs involved in CRC. For the in vivo study, researchers have chosen various types of animal models to establish the role of miRNA in discovering and establishing the role in various disease pathogenesis. Some act as tumor suppressor miRNAs whose expression is downregulated in CRC conditions, such as let-7 miRNA, miR-7, miR-9, miR-122, miR-125a-5p, miR-143, miR-144, miR-145, miR-101, miR-18a, etc., and all are experimentally proved. Some act as oncomicroRNA whose expression becomes upregulated in CRC, such as miR-10b (Xie et al. 2019), miR-17, miR-23a, miR-92a miR-106, miR-141, miR-155, miR-224, miR-372, etc. Some of these miRNAs target various signaling pathways like the RAS pathway, Wnt/β-catenin pathway, TGF- β pathway, Hippo pathway, EGFR pathway, etc., to control CRC development (Chen et al. 2019b). Some clinical studies of CRC have shown that miRNA plays a diverse role as a noninvasive biomarker in diagnosing, prognosis, and metastasis prediction of CRC. miRNAs are aberrantly found in the tissue sample, plasma, serum, saliva, and fecal matter. In the plasma of CRC patients, miR-21, miR-6826, miR-122, miR-129, and miR-24 have been found. Among them, miR-21 can be used as a diagnostic and prognostic biomarker; miR-6826 can be used as a predictive biomarker; miR-122 can be used as a prognostic biomarker, and miR-129 and miR-24 can be used as diagnostic biomarkers. Exosomes isolated from plasma also contain miRNAs such as miR-21, miR-6803-5p, miR-17-5p, and miR-92a-3p, which could be used for predicting prognosis and diagnosis of different tumor node metastasis stages (TNM) of CRC patients. Some serum-based miRNAs have been reported for potential detection of early-stage CRC, such as miR-21-3p, miR-24, miR-202-3p,

miR-320a, miR-423-5p, miR-532-3p, miR-1229-3p, and miR-1246. It has also been reported that serum exosomes contain miR-17-5p, miR-92a-3p, and miR-6803-5p. Circulating exosomal miRNAs found in plasma and serum can serve as a novel diagnostic and prognostic noninvasive biomarker for CRC (Chen et al. 2019a).

Various types of piRNAs are involved in tumorigenesis and metastasis in CRC. Circulating piRNAs are mainly found in serum, and it has also been found that in CRC conditions, it is deregulated and differentially expressed. One study reported that they had constructed a panel by taking 5 piRNAs for diagnosis of CRC, including piR-001311, piR-004153, piR-017723, piR-017724, and piR-020365 (Qu et al. 2019). piRNA can also act as both oncogene and tumor suppressor genes. Some piRNAs such as piR651, piR823, piR59056, piR54878, and piR62701 are highly expressed in CRC, and some are expressed in lower levels such as piR015551 (Sadoughi et al. 2021). Wang et al. have reported that piR-1245 is a vital oncogene that directly targets and inhibits several tumor suppressor genes such as activating transcription factor 3 (ATF3), B-cell translocation gene 1 (BTG1), dual specificity protein phosphatase 1(DUSP1), FAS, nuclear factor -kappa-B inhibitor alpha (NF-κ-BI-α), uridine phosphorylase 1 (UPP1), sestrin-2 (SESN2), and tumor protein 53-induced nuclear protein 1 (TP53INP1). Thereby it induced cell proliferation, migration, and metastasis of CRC cells. It has been concluded that these piRNAs could be used as a prognostic biomarker in CRC (Wang et al. 2018). Mai et al. have demonstrated that piR-54,265 played a carcinogenic role in the promotion of CRC by activating the STAT3 signaling pathway with the generation of PIWIL2/STAT3/phosphorylated-SRC (p-SRC) complex, and it could be used as a potent therapeutic target and to predict the chemotherapeutic response in CRC patients (Cheng et al. 2019; Mai et al. 2020).

Various studies have reported the role of snoRNAs in the regulation of human malignancies and demonstrated that snoRNAs could serve as prognostic, diagnostic, and therapeutic biomarkers role in CRC. snoRNA can act as both an oncogene and tumor suppressor gene. In CRC conditions, some snoRNAs such as SNORA43, SNORA21, SNORA24, SNORA15, and SNORA44 become increased and function in an oncogenic manner. SNORD33 and SNORD44 are essential tumor suppressor snoRNA whose expression decreases during CRC conditions. The snoRNAs derived from growth arrest specific-5 (GAS-5) are controlled by p53 tumor suppressor genes, which mediate p53 response against DNA damage in CRC. SNORD126 and SNORD42 have studied the xenograft mouse model of CRC and have shown that its expression increases in CRC. SNORD126 promotes CRC by upregulating fibroblast growth factor receptor-2 (FGFR2), which further activates the PI3K-AKT pathway (Fang et al. 2017). Studies have reported that SNORD21 and SNORD42 could be used as predictive biomarkers for CRC prognosis, and SNORD44 could be used as a therapeutic target for CRC in the near future (Liang et al. 2019). For CRC treatment, various chemotherapeutic drugs have been developed, but CRC developed multidrug resistance (MDR) properties against them. So to overcome this MDR gained by the cancer cell, siRNA treatment is indispensable to diminish this chemoresistance of various types of human cancer. One significant limitation of siRNA is that serum nuclease reduces its stability and cellular uptake by enzymatic

degradation. For this reason, various delivery approaches have been made for siRNA; among them, nanoparticle- and liposome-based delivery have shown greater treatment efficacy of CRC (Lee et al. 2013). Various studies have found that tRFs have role in tumorigenesis and it could be used as a promising target for diagnosis of human cancer and therapeutics for treatment of cancer. Till now, 13,823 tRFs have been identified which fall under six different types of tRFs such as 3′-half, 5′-half, i-tRF, tRF-1, tRF-3, and tRF-5. Among them, 16 tRFs are differentially expressed in colon cancer. Studies have shown that tRF-24-NMEH623K25, tRF-30-XSXMS-L73VL4Y, tRF-29-QU7BPN6ISBJO, and tRF-27-Q99P9P9NH5N are expressed highly in colon cancer tissue compared to normal colon mucosa (Xiong et al. 2019). Colon cancer cell line studies have shown that tRF-29-QU7BPN6ISBJO and tRF-27-Q99P9P9NH5N are also overexpressed.

Apart from small RNAs, LncRNAs (long noncoding RNAs) have been linked to a variety of malignancies, including colon cancer. LncRNA signatures have been used for determining the risk of recurrence in patients with colon cancer; the six lncRNA-signature-associated coding genes are considerably enriched in proliferation and angiogenesis, cell death, and essential cancer pathways, according to in silico functional analysis, all of which potentially play crucial roles in colon cancer recurrence. The six lncRNA profiles (LINC0184, AC105243.1, LOC101928168, ILF3-AS1, MIR31HG, and AC006329.1) have been taken together which have a lot of promise for predicting recurrence risk and providing individualized treatment for colon cancer patients (Zhou et al. 2018). The most common cause of mortality from colon cancer is liver metastases. On the other hand, the roles of lncRNAs in colon cancer liver metastases are still largely unknown. Wang et al. discovered a new lncRNA, B3GALT5-AS1, that is decreased in colon cancer tissues and even more so in colon cancer liver metastasis tissues (Wang et al. 2018). Competing endogenous RNAs (ceRNAs) networks have been constructed with various types of lncRNAs. It explains the interactive mechanism between lncRNAs and other molecules and also describes how these ceRNAs are involved in the alteration of expression of protein levels in CRC. Nowadays, various analytical studies on ceRNAs have been done and reported that they could be used as a diagnostic and prognostic biomarker of CRC (Vieira et al. 2021).

Mouse Model in CRC Research

Building of a mouse model has a goal because mouse models are used as a preclinical study tool for recapitulation of human cancer and other disease with the hope of formulating novel treatment of human cancer and developing novel anti-cancer drugs. Mouse models are a basic foundational tool and resource for the scientific study of basic biological processes, disease pathogenesis, novel therapies and techniques, and toxicological research. It provides invaluable information in the accomplishment of medical knowledge and mitigations of human suffering with any disease condition, most importantly in cancer and other neurodegenerative disorder (Matano et al. 2015). Despite of much advancement in the treatment of CRC, still

many therapeutic improvements are needed in the recent era, and preclinical in vivo mouse models played an indispensable role in the discovery of novel treatment approaches. An ideal CRC mice model allows researchers for studying each evolutionary stage of CRC development via radiology and endoscopic methods. Over the past century, the mouse has become preferred as a mammalian model, and it shares some common genetic features with the human genome, physiological anatomy, and metabolism. By doing experiments on these common features, researchers can generate valuable insights into the functions of these things within human beings. In biomedical research, different mouse models have been made with the desired character by breeding with a specific strain of mice. Any experiments could be done very easily on the mouse because its maintenance cost is very cheap; as it is small, it is easy to maintain it in the house, easy to manipulate the gene which provides a potential tool for generating a specific disease model to know the function of the manipulated gene (Yee et al. 2015). Mouse models are very flexible for the creation of research-specific models such as the genetically engineered models, xenograft models, cell culture models, peritoneum models, and chemically induced models which have led to the discovery of the molecular basis of tumor initiation, growth, and metastasis, as well as being utilized for anticancer drug discovery and testing (Li et al. 2021).

Chemical Induced Mice Models

Chemical carcinogen could be introduced via oral administration, ad libitum, and through injection such as intraperitoneal, intradermal, intravenous, subcutaneous, and intramuscular injections. For the generations of chemically induced CRC mouse or rat models generally, dextran sulfate sodium (DSS) and azoxymethane (AOM) and sometimes the precursor of AOM such as 1,2-dimethylhydrazine (DMH) and methylazoxymethylacetate (MAMA) are used for the induction of colon cancer in mice and rats (https://www.yourgenome.org/facts/why-use-the-mouse-in-research, 21- july-2021). Apart from this, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) carcinogens are used rarely for colon cancer induction in mice or rats. Depending on the administrative route and strain, different chemicals show different carcinogenic potential for CRC formation. Sometimes, these chemically induced carcinogenic models are not homogenous and rarely show invasive properties and metastasis (Fu and Lawrance 2015).

Peritoneal Model

It is the first method to develop tumor in the peritoneum and peritoneal cavity of immuno-compromised mice by directly injecting human CRC cell to this layer and cavity for studying about the growth and localization of peritoneal metastasis. As this model does not mimic the biological behavior of human cancer properly, it is not effectively used for CRC research (Bastiaenen et al. 2020). This model is usually

used for the study of nonsolid tumors like leukemia and to assess other pharmacological responses (Heijstek et al. 2005).

Xenograft Mice Model

A xenograft is the transplantation of tissue or cell between different species, and for the development of a xenograft mouse model, immunodeficiency or nude mice is required. Various types of xenograft models have been built up such as the ectopic xenograft model in which the transplanted site is different from the origin of the transplanted cell, and the orthotopic xenograft model in which the transplanted site is the same as the origin of cultured cells (Oliveira et al. 2020). Other types are cell line-derived xenograft mice models (CDX) and patient-derived xenograft models (PDX). CDX mice model is a very primitive traditional xenograft model. This model is developed by giving subcutaneous injection of a stable cancer cell line of the cultured cell of specific human cancer into the immunocompromised mice. This model has many advantages such as easy repetition of the experiments, and obtaining the tumor cell lines is also very easy and less cost effective. Limitations of this model are like the tumor microenvironment which is not the same as where it was collected from humans and does not reflect the accurate and original characteristics of the tumor (Keysar et al. 2013). PDX mice model is developed by directly transplanting or grafting fresh tumor tissue samples from a cancer patient through surgery into immunocompromised mice. Compared to the CDX mouse model, this model has many advantages like it can retain the original tumor microenvironment, diminishes the heterogeneity of repeated passage, and helps in analyzing different stages of development of CRC. Limitations are the following: As mice are immunocompromised, the life span will become short; they are prone to be affected by many diseases and infection because there may be some interaction between immunity and tumor as the immune system is different in both species (Li et al. 2021) (Fig. 1).

Transgenic Mice Model

Transgenic mice models are widely used in cancer research in the study of tumorigenesis and pathogenesis in which oncogenes can be constitutively or conditionally expressed and tumor suppressor genes could be silenced by using conventional methods, such as retroviral infection, microinjection of DNA constructs, and the so-called “gene-targeted transgene” approach. Transgenic mice are created by using a glass micropipette that contains a solution of genetically modified gene either in knock-in or knockout form and directly injecting it into the nucleus of fertilized egg of the mouse. Then this zygote is transferred into blastocyst of foster female mouse (Walrath et al. 2010). Recently, CRISPR-based technology has come into focus for the generation of transgenic mice models, and

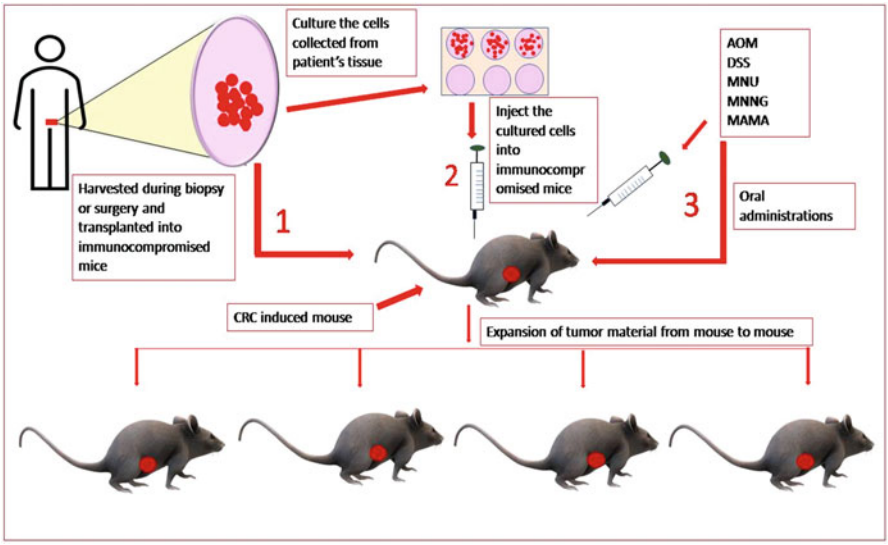

Fig. 1 (1) Patients-derived xenograft (PDX) mouse model; (2) cell line-derived xenograft (CDX) mouse model; and (3) chemical induced mice model creation process

CRISPR/CAS-9 is one of the best technologies for the generation of transgenic mice model in the study of carcinogenesis of colorectal cancer (Takeda et al. 2019). For the generation of transgenic mice model, the Cre-LoxP recombination system is also used, where cre-recombinase plays a crucial role. Knockout transgenic mouse model could be generated by using this system, where the target gene has undergone deletion, invertion, and translocation process. In the study of the development of CRC, various transgenic models have been established such as the knock-in transgenic mice model where one nucleotide could be substituted with the addition of another or by adding a sequence of genes that are not normally present in the mice, which is mainly used for studying the role of oncogenes and knockout transgenic mice models where the function of a normal gene is silenced or makes it inoperative intentionally which is also used for studying the role of oncogenes, tumor suppressor genes, and housekeeping genes (LamprehtTratar et al. 2018) (Figs. 2 and 3).

A Mouse Model for Small RNA Research

Mice as mammalian models express various types of small noncoding RNAs (sncRNA) such as miRNA, siRNA, snoRNA, piRNA, small nuclear RNA, piRNA, and tRFs. The expression of these sncRNAs largely depends on cell types, tissue types, cell state, and sex also. Studies about tissue-specific expression of sncRNA have begun a decade ago with the description of miRNA by using pioneering techniques such as

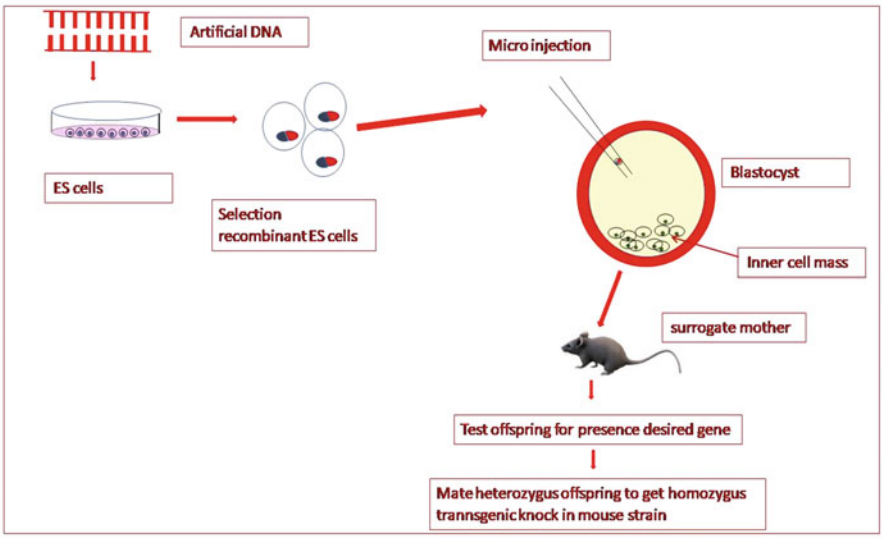

Fig. 2 Knock-in transgenic mouse model creation process

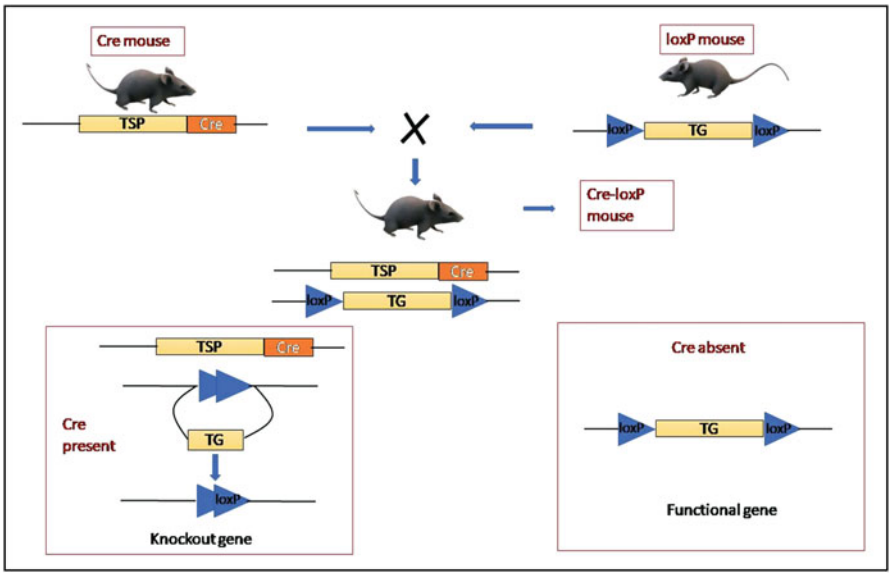

Fig. 3 Knockout mice creation by sing Cre-loxP recombination system (TSP = tissue specific promoter, TG = target gene)

microarray, quantitative real-time PCR (qRT-PCR), and the sanger di-deoxy sequencing method. These techniques are limited to detecting only upregulated miRNA, but now a new advanced technology such as RNA sequencing technology is used to

characterize both upregulated and downregulated miRNA (Liang et al. 2007; McCall et al. 2017). One study has been done to evaluate the expression of different sncRNAs in a mouse tissue-dependent manner. According to this study, they have analyzed the expressions of sncRNA in 10 adult male and 11 adult female mice. They have used GENCODE M20, GtRNAdb, and miRBase to map the expression sncRNA. They identified 1317 distinct miRNA, 733 snRNA, 583 snoRNA, 25 scaRNA, 346 tRNA, 22 mitochondrial tRNA, and 193 other small ncRNAs. From their study, they have found that miRNA is most abundant and shows tissue-specific expression. Tissue-specific snoRNAs can separate the analyzed tissue based on their transcript level, and over 200 snoRNAs have shown tissue-specific expression. They have also identified other snoRNAs whose function is unknown, such as SNORD53, Gm24339, Gm26448, SNORA73a, and SNORD104 in lymphoid tissues; SNORD34 Gm24837 in testes (Liang et al. 2007). They have found other snoRNAs involved in neurodevelopmental disorders such as SNORA35 and SNORD116 and involved in pancreatic cancer such as SNORD123 (Isakova et al. 2020). In CRC-induced mouse model, SNORD126 and SNORA42 (Table 1) have been studied, and it has shown upregulated expression (Fang et al. 2017; Okugawa et al. 2017). Nowadays, greater efforts are given to prepare a mouse model for evaluation of the therapeutic effects of miRNA in the preclinical study. For functional research of miRNA, various transgenic mouse models have been made such as knock-in and knockdown mice models; xenograft mouse models are also being used after transplanting fresh tumor tissue from patients' tumor samples after surgery (Pal and Kasinski 2017). Various miRNAs have been studied in a mouse model that is described in Table 1; some of them promote CRC growth (such as miR-10b, miR-155, miR-26a, miR-31, miR-335-5p, miR-21, and miR-135b), and some of them inhibit proliferation, invasion, and metastasis of CRC (such as miR-let-7a, miR-9, miR-27b, miR-214, miR-18a, miR-143, miR-145, miR-802, and miR-375). A study was done by taking mouse tissue to check the tissue-specific expression of different sncRNAs and found that about 400 miRNAs are differentially expressed in different tissues which has been done by using the deep sequencing method. Among them, very few miRNAs have been found expressed in intestines such as miR-194-1, miR-194-2, miR-192, miR-490, miR-203, miR-20b, and miR-802 (Isakova et al. 2020). Studies of piRNAs from CRC patient's tissue samples have shown aberrant expression of piRNAs. Various in vitro studies have investigated the role of piRNAs in CRC. Some of them are upregulated piRNAs such as piR-651, piR-54,878, piR59056, piR-62,701, piR-823, and piR-54,265, and some are downregulated such as piR-015551and piR-1245 (Sadoughi et al. 2021). Very fewer studies have been done on in vivo mice models by considering piRNA as an important regulator of CRC. Only two mice model studies have been done to explore the role of piRNA in the regulation of CRC such as piR-54,265 and piR-823 (Table 1) (Mai et al. 2018; Yin et al. 2017). Some in vitro study has proven that tRFs could be used as a prognostic and therapeutic biomarker, but very few in vivo mice model studies have been done to establish tRFs as an important biomarker for CRC. One in vivo mice model study has shown that tRF/1280 (Table 1) can inhibit the progression of CRC and 5'tRF GlyCCC (Table 1) is involved in CRC progression (Huang et al. 2017; Wu et al. 2021).

Table 1 Different types of small noncoding RNAs have been studied on CRC-induced animal model

Name	Expression	Function	Reference
miRNA			
Let-7a	Downregulated	By suppressing Np95 ICBP90 RING finger (NIRF) which is an ubiquitin ligase molecule, it inhibits the proliferation of colon cancer	(Wang et al. 2012)
miR-9	Downregulated	Directly target C-X-C chemokine receptor type 4 (CXCR-4) to prevent CRC cell proliferation and epithelial-mesenchymal transitions	(Xiong et al. 2018)
miR-27b	Downregulated	Directly target vascular endothelial growth factor-C (VEGFC) to inhibit tumor progression and angiogenesis of CRC cell	(Georges et al. 2019)
miR-10b	Upregulated	Suppress fibroblast growth gactor-13 (FGF-13) to inhibit the cell growth, migration and invasion in CRC	(Song and Li 2019)
miR-214	Downregulated	Mediating inhibitory effects on CRC cell proliferation, invasion, and metastasis by direct targeting Mediator complex subunit 19 (MED19) which inhibit forkhead box protein D3(FOXD3)	(He et al. 2016)
miR-155	Upregulated	Inhibit protein tyrosine phosphatase receptor J (PTPRJ) and thereby activating AKT which promote CRC cell proliferation and migration	(Zhang et al. 2017)
miR-18a	Downregulated	Directly target heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) and thereby inhibiting its oncogenic function and promoting apoptosis of colon cancer cell	(Fujiya et al. 2014)
miR-26a	Upregulated	Involved in CRC development by inhibiting the expression of retinoblastoma 1 (Rb-1)	(López-Urrutia et al. 2017)
miR-143 and miR-145	Downregulated	Down-regulate G1 regulators such as K-Ras, MYC, cyclin D2, cyclin-dependent kinase-6 (CDK6), and E2F3	(Zhu et al. 2011)
miR-802	Downregulated	Prevent the tumoriogenesis of CRC by inhibiting the expression of ubinuclein-2 (UBN-2) on posttranscriptional level	(Yang et al. 2020)
miR-31	Upregulated	Promote CRC development by suppressing the expression of Special AT-rich sequence-binding protein 2 (SATB-2)	(Yang et al. 2013)
miR-335-5p	Upregulated	Promote tumoriogenesis by inducing epithelial mesenchymal transition of CRC via direct inhibition of RAS p21 protein activator 1 (RASA-1)	(Sun et al. 2021)
miR-21	Upregulated	Promote CRC by inhibiting the expression of tumor suppressor gene phosphatase and TENsin homolog (PTEN)	(Wu et al. 2017)

(continued)

Table 1 (continued)

Name	Expression	Function	Reference
miR-135b	Upregulated	Promote tumor growth of CRC cell by deregulating PTEN/PI3K pathway and inducing the expression of SRC family kinase	(Valeri et al. 2014)
miR-375	Downregulated	Inhibit tumor growth of CRC cell by inhibiting PI3K/Akt signaling pathway	(Wang et al. 2014)
piRNA			
piR-54,265	Upregulated	Bind to piwi-like protein 2 (PIWIL2) which activates signal transducer and activator of transcription (STAT) signaling pathway and activate BCL-XL antiapoptotic protein	(Mai et al. 2018)
piR-823	Upregulated	Promote colorectal carcinogenesis by increasing the transcriptional activity of heat shock factor 1 (HSF1)	(Yin et al. 2017)
snoRNA			
SNORD126	Upregulated	Promote cancer formation by phosphorylating AKT, glycogen synthase kinase-3β (GSK-3β), p70S6K, and elevating fibroblast growth factor receptor-2 (FGFR2)	(Fang et al. 2017)
SNORA42	Upregulated	Induce CRC cell proliferation, migration, and invasion	(Okugawa et al. 2017)
tRFs			
tRF/miR-1280	Downregulated	Prevent tumor growth via inhibition of notch signaling pathway by targeting JAG2	(Huang et al. 2017)
5′-tRF-GlyCCC	Upregulated	In CRC, its level found becomes upregulated by the action of one tRNA demethylase AlkB homolog 3 (ALKBH3) involved in progression of CRC	(Wu et al. 2021)

Advantage of a Mouse Model in CRC Research

There are many advantages of using mice as a model organism in the study of colon carcinogenesis. Reproducibility of mice makes this model more preferable and suitable to use in research. Their genome could be manipulated easily to generate a model with different genetic backgrounds, and the pathogenesis of human CRC could be recapitulated. From the very beginning, mice are being used widely as a model for studying colorectal carcinogenesis mechanism (Rosenberg et al. 2009). The existence of recombinant mouse models, transgenic mouse models, knockout mouse models, and knock-in mouse models genetic mouse models could be used for the screening of anticancer drugs and explorations of novel gene therapy. It could be used for establishing the role of a gene as an oncogene or tumor suppressor gene (Chandra et al. 2020). In recent years, different xenograft models have become a research hotspot. PDX mouse model has the ability to retain the exact tumor microenvironment as like patients and some common characteristics of tumor cells

(Li et al. 2021). In CRC, CDX mouse model provides an advantage for generating a budding tumor like human CRC tissue (Georges et al. 2019). Carcinogen-induced mouse models in CRC help in studying the generation of several sncRNAs and their role in colon carcinogenesis which can set a basic foundational tool for human sample studies because both are mammals and express the same types of sncRNA (miRNA, siRNA, piRNA, tRFs, snoRNA, etc.). After evaluating the role of any particular sncRNA in response to CRC in a mouse model, clinical study could be done to establish the role of this particular sncRNA as a diagnostic or prognostic, or therapeutic biomarker of CRC.

As an excellent tool to study the pathogenesis of human diseases and explore the principles of disease prevention and treatment, animal models are constantly providing new ideas for cancer research.

Issues of Using Mouse Models in CRC Research

Usually, mouse models are used as preclinical tools to study human carcinogenesis, but there are lots of issues present that impede the creation of an ideal cancer mouse model. One issue is the limited recapitulation of human cancer because in human cancer occurs mostly due to stochastic somatic mutation and very rare due to germ line mutation, but in mice, most cancers occur typically due to mutations of the germ line, and a large portion of somatic cell. In human cancer, point mutations generally happen in alterations of gene functions, but in the case of mouse models, genetic alterations occur due to overexpression or deletion of certain genes (Cheon and Orsulic 2011). Induction of CRC in healthy mice sometimes fails to form cancer in the colon, but the chances of other cancer or any disease formation increase. The issue in the generation of PDX CRC models is when tumors fail to progress or metastasize and therefore do not retain all patterns of the disease course that were observed in patients. Limitations of using CDX CRC mice models are that they do not accurately mimic the tumor condition in human genetic heterogeneity and the tumor microenvironment (Pan et al. 2022). The mice are immunocompromised in this xenograft mouse model, so immune cells of mouse hinder the formation and growth of colon tumors and patients' response properly (Pompili et al. 2016). Designing of study and interpretation of data are a major issue when the data coming from mouse model trials is inconsistent, nonrandomized, inadequate, and absent of proper statistical analysis tools. One systematic review, based on animals, has reported various methodological problems such as poor experimental design, inconsistent and lack of adequate data, and lack of established statistical analysis (Kilkenny et al. 2009). Most of the studies do not follow "the three Rs" of animal research such as Replacement, Reduction, and Refinement. Heterogeneity is also an important issue because both humans and mice have some differences in their genes, disease pathogenesis, and medications. A contributing factor to this heterogeneity is the selection of mice which is not always based on previously established studies. Due to this heterogeneity, the result of these mouse model studies does not always carry forward to human trial experiments. One very important issue is the translation

of data from preclinical studies into the human clinical trial which cannot be done easily. The mouse model provides limited advancements in clinical studies, and data from this model are heavily biased because of improper analysis (Robinson et al. 2019).

Conclusion

As an excellent tool to study the pathogenesis of human diseases and explore the principles of disease prevention and treatment, mouse models are constantly providing new ideas for cancer research. However, there are some differences in physiology, heredity, and immunity between animal models and human beings. From the past two decades, various mouse models have been developed for studying colorectal carcinogenesis. For generation of genetic information, transgenic knock-in and knockout mice are very indispensable before the clinical study. PDX and CDX mouse models are very useful for identifying novel anticancer treatments against CRC, as they provide a faithful representation of human cancer. This study evaluated that the mouse model could be used as a prediction tool to predict and validate something which cannot be done directly on human beings. Although there is much advancement discovered for the treatment of CRC, still biomedical researchers are facing some problems in findings of the exact treatment regimen to control colorectal carcinogenesis. Among various sncRNAs (miRNA, siRNA, piRNA, snoRNA, and tRFS), miRNAs are most extensively studied in CRC and some miRNAs are already being established as a diagnostic, prognostic, and therapeutic biomarker. For diagnosis, prognosis, and treatment purpose, snoRNA, piRNA, and tRFs could be used, but before establishing these as a biomarker, snoRNAs need more focus and study. So mouse model is one of the important indispensable foundation tools for testing hypothesis and validation of human data.

Future Perspective

The use of mouse as model organism will be very helpful for the foreseeable future and to drive scientific discovery into a variety of medical fields. To limit current animal-based studies and minimize animal suffering, the governmental and institutional boards have developed some legislation and ethical approval processes. To study the role, these sncRNAs are involved in the pathogenesis of CRC in mice; these different types of tools could be designed by recapitulating the exact tumor microenvironment like CRC patients. In the future, PDX model could be used for drug screening and evaluation of preclinical therapeutic. Patients' specific PDX tumor model could be generated for better evaluation of preclinical therapeutic against CRC. PDX model could be used to define the association between drug resistance and gene mutations in CRC. Compared to the PDX model, the CDX model is not very accurate and unable to mimic the exact human malignant tumor. Still patient-derived CRC cell culture provides a high-throughput drug-screening valuable tool. In recent years, more complex

CRC cell-culturing methods have emerged where patient-derived CRC cells are cultured to form three-dimensional spheres, i.e., differing from conventional 2D culture. Recently, patient-derived organoids (PDO) have been generated by collecting patients' tumor cells and culturing them in an extracellular matrix and transplanting them into mice to create patient-derived organoid xenograft (PDOX) which is quite similar to PDX CRC mouse models. Various studies have demonstrated that PDOs reflect more faithfully the biological virtue and drug response of the parental tumor compared to 2D culture (Bürtin et al. 2020). In the future, these different types of models could be used for prediction and validation of the role of sncRNAs involved in colorectal carcinogenesis.

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Radiopharmaceuticals: A New Era in Cancer Therapy – Light on Initial Findings on Animal Model

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Ahamed Basha Abdul Bari, Prince Johnson Samuel, and Siva Shankar Rangasamy

Contents

Abstract

In recent years, the worldwide cancer burden has increased dramatically. The treatment modalities for cancer are surgery, radiation therapy, and chemotherapy using anticancer drugs. Other treatment options include immunotherapy, stem cell transplantation, photodynamic therapy, etc. Even though available therapies offer a significant protective effect, scientists are still seeking a breakthrough in cancer treatment. One such therapeutic approach is radiopharmaceutical therapy, treating cancer with known medicinal radio compounds. Numerous radiopharmaceuticals

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with anticancer potential have a broad scope in cancer research and drug development. But, the development of a novel radiopharmaceutical must fulfil stringent regulatory criteria, which means that its physiochemical and biological qualities and therapeutic potential must be validated in animal models before it can be used in clinical trials. This chapter discusses the importance of radiopharmaceuticals, their dosimetry, biological properties, transition from diagnostic to therapeutic importance, challenges, animal models, preclinical assessment, and the recent advances that are being adopted in the field of radiopharmaceutical cancer research. Hence, this chapter will be very helpful for clinicians and researchers who are working with therapeutic radiopharmaceuticals.

Keywords

Radiopharmaceutical therapy · Preclinical assessment · Animal models and cancer research

Introduction

Cancer is a leading cause of mortality in the world, with approximately ten million fatalities in 2020 (Sung et al. 2021). There are many different types of cancer that affect various organs, and the management of cancer varies as well. Surgery, radiation therapy, and chemotherapy with anticancer medicines are standard treatment techniques for nearly all forms of malignancy. Additionally, recent advances in the management of cancer include immunotherapy (Abbott and Ustoyev 2019), stem cell transplantation (Najafi et al. 2019), and photodynamic therapy (Li et al. 2020). Oncologists have used studies comparing the outcomes of various treatment methods to determine which technique is most likely to produce a better prognosis in the long term. However, scientists and clinicians are working day and night searching for a breakthrough toward better cancer care. One such solution is radiopharmaceutical therapy (RPT) (Sgouros et al. 2020), which has contributed to tremendous advances in cancer treatment. There are plenty of radiopharmaceuticals (RPs) that are currently proposed as drug candidates for cancer. However, before administration of the new radiopharmaceutical (RP) drug for human use, it has to undergo several research processes like analyzing the complete profile of physiochemical and biological properties and being thoroughly tested preclinically in animal models. Analyzing a RP drug for its biochemical properties is one of the initial steps in the process of drug development, and it is being carried out by researchers around the world. When it comes to RPT drug testing in animal models, it has its own set of advantages and disadvantages, so the choice of animal model, dose calculation, genetic factors, toxicity studies, and so on must all be considered before estimating the efficacy of a new RPT drug for cancer research. Recently, the RPT and its testing on small animals have become standard practice, and experimental animal models have been found to be suitable candidates in elucidating several biochemical pathways involved in cancer development, and their findings

can be extrapolated to the scientific findings in the drug development process for humans. Thus, the development of tumors and testing of novel RPT drugs in experimental animals served as an important tool before clinical testing. This chapter discusses the importance of RP, its current status of therapeutic research using animal models, and its efficacy for human application in the management of cancer.

Radiopharmaceuticals: An Overview

Radiations are emitted by a source and travels through space at various speeds. The physical characteristics of the emitted rays varies in mass, energy, and the degree of penetration on biological tissues and others materials. There are different types of radiations, each with its own set of features and consequences. They are broadly classified into nonionizing radiation and ionizing radiation. Nonionizing radiation doesn't have sufficient energy to take away an electron from an atom (e.g., visible light). On the other hand, the ionizing radiation can eliminate an electron; it can make an atom as an ion (e.g., X-ray). Thus, when radioactive elements emit ionizing radiation, it undergoes radioactive decay which is loss of energy as ionizing particles and radiation. Among the ionizing radiations, α, β, Auger, and γ electrons are the common therapeutic/theragnostic emitters that are being used in the management of various diseases including cancer (Herrero Álvarez et al. 2021). Elements that emit ionizing radiation are called radionuclides (RNs). In the current decade, apart from the diagnostic utility of RN, it is being analyzed more for its therapeutic potential in the management of cancer.

RPs are also known as medicinal radio compounds. They are a class of medications that contain radioactive isotopes with therapeutic potential. RPT is defined as "administration of RN in the treatment of diseases." But here, this chapter is dealing with RPT only in relation to cancer. RP, apart from its diagnostic utility like γ scan, positron emission tomography (PET scan), and hybrid scanning method, plays a pivotal role in the interventional nuclear medicine. In the clinical approach of cancer, RPT plays an important role and offers several added benefits over and above regular cancer treatment. In a routine radiation therapy (RT), the radiation is given from outside the cancer cells. However, in RPT, the radiation is delivered to the cell locally either directly or through a mediator, i.e., linking molecule/receptor (Sgouros et al. 2020). Further, one of the advanced cancer management modalities is immunocancer therapy where it mainly targets signaling pathways of the cancer cells, but RPT is found to be independent of those signaling pathways (Sgouros 2019). Similarly, in chemotherapy, patient has to undergo treatment for several cycles over a few months, but in RPT, the patients have to take only a few injections or oral drugs with minimal side effects compared to chemotherapy (St James et al. 2021). This makes RPT a better therapeutic offering in the management of cancer.

In RPT, a chemical compound having one or more atoms replaced by a RN is used. It has wide range of emission properties which traces biological reactions through α or β emission by using radioisotopes of hydrogen, carbon, phosphorus, sulfur, and iodine. Further, RP preparation is grouped under four categories, i.e.,

(1) it is prepared as an RN-containing medical product that comes in a ready-to-use form for therapeutic/theragnostic human usage. (2) It is generated as a short half-life daughter RN which is separated from a longer half-life parental RN, and later it is used for the preparation of RPs. (3) It is prepared as a precursor RN that is being used in the process of radiolabeling. (4) It is available as a ready-made multidose vial which can be used for the preparation of RPs. It is produced either by nuclear fission, charged particle/neutron bombardment, or through generator systems in adherence to good manufacturer guidelines.

Nuclear medicine imaging tools can visualize RNs, and their activity can be assessed by variety of techniques. It provides a better control and outcome over the current therapy, and it acts as precision tool in cancer management. In cancer patients with several metastasis, current available therapies like chemotherapy or RT or combination of both do not provide significant benefit in the disease outcome. Here, RPT is found to be a better option. However, RPT is not a novel concept in delivering radiation straight to cells, as radioactive iodine has been used in the management of thyroid cancer since decades. But newer RPs are emerging in cancer care. Recently, after the approval of β- and α-particle-emitting RPT for neuroendocrine and bone cancer, respectively, RPT is now considered as a potential, safe, and viable treatment approach in the management of cancer compared to the other treatment options currently available. Thus, considering its growing importance in recent years, there has been a surge in research and clinical trials involving novel RPs. The results obtained from them proved that RPs could lower the danger posed by cancer cells throughout the body.

RP Nomenclature

RPs are identified using a universal drug nomenclature. Several other nomenclatures also coexist. The international nonproprietary name (INN) designates a radioisotope as mass number without space followed by element symbol in parentheses with no superscript, followed by the ligand (if any), for example: technetium (99mTc) sestamibi. However, in United States Pharmacopeia (USP), name includes the basic drug name, followed by the radioisotope (as element symbol, space, and mass number) with no parentheses, hyphen, or superscript, and finally the ligand (if any), for example: technetium Tc 99 m sestamibi. The purpose for noting the RP nomenclature here is that both styles are used in scientific research publications (Ballinger 2005).

Diagnostic RPs

In diagnostic RPs, minimal dose of RP drug is administered to patients for the diagnosis of clinical conditions. Several types of RPs are being used in the imaging studies. Depending upon the route of administration of RP, it either passes through or is absorbed by tissues/organ. The radiation is then detected, and images are captured

using specialized imaging techniques. Later, the images are examined for diagnosis and to detect any abnormalities. Radioactive ^{47}Ca is used to investigate bone metabolism-related disorders (Denk et al. 2007), ^{14}C urea for gastrointestinal microbial growth (Atli et al. 2012), and ^{51}Cr for red cell volume determination (Gallet et al. 2020), and there are many other such applications. The same RP is also being used in imaging studies of various types of cancer, for example: F18-FDG (fluorodeoxyglucose) in PET scan (Stefan Morariu et al. 2020).

Transition of Diagnostic to Therapeutic RPs

Diagnostic RP imaging used to assist doctors in making decisions has grown at a breakneck pace. Now scientists and clinicians around the world are in the process of developing RPT for a wide range of cancers from melanoma to colorectal cancer. The scientists are aware that every cancer cell is having a targetable molecule on its cell surface. In addition to this, cancer cells have a good blood supply which is sufficient to carry the radio drugs and thus destroy the cancer cell. However, these novel drugs being used for therapeutic purposes are not really new medications but only reengineered products of the currently existing compounds that are being used in diagnostic imaging techniques. These targeting molecules have now been repurposed to carry radioactive compounds, or isotopes, that can kill cancer cells rather than just help visualize them. If the imaging radioactive compound reaches cancer cells and is visualized on a PET scan, then we say confidently that the RP treatment will also hit the target cancer cell and for sure it will be a better option among the treatment modalities of cancer. This method of repurposing imaging radioisotope was tested on the experimental prostate cancer. Prostate-specific membrane antigen is a protein discovered in huge amount on prostate cells (Hofman et al. 2018). Since prostate cancer cells are vulnerable to radiations, targeted RP that was injected gave a better result. One of the greatest advantages is that both imaging and treatment molecules are the same.

RT has been utilized in the treatment of cancer for several decades, and at least half of cancer patients receive or have received it during their treatment by using external radiation beams to kill malignant cell within the body. RT, while beneficial, has its own adverse effects such as the passage of radiation beams into normal tissues besides cancer cells. Its adverse effects include ageusia, cutaneous color changes, hair loss, and decrease in reproductive functions (Brook 2021). Hence in order to overcome the side effects of the RT, several researchers developed RPT, which deliver radiation therapy directly and specifically to cancer cells. Radiobiological differences between RT and RPT are given in Table 1.

RPT can have both direct and bystander cell cytotoxic effects like mutation, cell death, etc. which depends on the RN biological properties. The amount of RN that causes cell death is determined by the cell's radiation dose. When a high dose of radiation is followed by a low dosage, adaptation to the radiation can reduce the effect of radiation over time as indicated by a decrease in the extent of radiation-induced damage.

Table 1 Difference between RT and RPT

S. No	RT	RPT
Treatment modality	Based on the tumor location	Based on the tumor biology
Energy	Usually given with low linear energy transfer photons and electrons (0.2 keV/um)	Usually RNs (α or β particles, auger electrons, and γ emissions) conjugated to a carrier – Antibody, peptide, or ligand
Dosimetry	Well-defined	Not well-defined – Depend upon the target
Dosage rate	High (1–2 Gy/min)	Low, less than 0.5 Gy/hour
Dose distribution	Fairly homogeneous in the targeted field	Heterogeneous secondary to the emission properties
Treatment regimen	Fractionated	Usually, continuous

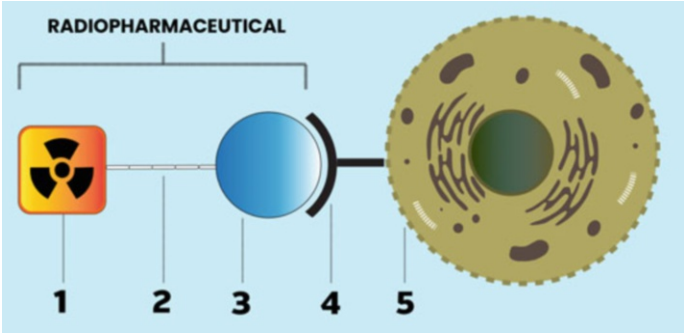

Fig. 1 RP and its functional units. 1. Radionuclide, 2. linker, 3. target molecule, 4. target protein, and 5. cancer cell. (Image modified and redrawn from the original image source: (Credit NIH – National cancer Institute))

Mechanism

Radioactive compounds find their way to cancer cells on their own. But how they act is well established, i.e., the RPs contain three connecting units: (1) radioactive compound, (2) linker, and (3) target cell. All the three act as a single unit (Figure 1) in delivering the effects toward a cancer cell. It is similar to the antibody-drug interaction, and it could be injected intravenously or be given through oral route. Once the RPs reach interior of the cancer cell, the radioactive compound kills the cancer cell by attacking the DNA of the cancer cells, i.e., radiation-induced DNA damage.

Depending on the type of radioactive substance utilized, the resultant energy can penetrate the cell attached to the RP as well as around 10–30 cells surrounding that cell. This increases the amount of cancer cells that can be destroyed with a single RP molecule (Vermeulen et al. 2019).

Preclinical Research on RPT: Initial Findings in an Animal Model

Small animal imaging through RN plays a major role in the diagnostic RPs which help in the assessment of regional blood flow, cell growth, receptor function, and the prognosis of cancer. This small animal imaging technique also creates a road map in the development of the initial stage of RPT. Recently, the RPT and its testing on small animals have become a standard practice in modern medicine. However, using small animals in RPT research requires proper analysis of genetic factors and their biological effects. The interpretation of data collected in tiny animals as well as a successful translation to the bedside are both critical. Thus, a perfect RPT for therapeutic research in small animals should respond specifically to the malignant cell; it should reach all cancer cells irrespective of location in the body. It should not affect or cause any harm to the normal healthy living cell, even at high doses of RP, because it has to destroy the malignant cell to a major extent.

In RPT, the drug candidate is usually given through an intravenous route, and the injected drug will offer cytotoxic radiation on the specific sites of the tumor. The abovementioned drug could be a peptide or an antibody that always offers cytotoxic radiation to tumor areas and selectively accomplishes targeted distribution. Many RNs reach specific organs or tissues without the use of ligands. Radium, a bone-specific compound, and iodine, a thyroid-specific compound, are two common examples of the above, and they target cancer cells more effectively. Targeted RPT exhibits significant positive effects both in a tumor of a single organ or tissue and in an organ with metastasis. Considering its importance, validation of RPT is necessary before it is applied for clinical use. In order to develop novel RPT drugs, in vitro studies must be conducted to characterize a new RPT antitumor compound and its mode of action. Preclinical biodistribution and toxicology studies are needed urgently to identify corrective dosage and toxic effects of RPT.

Biodistribution and Dosimetry in Animal Research

Research using animal models in the field of experimental RNs must adhere to specific dosimetry (González García and Peinado Montes 2020). That too has to be started with a single dose administration to the experimental animals, so that it will give an idea for human usage. Next, for any one animal species, whether it's a laboratory rat or mouse or a large animal like a monkey, the choice of animal species for the purpose of RPT research has to address the tissue cross-reactivity for the radioactive product (Soyluoglu and Durmus-Altun 2021). Its pharmacological aspects have to be compared with those of humans. Scientists intending to work on RPT animal models must assess the degree of radioactivity in the tissue studied for different durations (e.g., 5–6 × half-lives) with adequate number of samples to record the cumulative effect of the drug candidate. Timing of data collection, the amount of radioactive and nonradioactive compounds in a dosing mixture, daughter decays, RN purity, pH, microbial control, and half-lives must be studied (Morris et al. 2021). Apart from good manufacturing practices, the compound has to adhere

to good radiation practices (Gillings et al. 2021). The RPT study design in laboratory animals must be planned in such a way that the same study design can be applied to a human clinical trial. RPT effects must be studied over total body effects in an animal and in vital organs like the liver, heart, lung, kidney, brain, etc. Its effects must also be studied in adrenal glands, reproductive organs, muscle, bone, bone marrow, skin, eyes, etc. and any other organ/tissue if needed. RPT compound excretion data in the urine and feces has to be studied. Both sexes (males and females) of the animals have to be incorporated into the RPT study with the required number at a specific point in time. The RPT drug activity curve in animals must be compared with human organs for the percentage of administered activity and residence time, so that the animal studies can be extrapolated to humans. Values generated in humans from the various organs have to be developed into mathematical models and software in order to give acceptable evidence for the calculation of the dose (radiation absorption) of a radioactive compound.

Animal studies have shown that the pharmacokinetic target for assessing efficacy varies depending on the emission of the RPT particle. For example, RPT with α-particle emitters allows highly damaging radiation to be delivered to target cells. α particles primarily induce irreversible DNA damage (Pouget and Constanzo 2021). Hence, this α-emitting RPT is considered to provide better results than standard cancer treatment, which frequently results in resistance mechanisms. Several animal studies have been reported with a journey toward studying therapeutic benefits. A few animal studies also explain the acute and chronic radiation/ligand-induced toxicity, as well as the genetic alterations that are caused due to RPT. The key reason for the need for pharmacokinetic studies in preclinical RPT research is to calculate the dose-limiting organ (DLO) and maximum tolerated absorbed dose (MTAD) (Sgouros et al. 2014). Absorbed dose (AD) is nothing but energy absorbed by a tissue divided by its weight. The association between AD and the DLO indicates the degree of toxicity rather than an administered dose of a RN. Hence, the administered dose can be gradually increased till the animal model exhibits toxicity. Furthermore, if the dose-limiting organ is known, then absorbed dose toxicity can be calculated. Hence, its association can be kept as a reference value for the escalation variable during a phase I trial with human subjects. Apart from the maximum tolerated absorbed dose, with phase I data analysis, future trials can be done, particularly in combination therapy research. Finally, it can be said that research studies using animal models lead to clinical trial studies that may end up in precision medicine in the management of cancer care for humans.

RPT mainly exerts its beneficial effects in the management of cancer by means of three types of radiation particles as mentioned earlier – β particles, α particles, and Auger and γ electrons. However, the γ emitters play a major role in the diagnosis of cancer rather than treatment. The majority of radiation-emitting isotopes used in treatment are tagged with peptides or antibodies to target specific tumors, and they are only used as carriers to deliver ionizing radiation to tumor tissue. Depending on their irradiation capacity, the RN particles can independently irradiate a variety of cells, tissues, and organs. For example, the penetration of α particle is in a range of 50–80 microns with a 5–8 Mev. However, β particle can penetrate up to 1–10 mm

with 0.1–1 MeV (Kassis 2008). Furthermore, in a few cases, the combination of emitters for imaging and therapeutic purposes can be accomplished using the same or a different RN, such as ^{131}I, which is a well-known mixed β/γ-emitting compound (Wyszomirska 2012). The research on the usage of RP compound was done on ^{131}I for the diagnosis and management of thyroid disorders, including thyroid cancer. Thus, in the emission of electrons and, based on their decay, RPT, electrons are divided mainly into α, β, and Auger electrons with regard to the management of cancer. In order to justify the therapeutic efficacy of the RPT to accomplish human cancer, we need several analyses that have to be done in various animal models. If it shows a promising protective effect in the animal model of malignancy, then we can further extend it to humans confidently. In the following paragraphs, RN-based experimental preclinical studies using various animal cancer models are discussed with regard to therapeutic approach.

RP has progressed through several stages of development over the last few decades, and it now competes in a multibillion-dollar global market. Thus, several researchers are showing keen interest in RP both for diagnostic and therapeutic purposes. Beyond diagnostic, therapeutic RP attracts more scientists across the world because of its beneficial effects in the management of several cancers. However, the development of a new RP has to meet strict regulatory requirements, i.e., before it has to be used in clinical trials, its physiochemical and biological properties have to be tested in animal models. Before any RP drug development, it must adhere to characteristics such as the RP being rapidly absorbed and acting in the target organ for a specific period of time, the target RP not affecting healthy tissues, and being stable with minimal toxic effects.

RP extrapolation to humans is not always an easy task because of the limitations in translatability. Cellular structural modification has to be taken into account as one of the major criteria in the selection of animal species. Next, a metabolic variation, its kinetics, and its pathways may vary from one species to another and have to be studied thoroughly before the selection of animals in the RP research study. Larger animals exhibit slower metabolisms, and small laboratory animals are found to have higher metabolic activity. Next, before the start of the small animal testing for RP, in vitro RP testing in cancer cell culture has to be done to establish a toxic compound or any hazardous material.

Study design in relation to RP in experimental small animals must consider the following factors (RADIOISOTOPES and SERIES 2021)

- The affinity of RP toward its targets via ligand and its kinetics must be evaluated using a saturation or competition assay with a dissociation or inhibition constant. It can be used in the selection of RP in experimental animal research. It is necessary to understand how specifically and selectively the RP compound binds to the target protein. Association and dissociation rates of the binding molecule have to be determined for a research drug candidate by a time-resolved assay. Specificity of the RP therapeutic drug can be studied in laboratory animals by administering an unlabeled ligand (blocker) to particular RP. It is an index of the saturability of the binding which shows RP binding to a specific target protein.

- The RP drug has to be studied for its distribution into the cell and whether its movement is active or passive transport. Thus, internalization may provide a way to understand the concept of intensity of RN internalization, which helps to select the choice of RP drug selection, its mechanism of action, and efficacy.
- Cell uptake studies in RP will give an idea of whether the research RP drug has been taken up by the target completely or not. Analyzing the time duration of cell uptake of the known RP helps us easily to calculate the difference in timing of injection to accumulation of the drug.
- Tests for functional property, permeability assays, metabolic markers, drug stability, and its stability in body fluids must be done in small animal RP cancer research studies. The battery preclinical assessment in animal models in relation to RPT research is depicted in Figure 2.

The animal models used in the cancer studies include solid tumors induced through a xenograft obtained from human cell line, and then cancer is developed in immunocompromised mouse models. Other models include spontaneous development of a cancer in a genetically engineered mouse (Cheon and Orsulic 2011). However, each model has its own pros and cons, but to a greater extent, it resembles the process of carcinogenesis in humans. Initially RP was used in small animal imaging studies, and small animal imaging through RN plays a major role in the diagnostic RPs which helps as a noninvasive tool in the assessment of regional blood flow, cell growth, receptors function, and in the prognosis of cancer. This small animal imaging technique also creates a road map in the development of initial stage of RPT.

Fig. 2 Preclinical assessment in animal models in relation to RPT research. Figure indicates battery of assessments that are required for RPT research in experimental animals. DLO, dose-limiting organ, and MTAD, maximum tolerated absorbed dose. AD – absorbed dose for RN

The interpretation of data collected in tiny animals and a successful translation to the bedside are both critical. Thus, a perfect RPT for therapeutic research in small animals should respond specifically to the malignant cell; it should reach all cancer cells irrespective of location in the body. It should not affect or cause any harm to the normal healthy living cell, even at high doses of RP, because it has to destroy the malignant cell to a major extent.

Common RPT Agents Under Research

S. no	Radionuclide	Cancer type/organ	References
1	^{131}I and its related compounds	Thyroid cancer, bone cancer, and adrenergic receptor-mediated cancer	Sgouros and Goldenberg (2014); Sgouros (2019); Herrmann et al. (2020); St James et al. (2021))
2	^{212}Pb and its related compounds	Breast cancer, somatostatin receptor-mediated cancer, endothelial tissue, and blood cancer	
3	^{225}Ac	Leukemia and lung cancer	
4	^{90}Y	Hepatic malignancies	
5	^{177}Lu and its related compounds	Somatostatin receptor-mediated cancer, prostate cancer, and GI cancer	
6	^{223}Ra	Cancer of the bone	
7	^{227}Th and its related compounds	Prostate cancer, breast cancer, cancer originated from mesothelioma and lymphoma	

Targeted Cancer Therapy in Experimental Animal Research

Innovative approaches through targeted RNs in cancer management have been made possible by the availability of potential α emitters. Studies have shown that there is significant progress in the application of α emitters like ^{225}Ac, ^{224}Ra, ^{212}Pb, etc., directly to the site of the cancer without affecting the healthy cells. In one study, it was reported that a single injection of ^{212}Pb-trastuzumab decreased the overall prostate cancer growth by more than 60% in a nude mouse (Tan et al. 2012). In another neuroendocrine tumor model study in mice, treatment with 213 Bi-DOTATATE emitters and L-lysine provided significant protection and improved laboratory animal survival (Chan et al. 2016). Another in vivo study demonstrates that α treatment with ^{149}Tb of single cancer cells offers a promising protective effect in the mouse model of leukemia (Beyer et al. 2004). Adding to this, in a mouse model of ovarian cancer, DOTAylated-huCC49 was tagged with the-^{225}Ac, and it offered greater protection against the damaging effects of malignancy (Minnix et al. 2021). Supporting this, another reference literature established

an RPT in an exocrine pancreatic mouse model, where ^{211}At-labeled methyl tyrosine showed an arrest of tumor growth and inhibited the decrease in body weight of the animals (Kaneda-Nakashima et al. 2021). Next, another preclinical investigation in glioma-bearing mice proved that usage of astatine ^{211}At-labeled phenylalanine, one of the α emitters, was reported to offer good protection in a dose-dependent manner by specifically targeting L-amino acid transporters (Watabe et al. 2020). Similarly, in rats with intracranial glioblastoma, systemic therapy with ^{211}At phenylalanine improves the survival rate of the animal (Borrmann et al. 2013). Hence, these data suggest that it can be used as a better therapeutic management for cerebral glioblastoma, either by recurring local injections or intracranial delivery after excision of the tumor. In the same manner, in another study, ^{211}At-NaAt exhibited significant protection in thyroid cancer (Liu et al. 2020) and ^{211}At-trastuzumab in metastatic gastric cancer using mouse models (Li et al. 2021). Thus, RPT holds a strong promise for the betterment of various cancers in experimental animal models, and we can say that these RPTs have a greater potential that can be extrapolated to the human cancers of various organs and tissues.

β electrons are the most common type of emitter that has been employed in cancer research in the field of RPT emitted from the nucleus. The main reason for the adoption of β is its wide availability. A study reported that targeted RPT with a known RN, ^{177}Lu, showed promising results in the treatment of small lung cancers in nude mice (Schmitt et al. 2003). Another study reported that ^{188}Rh and ^{177}Lu, a β emitter, were found to be highly beneficial in the radio immunotherapeutic approach of HPV-positive cervical cancer in an experimental mouse model (Phaeton et al. 2016). Its efficacy was compared with α as well as Auger electrons, and in several comparative studies, β emitters were reported to be less effective when compared to the α or Auger electrons. A comparative study proved the efficacy of high-linear energy transfer α emitter versus low β emitters in radioimmunotherapy of solid tumors in mice with colon cancer and reported that α emitters are therapeutically highly effective when compared to the β emitters (Behr et al. 1999). Similarly, another study suggested that the anticarcinogenic effect of β emitter (^{177}Lu)-labeled FAPI-46 (fibroblast activation protein targeted radiotracer) was relatively slow, but it lasted longer when compared to α emitter (^{225}Ac)-labeled FAPI-46 in pancreatic cancer models of experimental animals (Liu et al. 2022). Thus, there is a lot of debate, and research work is going on to prove the efficacy of β emitter when compared to others. It is the researcher's responsibility to bring clarity regarding the efficacy of the β-emitter RN in the management of cancer.

Auger electrons (AEs) are low-energy electrons released by RNs decaying through electron capture (e.g., ^{125}I and ^{123}I) (Ku et al. 2019). During its decay, the energy stays for a few nanometer-to-micrometer distances, resulting in a high-linear energy transfer that can kill malignant cells. In this way, AE is found to be a promising drug candidate in the management of cancer and its developmental research. Further, AE-tagged RN is linked to monoclonal antibodies or other targeting ligands (with or without nanoparticles) in order to act as a therapeutic agent for cancer treatment. AE compounds are documented as the most destructive RN to DNA when emitted close to the DNA. Hence, several researchers have

conducted plenty of preclinical studies to prove the efficacy of AE's tagged RN using experimental animal models. An experimental study reported that poly-adenosine diphosphate-ribose polymerase-targeted AE therapy was found to show a beneficial effect in p53 mutant colon cancer xenograft mouse models (Wilson et al. 2021). Further, a group of scientists from Johns Hopkins University School of Medicine proved that AE RPT targeting prostate-specific membrane antigen caused a considerable delay in the progress of metastasis and caused a longer survival rate without any chronic toxic effects in a murine model of prostate cancer (Shen et al. 2020). Another preclinical study from a Canadian research team showed that trastuzumab-resistant HER2-positive breast cancer was treated effectively with ^{111}In-Bn-DTPA-nimotuzumab in mouse models. Further, its exposure causes minimal body weight loss without any damage to the normal (non-tumor) adjacent tissues (Ku et al. 2019). In concordance to the beneficial effects of RPT in experimental animals, ^{161}Tb-PSMA (prostate-specific membrane antigen)-617 reported a better in vitro and in vivo positive response when compared to ^{177}Lu-PSMA-617 tumor-bearing mice (Müller et al. 2019). In another study, an Auger-emitting PARP inhibitor was validated with a higher survival rate in experimental mouse models of glioblastoma multiforme and also revealed the significant potential of ^{123}I-MAPi (iodine-123 Meitner-Auger PARP1 inhibitor) for human patients (Müller et al. 2019). Similarly, in a mouse bone cancer model, it was established that ^{195}mPt-bisphosphonate increases the death of metastatic malignant cells and opens up new research ideas for AE RPT bone metastases (Nadar et al. 2021). In yet another combination of therapeutic research, it was observed that when an AE emitter (^{195}mPt) is used with cisplatin treatment, the therapeutic impact of cisplatin on tumors is considerably enhanced without affecting the normal tissue damage in a mouse model of adenocarcinoma (Dikiy et al. 2008).

Therapeutic Radiopharmaceuticals from Animals to Humans

Available literature shows that several therapeutic RPs have been reported and are being used in higher dosages to treat certain types of cancer and other disorders. Among them, one of the RNs is ^{223}Ra (Parker et al. 2013), which is being used in the treatment of prostate cancer with bone metastasis when surgical intervention is not recommended. Supporting this, in a European study, patients with prostate cancer who were given alpha emitter ^{223}Ra intravenously at a dose of 50 kBq per kilogram of body weight had a higher overall survival rate, less myelosuppression, and fewer side effects. Another study discovered that giving men with bone metastatic castration-resistant prostate cancer ^{223}Ra improves their overall survival (Jarvis et al. 2021). Another compound, namely, samarium-153 ethylenediamine tetramethylene phosphonate, ^{153}Sm-EDTMP injection, is used to treat bone pain caused by various types of cancer. In Mexican patients with severe metastatic bone pain caused by kidney, prostate, and breast cancer, ^{153}Sm-EDTMP was found to be a palliative drug and the best alternative option in pain management (Correa-González et al. 2014). One RN, namely, ^{32}Cr phosphate, when administered through a pleural or peritoneal catheter, decreases fluid leakage caused by

cancer. Its injectable dosage showed beneficial effects on lymphoid tissue, ovarian, and brain tumors. In an Asian study, after lung cancer resection, treatment of ^{32}Cr phosphate inhibits postoperative occult lymphatic metastases and improves the patients' overall survival outcome (Gao et al. 2009). Additionally, another therapeutic RPT, namely, strontium chloride (^{89}Sr), is used to treat bone pain caused by malignancy. Combination therapy with strontium-89 chloride (^{89}SrCl2) has been found to be effective in treating painful bone metastases and destroying tumor cells in Japanese patients (Baba et al. 2018). A radio drug, namely, Lu-tetraazacyclododecane tetraacetic acid-octreotide (^{177}Lu-DOTATATE), is used to treat tumors of the gastrointestinal tract (Mittra 2018). The efficacy of ^{177}Lu-DOTATATE is supported by a study conducted in Oregon, which found that the abovementioned candidate medicine is quite helpful in the treatment of neuroendocrine tumors. A case study done in Minnesota proved to have a beneficial effect on recurrent meningioma (Mittra 2018). Sodium iodide ^{131}I is another RN being used clinically to treat thyroid cancer. Following thyroid cancer surgery, larger dosages of radio iodide are typically administered to eliminate any residual diseased thyroid tissue and its metastasis (Weeks and Grossman 2022). Iodine 131 tositumomab is also being used in the management of mantle cell lymphoma (Zelenetz et al. 2020). In addition to all the above, there is yet another radiochemical, namely, iobenguane ^{131}I, recommended as a good candidate drug in the treatment of pheochromocytoma and ganglioma (Ilanchezhian et al. 2020). Thus, it is evident from the above literature that RPT is really a boon to cancer patients, especially in increasing their survival rate.

Challenges

The usefulness of the RP is well known, and there is no doubt about its efficacy. However, many of these drugs are still under research, and they have to get approval from the drug regulatory bodies across the globe. Another challenge is the production of many therapeutic radioisotopes, and it has to be addressed by easing the regulatory guidelines for large-scale industrial production and marketing. Additionally, many of the drugs are covered under insurance coverage, and steps have to be taken to add these drugs under insurance schemes.

Conclusion

RPs are resurfacing as promising anticancer therapeutic agents apart from their well-established diagnostic usage. However, there are no universally accepted protocols or standardized techniques for assessing the validity prior to the start of early-phase trials. Even though RPT is a one-of-a-kind therapeutic approach with a well-understood mechanism of action, plenty of animal studies with preclinical models are necessary to assist RPT deployment in clinical settings. As mentioned earlier, RPT has very minimal adverse effects when compared to the regularly recommended treatment procedure. Considering disadvantageous therapeutic effects, more insights into animal models will pave the way forward toward

implementation of precision medicine in RPT. Precision medicine can be used in RPT to tailor individual patient therapy by changing the treatment protocol between patients or groups of patients. As more animal research for RPT has been elucidated, more specific and detailed advice on how to optimally combine RPT with surgical management and other routine therapies like chemotherapy, hormonal therapy, etc. may be provided. Thus, it can be concluded that animal model research on RPT leads to the development of novel RPT drugs, and, ultimately, it may not only decrease the mortality rate of cancer patients but also be a boon toward increasing the therapeutic effectiveness of cancer patients.

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Cancer Modeling: Modern Imaging Applications in the Development of a Unique Animal Model System to Analyze Cancer Advancement and Treatment

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Abstract

Nanoparticles are theranostic agents exerting both therapeutic and diagnostic purposes. Metallic nanoparticles can image tumor tissues by means of active and passive targeting. Many magnetic nanoparticles are currently approved by FDA. Hydrogel magnetic nanoparticle can carry chemotherapeutic agents and tumor-associated biomolecular binding with good magnetic susceptibility. Dextran-coated magnetic nanoparticles caused accurate cancer nodal staging. Many chemotherapeutics, e.g., methotrexate, doxorubicin, and paclitaxel were formulated with metallic nanoparticles. The uptake of gold and iron oxide nanoparticles conjugated with antibody against cancer antigens increased the precise cancer cells targeting. Multimodal multifunctional nanoparticles (as magnetic nanoparticles) are nanoparticles having different functional abilities in a single stable unit, e.g., a core nanoparticle attached to specific targeting ligands (for the surface molecules on target cells) and an imaging agent to trace the transport progress. Magnetic nanoparticles are excellent biosensors and bioimaging (contrast agents) agents upon using multiple imaging modalities. We suggest using multimodal multifunctional magnetic nanoparticles formed of magnetic nanoparticles conjugated with anti-ferritin antibody (to target surface cancer cells' ligands), positive charges (to target lactate of Warburg's effect), and anti-integrin antibodies (to target integrins on cancer cells surface) and to be coupled with high MRI resolution. Adjusting the size and surface coatings of magnetic nanoparticles using future research may reduce toxicity and improve magnetic behaviors. By focusing on improving their drug loading capacity, and increasing their specificity and affinity to target cancer cells, magnetic and multimodal nanoparticles may become suitable for clinical use with integrated imaging and multimodal therapy in the near future and dramatically impact the treatment of cancer.

Keywords

Theranostic agents · Metallic nanoparticles · Multimodal multifunctional magnetic nanoparticles · Tumour imaging · Animal model · Warburg effect

Introduction

Metallic nanoparticles emerge as new drug carriers, drug delivery systems, and contrast agents in both cancer diagnosis and treatment. Such nanoparticles are called theranostic (therapeutic and diagnostic) agents. Metallic nanoparticles can image tumor tissues by means of active and passive targeting. Iron nanoparticles are theranostic agents and contrast agents helping magnetically targeted drug delivery and MRI detection of tumors. Iron oxide nanoparticles include two imaging agent

Table 1 Criteria of optimal *in vivo* imaging agent should achieve the following goals and

Safe
Selective
Stable
Cheap
Easily prepared
Allows for follow up of given treatments
Can be traced and stopped when needed

categories: superparamagnetic iron oxide nanoparticles (SPIONs) and ultra-small superparamagnetic iron oxide nanoparticles (USPIONs). Hopefully, SPION nanoparticles have biocompatible and biodegradable properties of iron and follow the normal biochemical pathways of iron metabolism (Ahmad et al. 2010).

Unfortunately, an ideal *in vivo* imaging agent for proper cancer diagnosis and treatment is still beyond capabilities despite intensive research efforts. An optimal nanovehicle for both tumor imaging and treatment should achieve many criteria listed in Table 1.

Toward Ideal Diagnostic and Therapeutic Agents Based on Cancer Biology

Better understanding of cancer biology helps optimal preparation of tracing agents for better tumor targeting. This can be traced by commonly available radiological tools as CT, MRI, and PET-CT. The hallmark of cancer is the aerobic glycolysis-induced production of lactate (not pyruvate) even in the presence of oxygen (Warburg effect). Moreover, better understanding of surface agents that characterize cancer cells and distinguish them from normal cells is quite vital for proper drug delivery to cancer tissue with minimal off-targeting. Cancer cells exhibit the glycolytic phenotype (compared to normal cells), and extrude lactate outside cells exhibiting a negative surface charge. In addition, hyaluronan is expressed on cancer cells exterior. Collectively, this facilitates cancer cell targeting while sparing normal cells. Moreover, expression of CD44 on cancer stem cells (responsible for tumor recurrence after treatment) compared to normal stem cells helps targeting cancer stem cells and minimizing the recurrence possibilities. Designing imaging modalities linked to ligands (positive charges, anti-CD44, anti-hyaluronan, etc.) helps proper *in vivo* diagnosis, treatment, and follow up.

Reporter Systems

It is a big hope to generate reporter mice models for proper cancer diagnosis and treatment. Non-invasive *in vivo* imaging techniques help tracing single molecular events. This also allows to spatiotemporally investigate the molecular events causing neoplasia.

Recently, imaging techniques progress from detecting anatomical structures to dynamically analyze the molecular processes in viable organs (Massoud and Gambhir 2003). After that, many reporter systems successfully studied reporter gene expression that could be traced using *in vivo* non-invasive imaging techniques to investigate many biological processes in complex living organisms. Studied biological targets include transcription of target genes, protein-protein interaction, and signal transducers activation, e.g., proteases or protein kinases and calcium mobilization. Reporter mice giving those vital data may help studying the molecular modifications causing neoplastic transformation and growth for better studying of human cancers (Sharpless and Depinho 2006). This allows a better molecular study of tumor development from transformed state to metastatic disease.

Imaging Cancer Cells and Their Environment in Living Organisms

In the beginning, *in vivo* imaging modalities studied cancer cells injected into immunodeficient mice. Many injected tumor cell types expressed reporter genes. Different radiological investigations used optical imaging, magnetic resonance imaging, and positron emission tomography for imaging *in vivo* tumors. Reporters include fluorescent proteins (Green fluorescent proteins) or luminescent (Luciferases) proteins (Lowik et al. 2005), protein-trapping paramagnetic compounds (as ferritin chains that promote intracellular iron accumulation), and radiotracer-binding proteins (Genove et al. 2005). Such reporters were used for *in vivo* imaging. Tumor imaging usually aims at assessing animal survival, tumor dimension, and number of metastases. In addition, periodic serial imaging of tumors behavior (subjected to different experimental treatments) in xenograft animal models is done. This also allows early detection of micrometastatic secondaries and investigating the effects of given treatments. Assessing those parameters was not optimal using conventional imaging methods alone (Guller et al. 2021).

Biosensors of Key Genetic Processes in Cancer Cells

Reporter-based biosensors can detect the following:

(a) Gene transcription activity (via sensing transcriptional factors or gene promoters)
(b) Protein–protein interactions
(c) Sub-cellular localization
(d) Proteases activity

These biosensors are available commercially for use in animal models.

Table 2 Merits of superparamagnetic iron oxide nanoparticles (SPIONs)

Easily visualized using MRI
Magnetic field gradients provide a source of contrast for MRI.
Can be guided to target cancer cells using external magnetic field,
Can be heated to provide hyperthermia for cancer therapy (Yu et al. 2008).
Their magnetism vanishes in the absence of applied magnetic fields (Landmark et al. 2008; Gupta et al. 2007).
SPIONs are biocompatible (degraded into nontoxic iron ions *in vivo* that are metabolized physiologically).
SPIONs' efficacy is not affected by surroundings (Landmark et al. 2008).

Magnetic Nanoparticles Are Promising Cancer Theranostics

With early cancer detection, the cure rate is greatly better helping decreasing both morbidity and mortality rates of patients. Tumor imaging helps cancer diagnosis and selecting best clinical treatment options. Nanoparticles play vital roles in cancer imaging, diagnosis, drug delivery, and treatment. Magnetic nanoparticles present in the superparamagnetic state (Briguet et al. 1988) were extensively researched in cancer imaging. They have a lot of merits (Table 2).

Using xenograft animal models (both orthotopic and ectopic) expressing a reporter system eases the imaging and measurement of tumor parameters. Updating and optimizing the chosen reporter/imaging system accelerates the preclinical development of novel anticancer drugs. Magnetic nanoparticles are fine nanomagnetic materials having magnetic response, small particle size, large "specific surface area," and superparamagnetism (Yarar et al. 2016). Magnetic nanoparticles are constructed under a constant magnetic field, where heat is absorbed by the electromagnetic field.

Application of Nanotechnology in Cancer Imaging

This occurs via nano detection for sensing proteins and cancer cells and nano vector formulation for high-contrast imaging (Mccarthy and Weissleder 2008; Mccarthy et al. 2007). Dual-mode nanoparticles can be imaged with MRI and optical imaging. More than 60% of patients with breast, lung, colon, prostate, and ovarian cancer have hidden or overt metastatic colonies at the time of presentation (Menon and Jacobs 2000). Currently MRI, positron emission tomography, single photon emission tomography, and computed tomography are noninvasive imaging modalities used for cancer detection in humans enabling earlier detection of metastases. Nanoparticle-enhanced MRI improved identification of lymph node metastasis in solid tumors via identifying histologically positive nodes outside the usual field of resection (Harisinghani et al. 2003; Harisinghani and Weissleder 2004).

Nanoparticles used for diagnostic imaging, e.g., nanorobotics are quite vital for cancer diagnosis and follow up of cancer treatments. Novel nanotherapeutics as nanoemulsion, solid lipid nanoparticles, dendrimers, polymeric nanoparticles, nanostructured lipid carriers, nanocapsules, and nanosponges-based approaches are also of utmost importance for healthcare (Wang et al. 2016).

Magnetic Particle Imaging

Limitations to MRI in diagnostic imaging include long scan times, lack of standards to quantify MRI clinical use, inefficient identification of false positive results, a possible risk to patients (radiation exposure), and systemic accumulation or nephrogenic systemic fibrosis in certain patients. Based on that, there is a true need for more ideal imaging modalities for efficient, safe, specific, ultra-sensitive, multipurpose imaging system to get quantifiable 3D images.

Cheng et al. reported successful *in vitro* and *in vivo* destruction of glioma cells using spin-vortex, disk-shaped permalloy magnetic particles in a low-frequency rotating magnetic field (Cheng et al. 2016). Magnetic hyperthermia increases the efficacy of chemotherapeutic drugs to some extent, e.g., using SPIONs (MF66) functionalized with nucant multivalent pseudopeptide (N6L), doxorubicin, and magnetic hyperthermia increased the therapeutic effects of magnetic hyperthermia in breast cancer (Kossatz et al. 2015). Moreover, dual-functional Pt-Fe-HAP was developed for magnetic nanoparticles application for chemo-hyperthermia treatment of lung cancer (Tseng et al. 2014). Interestingly, magnetic nanoparticle hyperthermia enhances radiation therapy in a study in mouse models of human prostate cancer (Attaluri et al. 2015).

Benefits of Magnetic Particle Imaging Include

1. Better quantitative distribution of magnetic nanoparticles quantity with high spatial and temporal resolutions using magnetization of SPION tracers.
2. Creation of more clear images via making use of diamagnetic properties of tissues and organs.
3. Better quantification of the location and amount of SPIONs irrespective of tissue depth.
4. Better tissue penetration.
5. Diversity in cancer applications (both diagnosis and treatment).

Magnetic particle imaging allows high spatial resolution and sensitivity as a tomographic imaging technology. A recent report confirmed that super paramagnetic iron oxide nanoparticles (SPIONs) for magnetic particle imaging of head and neck cancer cells where SPIONs are a promising tracer material for use in innovative tumor cell analysis in magnetic particle imaging (Lindemann et al. 2014).

A breast imaging technique was presented combining near-infrared dye-labelled amino-terminal fragments of urokinase plasminogen activator, receptor-targeted magnetic iron oxide nanoparticles with high-resolution near-infrared light-induced photoacoustic tomography for imaging breast cancer using a murine model of orthotopic mammary cancer (Xi et al. 2014). Other reports confirmed that ultrashort echo time imaging (Wang et al. 2014) and magnetic particle imaging (Nishimoto et al. 2015) may improve the detection of magnetic nanoparticles in cancer.

Using single-photon emission computed tomography (SPECT) and MRI helped dual imaging of pancreatic and breast cancer (Rosenberger et al. 2015; Deng et al. 2015). A combination of MRI and optical imaging for diagnosing breast cancer was reported (Sun et al. 2014).

Controlling heat generation for tumor cell killing using magnetic hyperthermia needs improving the following determinants that include:

- Magnetic field strength and frequency
- Nanoparticles size
- Nanoparticles concentration
- Solution viscosity (Shah et al. 2015)

Examples of Nanoparticles Success in Different Organs Tumors

Pancreas Cancer

Conjugating chitosan-coated magnetic nanoparticles and survivin antisense oligonucleotides resulted in forming magnetic nanoparticles that are functionalized with antisense oligonucleotides causing targeted localization in pancreatic tumors, e.g., nanoparticles targeting survivin can be used by MRI for detecting pancreatic tumors (Wang et al. 2016).

Breast Cancer

An optimization of the optimal measurement configuration for magnetic nanoparticles-enhanced breast cancer microwave imaging was reported. The authors outlined some guidelines for the design of the imaging device for magnetic nanoparticles to enhance the microwave imaging of breast cancer. Magnetic nanoparticles-enhanced microwave imaging may reliably detect cancer lesions even using low-complexity arrangements (Bucci et al. 2014). Interestingly, nanoparticles encapsulation inside liposomes improved the delivery and retention of SPIONs in breast tumors and significantly increased nanoparticles quantities inside tumors. That may be ideal for MRI detection of breast tumors (Kato et al. 2015). Moreover, promising results were reported regarding tumor imaging using nanoparticles formed of magnetic nanoclusters coated with ruthenium (II) complexes with silica (fluorescent magnetic nanoparticles); magnetic nanoparticles; and cyclic-arginine-glycine-aspartic acid peptide. Magnetic nanoparticles

accumulated around the tumors. The result indicated that such nanoparticles can be of diagnostic imaging significance for detection of breast cancer using MRI and optical imaging (Sun et al. 2014). Interestingly, a compressive sensing approach for three-dimensional breast cancer microwave imaging was reported using a compressive sensing algorithm to assess the maximum concentration of magnetic nanoparticles that may be targeted in human tissues (Bevacqua and Scapaticci 2015).

Prostate Cancer

MRI is the best imaging modality for patients with prostate cancer providing the best soft tissue resolution (Guneyli et al. 2016). Patients underwent MRI before and one day after SPION injection. This was the first study to use intraprostatic injection of SPIONs to visualize Sentinel lymph nodes by MRI in patients with prostate cancer (Winter et al. 2016). Using a transrectal intraprostatic injection of SPIONs for magnetic marking in prostate cancer is safe, feasible, and reliably identifies lymph node metastases in the majority of patients (Winter et al. 2015).

Lung Cancer

To improve the sensitivity of detection of metastasis of lung cancer, immune SPIONs (coated with oleic acid and carboxymethyl dextran and then conjugated to mouse anti-CD 44v6 monoclonal antibody) were reported in magnetic resonance immune imaging (Wan et al. 2016). SPIONs help lung tumor-targeting diagnosis and can be used via pulmonary inhalation for aerosol-based delivery helping direct controlled entry of the drugs to the lungs. Interestingly, the application of magnetic nanoparticles-based imaging to pulmonary tissues was reported using nebulized magnetic nanoparticles (Nishimoto et al. 2015).

Photodynamic therapy is a minimally invasive line for cancer treatment involving systemic or local application of photosensitizing drugs (photosensitizers), followed by photoexcitation of the photosensitizers in the tissues using light of an appropriate wavelength and power (Li et al. 2014). In the presence of oxygen, photosensitizers are excited from ground state to excited state where electrons are transferred forming free radicals causing cancer cell damage. Targeted drug delivery system with magnetic nanoparticles is of interest to potentiate the effects of photodynamic therapy (Cheng et al. 2016). Fe_3O_4@HP particles exhibited potent photodynamic anticancer activity, and strong anticancer effects against human prostate cancer cells and breast cancer cell lines (Nam et al. 2016).

Photothermal Therapy

The photothermal effect of magnetic nanoparticles clusters was initially reported for the photothermal ablation of tumors *in vitro* and *in vivo*. Magnetic nanoparticles

clusters are effective for photothermal therapy with near-infrared irradiation. Compared to individual magnetic iron oxide nanoparticles, clustered Fe_3O_4 nanoparticles may result in higher temperatures that are more cytotoxic against lung cancer cells (Shen et al. 2015). The size of magnetic nanoparticles is an important factor for photothermal therapy. Small iron oxide nanoparticles caused greater cellular internalization, thus facilitating a higher photothermal ablation efficacy *in vitro*. In addition, 120 nm may be the optimal diameter of iron oxide nanoparticles for MRI and photoacoustic tomography *in vitro* (Guo et al. 2016b).

Bhana et al. (2015) reported the novel use of near-infrared-absorbing gold nanopopcorns (magnetic-optical hybrid nanosystems) with iron oxide cluster core for magnetically amplified photothermal and photodynamic drug delivery for cancer therapy (magnetic-field-guided) (Bhana et al. 2015). Those composite nanoparticles may generate heat and reactive oxygen species (ROS) simultaneously upon laser irradiation that can be directed selectively toward cellular mitochondria (mitochondria-targeting magnetic composite nanoparticles for enhanced phototherapy of cancer) (Guo et al. 2016a).

Magnetic Nanoparticle-Guided Drug Delivery

Magnetic-guided drug delivery helps improving the therapeutic effects of drugs and minimizing drugs side effects associated with cancer chemotherapy. Magnetic nanoparticles constitute a high-efficiency drug delivery system for better drug targeting. Magnetic nanoparticles with a supporting shell are good contrast agents for MRI (Khalkhali et al. 2015). Magnetic nanoparticles are quite vital for drug delivery particularly when specific targeting is enhanced via attaching magnetic nanoparticles to antibodies, chemotherapeutics, or other drugs. Magnetic nanoparticles may be used as drug carriers. Nigam and Bahadur demonstrated the fabrication and characterization of the dendrimerized magnetic nanoparticles as carriers for the anticancer compound, epigallocatechin gallate (Nigam and Bahadur 2016).

Recent studies had demonstrated that antibody-conjugated magnetic nanoparticles linked to antibodies help ovarian cancer diagnosis using multiplexed magnetic nanoparticle-antibody conjugates (magnetic nanoparticles-based prognostic detection of ovarian cancer biomarkers). Antibody conjugated magnetic nanoparticles linked to antibodies help also cancer treatment owing to their profuse accumulation within cancer cells (Pal et al. 2015). A promising report by Wang et al. confirmed the successful *in vivo* targeting of gastric cancer using anti-α-subunit of adenosine triphosphate synthase monoclonal antibody that was conjugated to fluorescent magnetic nanoparticles (Wang et al. 2009).

Rasaneh and Dadras suggested combining magnetic nanoparticles with herceptin antibody to increase its therapeutic efficiency via increasing magnetic nanoparticles accumulation at the tumor site (Rasaneh and Dadras 2015). Coating multifunctionalized iron oxide magnetic nanoparticles to anti-CD44 antibody together with gemcitabine derivatives was reported for treating CD44-positive cancer cells (Aires et al. 2016). Moreover, Huang et al. reported ovarian cancer targeting using

magnetic Fe_3O_4 nanoparticles carrying docetaxel loaded β-cyclodextrin and coated with single-chain antibody for treating ovarian cancer (Billings et al. 2021). Doxorubicin is the most widely used chemotherapeutic agent in targeting delivery systems for treating cancer. Unfortunately, magnetic nanoparticles have limited stability owing to their hydrophobic coating (Singh et al. 2013). Interestingly, a reducible copolymer formed of polyamidoamine-magnetic iron oxide self-assembled nanoparticles (SPIONs) was designed for doxorubicin delivery for cancer therapy (Chen et al. 2014). The application of iron oxide magnetic nanoparticles improved doxorubicin-nanoparticles cell penetration compared to doxorubicin alone with the same therapeutic effects of doxorubicin alone (Augustin et al. 2016). Cicha et al. reported the therapeutic benefits of SPIONs as drug delivery system via analyzing cellular responses to mitoxantrone-carrying SPIONs and mitoxantrone released from SPIONs.

Alternative and integrative medicine also shared in drug delivery systems. Polymer-coated magnetic nanoparticles for curcumin delivery to cancer cells was reported (Mancarella et al. 2015). In addition, multilayer coated magnetic nanoparticles was reported as biocompatible curcumin delivery platforms for drug carriers for the delivery of curcumin, to treat breast cancer (Akrami et al. 2015). Curcumin has been widely applied in the drug delivery of magnetic nanoparticles in breast and ovarian cancer where Mancarella et al. (2015) reported a layer by layer functionalization of ferric oxide nanoparticles via coating them in dextran and poly (L-lysine). That resulted in increased upload of curcumin in ferric oxide nanoparticles for treating ovarian cancer (Mancarella et al. 2015). Furthermore, magnetic ferric oxide nanoparticles with hydroxyapatite-PEI-b-cyclodextrin were reported to be effective for pH-sensitive and magnetically guided drug delivery (Kalita et al. 2016).

Nanoparticles for siRNA Delivery for Cancer Treatment

Nanosystems exert a dual-purpose for both *in vivo* transfer of siRNA and imaging of its accumulation in cells using MRI and near-infrared optical imaging (Medarova et al. 2007).

Magnetic Nanoparticles for Dual Imaging and Drug Delivery

Lee et al. (2007a) used cross-linked superparamagnetic iron oxide nanoparticles for dual cancer imaging using MRI and optical techniques. SPIONS effectively detected tumors *in vivo* by dual imaging even without having any attached targeting ligands. This preferential concentration at tumor sites was explained by the EPR effect (Lee et al. 2007a).

Yu et al. successfully used SPIONs for delivering doxorubicin to lung carcinoma cells-bearing mice (causing 63% inhibition of tumor growth) versus 38% inhibition

in free doxorubicin group (without SPIONs) (Yu et al. 2008). Importantly, SPIONS were non-toxic and do not cause myelosupression.

Tumors overexpress integrins (as αvβ3 integrins) that are attractive targets using nanoparticles. Interestingly, Arginine-glycine-aspartic acid is a tripeptide sequence that binds to integrins. Linking this tripeptide fraction to a fluorescently labeled iron oxide nanoparticle helped targeting human breast cancer cells and rat gliosarcoma cells overexpressing αvβ3 integrins (Montet et al. 2006).

Interestingly, superparamagnetic nanoparticles conjugated with Cy5.5 dye were excellent dual purpose probes that transferred siRNA *in vivo* and simultaneously imaged its accumulation within the tumor cells dextran-coated. Interestingly, that was monitored by MRI and optical imaging *in vivo* where the silencing process was detected by optical imaging (Medarova et al. 2007).

Multifunctional/Multimodal Nanoparticles

Multimodal multifunctional nanoparticles (as magnetic nanoparticles) are nanoparticles having different functional abilities in a single stable unit, e.g., a core nanoparticle attached to specific targeting ligands (for the surface molecules on target cells) and an imaging agent to trace the transport progress. Functions exerted by magnetic nanoparticles depend on the components attached, e.g., tumor targeting moieties, fluorescent molecules, anticancer drugs, or siRNA (Liong et al. 2008). Magnetic nanoparticles are excellent biosensors and bioimaging (contrast agents) agents using multiple imaging modalities as X-ray, ultrasound, MRI, and fluorescence. Magnetic nanoparticles facilitate intra-operative imaging for precise tumors resection.

Trastuzumab is a monoclonal antibody binding to HER2, (an overexpressed marker in breast and ovarian cancer) is also conjugated to magnetism-engineered iron oxide nanoparticles. That enables this multimodal nanoparticle to detect cancer upon using MRI imaging (Lee et al. 2007b). Moreover, SPIONs exceed radioisotopes for better preoperative MRI images for mapping sentinel lymph nodes.

Bagalkot et al. designed a nanoparticle composed of quantum dots, an aptamer (for cell-specific targeting) and doxorubicin that did excellent *in vitro* targeted imaging and sensed drug release (Bagalkot et al. 2007). Wang et al. designed magnetic nanoparticles using Fe_3O_4 (magnetite) nanoparticles coated with gold nanorods (PEGylated Aurods that have stronger magnetization than bare Fe_3O_4 particles as MR contrast agents and fluorescence-imaging agents) and trastuzumab to target breast cancer cells. That caused a significantly increased rate of internalization inside cancer cells (Wang et al. 2009). Medarova et al. designed magnetic nanoparticles consisting of SPION particles and Cy5.5 attached on a peptide recognizing uMUC-1 (a tumor antigen in 90% of breast cancers that predicts chemotherapeutic response) that monitored antigen expression, tumor size, and patient's response to chemotherapy using MRI. That resulted in better visualization, reduction of tumor mass, and decreased uMUC-1 expression (Medarova et al. 2007).

Personalized Therapeutic Response

Light-activated theranostic nanoparticles helped imaging and treatment of brain tumors and improved the survival in tumor-bearing animals from 13 to 33 days, with 2 animals having disease-free survival within 180 days of therapy (Reddy et al. 2006). Makino et al. successfully coupled near-infrared fluorescence (penetrated deeper into the tissues) and nanoparticles in targeting drug delivery for liver cancer (Makino et al. 2009). Interestingly, 100% cancer cells death was imaged when human transformed macrophage were incubated with nanocarriers for 60 min with laser illumination (Mccarthy and Weissleder 2008). Moreover, Alexa 750 near-infrared fluorescence dye coupled with phospholipid micelle facilitated rapid imaging of tumor in breast cancer models (Papagiannaros et al. 2009).

SPIONs conjugated with monoclonal antibodies helped diagnosing different cancers. SPIONs conjugated with peptides (as transferrin and pancreatic receptors that are overexpressed in many tumors) helped targeting these receptors and imaging cancer cells. Many magnetic nanoparticles are currently approved by FDA (Wang et al. 2001). Hydrogel magnetic nanoparticle can carry chemotherapeutic agents and tumor-associated biomolecular binding with good magnetic susceptibility (Anderson et al. 2010). Moreover, dextran-coated magnetic nanoparticles caused accurate cancer nodal staging (Sunderland et al. 2006; Harisinghani and Weissleder 2004). Many chemotherapeutics, e.g., methotrexate, doxorubicin, and paclitaxel were formulated with metallic nanoparticles. Also, SPIONs were conjugated with Herceptin antibody and targeted Her2/neu receptor in breast cancer (Ross et al. 2004).

The uptake of hybrid (gold and iron oxide) nanoparticles conjugated with antibody against A33 cancer antigen on cancer cells was five times higher in A33-expressing cells than normal cells (Kirui et al. 2010). Gold nanoshells conjugated with hER2 antibody effectively targeted breast carcinoma cells (Loo et al. 2005). Hainfeld et al., reported that combination of gold nanoparticles followed by X-ray treatment reduced the size of tumors in mice (Hainfeld et al. 2004). Huang et al. (2006) reported increased cellular uptake of gold nanorods (conjugated with antibody targeting ant epidermal growth factor receptor) in malignant cells (Huang et al. 2006).

Best Animal Model for Tumor Imaging and Therapy

We suggest using multimodal multifunctional magnetic nanoparticles formed of magnetic nanoparticles conjugated with anti-ferritin antibody (to target surface cancer cells' ligands), positive charges (to target lactate of Warburg's effect), and anti-integrin antibodies (to target integrins on cancer cells surface) and to be coupled with high MRI resolution. That should be attached to sensitive and semi-quantitative tracer detection provided by an optical imaging modality. Reporter half-life is a vital parameter that should be precisely evaluated in animal models.

Future Perspectives

Magnetic nanoparticles are promising contrast agents and adjuvants for both cancer model imaging and therapy. Surface coatings of magnetic nanoparticles help more precise cancer cells targeting. However, toxicity issues related to magnetic nanoparticles should be balanced to their benefits. Adjusting the size and surface coatings of magnetic nanoparticles using future research may reduce toxicity and improve magnetic behaviors. Despite many successful studies using magnetic nanoparticles as a theranostic material, there are still some challenges. While many magnetic nanoparticles formulations have demonstrated excellent results in small animal models, they cannot satisfy the targeted clinical requirement. By focusing on improving their drug loading capacity and increasing their specificity and affinity to target cancer cells, magnetic and multimodal nanoparticles may become suitable for clinical use with integrated imaging and multimodal therapy in the near future and dramatically impact the treatment of cancer.

Conclusion

In conclusion, nanoparticles are theranostic agents exerting both therapeutic and diagnostic purposes. Metallic nanoparticles, hydrogel magnetic nanoparticle, and dextran-coated magnetic nanoparticles carry a lot of hope in improving cancer imaging. Many chemotherapeutics, e.g., methotrexate, doxorubicin, and paclitaxel, were formulated with metallic nanoparticles. Multimodal multifunctional nanoparticles improved specific cancer imaging and targeting via attaching to specific ligands. Magnetic nanoparticles are excellent biosensors and bioimaging (contrast agents) agents upon using multiple imaging modalities. More research efforts are needed to optimize cancer imaging and treatment.

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The Molecular and Biochemical Variations During Cancer Prognosis in Mouse Models

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Contents

Abstract

Cancer is a dreadful disease that causes morbidity and mortality; cancer is caused due to metabolic reprogramming and genetic variations. Experimental studies have shown that some cancers are associated with translocation of genes; furthermore, mutations in tumor suppressor gene lead to disruption of cell division, loss of apoptosis, initiation of angiogenesis, and inhibition of endogenous inhibitors. Soon it became important for the researcher and clinicians to carry out studies to understand the physiological changes, signal transductions, and gene expression in cancer development and metastasis. Further, using animal models was endorsed; employing animal models for cancer research have had paved the

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way for other discoveries such as the experimental studies performed by George snell by transferring tumor cells from one mice strain to another led to the pioneering of MHC molecules that caused the rejection of transplants; further, the ornithological similarities were contemplated in humans and mice. Thus, this led to the establishment of mouse models for performing tumor immunotherapy. Amidst performing preclinical cancer studies of specific protein molecules, researchers confronted various other phenotypic changes, for example, CTL4 lacked mice-expressed autoimmune phenotype. Recently, a knockout mice miRNA21 was developed to study the changes in the lack of miRNA21 as it is highly expressed in all types of cancer; further antisense sequence was designed to silence the miRNA in the mice model leading to the gradual decrement of tumors in mice bodies. Hence, animal models could be flexibly used to perform studies at the molecular level. Considering the genetic similarities between humans and mice, preclinical oncogene-targeted drugs can be tested which provides us with insight regarding the cytotoxicity and efficacy of the drugs. Based on the studies, suitable animal models can be developed like patient-derived xenografts mice models were used to prognosticate cogency of anticancer drugs. Therefore, various alternatives are opted in modifying the animal model which is suitable for diverse study types such as transplanting human cells derived from the specific organ to the orthotropic organ of mice, and use of syngeneic or transgenic mice is also a preferable option. Therefore, the objective of indulging animal models for cancer research is to advance new effective and non-toxic cancer therapies with minimal side effects; results of preclinical studies have paved the way in speculating the cellular changes confined to cancer types in humans.

Keywords

Transgenic mice · Knockout mice · Knock-in mice model · Immunocompromised mice · Oncogene · Anticancer drugs · Monoclonal antibodies · Patient-derived xenografts

Introduction

Conducting human research under laboratory conditions has always been challenging; maintaining accurate human physiological conditions in vitro has led to unsuccessful outcomes. The limitations and outcomes led to the increment of augmented demand for animal models which provided the researchers to carry out our preclinical studies. Consequently, the mouse was a well-defined and convenient model for human biology; besides sharing genetic similarities with humans, mouse models were flexible for in vivo research, and the other remarkable benefits were the mouse models that could be genetically modified to sustain studies of our interest. Over the period, various methodologies were inculcated to develop mice models with human tumor tissue grafts; multiple mice strains were developed which proved to be

exclusive for cancer research. Despite genetic similarities, several restraining factors in the host prevent the approval of human xenografts; to overcome such constraints, variations have been made at the genetic level to archive a useful xenografts engraftment for an example targeted mutations at IL-2 receptor γ chain locus in NOD/SCID strain of mice which constitutes lack of NK cells; therefore, an improvised immunocompromised model is best suitable for preclinical cancer studies for immunotherapy and other immunological studies such as inhibition of CTL4 using monoclonal antibodies. Another methodology used for developing mice models is the transgenesis process used to introduce a new gene into the genome of mice such as mice with the addition of new gene of interest are termed transgenic mice; these models are widely used to conduct studies such as gene regulation, molecular and biochemical mechanism of disease development, and immunological studies. Oncogenes have been known to play a critical role in cancer cell transformation and prognosis; knockout mice were used to study the function of oncogenic genes of human cancer with orthogenic oncogene in mice models. In knockout mice, the functional gene was replaced by a nonfunctional gene, and further, the physiological significance of the functional gene in its absence was capsulized. Therefore, developing mice models with mutations in oncogenes has allowed us to explicate the function of gene products in vivo which gives us the insight to predict the responses of such mutation in human oncogenes and find an optimum therapy for the cancer types. Cancer is a cause of multifactorial events occurring within human cells; furthermore, oncogene products aid the process of tumorigenesis in certain types of cancer; cancer cells have the potential to secrete several growth factors and signaling molecules to support and sustain invasion and metastasis. Cancer can be defined as a disease in which there is an uncontrolled growth and spread of cells observed. Generally, cells divide and grow whenever required by the body, and as soon as those cells grow old, they die by the process of apoptosis, but in the cancerous cell, the apoptosis does not occur, and the uncontrolled growth of cells leads to the formation of lumps of tissue known as tumors. These tumors can be malignant, i.e., cancerous, or benign, i.e., non-cancerous. The tumors can spread to different parts of the body and start forming new tumors; this process is known as metastasis. Abnormal changes in the genetic material lead to cancer. Proto-oncogenes, tumor suppressor genes, and tumor suppressor genes are the genes that when affected contribute to cancer. There are many types of cancer depending on the organ and tissue getting affected; some of them are as follows:

- Carcinoma: This type of cancer occurs due to the epithelial cells getting affected and is one of the most common types of cancer. Epithelial cancer is also of different types depending upon which type of epithelial cell it gets affected like the squamous cell carcinoma which affects the squamous cell (cells that line many organs like kidneys, bladder, etc.).
- Sarcoma: This rare cancer occurs in the bone and soft tissues. The most common type of soft tissue cancer is Kaposi sarcoma, while osteosarcoma is the most common type of bone cancer.

- Leukemia: Cancer that affects the blood-forming tissue of bone marrow is known as leukemia. In this type of cancer, large numbers of abnormal white blood cells build up in our blood instead of the formation of solid tumors which then leads to the crowding out of normal blood cells, due to which it is tougher on the body to deliver oxygen to its cells. Leukemia is grouped depending upon how quickly cancer gets worse, i.e., acute or chronic or on what type of blood cell cancer first starts in.
- Lymphoma: Lymphoma begins in lymphocytes which are the T and B cells. These abnormal cells build up in lymph nodes, lymph vessels, and other organs of the body. Hodgkin lymphomas that normally form from B cells and non-Hodgkin lymphoma are the two main types of lymphomas.
- Melanoma: This is the type of cancer that begins in the cells that make melanin. Melanomas are generally seen on the skin but sometimes can also be formed in pigmented tissues like the eyes (https://www.cancer.gov/about-cancer/understanding/what-is-cancer).

The therapeutic progress entirely relies on the preclinical studies to understand various physiological mechanisms supporting pre- and post-tumorigenesis of cells subjected to oncogene mutations or mutations in tumor suppressor genes, abnormal cell division, and proliferation at in vivo conditions using mice models. Such studies at molecular and sub-molecular levels have paved the way in designing protein-specific monoclonal antibodies for immune checkpoint inhibition; combinational therapy, oncogene-targeted drugs, and stem or bone marrow transplantation have been multiple alternatives for curing cancer which could be possibly derived based on the studies performed in vivo using animal models.

Transgenic Mice

In the 1980s, the first transgenic mice were established which was genetically modified to express oncogenes of interest; the cloning of foreign genes into the mouse genome was a useful discovery. Molecular cloning of viral and cellular oncogenes showed that mice carrying foreign could develop cancer. Consolidating all the results obtained from experimental studies, it was further hypothesized that oncogenes expression in noncancerous cells of mammalian tissue led to the development of cancer. By 1992, knockout mice were eventuated with the generation of mice that lacked tumor suppressor genes; this allowed the researchers to study the functionality of tumor suppressor genes and their role in cancer progression which also helped to predict their role in human cells (Jacks 1996). The knockout mice helped the researchers to study the certain genes coding for a specific protein a whole genome, one or more gene expressions can be validated at a time, and its relevance in cancer disease is studied. The development of knockout mice is usually done by deleting the exon that consists of the start codon thereafter blocking transcription and translation. A careful and steady molecular characterization of genetically altered alleles is important as in some cases, despite blocking the start codon, alternative

start codon or alternative splicing can produce a truncated protein which functions to induce premature termination of mRNA translation and thus conceals the significance of the gene of interest. It is important to corroborate whether the function of a gene is completely lost or inactivated while performing biological loss-of-function studies (Walrath et al. 2010). Translational research focuses on developing treatments, interpreting experimental data, testing the efficacy and potentiality of the therapeutics, and further implementing the research of clinical studies; about this, gene knockout mouse models have paved its way in evaluating the potentiality of targeted therapies for cancer treatment. Creating cancer models that mimic the accurate wild type of cancer environment found in humans is of a great deal as some of the gene function differs in mice and humans. For example, if the mouse can utilize an alternative gene product for cancer, the development pathway contrary to this humans might not utilize the same gene product; thus, based on this, the experimental results will not be coherent. Knockout models have been crucial in understanding the cause and factors supporting cancer developed at the molecular level by assessing the genes categorized as oncogenes which function as proto-oncogenes in normal cells, tumor suppressor genes, and metabolic genes. Further, to classify certain genes as tumor suppressor genes would rely on the development of tumor under the mutated state of the gene that can impart or instigate tumor development. During the preclinical in vivo studies, it has been noticed that deletion or mutation of the gene of interest via various recombinant techniques at the embryonic stage does not depict the accurate tumorigenesis residing in humans; therefore, such deletions or modifications of the gene from the entire body lead to subsequent abnormalities leading to the death of the animal model at an embryonic stage which halts the study of the gene of interest. To subdue such difficulties, genetic engineering techniques have been developed to induce changes at the genetic level which is specific to the tissue and gene. Mouse models with conditional gene mutation were developed to replace the conventional knockout models by using a recombinant strategy such as the Cre-lox P recombinant system which is a site-specific recombinase that catalyzes the site-specific homologous recombination between defined 34 bp of lox P region (Branda and Dymecki 2004). This recombinant process proved to be a useful tool for studying genes of interest at specific tissue; the function of Cre was first observed in bacteriophage P1 which infects *E. coli*. Furthermore, one of the key features of Cre recombinase is to perform site-specific recombination, that is, it catalyzes recombination process at the lox P site as shown in Fig. 1. This system inculcated to modify the traditional process in which knockout gene is carried by every cell of transgenic mice; this proposed various limitations such as death at embryonic state or abnormalities in other organs which were not suitable for performing studies relevant to human cancer.

Now the protocol to develop a double transgenic mouse had various steps, and the first step was isolation and insertion of Cre gene at the cell-specific promoter region, therefore keeping it under the control of the promoter, and the next process was delivering the Cre gene into the mice. Diverse types of Cre delivery methodologies have been reviewed, and tetracycline-induced delivery system was extensively used to control Cre gene expression temporally and structurally in mice models (Berlinger

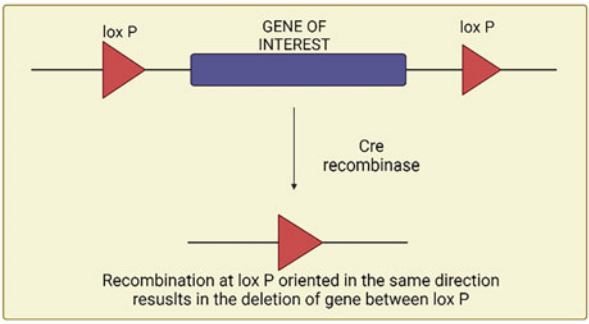

Fig. 1 Site-specific catalysis of Cre recombinase

et al. 2000). Here the expression of transgene which is Cre, in this case, is based on the presence or absence of tetracycline; for the experimental purpose to avoid cytotoxicity, non-toxic analogue doxycycline is used which functions as a regulator for transgene expression. This system consists of two functional units; the product of one unit is required for the initiation of gene expression on another unit. Being an inducible system, it can be studied under two different conditions. The first condition, which is termed tet-off, is caused in the presence of doxycycline; the expression of Cre is turned off. The tet-off system is dependent on the hybrid protein which is composed of tetracycline repressor and an amino acid sequence; this protein hybrid is crucial for transcription functioning as a transactivator (tTA) (Gossen and Bujard 1992). The function of tTA is controlled by doxycycline, and gene encoding tTA is under the control of the cell-specific promoter. The promoter that directs the transgene consists of set of tetracycline operating sequences (tet O) which is situated upstream of the eukaryotic promoter. Hence, binding of tTA to tet O is necessary for transgene transcription; in addition, binding of doxycycline to tTA leads to conformational changes and inhibiting it from binding to the tet O; as a result, there is no expression of the transgene as depicted in Fig. 2. Doxycycline acts as a switch for the transactivator (Hadjantonakis et al. 2008). On the other hand, a tet-on system is also devised in which the tTA is mutated which is termed as reversed tetracycline transactivator (rtTA); this system works in the presence of doxycycline which binds to the rtTA causing structural modifications which further induces its binding with tet O resulting in transgene expression (Das et al. 2016).

Therefore, this leads to the development of transgenic mice with tissue-specific Cre genes. Further, transgenic mice carrying lox P flanked sites are developed; lox P site with repeat sequence towards the same direction is inserted at either side of the exon carrying the gene of interest. Lox p floxed sequence is then inserted into the chromosome of embryonic stem cells by homologous recombination. ESC carrying the genetically modified gene is cultured and selected and further microinjected into the blastula which is then implanted into the mouse. Transgenic mice carrying the tissue-specific Cre gene is mated with transgenic mice inserted with lox p flanked

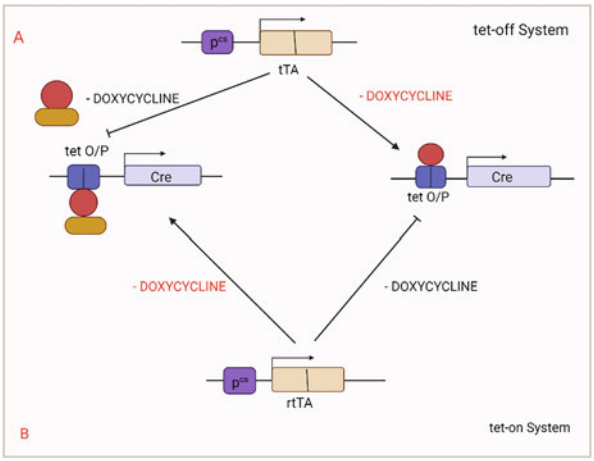

Fig. 2 Tetracycline inducible system for Cre transgene expression. Expression of Cre recombinase in double transgenic mice depends on the binding of transactivator tTA to tet O, and the transactivator is under the control of inducer which is doxycycline in this system. A. In a tet-off system, the addition of doxycycline inhibits the binding of tTA to tet O, and in its absence, transgene expression is not halted; this was an expression of Cre that can be controlled to produce conditional knockout gene at tissue in a specific site. B. In the tet-on system, the transactivator is mutated forming a reverse process in which the addition of doxycycline promotes the binding of rtTA to tet O as binding of doxycycline with rtTA causes conformational changes leading to transcription of Cre gene; furthermore, the absence of doxycycline inhibits the binding of rtTA to tet O

sequences. The offspring produced will contain lox p sequence with the deletion or inactivation of gene between lox P sequence upon the expression of Cre recombinase as represented in Fig. 3 (Kim et al. 2018). The expression of Cre recombinase can be controlled in double transgenic mice by adding inducers while feeding the mice as Cre expression is under the control of the transactivator which is sensitive to inducers as explained in the tetracycline-inducible system.

Several models using recombinant technology tools have been developed based on the studies to be performed and evaluated. Further for constructing the knockout or knock-in transgenic mice Cre-lox P inducible system, the tissue type and tissue-specific promoter are subjected to differ depending on the cancer type. Contrarily, knock-in models have been extensively used to study oncogenes specific to tumorigenesis. Random insertion of desired genes can be avoided in these models as the commonly used insertion site in mice is Rosa26 locus at chromosome 6; this region is destitute of the essential gene (Friedrich and Soriano 1991); this minimizes the limitations, and gene expression occurs without any disruption which circumvents the functionality of oncogenes in cancers of different origins. Considering these characteristic features of knock-in models, a Cre-dependent Cas 9 knock-in mouse has been developed that serves the purpose of genome editing via Cas 9 which catalyzes the site-specific gene insertion. Various molecular modifications must be induced which provides an accurate model for studying human cancer with

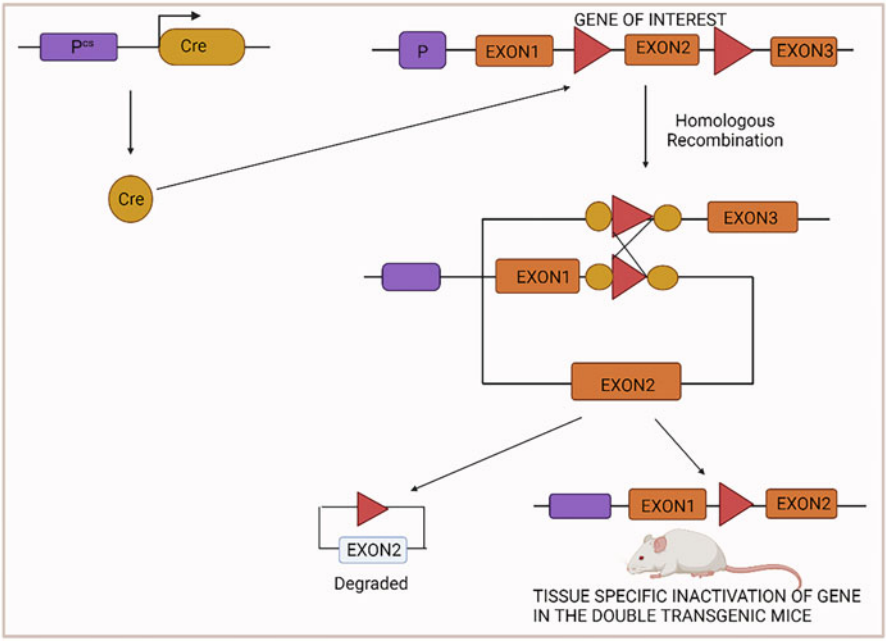

Fig. 3 Molecular mechanism of conditional gene knockout via Cre recombinase-lox P system in double transgenic mice – two molecules of Cre protein bind to each lox P site to carry out homologous recombination; the exon carrying the gene of interest is circularized and degraded. Further, the biological function of a knockout gene is evaluated

similarities. **Development of Cre-dependent Cas 9 Knock-in mice** – The homology arm consists of the transgene floxed with lox P sequence, Cas 9 linked with peptide 2A sequence, and enhanced green fluorescent protein to track the expression of Cas 9 in transduced cells; further, the transcription of the transgene is regulated by CAG promoter. The entire system is induced by Cre recombinase which is necessary for the expression of Cas 9. This homology arm is further inserted into the Rosa 26 via homologous recombination. Further, AAV-vector (Adeno-associated virus) is used to deliver the integrated guide RNA for the genes including Cre recombinase expression into the knock-in mice. Thus, transduced cells experience the gene expression under controlled conditions, Cre catalyzes the excision at lox P site, and the activity of Cas 9 endonuclease is to institute double-strand break at the target site which is guided and directed by guidance RNA as depicted in Fig. 4 (Rocha-Martins et al. 2015). Tumorigenesis is a multigenic disease process that includes a gain of function mutations in proto-oncogenes and loss of function mutation in the tumor suppressor gene. These models were further used to study various tumor suppressor genes such as p53 and LKB1 and an oncogene KRAS as these genes are profoundly undergoing mutation in lung adenocarcinoma (Cancer Genome Atlas Research Network 2014).

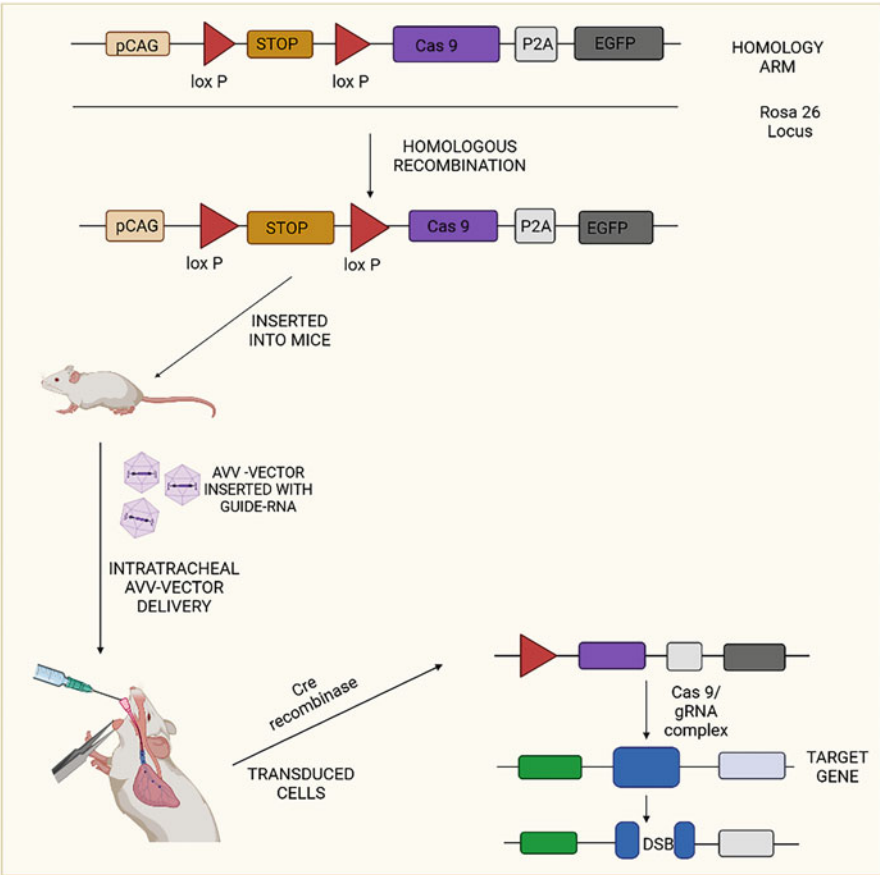

Fig. 4 Development of Cre-dependent Cas 9 knock-in mice. Insertion of homology arm into Rosa 26 locus through homologous recombination. The recombinant chromosome is inserted into the mice leading to the development of Cre-dependent Cas 9 knock-in mice. These models are used to study multigenic cancers such as lunch adenocarcinoma as knock-in mice models circumvent the study of significant oncogenes and tumor suppressor genes. To enhance the activity of Cas 9 guide RNA, sequences are delivered using AAV-Vectors via intratracheal. The Cas 9-gRNA complex is essential for the double-strand break at the target site

Patient-Derived Xenografts

Xenografts are the tissues or organs of a species that are transplanted into an organism belonging to different species. Patient-derived xenografts (PDX) are those tissues or organs which are derived from a patient and are transplanted into another organism like mice to study more about cancer and how a particular treatment would work on that patient.

In cancer research, the preclinical model plays an important role in helping the researchers have a better understanding not only on how the disease works but also on how a particular treatment will work on the body and cancer. For this purpose, cancer cell lines were being grown indefinitely in an artificial condition and were being used, but even though these cell lines were easy to use, limitations were also causing a hindrance in the preclinical drug development. Several studies have shown that the activity predicted by the cell lines for certain diseases is not accurate (Johnson et al. 2001); moreover, there is evidence suggesting the alteration of biological properties in these cell lines like loss and gain of genetic information, etc. (Gillet et al. 2011). So, to circumvent these limitations of cell lines, researchers have been working on various advanced preclinical models like PDX. The general method of developing a patient-derived xenograft is done by collecting bits and pieces of primary/ metastatic solid tumors by the process of surgery or biopsy that have been maintained as tissue structure. These tumors are then implanted into the dorsal side of the mice as a single-cell suspension or as pieces or mixed with human fibroblasts or mesenchymal stem cells. Selection of the perfect mouse is a very important step for the generation of PDX models, as more severely immunosuppressed models such as the non-obese diabetic model are given first consideration due to their higher engraftment rate (Nemati et al. 2010; Sivanand et al. 2012).

Based on clinical manifestations of individual cancer types, various chemotherapeutic or cancer vaccines can be tested using PDX models which further leads to the development of personalized medicines. PDX models have been extensively used for preclinical examinations of colorectal cancer as they help in maintaining an accurate tumor microenvironment; therefore, heterogeneity is an ideal condition to study cancer (Xie and Lin 2020). The implantations of tumor fragments are done heterotopically in which the tumor fragments are implanted into an organ site which is originally unrelated or different from the site of origin or anatomical organ of the host, basically implanted to subcutaneous or subrenal capsular sites. Contrarily orthotopic implantation the host's cancer tissue is transplanted into the coinciding anatomical organ (Jung et al. 2018). The PDX models for colorectal cancer have been heterotopic and orthotopic, cancer metastasis studies of colorectal cancer can be carried out in orthotopic engraftments (Xie and Lin 2020). Furthermore, studies have shown that PDX models can be used to demonstrate the progression of metastasis which is associated with ascending proliferation and expression of MYC which could be halted by using cyclin-dependent kinase inhibitors treatment (Lawson et al. 2015). Usage of PDX models is not confined to colorectal cancer, but it is also been used to develop potential biomarkers and for drug screening, especially for BrCa prognosis in breast cancer (Kawaguchi et al. 2017).

Some of the salient features of PDX models are the ability of these models to be a potential personalized treatment for cancer, these models also retain the primary characteristics of the tumor like fine tissue structure (Li et al. 2013), the models also depict the actual mutation frequency that has been observed in the human genes (Rubio-Viqueira et al. 2006), etc.

Applications/Advantages of PDX Models

The best way to understand the salient features and applications of PDX is through some examples which are as follows:

- **Utilization of PDX in Leukemia**: The most common type of leukemia observed in adults is AML or acute myeloid leukemia which starts from a rare leukemia stem cell and is characterized by the expansion of immature myeloblasts. Even though there are some highly effective chemotherapy treatments for leukemia, there are still cases of relapses observed in the majority of them (Lübbert et al. 2008). Due to this unfavorable situation, a need for a better therapeutic strategy was needed, and a need for a better model to test them on that is where the PDX model came into play. The human AML cell lines were maintained in a minimum essential medium that contained 10% fetal bovine serum. These cultured AML cell lines were first washed using PBS (phosphate-buffered saline) to remove any debris and then were again suspended in PBS after which they were intravenously injected into an adult mouse which was irradiated sublethally 24 hrs before the injection of leukemia cells was given. These cells after being injected into the mice produced xenograft tumors, and daily monitoring was done till the time of the death of the mouse to study the symptoms it showed. The hematopoietic cells count, and study of leukemic engraftment was done after collecting the blood by retro-orbital bleeding and by dissection of spleen and bone marrow after the death of the mice. The results of the study were that the irradiation conditioning step did not cause any change to the efficacy of xenotransplantation of AML cells; the result obtained through PDX also depicted the diversity seen in engraftment capacities and tissue tropism as well as the aggressiveness in these cell lines that are generally observed in AML patient. Moreover, the results also showed that the immune phenotype of these AML cells was conserved in vivo and showed the same level of expression markers that were depicted before the xenotransplantation (Saland et al. 2015).
- **Drug Resistance Screening Using PDX**: PDX tumors show a great deal of drug resistance and provide help in drug screening due to these tumors depicting cellular and molecular heterogeneity which is a feature that is recognized as a key components for drug resistance observed during prolonged treatment. This has been observed due to making a PDX tumor out of the metastatic cell of a patient immediately after their death that has been showing drug resistance during their autopsy in a patient suffering from pancreatic cancer or prostate cancer (Rosfjord et al. 2014).
- **PDX Models in Breast Cancer Research**: Human breast cancer is a heterogeneous collection of diseases that depicts a huge amount of diversity in its genomic alterations, gene expression, metastatic behavior, and treatment response (Allred et al. 2008). Breast cancer is of three types, the first is the type that shows estrogen receptor alpha (ER+) and also typically exhibits the expression of the progesterone receptor (PR+), the second one is the type of breast cancer that shows overexpression of ERBB2 (HER2+), and, finally, the last type of breast cancer

does not express any one of these markers, i.e., negative for all these markers, and is also known as "triple-negative" breast cancer. The development of PDX models for breast cancer have come as a huge boost for the researcher as they can grow cell lines for all the three types of breast cancer that depicts characteristics as observed in humans; moreover, they are also able to study the distinct treatment response and disease outcome observed in ethically different patients (Dobrolecki et al. 2016).

Limitations of PDX Models

Even though PDX has huge benefits and applications, it also has limitations to it which are as follows:

- There is need to define the best strategy that can be used to engraft in mice, i.e., whether the subcutaneous implantation should be done or the orthotropic implantation.
- There is need to consider the most appropriate tissue that can be utilized to construct the PDX model and how to process this tissue.
- There is a delay observed in the engraftment time in mice and clinical schedules for patients.
- A high chance of engraftment failure is observed in some types of tumor-like hormone receptor-positive HBC.
- For PDX research to occur, an immunodeficient host strain is required for tumor engraftment and propagation, but this limits the use of these models in the screening of immune-mediating agents like vaccines.
- Cost of using PDX models in drug screening (Hidalgo et al. 2014).

Conclusion

An animal model for cancer research has been designed to circumvent all the limitations; modifications were made to mimic the tumor microenvironment similar to human cancers. Cancer is a multifactorial disease, and several hallmarks have been deduced which were identified to support tumor growth. Transgenic mice and PDX models were extensively used to study the cancer hallmarks individually under specified tissue, receptors, oncogenes, and tumor suppressor genes. These studies have further shed light on the signaling transduction pathways and biochemical mechanisms that drive tumorigenesis as well as give insights into the creation of personalized medicine to treat cancer. The selection of mice strain or development of transgenic mice is entirely dependent on the study protocol and the cancer type. To further narrow it down, models are developed for the specific process to be reviewed under in vivo conditions; for example, K14-HPV 16 mouse and RIPTag mouse of pancreatic islet carcinoma (Walrath et al. 2010) has been extensively used to study and identify the angiogenic factors supporting cancer invasion and sustaining

angiogenesis. Vascular sprouting is observed early in dysplasia in these models which contributes to tumor development. One of the classic examples of transgenic models developed for cancer research was p53 null models; these models are analogous to human cancer predisposition Li-Fraumeni Syndrome. Mice models were also developed using recombinant techniques to produce human monoclonal antibodies via YAC transgenesis. Monoclonal antibodies are effective agents to halt cancer cells proliferation, and also can be used efficiently in immunotherapy to block CTLA-4 which would unleash T-cell brake allowing the immune system to attack cancer cells. Further to obtain a more accurate cancer microenvironment similar to human cancers, human xenograft mice were developed using Xenomouse technology. Xenomouse technology was also used to produce human monoclonal antibodies using YAC transgenesis which consists of human heavy and light chains. One such example is ABX-EGF monoclonal antibody against EGFR which is observed to overexpress in tumors. Therefore, using animal models minimized the risk of cytotoxicity and side effects of anti-cancer drugs in humans. The models have persuaded researchers to develop diverse cancer therapies.

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Animal Model-Based Studies to Evaluate the Lipid-Based Drug Delivery Nanocarriers for Cancer Treatment

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Jino Affrald R. and Shoba Narayan

Contents

Abstract

In an effort to find products and processes for human healthcare, experimental objects and materials that can simulate the human body have been used in medical research since time immemorial. Cancer is the second largest disease condition, and therapies and therapeutics including those based on nanomedicine are being

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developed. This chapter provides an understanding of the various types of animal models that have helped in understanding the disease and the levels of success of various therapeutics. Animal models such as mice, hamsters, rabbits, zebrafish, and amphibians have been discussed in detail. From the available literature, it can be seen that animal models can be used for understanding the biochemical and physiological processes relating to occurrence of cancer and also for screening of drugs. The chapter also provides an insight into current trends in cancer management such as microarray, nanotech products, and lipid nanoparticles that are employed both as therapeutics and diagnostics (theranostics). To understand the efficacy and biocompatibility of these theranostic agents, animal models are employed. Some of them are xenografts, chemically induced animal models, and genetically modified animal models. From the chapter, it can be seen that there is a significant progress in the understanding of occurrence and spread of cancer and thus the therapeutics for cancer management. The progress to a large extend is attributed to the choice of right animal models.

Keywords

Cancer · Animal models · Xenograft · Lipid nanoparticles · Theranostics

Introduction

Cancer is one of the most deadly diseases of the twentieth century, and evidence of cancer among ancestors of modern humans extends back over million years (Hausman 2019). Many high-income countries are seeing a decrease in cancer-related deaths, whereas low and low middle-income countries are seeing an increase. Low-income countries report more stomach, liver, esophageal, and cervical cancer incidences. Although rates in high-income countries remain high, rates are leveling off or declining for the most prevalent malignancies by reducing recognized risk factors, developing better screening and early identification methods, and improving treatment strategies (Torre et al. 2016).

Longitudinal monitoring of tumors being impractical and the temporal dynamics of cancer evolution remain mysterious. A single moment in time – cancer's endpoint – when it is taken from the body and analyzed in the lab is primarily the basis of conclusions drawn. Fortunately, the cancer genome provides a rich record of cancer progression due to continuous mutations that uniquely designate clonal lineages within the tumor (Graham and Sottoriva 2017). These tumors become extremely heterogeneous as cancer progresses, resulting in a mixed population of cells with various biological characteristics and therapeutic responses. The variability is at geographical and temporal levels and is essential in developing resistance phenotypes. Resistance phenotypes facilitate the application of selective pressures during cancer therapy (Pucci et al. 2019). Surgical removal, chemo- or alternative therapies, radiation, and auto-cell death are considered as some of the requirements to stop the spread of cancer (Roy and Saikia 2016). Thus, cancer therapy has always been based on neoplastic cells. Surgical,

radiation, chemotherapy, and immunotherapy treatments target the quickly multiplying mutant tumor cells (Wang et al. 2018). Treatment of cancer is through surgical intervention, radiation, and chemotherapeutic medications. Chemotherapeutic medicines harm healthy cells and induce toxicity in patients.

As a result, researchers seek strategies to target just malignant cells (Zaimy et al. 2017), using information gathered via scientific breakthroughs. An integrated approach to cancer management is required. Currently, anticancer medications based on thousands of herbal and traditional components are in various stages of testing (Balachandran and Govindarajan 2005). However, nanotechnology has recently received a lot of interest for cancer therapy worldwide. Nanobiotechnology fosters the integration of diagnostics and treatments as a personalized approach to cancer treatment. Nanoparticles find use in diagnosing and treating various disorders, including cancer (Chaturvedi et al. 2019). High sensitivity, specificity, and multiplexed measuring capability are some of the aspects that have led to use of outcomes of nanotech research in early detection of extracellular cancer biomarkers and in cancer cell imaging (Zhang et al. 2019). Accurate early-detection devices, powerful imaging modalities, and improved radiation adjuvants are based on the adaptability and functionality of nanomaterials (Kemp and Kwon 2021). Nanotechnology-based chemotherapy has demonstrated its potential to provide further effectiveness and safety among diverse branches of cancer research (Desai et al. 2020).

Historical Perspective of Animal Models

Animals were first used as human anatomy and physiology models in ancient Greece. These early comparative researches mainly were observational, with the goal of better understanding human ontogeny and physiology. Trade channels transmitted the knowledge to European and Arab physicians. Animal models contributed to a significant paradigm shift in understanding human physiology during the early period, which witnessed many notable discoveries (Ericsson et al. 2013). Preclinical studies employing numerous animal models have laid the groundwork for the fundamental understanding of disease pathophysiology and human anatomy (Robinson et al. 2019). These animal models are frequently chosen based on convenience or tradition, while technical or scientific rationale plays a role in these selections. More sophisticated mammalian brains and genetic model species such as zebrafish have received less attention, yet this model offers significant benefits that are becoming acknowledged (Grone and Baraban 2015).

The science of oophorectomy grew from the studies of Lathrop and Loeb (1913–1916) and further progressed to preventing chemical-induced carcinogenesis in mouse models (1929). Further developments include the 1940 studies on energy restriction and associated discoveries in the 1950s. Molecular level characterization of cancer progression and multistep carcinogenesis led to studies on effect of angiogenesis inhibition.

This timeline has an exciting collection of preclinical and clinical chemoprevention, vaccination, surgery, and behavioral science research (Lippman and Hawk 2009). Cancer models in mice are well-known and widely utilized in cancer research (George et al. 2021). Understanding the molecular pathways and processes as well as gene and protein activities has been possible through rodent cancer models such as xenograft and generated (chemical/genetic) mouse tumors (Cekanova and Rathore 2014). For stromal responses, metastatic behaviors, and therapeutic effects, the rabbit VX2 allograft cancer model is used. This model has cancer cells that develop in a host rabbit with natural immunity, making it appealing and distinctive (Oshiro 2014).

Experimental Animal Model in Cancer Research

In a phase of cancer, the immune system serves significant purposes – the immune system of the host guards against tumor growth by immunosurveillance. Nonetheless, immune cell tumor detection triggers sculpting processes, resulting in a Darwinian selection of tumor cell types with lower immunogenicity (Overgaard et al. 2018). Initiation, progression, and metastasis studies as well as preclinical validation of cancer therapeutics adopt animal models as the same permits the recreation of tumor in a pathophysiologic environment (Mendes et al. 2020). There are still substantial drawbacks to using mice to mimic human cancer, such as species-specific variations and a false representation of de novo human tumor growth. In the future, the mouse models need to be expanded to in vivo imaging and high-throughput screening (Pathak et al. 2020). This would lead to generation of clinically relevant data on molecular, cellular, and genomic events of human cancer (Cheon and Orsulic 2011).

Nude Mice

Hairless, devoid of a normal thymus gland, and a weak immune system are characteristic features of a nude mouse that helps them accept tumor cells from species such as mice and athymic nude mice. Athymic nude mouse is a murine where the Foxn1 gene undergoes a spontaneous deletion, leading to deteriorated or absent thymus. This absence of thymus leads to suppressed immune system and a reduction in number of T cells. The successful use of nude mouse for in vivo studies relating human cancer is attributed to the ability to transplant human cancer into nude mice, with a good success rate in creation of tumor lines. Additional immunosuppression of mice or extremely young animals can boost tumor development and malignant expression (Giovanella and Fogh 1985). Researchers highlighted the histological and functional closeness of the xenografts to the original tumor in early reports of tumor transplantation in nude mice. However, further use of this model system has shown cases in which nude mouse transplants varied from the original tumor, raising concerns about the transplant's comparability to the parent tumor (Sharkey and Fogh

1984). Researchers have used CRISPR-Cas9 to create FOXN1 mutant nude rabbits to illustrate the functional applicability of nude mice in biomedical research.

Even though each model, including naked mice, has its own set of drawbacks, models are nevertheless widely used in cancer research, particularly in the quest for novel medications that can more effectively battle cancer or improve the effectiveness of current therapies. Anticancer medication testing will be more effective in new mouse strains with a severely weakened immune system (Szadvári et al. 2016). 2–5 million established neuroblastoma cell lines should be implanted into 20 nu/nu Swiss mice to generate a repeatable model of human neuroblastoma (Helson et al. 1975).

Hamsters

The immune-privileged cheek pouch of the hamster is one of the best-defined animal models for investigating oral squamous cell cancer. Chemical-induced carcinogenesis in hamsters helps explore distinct phases of tumor formation and cellular alterations during the multistep process of oral carcinogenesis. Such studies are comparable to those seen in human mouth cancer (Yapijakis et al. 2019). The hamster buccal pouch carcinogenesis model is one of the best-known animal tumor models for studying multistage oral carcinogenesis and evaluating the efficacy of chemo-intervention (Nagini and Kowshik 2016). These hamster models are widely utilized study subjects and are readily available and easy to produce, develop quickly, have short life cycles, and are sensitive to various infections. Besides cancer research, hamsters are used in several investigations, including infectious illness research and behavioral studies. According to one fascinating analysis, Syrian hamsters are a few animal models regarded as semi-permissive for human adenovirus. The golden Syrian hamster is a good model for studying malignant illness in a wide range of both spontaneous and induced malignancies (Fabrizio 1965). Oncolytic adenovirus driven by the viral E3 promoter could cure syngeneic pancreatic tumors while eliciting an anticancer immune response, as measured by T-cell proliferation. This study employed murine IL-12 and revealed partial cross-reactivity between mouse and Syrian hamster cytokines. Another study on human PC3 prostate xenografts or TRAMP-C1 tumors with the Ad5-IL-12 and mifepristone combination showed considerably greater therapeutic effectiveness than controls in another trial (Cerullo et al. 2012).

Rabbits

The rabbit VX2 tumor model has been used in cancer research for a long time. The VX2 tumor, developed by Rous et al. between 1930 and 1940, is a virus-induced anaplastic squamous cell carcinoma with hypervascularity, fast development, and facile dissemination in skeletal muscle. The VX2 tumor has been used to simulate head and neck, kidney, brain, lung, urinary bladder, uterine, liver, bone, and pancreas

tumors. The vigorous development rate and relatively big rabbit vasculature make the model ideal for interventional radiologists. It has been used in several investigations in recent years (Parvinian et al. 2014). In Interventional radiology, the rabbit VX2 tumor is a used animal model for translational research on hepatocellular carcinoma (Khabbaz et al. 2019). Implanting VX2 frozen tumors through surgical implantation in para-renal area of rabbits is one of the methods suggested to study the tumor response to localized/regional treatment of solid malignancies (Bimonte et al. 2016). Studies indicate that the loss of function mutations in forkhead box N1 gene leads to athymia, T-cell immunodeficiency, hereditary baldness, and nail dystrophy (Song et al. 2021).

Zebrafish and Amphibians

The zebrafish has long been a popular model for developmental biology and cancer biology in recent years. Like in the previous animal models, human tumors can be transplanted into zebrafish and real-time invasion and spread of tumor monitored (Cagan et al. 2019). For this, creating genomic instability, inactivation of tumor suppressor genes by target-selection mutagenesis, and generation of transgenics to express human oncogenes have been reported alongside transplantation (Feitsma and Cuppen 2008). Researchers have also employed the zebrafish rag2 promoter to regulate expression of mouse Myc open reading frame. This method lets up to 5% of F0 zebrafish acquiring cell acute lymphoblastic leukemia. Germline transfer increases the penetrance of the transgene by 100%, along with a decrease in tumor latency. Germline transfer model produced a great proof of concept for cancer modeling in zebrafish and created the groundwork for new leukemogenesis processes to be discovered (Fazio et al. 2020). Xenopus model system has contributed to cancer research owing to similarities between tumor pathology and early embryo development. Cancers commonly disrupt cell cycle regulation, signaling networks, and cell behaviors such as migration. Because of its speed, diversity, and accessibility, the Xenopus model has emerged as a promising tool for improving the molecular understanding of oncogenesis and providing an early in vivo model for chemotherapeutic development (Hardwick and Philpott 2018).

Figure 1 provides an insight on induction of cancer in mouse model. Tumor models are chosen based on the distinct disease pattern in humans (Onaciu et al. 2020). As transplantation of genetically produced cancer models is time consuming and expensive, the injecting of malignant cells locally or systemically is a suggested alternative. To inoculate, cancer cell suspensions are injected through the tail vein, retro-orbital, subcutaneous, or orthotopic routes. The tail vein and retro-orbital methods are less invasive and safer to the animal, especially in the case of hematological malignancies. Compared to retro-model, which is stressful to the animals, the tail vein method can be employed for therapeutic administration and subsequent fluid collection. If the goal is ease of execution and obtaining rapid results, the subcutaneous implantation of cancer cells is preferred over orthotopic process. To imitate living organism settings during tumor growth, the orthotopic microsurgery is

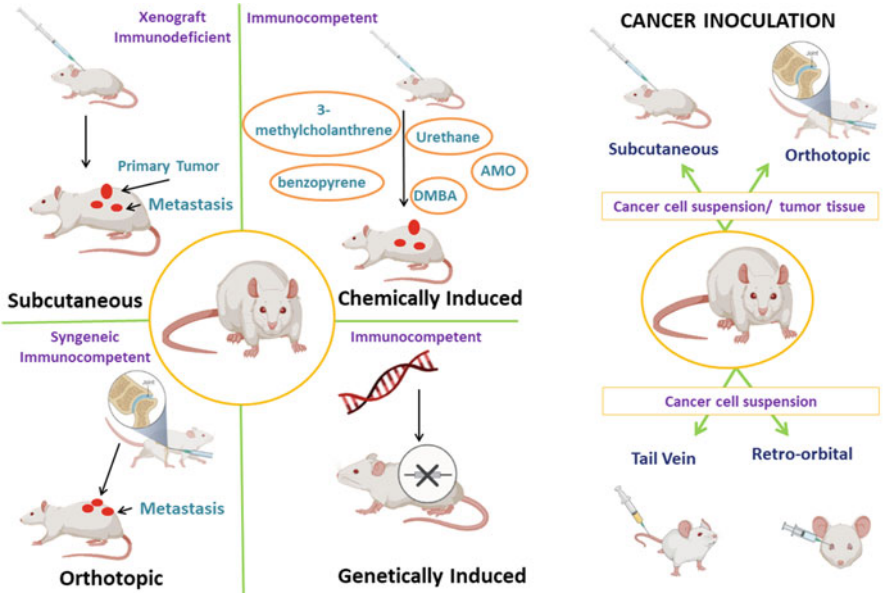

Fig. 1 Cancer induction in a mouse model

ideal. In both the methods, implantation of cell suspensions and tumor tissues is possible. During implantation, care is taken to choose locations that would promote metastatic profile by means of growth and migration of cell networks. The ideal locations are layered structures. Forward thinking in this direction includes the implantation of electrical devices and materials for molecular level assessment.

Current Trends in Cancer Management

The discovery of biomarkers for accurate prediction of how effective cancer management and treatment has been traces back to the quantitative proteomic methods. Mass spectrometry with high resolution, speed, and sensitivity, protein arrays, and data handling and interpretation through advanced bioinformatics have all contributed to the biomarker development (Cho and Cheng 2007).

Microarray

Microarray is a powerful new tool for researching the molecular basis of interactions at a scale that is hard to achieve with traditional methods. This method allows researchers to look at the expression of thousands of genes simultaneously. This technology can promote the development of rational therapeutic approaches and

cancer diagnostics and prognosis, ensuring its implementation in specialized centers and hospitals within the next few years. Membrane microarray findings are hampered by the technique's relative insensitivity to detect low-level transcripts and the limited number of spots on the membranes. Patterns of gene expression in prostate cancer are better described using microarrays (Russo et al. 2003). Technological changes and advancements have altered reproducibility. The statistical challenges of linking many variables to a limited number of observations have added to the microarray technique's limitations (Tinker et al. 2006).

Nanotechnology

Nanotechnology is one of the most extensively employed methods in cancer research today. Drug delivery, gene therapy, detection and diagnostics, drug carriage, biomarker mapping, targeted therapy, and molecular imaging are intriguing uses of nanotechnology in cancer diagnosis and treatment. Treatments based on nanotechnology, such as the creation of nanoscale drug delivery systems, can enable accurate malignant tissue targeting while minimizing adverse effects. Nanomaterials can easily overcome cell barriers due to their biological origin. Treatment of malignancies through active and passive targeting of nanomedicine has been known for the last two decades. The sensitivity of medications results in poor outcomes and can have various adverse effects, including harm to the body (Jin et al. 2020). Polymeric nanomaterials, metallic nanoparticles, nanocrystals, carbon-based materials, nanostructured lipid carriers, and nanoemulsions have been developed. Nanoscale size and unique physicochemical characteristics improve pharmacokinetics and pharmacodynamics over conventional formulations (Navya et al. 2019). Biocompatible hydrogels are promising materials in medicine and biology because their porous structure, capacity to entrap a considerable quantity of water, and tenability of mechanical and tissue adhesion qualities make them appropriate for many applications (Piantanida et al. 2019).

Especially with the development of multifunctional systems combining therapeutic and diagnostic capabilities, "nanotheranostics" provide a unique and exceptionally adaptable platform for both illness diagnosis and cure. One of the most intriguing parts of nanotheranostics is the pharmacokinetic profile monitoring, leading to understanding of the efficacy of the therapy. Because of its therapeutic and diagnostic capabilities, nanotheranostics is appealing for optimizing cancer treatment results. This can be considered as more towards personalized medicine, where the goal is to provide the most appropriate drug in optimal dosage for the patient at the right time (Valetti et al. 2013). Figure 2 provides an illustration on lipid-based nanocarriers and its role as multifunctional carrier.

Lipid-Based Nanoparticle for Cancer Therapy

The most prominent among lipid nanoparticles are the solid lipid nanoparticles, nanostructured lipid carriers, lipid–drug conjugates, and lipid nanocapsules (Battaglia and Gallarate 2012) that have been developed as mRNA delivery

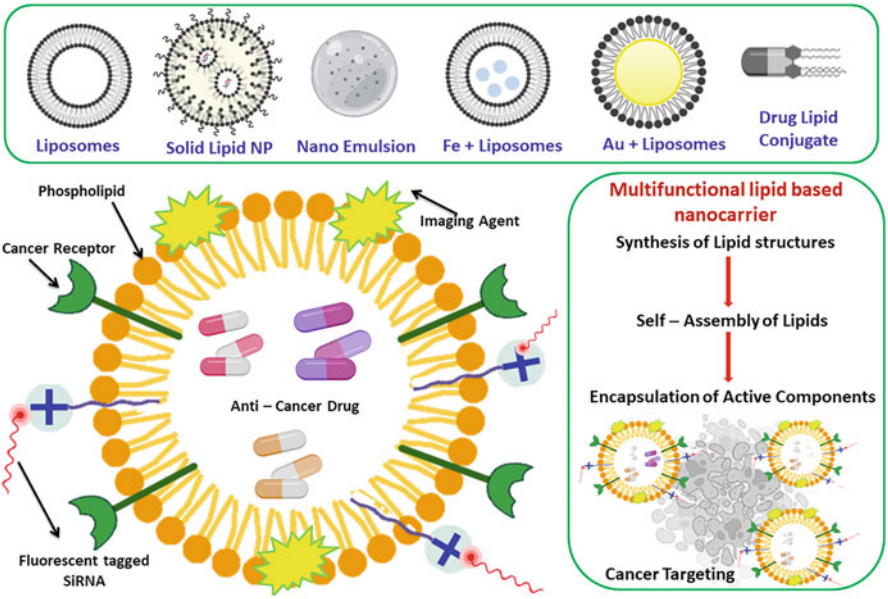

Fig. 2 Multifunctional lipid-based nanocarrier

materials. Lipid nanoparticles, in particular, have undergone extensive research and have effectively reached clinical trials for the delivery of small molecules, siRNA medicines, and mRNA in cancer therapy (Hou et al. 2021).

Lipid Nanoparticles

Lipid nanoparticles are the most sophisticated non-viral nucleic acid delivery systems that have been studied extensively (Qiu et al. 2021). Combinatorial lipid nanoparticles with various chemical topologies provide for intracellular protein delivery. Researchers developed a combinatorial library of cationic lipids by creating simultaneous Michael addition or ring-opening reactions of aliphatic amines, leading to nanoparticle formulations for intracellular protein transport (Chang et al. 2019). When used in conjunction with VEGF siRNA, the chemotherapy medication – paclitaxel – may synergize cancer development. On the other hand, effective and safe delivery methods with high paclitaxel encapsulation efficiency and long-term drug release are required. Researchers employed a tripeptide lipid nanoparticle to deliver paclitaxel and anti-VEGF siRNA to boost antitumor efficacy in lung cancer therapy (Zhang et al. 2020).

Solid Lipid Nanoparticles

Solid lipid nanoparticles are gaining attraction as a targeted delivery alternative to colloidal systems. These comprise submicron-sized (50–1000 nm), biocompatible,

and biodegradable materials that integrate lipophilic and hydrophilic medicines (Manjunath et al. 2005). Drug release can be controlled and targeted in solid lipid nanoparticles while maintaining drug stability. It can hold a higher concentration of drugs than other carriers, leading to the possibility of transporting lipophilic and hydrophilic medications. Biocompatibility in solid lipid nanoparticles arises from its biodegradability.

Moreover, it is simpler to scale up and cost-effective than polymeric/surfactant-based carriers. Validation and regulatory approval are easier to get (Mukherjee et al. 2009). It is expected that solid lipid nanoparticles will be adapted shortly to deliver anticancer chemicals in a more efficient, precise, and safe manner (Wong et al. 2007) as the nanoparticles would pass through the physiological barriers and escape the multidrug resistance mechanism of cancer cells. Drug delivery through solid lipid nanoparticles involves mechanisms such as passive targeting and active targeting. Active targeting is through modification of lipid surfaces and has been successfully demonstrated for different malignancies (Bayón-Cordero et al. 2019). For example, cationic solid lipid nanoparticles were utilized to transport a particular miRNA-200c to avoid breast cancer cells becoming resistant, to improve responsiveness to the medication delivered by nanostructured lipid carriers. The breast cancer stem cells have a lower level of miRNA, and thus delivery of miRNA through solid lipid nanoparticles reduces the production of class III beta-tubulin, implying that microtubule-targeting medicines would be more effective (such as paclitaxel) (Bayón-Cordero et al. 2019). Recent reports show that DHA-dFdC or 4-(N)-docosahexaenoyl 2′, 2′-difluorodeoxycytidine is a potent new lipophilic molecule with broad-spectrum anticancer action (Valdes et al. 2019).

Liposomes

Liposomes have been employed in the administration of anticancer medications and can regulate biodistribution and clearance of the drug molecule. Liposomes are taken up by reticuloendothelial system after being injected into the body. Liposomal medications aggregate in various organs to increase drug therapeutic index and minimize adverse effects (Feng et al. 2017). Following the successful authorization of liposomal version of doxorubicin modified with PEG, abraxane, which is paclitaxel loaded on modified albumin nanoparticles has been verified for treatment of breast cancer (Alavi and Hamidi 2019). Liposomal nanoformulation possesses enormous advantages over conventional drug delivery systems. Liposomes are functionalized with aptamers or any targeting ligand in general (Moosavian and Sahebkar 2019). The method allows the drug to accumulate in tumor cells with high selectivity and efficiency, no harmful impact on other organs, and no deleterious effects on liposome pharmacokinetics. Effectiveness of liposomal medications is improved through ligand targeting of tumors (Torchilin 2008).

Among other gene expression modifiers, liposome has been employed in gene therapy to deliver plasmids, antisense oligonucleotides, and siRNA. Targeted liposomes outperform untargeted liposomes in delivery through increased interaction

with the target cells, leading to fusion with the cellular membrane or endocytosis-mediated internalization (Sofou and Sgouros 2008).

Lipid-Based Nanocomposite Carriers

Ternary nanocomposite carriers made of organic clay and lipid vesicles showed to be beneficial in increasing drug cellular absorption and anti-inflammatory effects. Organic clay – budesonide – liposome had a more excellent colonic distribution, longer residence duration, and a lower C_{max} in bio-distribution and pharmacokinetic investigations. It thus may be a viable colon-targeted delivery strategy (Kim et al. 2020). A magnetic component in a biocompatible magnetic lipid nanoformulation allowed for non-invasive MRI and remote administration of magnetic hyperthermia. Its systemic injection increased antitumor activity while allowing in vivo MRI monitoring. Magnetic hyperthermia and magnetic ablation worked together to improve the thermal release of chemotherapeutics (García-Hevia et al. 2022). Reports indicate that *s*-adenosyl-L-methionine-loaded SLN escapes from the stomach environment and achieves passive targeting by increasing intestinal *s*-adenosyl-L-methionine permeability (Amasya et al. 2021).

Animal Models Used in Lipid Nanoparticle-Based Cancer Therapy

To satisfy unmet needs in delivering therapeutic agents and imaging agents for cancer therapy and diagnostics, researchers are continually inventing novel nanomaterials, nanodevices, and nanoparticles. Lipid-based nanoparticles are genuine particles with a diameter of about 100 nm and assembled from various lipid and other chemical components. The components work together to overcome the bio-barrier, and the lipid-based nanoparticles accumulate preferentially in or around disease-target cells to deliver therapeutic or imaging agents for diagnosis (Miller 2013). Lipidic nanocarrier systems containing endogenous high-density lipoprotein have emerged as prospective cancer theranostic solutions because of their non-immunogenicity, biocompatibility, and low reticuloendothelial system absorption. Recently, researchers have discovered HPPS (HDL-like peptide-phospholipid scaffolds) that mimic plasma-derived HDL's structural and functional characteristics. TfR mAb (monoclonal antibody) customized nanomedicines for enhanced tumor targeting were also discovered (García-Pinel et al. 2019).

Lipid Nanoparticle-Based Chemotherapy in Xenograft Animal Model

Folic acid-conjugated carbon nanotube-lipid-paclitaxel increases cell penetration capacity and improves drug efficacy in vitro compared to free drug Taxol and non-targeted carbon nanotube-lipid-paclitaxel at 48 h. The human breast cancer xenograft mouse model was used for the studies (Shao et al. 2013). MiR-122,

liver-specific tumor suppressor microRNA, is typically downregulated in hepatocellular carcinoma. A lipid nanoparticle-encapsulated miR-122 mimic administered intratumorally resulted in a 50% growth reduction of hepatocellular carcinoma xenografts within 30 days, connected with the suppression of target genes and angiogenesis impairment (Hsu et al. 2013). Luciferase expression in PC-3M-luciferase subcutaneous xenograft and metastasis models could be inhibited using lipid nanoparticles of luciferase-siRNA. By combining the nanoparticle with androgen receptors, the nanoparticles silenced the clusterin and thus the progression of enzalutamide-resistant prostate cancer (Yamamoto et al. 2015). Using similar xenograft models, artemisinin dimer lipid nanoparticles were demonstrated to reduce breast cancer. Compared to normal drugs, the lipid bound particles suppressed tumors more effectively, possibly through release of the cargo throughout the cytoplasm (Zhang et al. 2015).

Lipid Nanoparticle-Based Chemotherapy in Chemically Induced Animal Model

There are numerous examples of chemically induced mice models each with benefits and drawbacks. DMBA-induced rat models of breast cancer (Brown et al. 2010), 1,2-dimethylhydrazine-induced colon cancer (Dudhipala et al. 2018), Benzo(a)-pyrene-induced lung cancer in Swiss-albino mice (Naseema et al. 2018), and *N*-butyl-*N*-(4-hydroxybutyl) nitrosoamine-induced urinary bladder carcinogenesis (Miyazaki et al. 2011) in rats are some examples of chemically induced animal models. It has also been reported that dimethylnitrosamine in the diet induces tumors in the liver and kidney of rats, while a single injection induces only kidney tumors (Craddock 1973). In all these models, the pharmacokinetic tests have demonstrated a multifold reduction in tumor when treated with lipid formulations, possibly because of the increased bioavailability. Table 1 provides few examples on the use of lipid nanoparticle for cancer therapy in animal model. Table 2 provides few examples of clinically approved lipid-based carriers.

Lipid Nanoparticle-Based Chemotherapy in Genetically Modified Animal Model

Genetically modified animal models such as mice have aided in studying the fundamental pathways involved in cancer. Genetically modified mouse models may be beneficial for hazard detection, but are ineffective for assessing dose-response relationships. As a result, caution should be exercised when utilizing genetically modified mouse models to evaluate the carcinogenic risks of substances (Eastmond et al. 2013). Researchers introduced cloned cancer genes, popularly known as oncomice, into the genomes of transgenic mice in the early 1980s. The first oncomouse was a genetically engineered mouse model with transgenic expression of a specifically activated oncogene (vHRas) under the control of a mammary

Table 1 Animal models used in lipid nanoparticle-based cancer therapy

Nanoparticle	Cancer induction method	Animal and cancer model	Nanoparticle	Reference
Lipid nanoparticle	Cell line-derived cancer	BALB/c nude mice, prostate cancer	Bioconjugated solid lipid nanoparticles	(Akanda et al. 2021)
	DMBA-induced animal model	Female Sprague-Dawley strain, breast cancer	Fucose-decorated solid lipid nanocarriers with methotrexate	(Garg et al. 2016)
	Xenograft Rb model in rats	Male Sprague-Dawley rats, retinoblastoma	Lipid nanoparticles, MiR-181a, and melphalan cargo	(Tabatabaei et al. 2019)
	In vivo P388/ADR leukemia mouse model	Male athymic ncr-nu/nu mice – pgp-mediated multiple drug resistant leukemia	Idarubicin and doxorubicin solid lipid nanoparticles	(Ma et al. 2009)
Solid lipid nanoparticle	B16F10 cancer cell line-derived cancer	C57/BL6 mice, breast cancer	Bombesin-conjugated solid lipid nanoparticles epigallocatechin gallate as cargo	(Radhakrishnan et al. 2019)
	TPA-induced and benzo (a)pyrene-induced tumor	Laca Mice, skin cancer	Sesamol-loaded solid lipid nanoparticles	(Geetha et al. 2015)
	Glioblastoma-induced syngeneic mouse model	C57bl/6 mice, glioma	Peptide-coated and docetaxel-loaded lipid nanoparticles	(Kadari et al. 2018)
	DMBA-induced breast cancer	Female Sprague-Dawley strain, breast cancer	Fucose-decorated solid lipid nanocarriers, methotrexate as cargo	(Garg et al. 2016)
	Melanoma-induced mice model	Balb/C mice, skin cancer	Dermal delivery of doxorubicin-loaded solid lipid nanoparticles	(Tupal et al. 2016)
Liposomes	Hepatocellular carcinoma model	Nude mouse, liver cancer	Paclitaxel-loaded EGFR peptide-conjugated magnetic polymeric liposomes	(Lin et al. 2020)
	Dalton's ascites lymphoma mice model	Mice, cancer	Quercetin-decorated curcumin liposomes	(Ravichandiran et al. 2017)

(continued)

Table 1 (continued)

Nanoparticle	Cancer induction method	Animal and cancer model	Nanoparticle	Reference
Lipid composite nanocarriers	Mice-bearing SGC7901 cell xenografts	Mice, gastric cancer	Etoposide-loaded nanostructured lipid carriers	(Jiang et al. 2016)
	Colorectal cancer model	Mice, colorectal cancer	Hyaluronic acid-capped, irinotecan, and gene co-loaded lipid-polymer hybrid nanocarrier	(Wang et al. 2020)
	Subcutaneous xenograft model	Nude mice, pancreatic cancer	Lipid-coated mesoporous silica nanoparticles, irinotecan cargo	(Liu et al. 2016)

specific promoter (MMTV), rendering the mouse susceptible to mammary tumor development. The first oncomice generated a lot of buzz in the cancer research community because it offered explicit confirmation for the concept that oncogene expression in normal cells may lead to tumor formation (Kersten et al. 2017).

Through use of BALB/c nude mice models, the effectivity of estrogen targeting using long circulating liposomal formulations has been examined. The studies reveal an effective estrogen targeting and a viable reduction in gastric cancer (Sun et al. 2021). Similarly, curcumin-loaded solid lipid nanoparticles suppressed the NF-B activation and IB degradation levels (Wang et al. 2015). By delivering IL-12 messenger RNA through lipid nanoparticles, MYC oncogene-driven hepatocellular carcinoma could be reduced through enhanced interferon production (Lai et al. 2018). A mouse model of hepatorenal tyrosinemia Type 1 was employed to understand the effectivity of dendrimer-based lipid nanoparticles having FAH mRNA as cargo. It was demonstrated the drug carrier improved the liver functions and extended the survival rate, leading to its potential as a treatment strategy for genetic liver disorders (Cheng et al. 2018).

The usefulness of mice models for fundamental understanding of a disease condition or its cure has been established beyond doubt. However, translation of such results to take up human trials has been difficult as they share little similarities. This lacunae is overcome by employing pig models as they share several similarities, such as size of organs and pathophysiology. The field of porcine oncology is rapidly developing where oncogenic mutations are being replicated on pigs, leading to imitation of human disease conditions (Kalla et al. 2020).

Conclusion

Animal models have served as an excellent tool to study the pathogenesis of diseases and thus develop disease prevention and treatment. The right choice of animal models and induction methods of cancer is vital for ironing out the differences in

Table 2 Approved lipid-based drug carriers

Nanoparticle	Drug	Use	Status	Reference
Bilamellar liposome desaturated phosphatidylcholine: distearylphosphatidylglycerol: cholesterol	Cytarabine and daunorubicin (5:1)	Myeloid leukemia	FDA approved in 2017	(Maakaron and Mims 2019)
Pegylated liposome	Irinotecan	Pancreatic cancer (metastatic)	FDA approved in 2015	(Ko 2016)
Pegylated liposome	Doxorubicin conjugation with cyclophosphamide	Breast cancer	FDA approved in 2001	(Shafei et al. 2017)
Liposome	Daunorubicin	HIV-associated Kaposi's sarcoma	FDA approved in 1996	(Krown et al. 2004)
Pegylated liposome	Doxorubicin	Ovarian/breast cancer	FDA approved in 1995	(Barenholz 2012)

physiology and immunity levels between animal and human beings. Some examples include the cell line-derived xenografts. Progress in the understanding of role of nanomedicine in diagnosis and management of cancer has been possible through appropriate animal models, where research has progressed in areas of gene editing, chemically induced cancer, and so on. The way forward for cancer therapy is the combination of diagnostics with therapeutics or in other words theranostics. In this, a combination of in vitro and in vivo studies to understand the biocompatibility and toxicity of the theranostic nanoparticles is of high importance. Lipid nanoparticles or liposomes carrying drug and diagnostic cargo seem to be way forward in cancer management.

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Zebrafish: A Versatile Animal Model to Study Tumorigenesis Process and Effective Preclinical Drug Screening for Human Cancer Research

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Darshee Baxi

Contents

Abstract

Animal model systems are very important for preclinical evaluation of drugs and also to understand underlined pathophysiology and cellular cross talks associated with different disease conditions. Rodent animal model system has been of immense scientific interest but has its own concerns in terms of management and handling. Cancer research has seen a huge rise in the number of discoveries of preclinical candidates due to the increase in global burden of this disease. Hence, there is a need to expedite the screening of these candidate molecules such that clinical trials can happen, and the drugs can be available for treatment to patients. In this direction, zebrafish has caught attention of the entire scientific community owing to its similar genetic profile and tumor induction capability. Additionally, they have been easy to manage and experiment within the existing lab set ups. Not only are the adult fishes important model systems for cancer study but also are the unique properties of its embryo that make it one of the promising methods

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to study tumor cell biology and also evaluate drug candidates like several phytochemicals. This chapter reviews the different approaches that have been used recently for cancer research and phytochemical screening using zebrafish adult, juvenile, and embryos as model systems.

Keywords

Zebrafish · Animal model · Cancer · Drug · Phytochemicals

Introduction

Cancer has been the most concerning disease of the current decade. Despite several efforts, the disease is becoming challenging each day and pushing the scientific workforce to develop newer and more efficient drugs and treatment regimen. On one side, the pathophysiology of cancer remains a mystery, and on the other side, existing drugs are seemingly becoming useless with increase in the resistance to these existing bank of synthesis drugs. The clinical complications of drug resistance have set in a high demand for search of newer candidate molecules that could initiate better control over proliferating cells. Rat and mice models have been a long followed choice for in vivo studies, but having concerns of management and absence of sufficiently large dedicated facilities have made scientists look out for alternative model system that is not only easy to handle but also having capability for cancer induction. Vertebrate models are necessary to study cancer therapeutics, and fish to human all have a similar capability for cancer induction.

In this chapter, the benefits of the candidature of zebrafish for effective experimental evaluation of cancer therapeutics are summarized. There are several attributes of this model organism that makes it a very promising candidate for such studies. Zebrafish and human share similar biology of cancer, and so it can be an ideal study model for human cancer understanding. It has been scientifically co-related that teleost can develop different kinds of benign and malignant tumors in organs on being exposed to aquatic chemical carcinogens (Spitsbergen et al. 2000). Both fish and human have genetic basis for cancer development which becomes an additional factor of consideration (Amatruda et al. 2002). zebrafish and human share genomic equivalence and conservation of tumor suppressors, oncogenes, and cell cycle regulating genes. This allows specific targeting of these concerned cancer pathways that adds up to the advantages of this model system (Patton and Zon 2001). Zebrafish embryos have been another most effective ways of screening mutagens and carcinogens. Transparent and rapid development of zebrafish embryos is a unique opportunity to study these responses and also evaluate their efficacy in understanding adult onset targets of cancer. The short period of development is also a window of good opportunity to study developmental origin of diseases, and zebrafish provides this platform with ease of maintenance and detailed scientific insight (Baxi et al. unpublished data; Amatruda et al. 2002; Amatruda and Patton 2008).

Early exposure to environmental toxins is one of the major triggers to a thrifty phenotype and one of the key considerations for developmental origin of health and

diseases (Heindel et al. 2017). Rodent models have been used to study thrifty phenotypes and programming related to adult onset diseases; however, it is very difficult and cost intensive to maintain these animals till they become adult (Baxi et al. 2012). Scientists have looked for alternative models that could be easy to study generations and several stages within one generation to understand these long-term consequences of early life exposure. In this regard, zebrafish fits in perfectly well owing to its short life cycle and ease to study developmental stages. It is also important to note that being small in size they can be easily kept inside a laboratory set up and can be monitored on a daily basis without much concerns. Danio rerio, better known as zebrafish, has proved to be superior for its use in cancer research in the last decade. In order to establish a cancer model in zebrafish, several methods have been used like transplantation of mammalian tumor cells, carcinogenic treatment exposure, transgenic regulation, inducing gene mutations, or activating signaling pathways through chemical exposure which induce tumors in several zebrafish organs like liver, intestine, skin, muscle, vasculature, gonads, and pancreas (Zhao et al. 2015).

This chapter thus is an attempt to understand adult-, juvenile-, and embryo-based approaches of zebrafish as an animal model for throughput research in cancer biology and specific emphasis on phytochemical and drug screening. The attempt here is that scientists and researchers can get a holistic view of the different approaches that can be devised using this dynamic model also sometimes referred to as the new laboratory rat.

Zebrafish as animal model

Zebrafish, Danio rerio, is a tropical freshwater teleost belonging to the Cyprinidae family. It is available in wild in several regions of the world, and hence procuring them from the wild is easy and affordable. There are also mutants developed in labs, and these mutants are critical to study certain specific pathways of concern. There are several attributes of this model organism including relative ease to rear and breed them in captivity, short generation time, easy excess to genomic resources, rapid development, and high fecundity. We also have a complete understanding of genomic sequence, and that makes it easier for molecular understanding and genetic insights. Additionally, embryos are transparent and allow for live imaging which is a unique attribute to this animal model. Also these embryos could be seeded in well plates and be used for screening several chemicals in one short owing to the high rate of fecundity. 70 percent of human genome has similarity with zebrafish and thus again an attribute for better translation research capabilities (Zhao et al. 2015).

Zebrafish Life Cycle, Maintenance, and Breeding

The life cycle of zebrafish is simple and spans over a period of 0 to 90 days from zygote to adult. The embryo develops outside and optically clear that allows experimentations and observational studies. Blastula lasts for 3 hours, and gastrulation is completed in 5 hours in an 18-hour-old embryo. These embryos showcase

primary organ system and segmentation by 24 hours. At the end of 72 hours, the hatching takes place, and the larvae starts searching for food. Food sources can also be manipulated at this juncture for experimental evaluation. The larval window period lasts from 72 hours to 29 days post-fertilization. The larvae also exhibit metabolic accumulation of fatty content when fed on high fat diet; several phyto-chemicals and screening tests can be performed in this window period to evaluate an epigenetic profile. The juvenile form is observed at the window period of 30 days to 89 days post-fertilization. This form is a miniature adult and can easily exhibit morphological changes as well as developmental anomalies (Fig. 1). The adult zebrafish spans between time period of 90 days to 2 years and is also a very good model system to study several tumor-related pathophysiology. The zebrafish adult attains sexual maturity quickly, and sexual dimorphism is also distinct to understand and compare male and female phenotypes. The adult zebrafish has a high fecundity rate, and when maintained under optimum conditions, the zebrafish can lay about 200 eggs per week. In order to maintain suitable optimum conditions; zebrafish are placed in an aquarium system with RO water and aerator pumps. The temperature needs to be maintained at 28 ± 2 °C. Ph is maintained at 7.0 ± 0.5 and dissolved oxygen at 4.0 ± 2 mg/L. Feed is given twice a week and consists of commercial fish pellets or live feed. The water in the tank needs to be changed by 30% daily, and filters are to be cleaned every alternate day to ensure optimum conditions. The day night cycle can be maintained through temperature control. These are the simple lab set up requirements that can be accessed by any lab that is in its basic set up conditions. Additionally, there are fully automated zebrafish cabinets available which can be installed, but owing to its high cost, not all labs may be able to procure them. Male zebrafish have a streamlined body, while female counterparts have a protruding belly.

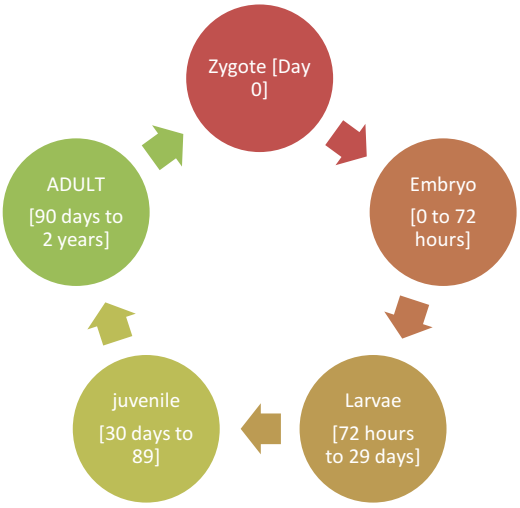

Fig. 1 Life cycle of zebrafish

Zebrafish Cancer Models

Zebrafish can be genetically manipulated for studies, and this kind of forward genetics showcases their utility in developing and predicting novel cancer markers. Spontaneous mutations and transgenetics have been used for inducing human cancers in zebrafish models for both adult and embryo. One of the major advantages is the transparent body of zebrafish that allows long-term evaluation of cancer easier and also helps evaluate environmental responses in them like inflammation and angiogenesis. Visible evaluations serve as one of the biggest assets of this animal model system. Moreover, it is also possible to transplant marked cancer cells into the zebrafish and study. These fishes are easy to house, and their eggs are used in pharmacological screening. In certain kinds of leukemia, zebrafish models were developed through overexpression of certain genes like the proto-oncogenes that have been known to be associated with cancer progression in human beings.

Tumor Induction Through Mutagenesis

There are several methods used for induction of tumorigenesis process in zebrafish. Chemical mutagenesis, irradiation mutagenesis, and insertional mutagenesis through use of transposons or vector mediated are used as ways to induce mutations and subsequent tumor development. Till these genomic interventions were available, the researchers used chemical carcinogens added to the water for cancer development. Some of these chemical carcinogens were N-ethyl-N-nitrosourea, N-methyl-N_-nitro-N-nitrosoguanidine (MNNG), dibenzo(a,l)pyrene, (DBP), 7,2-dimethylbenz(a)anthracene (DMBA), and N-dimethylnitrosamine (DEN) (Letrado et al. 2018). With advancement in genomic editing and engineering, scientists now deploy the use of designer endonuclease to imply inactivation of a concrete gene or modification of gene regulatory circuit through genome editing. Reverse genetics has been used to introduce gene level changes, and several techniques including RNAi and morpholinos have been studied. Ethylnitrosourea (ENU), DMBA, and MNNG have been associated with the development of several skin papillomas, hepatic adenomas, and various neoplasms. Emergence of transgenic technology helped develop transplantable tumor genes in zebrafish. Later, mutant models of zebrafish started to be produced such as mutant tpe p53, and they could be used for several typical kinds of cancer such as nerve sheath tumors. There are several conserved traits in zebrafish that are currently been focused upon, and manipulations in either adult or embryonic mutagenic microenvironment can reveal a lot of novel information of the onset and progression of tumorigenesis process (Bill et al. 2009; Nasevicius and Ekker 2000). A particular advantage that zebrafish provide is that the cancer progression and complications can be studied throughout the life span of this animal model, and each stage can also be individually monitored for experimental intervention.

Transgenic Lines for Tumor Studies

Developing transgenic lines of zebrafish is one of the recent methods adopted to alter the DNA, while the embryo is one cell staged. Such manipulations are done through microinjections of exogenous DNA into the single-celled embryo. Such method leads to altered expression of wild type or activation of oncogenes by manipulation of tissue-specific promotor. However, this method has its own concerns and one of them is being lack of stable lines owing to stronger deleterious effects of such oncogenic actions. In order to address this concern, there are spatial or temporal approaches adopted to restrict the expression of oncogenes (Halpern et al. 2008). Heat shock proteins have been utilized to inactivate tumor suppressor or control oncogene expression. Apart from these, there are several approaches based on genetic manipulations in embryo now available such that long-term studies of mutants can be easily studied, and markers related to underlined causes of metastasis can be well characterized (Langenau et al. 2005; Mosimann et al. 2013).

Tumour Cell Transplantation in Zebrafish

Another very important method that has been used for cancer studies using zebrafish is transplantation of tumor cells and generation of cancer. Several attributes of cancer like angiogenesis, migration, metastasis, and cell extravasation have been studied through this method. In order to carry out transplantation, several approaches are considered; however, the most common literature understanding is of use of cell line transplantation which is not well accepted and is criticized for this use for xeno-transplantation assays. The reasons for this critic are the fact that established cell lines do not necessarily produce similar conditions when transplanted in vivo like heterogeneity and its associated evolution into a metastatic state. There are several approaches for injecting these tumor cells; however, the most advantageous approach is through microinjections inside the yolk as it provides large site to house the transplanted stage specially performed in the first few days of embryo development. This is a better site than inserting cells in the caudal vein or any other regions in adult. Moreover, zebrafish development gives an advantage to escape immune rejection possibilities. The innate immune response takes almost 21 days to be developed in zebrafish, and hence transplantation when carried out in the earlier window of development helps the researcher to avoid use of immune suppressors or any added genetic manipulation (Letrado et al. 2018). Thus, when transplantation is carried out in adult zebrafish models, immune system ablation is required in order to carry out this assay. The techniques used are similar to that used in mouse model wherein suppression of T and B cells and natural killer cell is outlined in parallel to the transplantation protocol. Thus, the stage at which transplantation is carried out serves to be an important point for deciding the final assay (Macgregor et al. 2011; Liu et al. 2011; Zhu et al. 2012; Rudner et al. 2011). Methods that have been used for immune suppression are using sublethal radiation, gamma irradiation to ablate T

Table 1 Different types of graft techniques for implanting tumor cells

Type of graft	Method	Cancer type	Reference
Allotransplantation	Allografts from one to other individual of the same species	all	Letrado et al. (2018)
Xenotransplantation	Live tumor cell transfer from one to the other species	Melanoma, gastric, breast, neuroendocrine cancers	Zhai et al. (2017), Mercatali et al. (2016), Gaudenzi et al. (2017)
Orthotopic transplantation	Transplantation at the same site as the donor	Breast, brain, prostate, lung	Wertman et al. (2016), Eden et al. (2015), Killion et al. (1998)

cells, or dexamethasone to induce immune suppression which allows solid tumor transplantation; in 5 day old, a dose of 250 mg/ml is used 1 to 3 days prior to carrying out transplantation. There are also transgenic immune-suppressed zebrafish mutants developed for such assays. However, it is important to note that such mutants have not been commonly used in transplantation experiments as they are difficult to maintain (Table 1). Two other methods of transplantation that have been recently developed are selection of syngeneic fish as donor to avoid immune suppression. Another method is to irradiate human tumor cells into zebrafish embryo. Subsequently, the same zebrafish shall receive another serial transplant of re-transplanting nonirradiated cells in the same zebrafish after a gap of 3 months when they become adult (Wertman et al. 2016; Eden et al. 2015; Killion et al. 1998; Letrado et al. 2018).

Approaches for Preclinical Drug Screening Using Zebrafish as an Animal Model

In the earlier part of this chapter, detailed understanding of advantages of zebrafish embryos has been outlined. However, the emphasis was for the developing of a cancer model. In this section, we shall now emphasize on the use of this model in screening of preclinical drug candidates. Zebrafish embryos serve to be very good models to study toxicity profiles of drug. The global challenge in the field of cancer research is to come out with more clinical candidates such that cancer management would be better and effective when the number of patients is increasing globally. In this regard, use of zebrafish embryos has served an important discovery to shorten the time of drug discovery and facilitate it by better scientific merits of screening. Researchers have used zebrafish embryos as vertebrate model to develop whole animal bioassay to study preclinical drug candidates. In a typical set up, zebrafish embryos are seeded in 96 well plate, and chemicals are added to the wells, and standard assay protocols are used to study its efficacy (Zhao et al. 2015). The embryos are incubated in single wells, and they grow with the help of yolk without any need of external nutrient source. Small molecules including chemicals, peptides,

Table 2 Summary of different kinds of assays for testing toxicity, pharmacological and cellular understanding using zebrafish embryos

Screening Assay	Toxicity testing
•Varying drug concentrations were administered to the 96 well plate seeded with zebrafish embryos to determine LD 50 , dose was renewed daily for five days and Ph and ammonia were monitored.Each series was repeated four times for standard deviation calculations	
Screening Assay	Angiogenesis
•EAP staining activity checked at 24 hours and 72 hours post incubation of drug to evaliate angiogenic action of the drug molecule. Quantification was done using microplat reader	
Screening Assay	Apoptosis
•Assessment of wholeanimal apoptosis with a fluorescence microplate reader can be used as a primary screen to identify agents that modulate apoptosis in whole embryos.	

and fluorescent substrates simply get dissolved in water and freely diffuse in the presence of a carrier-like DMSO. These procedures allow screening of large number of drug molecules and an immediate statistical understanding that comes as an important advantage for drug testing. Also the amount of testing volume of sample required is small when compared to mouse models. Another very important point of advantage is the visualization capability of internal organs in zebrafish embryos. This provides a better understanding when compared to laborious histological procedures. Further studies may be required for validating the clinical relevance of zebrafish assays, but their conserved genome is a promising attribute to use for developing better evaluation strategies (Table 2).

Phytotherapeutic Testing Using Zebrafish Embryos

If we span through the literature, it is evident that compared to synthetic drug testing and screening using zebrafish embryos, there are lesser studies indicating herbal formulation testing using zebrafish embryos. However, the need for toxicity profiling remains as much for herbal formulations and phytomolecules. Here we emphasize on important studies that have been carried out using zebrafish embryos and their relative outcomes. The zebrafish toxicity model has been particularly advantageous in evaluating crude herbal preparations. Several plant extracts have been tested for embryo toxicity and have also been further co-related with mouse/rat model evaluation or computational methods (Xia et al. 2017a; Sun et al. 2017; Alafiatayo et al. 2019; Sajkowska-Kozielewicz et al. 2016; Romagosa et al. 2016; Ismail et al. 2017; Chen et al. 2017; Ding et al. 2015). Apart from crude extract testing, several phytocompounds and isolated molecules have been tested using zebrafish embryo model. These assays have been employed to evaluate the toxicity profiles of different extractions, compounds, and their constituents. There are reports of toxicity testing of several fractions like ethanolic, methanolic, acetone, and water (Yang et al. 2018; Zhang et al. 2018; Liu et al. 2017; He et al. 2018; Chen et al. 2018) (Table 3).

Table 3 Overview of applications of zebrafish in cancer pathology understanding

Applications of zebrafish model system
Modeling human cancer
Capability to study angiogenesis in tumor
Metastatic cancer study
Antineoplastic screening
Tumor immunotherapy
Prevention strategies for recurring tumor cells
Drug screening
Phytochemical screening
Ease to maintain
Transparent embryos
Microscopic examination of live embryos
Cytotoxic screening

Conclusions

The kind of complexity with human diseases is increasing day by day, and the incidences of cancer are also high despite some of the throughput treatments available. The problem is intense, and the pressure to find novel alternatives as therapeutics is immense. In such a scenario, there is a need to develop model systems that are easy to handle, low cost maintenance, and dynamic to study human cancer types. Rodent models were the choice, but its management and cost have a bearing along with other concerns of handling a large animal model. Additional point of difference between rodent and fish model is the generational time and ease to manipulate the microenvironment in fishes. The fact that they have ex utero development helps us utilize this capability for the scientific understanding. Generating mutants is also possible with these models, and moreover, they are available in the wild with high fecundity rate. This chapter has outlined some of the important attributes of zebrafish as model organism for cancer studies and drug screening. It is also important to note that the conserved nature of oncogenes is an added advantage, and the overall similarity with human genome makes it a classical choice which will remain an important model for in vivo studies in times to come. However, there is a need to further evaluate newer possibilities with this tiny being such that it can be further benefit the goal of overall human health and well-being through in-depth scientific understanding of the diseases and cancer.

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Preclinical Animal Models of Cancer: Applications and Limitations

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Girish Sharma, Yuvraj Goyal, and Simran Bhatia

Contents

Abstract

Despite the global advancement in availability of modern diagnostic tools and a variety of therapeutic modalities, many diseases have still been on the rise worldwide. Animal models serve as a valuable means in conducting preclinical research related to various human diseases including cancer. These models assist

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not only to understand the underlying genetic mechanism of a tumor but also the impact of several crucial genes that quite often gets mutated leading to deadly cancers. Scientists are striving for regulated and customized animal models which mimic the real cancers in human beings in their growth and development. For developing animal models of cancer, a variety of animals and personalized as well as precision medicine methods are being used to cater the demand of different patients. Of these animal models of cancer, several mice models have been used worldwide due to their uniqueness in mimicking human cancers. Here, we have discussed about the major benefits and constraints related with the use of such models.

Keywords

Cancer · Preclinical · Animal models · Applications · Limitations

Introduction

Uncontrolled growth of cells, i.e., tumor, has become one of the major diseases that fatally endanger an individual's health, as a result of proficient control of most important infection causing diseases and the extension of quality and disease-free human life. As per a 2015 World Health Organization (WHO) report, tumor formation is one of the main causes of death among individuals aged between 70 and 91 years in the developed countries (Bray et al. 2018). The number of new cancer cases per year is predicted to rise from 18.1 million in 2018 to 29.4 million in 2040 as an outcome of combined effects of growing and aging population (Wild 2019). Cancer has already become a serious problem in both developing and underdeveloped countries because of delayed detection of most cancers and inadequate prevention measures. To overcome this global burden, highly innovative approaches and new diagnostic techniques are required. Experimentation with animals and/or animal models acts as a bridge to fill the gap between cell culture (in vitro) experiments and clinical research. Animal diseases have analogous occurrence and development to human diseases under specific situations. Further, animal models encompass similar genetics, physiology, and anatomy to humans. Therefore, animal models are helpful to conduct research related to several human diseases including cancer. Animal models can aid in understanding the genetic foundation of a tumor and the impact of certain genes and gene mutations in the initiation and promotion/progression of tumor besides designing and testing of anticancer medicines (Schachtschneider et al. 2017). Scientists are striving for regulated and customized animal models that are more close to human cancers since personalized and precision medicine continues to advance (Xu et al. 2019). To develop animal models of cancer, a variety of methodologies have been used in several animals. Additionally in tumor studies, every tumor mice model has its own peculiarities.

Mouse Model

Prior to the expansion of animal genetic models, researchers have employed cell culture systems using cell lines obtained from human malignancies to study cancer, though use of such methodologies to obtain the useful details that are vital for cancer research has certain limitations too, such as inability to analyze physiological interactions between tumor cells and their environment in vivo. Some of these constraints can be overcome by using xenograft models where human tumor-derived cell lines are transplanted in mice (Cheon and Orsulic 2011). Most of these investigations must be conducted using subcutaneous implantation and immune-compromised mice to avoid immune responses and site-specific interactions. For both morphologic and genetic levels, tumors can be quickly generated favoring human tumor cells within a perfect mouse system. Several mice models have provided an invaluable tool for investigating tumor initiation, promotion/progression, and treatment response. Additionally, cancer biologists now have access to a variety of genetic manipulation tools (Walrath et al. 2010). Choosing the right technique for creating mice models of cancer has been a crucial initial step in any such research study. Furthermore, determining the deliberate aims of individual mouse models is also crucial.

Mice are considered to be the most valuable research animal and perhaps the most profitable vertebrate in the contemporary times due to the fact that they offer various advantages including individual cages that can hold multiple animals in a little area; they have a short life cycle as tiny as 9 weeks among generations for specific strains, also being prolific breeders. For mice, a huge range of tools and reagents are available, allowing researchers to investigate practically any component of the immune response. Additionally, the genomes of human beings and mice species have many similarities, such as amount of genes coding for the proteins (Baxter and Griffin 2016). The murine histocompatibility complex has been well understood, and many features of humoral, innate, and cellular immune responses were first reported in mice and later found in humans. Because of these qualities, the mouse has become the favored animal for studying the host immune response to a variety of virus infections. Despite significant progress in understanding the underlying causes of uncontrolled growth of cells and the identification of anticancer medicines in recent years, effective clinical translation of these techniques remains a challenge (Landgraf et al. 2018).

Humanized Mouse Model

The key reasons state that the majority of cancer research uses rodent models, which differ physiologically from humans in a variety of ways. As a result, rodent cancer models are unable to exactly mimic the constitution of cancer patients, necessitating the evolution of new cancer models that are superiorly suited to broadly signifying the compound features of human tumors, thereby allowing for betterment of central

and translational research (Perrin 2014). The first animals with malignancies of human origin created using this method were the nude mice with T-cell-deficient and immunized with human cancer cell lines, also known as cell-derived (CDX) models. These rodent models have become a common in vivo stage for studying oncogenesis related to human body and assessing the efficiency of anticancer drugs (Fogh et al. 1977). Furthermore, the evolution of mice strains with more severe immunodeficiency such as NOD/SCID mice and NOD/SCID IL2rg/mice made it easier to use rodents to competently repopulate primary human tumor cells and mirror the diverse features of these cancers in patients [patient-derived xenograft models (PDX models)] (Hidalgo et al. 2014).

In tumor studies, both human oncology investigations and anticancer medication intervention are greatly aided by the use of CDX and PDX models. Current studies have revealed that the lack of human-resistant components in these types of animals may critically limit their utility in advanced studies and the development of innovative human cancer therapies (Aparicio et al. 2015). Humanized mice are genetically modified to carry genes, defected cells, and tissues, allowing them to mimic human traits (Shultz et al. 2007). HIS mice (humanized mice with a functional immune system of humans) could be a useful animal to study the relations among human immunological mechanisms and tumors as well as helpful in the development of antitumor interventions (Hu and Yang 2012).

Immunodeficient Mouse Model

Human tumor cell engraftment in immunocompetent models is hampered by robust xenogeneic immune rejection (Yang and Sykes 2007). Various mouse strains which are enabled to produce sufficient immune responses have been created by interrupting key genes involved in resistant cell formation, endurance, and functionality. The capability to create these animals which are able to produce sufficient immune responses is essential for creating humanized mice that can be used to study human cancer progression. Immunodeficient mice were produced indeed to overcome the rejection of human cancer cells by the mouse adaptive (T and B cells) and innate (NK cells and macrophages) immune systems. For instance, forkhead box N1 (*Foxn1*) and protein kinase, DNA-activated, catalytic polypeptide (*Prkdc*) gene (Bosma et al. 1983) deletion causes T- or B-cell deficiency in mice; deletion of interleukin 2 receptor subunit gamma (*IL2rg*) (Ito et al. 2002) or β2-microglobulin (*B2m*) genes leads to the absence or functional impairment of mouse NK cells, recombination-activating gene 1 (*Rag1*) (Mombaerts et al. 1992), and recombination-activating gene 2 (*Rag2*) (Shinkai 1992), although selection of nonobese diabetic (*NOD*) or *NOD Sirpa* genes prevents phagocytosis by mouse macrophages (Table 1). Combinations of these genetic engineering strategies have been applied to develop the popular immunodeficient mouse strains such as NOD/Prkdcscid (NOD/SCID), NOD/SCID IL2rg−/− (NSG or NOG), and Balb/c Rag1−/− IL2rg−/− (BRG) that have all been used in human cancer research.

Single-gene mutation models such as nude mice (*nu*) strains and severe combined immunodeficiency (SCID) strains, nonobese diabetic strains, *RAG* (recombination-activating gene) strains with targeted gene deletion, and a variety of hybrids derived

Table 1 Various gene deletions and their effects on mice

S. No.	Deleted gene	Effect of deleted gene on mice	Reference
1.	Forkhead box N1 (*Foxn1*), recombination-activating gene 1 (*Rag1*), recombination-activating gene 2 (*Rag2*), and protein kinase, DNA-activated, catalytic polypeptide (*Prkdc*) gene	T- and B-cell deficiency in mice	Bosma et al. (1983); Mombaerts et al. (1992); Shinkai (1992)
2.	Interleukin 2 receptor subunit gamma (*IL2rg*), β2-microglobulin (*B2m*) gene	Functional impairment of mouse NK cells	Ito et al. (2002)
3.	Nonobese diabetic (*NOD*) or *NOD Sirpa* genes	Prevents phagocytosis by mouse macrophages	Ito et al. (2002)

from crossing double and triple mutation mice strains with additional defects in innate as well as adaptive immunity are among the immunodeficiency mouse models.

Patient-Derived Tumor Xenograft Models

Human xenograft models, which involve human cell lines transplanted into hosts with weakened immune systems such as SCID mice, are widely used models for evaluating cancer cell killing medicines. These models are very useful for the development of chimeric antigen receptor (CAR) medicines, which can grow xenografts for antitumor efficacy testing using either human cell lines or patient-derived materials (Siegler and Wang 2018).

The degree of immunodeficiency of the murine host is one of the essential parameters that determine the efficacy of human xenograft models for various immune system-enhancing uses. T-cell function is impaired in athymic nude mice because they lack normal thymic development. Many parts of the immune response, however disrupted, are present in athymic nude mice because functioning innate immune populations such as neutrophils and dendritic cells, as well as B cells and natural killer (NK) cells. As a result, in this model, engraftment of human hematopoietic elements and other primary human cells is extremely limited. SCID mice lack a DNA-dependent protein kinase that is necessary for T- and B-cell development, while Rag-deficient mice lack the *Rag1* and *Rag2* genes, which are also required for T- and B-cell function. The ablation of the *IL2r* chain results in concurrent impairments in the functions of the IL2, IL4, IL7, IL9, IL15, and IL21 receptors, as well as mice lacking NK cells. The immunodeficiency in the ensuing mice worsens as a result of combining genetic defects, and the engraftment of donor human immune cells improves as a result. Thus, mice produced from SCID, Rag1null, or Rag2null animals with a specific mutation in the *IL2r* gene are the best for engraftment of human hematopoietic stem cells (Hasgur et al. 2016).

The NOD/SCID *IL2r* chain knockout mouse, preclinical model with engineered combined immunodeficiency has been frequently used hosts for chimeric human-mouse immune reconstitution and various tissue chimaeras (Walsh et al. 2017). Although athymic nude mice were adequate for engraftment of human cancer cell lines, NSG mice and their counterparts are necessary for engraftment of real human tumors (Puchalapalli et al. 2016). These primary tumor samples were used to create PDXs that accurately model the complexity involved in natural tumor development, including genomic heterogeneity, tumor architecture, and microenvironmental factors, which is critical for developing an effective in vivo preclinical tumor model for therapeutic evaluation (Byrne et al. 2017).

Given the importance of immunotherapy in various human cancers and the disadvantages of both identical gene cell line-based models and GEMMs in producing tumors that accurately reflect the genetic and physiologic heterogeneity of human cancers, there has been a growing interest in developing methods of human immune reconstitution within PDX models in order to create an experimental humanized model for evaluation of immunotherapy. The regenerated human hematopoietic system and tumor from the same patient must theoretically match in this experimental setup. There are hurdles to the effective development of humanized PDXs.

For successful tumor proliferation, among several mice in a realistic timeframe, a PDX model requires high take rates. The hematopoietic system, unlike the PDX models, cannot be formed from a single tumor that is transmitted through several mice; therefore recurrent persistent sampling would be required to form individual humanized PDX mice.

Murine Tumor Mouse Models

Genetically Engineered Mouse Models

Our understanding of the hereditary basis for tumor has advanced dramatically in the last two decades, followed by the advancement in genetic engineering technologies. These advancements result in the development of mice that incorporate precise genetic changes to allow tissue-specific autochthonous tumor growth. These models primarily use tissue-specific promoters to induce tumor gene expression via either SV 40 vector or genes pertinent to tumor formation (Greenberg et al. 1995), such as tissue-specific recombinase enzyme expression to drive tumor suppressor gene deletion (such as *PTEN* and *TP53* in prostate cancer (Chen et al. 2005) or *APC* in colon cancer (Shibata et al. 1997), *Kras* and *MYC* in breast cancer (Sinn et al. 1987), and *BRAF* V600E in melanoma (Hooijkaas 2012)).

In prostate cancer models, these genetic changes can be used to drive autochthonous invasive development of cancer and to create precancerous lesions such as prostatic intraepithelial neoplasia or pancreatic intraepithelial neoplasia (*PanIN*) (Kaplan-Lefko et al. 2003) in pancreatic cancer models (Hingorani et al. 2005). For immunotherapeutic intervention, extensive windows are required to establish an efficient anticancer immune response and simulate immune-associated side effects that are made possible by the longer period of tumor formation and progression.

This prolonged genesis and spread of tumor allow the autochthonous establishment of a compound tumor microenvironment, as genetically designed models stimulate neoplastic transformation of healthy cells at the necessary organ location to drive tumor development. In comparison to syngeneic tumor models, this significant advantage of GEMMs makes them particularly useful for assessing immunotherapeutic modalities. The quantity and composition of immune cell infiltration are both governed by native immunosuppressive stroma and vasculature, both of which are present in the novel tumor microenvironments that develop in the setting of GEMMs. Models can also be utilized in which the alteration driving tumor development is related to the tumor immune microenvironment, which can impact immunotherapy effectiveness like *PTEN* loss which has been linked to an immunosuppressive tumor microenvironment in melanoma, making it possible to test therapeutic modalities making these malignancies more sensitive to immunotherapeutic intervention using models in which *PTEN* loss is the driving force behind tumor formation. This is in dissimilarity to human disease which is most commonly affected by the steady accrual of mutations in a lesser fraction of cells contained by the organ of origin, eventually leading to transformation (Peng et al. 2015).

GEMMs which exploit tissue-specific promoters stimulate development of cancer in entire cells of that lineage. Furthermore, the mutational load in genetically engineered models may not be equal to that described in the corresponding human illness due to overexpression or deletion of a specific number of genes. Increased mutational load and subsequent neoepitope production are a significant issue for evaluating the efficacy of ICB, so this is critical when evaluating immunotherapies (Goodman et al. 2017). It is conceivable to increase carcinogenesis while also driving the accumulation of new mutations by targeting genes linked with mismatch repair and genomic stability [such as *MLH1* (Germano et al. 2017), *BRCA1/2* (White et al. 2008), *APC* (Shibata et al. 1997), and *mTERT* (Bojovic and Crowe 2011)]. This enhanced mutational rate encourages the production of novel antigens that CD8+ T cells can detect, thereby making immunoediting easier (Yarchoan et al. 2017). Moreover, increasing genomic instability may enhance the coevolution of the anticancer immune response and tumor escape pathways, potentially leading to immunotherapy confrontation.

Challenges Faced by GEMMs

However, GEMMs have significant merits in the evaluation of immunotherapeutics; they face some of the logistical obstacles that prevent them from being used in the evaluation of cytotoxic drugs.

1. A major challenge when employing GEMMs is, consider the entrance of the model tumor phenotype and neoplastic development latency depending on the mechanisms, this can vary dramatically and be used to promote the growth of tumors (Ku et al. 2017). The development of GEMMs that target numerous tumor suppressors that can boost penetrance and decrease latency can help to overcome some of these limitations.

2. To monitor tumor progression, standardize treatment scheduling, and track the kinetics of anticancer immune responses, noninvasive imaging techniques such as ultrasonography or magnetic resonance imaging (MRI) are required.
3. It is vital to assess whether the murine immune target is cross-reactive with the comparable human target, just as it is with syngeneic models. This comprises antigens and surface markers found on human immune cells as well as malignancies not found in mouse cells.
4. Cross-reactivity is especially important in the development of immunotherapeutic vaccines that detect antigens in the context of human MHC class I in patients; GEMMs that integrate human MHC class I and MHC class II have been designed to analyze peptide-specific T-cell responses that are relevant to human antitumor immune responses in order to examine these peptide-specific responses (Pajot et al. 2004). Furthermore, expression models that incorporate target antigen expression into GEMMs (DuPage et al. 2011) have been established enabling the quick construction of models that may be used to test antigen-specific immunotherapies. Antigen processing differences occur between mouse and human antigen-presenting cells, affecting cross-reactivity with human epitopes and underlining the fundamental issues that afflict all preclinical models that employ murine vs human cancer cells. As a result, the best paradigm for evaluating immunotherapies would be responses to human cancers in immunocompetent models.

Tumor Cell Lines with Identical Genes

Tumor models with similar genes are the most well-known and widely used in preclinical trials for evaluating antitumor drugs. It is feasible to obtain impulsive, mutagen-induced, or recombinant cancer cell lines from inherent strains such as C57BL/6, BALB/c, and FVB mice which can then be extended in vitro and utilized to immunize wild-type hosts to generate a cancer-bearing system. These animals are mainly valuable in the evaluation of immunotherapy medicines because they can be used to evaluate the formation of new antitumor immune responses without the need for adoptive immune population transfer. The most noticeable applications of syngeneic tumor models are the use of mutagens to induce tumor growth in mice and then testing the anticancer efficacy of tumor immunotherapies in these tumor-bearing mice (Schreiber et al. 2011). These models were employed by Schreiber et al., to detect and characterize the process of transformation of normal cells into clinically detectable tumors using chemicals such as methylcholanthrene (MCA). The impact of mutagen-induced cell lines on tumor formation and antitumor resistant responses as well as the evaluation of immunotherapy can all be investigated (Uno et al. 2006). Carcinogen-induced cancer models, in contrast to genetically specified tumor models, have a higher level of gene instability, ensuing in the formation of a more "physiologically realistic" tumor microenvironment. However, this complexity comes with its own set of obstacles, such as penetrance of tumor, issues related to latency, and lack of shared tumor allergens.

Many of these mutagen-derived tumors were used to create cancer models which are frequently employed to create mouse tumor models with identical genes. The

most vital advantages of tumor cell lines with identical genes is their simplicity. Because they use tumor cell lines that can be rapidly and reproducibly expanded in large numbers prior to implantation into hosts, these syngeneic models can be used for studies that require large group numbers that are difficult to obtain using genetically engineered models or patient-derived xenografts (PDXs). Another advantage of using syngeneic tumor models is that they may be genetically manipulated to evaluate particular tumor cell-intrinsic immunotherapy sensitivity or resistance biomarkers. In research assessing antigen-specific vaccination methods, tumor cells engineered to express the target antigen, for example, can be used to investigate anticancer effector responses in vitro and in vivo. It can be challenging to target antigens whose expression is typically limited to organs for which there are no suitable preclinical models. It is also possible to weigh the relative relevance of a variety of factors that might affect immunotherapeutic effectiveness. This is especially true in checkpoint blockade, where checkpoint ligands may be removed or altered to determine their function in the anticancer response.

Challenges Faced by Tumor Models with Identical Genes

Tumor models with identical genes have turned out to be the most widely used preclinical model for the assessment of immunotherapy due to their ease of use and experimental reproducibility. However, these practical advantages also highlight one of the system's drawbacks. The genetic and microenvironmental heterogeneity that characterizes cancer is missing from these models. Tumor heterogeneity causes both intrapatient and interpatient heterogeneity, which makes each patient's malignancy distinct (Hanahan and Weinberg 2011). This is the most thought-provoking elements of creating effective cancer treatments; therefore an ideal preclinical system for researching immunotherapeutics would also correctly represent this diversity. Syngeneic tumor models, on the other hand, are woefully deficient in both areas. The cell lines are transplanted into a small number of inbred mouse strains that lack interpatient variability and lack mutational patterns that mirror human intrapatient genomic heterogeneity. The absence of cancer stem cells and other progenitor populations in the tumor microenvironment contributes to the lack of mutational heterogeneity in syngeneic xenograft tumors. This might be a long-term source of tumor mutational evolution (Shackleton et al. 2009). Furthermore, mutational heterogeneity requires clonal development of differentiated cancer cells, which may be problematic in many syngeneic murine models due to their lower levels of genomic instability than humans (Prowse and Greider 1995).

In addition, tumor models with identical genes have typically experienced considerable selection as a result of adaptation to severe in vitro or in vivo conditions, resulting in clonal diversity limitations. In vivo implanted syngeneic tumor cells can behave differently throughout tumor growth. Numerous lineages can be introduced into tumors to overcome these barriers, leading in tumors with multiple populations (Calbo et al. 2011).

During the immunoediting process, this artificial heterogeneity lacks the tumor cell-intrinsic functional flexibility that permits tumors to continually adapt and develop in response to the immune response. Another problem with animal models

with identical genes is that the implanted tumors develop as new poorly differentiated malignancies rather than going through the natural stages of tumor evolution that genetically modified models do like premalignant transformation, tumor development, and progression (Greenberg et al. 1995).

In most syngeneic tumor models, this causes tumor growth to be sped up, resulting in tumor expansion over a period of weeks. Because anticancer immune responses produced by immunotherapy normally have a latency period prior to growth and maturation, therapeutic advantage is often detected as improvement in overall survival rather than objective clinical reactions (Madan et al. 2010). As a result, the rapid kinetics of tumor growth in syngeneic models frequently provides an insufficient time frame for assessing immunotherapy efficacy. Furthermore, it prohibits the study of immunotherapeutics at initial stages of disease, which has been indicated as a possible best time to begin interventions by activating the immune system in some abnormal cells in order to maximize clinical benefit (Gulley and Drake 2011).

Zebrafish (*Danio rerio*)

Over the years, the zebrafish (*Danio rerio*) has become a popular and widely used animal model in preclinical cancer research. The zebrafish is a tropical freshwater fish belonging to the Cyprinidae family and the Actinopterygii class that lives primarily in the Ganga river in India.

The fish was initially utilized as a study replica in the year 1970 by a researcher George Streisinger, who chose it over the mouse model because of its simplicity and ease of genetic manipulation. However, in the 1990s, the usage of zebrafish as an animal model grew, as two scientists exploited the model to create two huge mutant lines (Dahm et al. 2006). Zebrafish and its capacity to faithfully mimic a range of human malignancies make them a valuable in vivo system for drug discovery and validation.

Transgenesis, gene inactivation, transplanting, and carcinogenic induction all have been used to create zebrafish models of human cancer that have shown to be molecularly and pathologically comparable to their human counterparts. The suppression of cancer-relevant traits allows researchers to find and test effective drugs in embryonic and adult zebrafish. Following the selection of suitable compounds, preclinical testing in mammalian models can be carried out, allowing lead compounds to enter human trials quickly and efficiently. From past decades, zebrafish has been utilized as a scientific animal. Because of the obvious advantages of zebrafish, including large clutch sizes, transparent embryos, and embryo development outside of the womb, the initial focus was on developmental biology. Studies have indicated that zebrafish may form practically every type of tumor on their own. There are various most prevalent targets for spontaneous neoplasia: testis, gut, thyroid, liver, peripheral nerve, connective tissue, and ultimobranchial gland. Furthermore, the zebrafish model provides a number of advantages for therapeutic nanoscale drug delivery systems, particularly in terms of optical transparency. Its

zebrafish embryo's transparency allows researchers to see within the fish body in real time, such as organ or tumor development and vascular expansion. The embryos of zebrafish remain translucent until 60 hours after fertilization (hpf), when the coloring process begins. To maintain their transparency beyond that, the embryos are treated with chemical called 1-phenyl 2-thiourea (PTU), which inhibits the coloring process, or transgenic mice like Casper, which lack pigments on their skin, can be utilized instead (McGarth 2008).

The existence of replicated factors resulting from a recent partial or total genome replication in teleosts is a recurring problem in zebrafish, which may alter the role of tumor genes and tumor inhibitors in tumor genesis. For example, in the zebrafish genome, there are two variants of pten that are functionally superfluous in growth but not in tumor genesis. There will be no defeat of pten a as found in the defected cells of homozygous pten b mutants (Feitsma and Cuppen 2008). Because zebrafish is an excellent model for molecular investigations of many human diseases, cancer is one of the most studied disorders utilizing this model. This is owing to the models and its ease of design, which includes genetic editing, xenotransplantation, and chemical exposures. In zebrafish, for example, overexpression of the *Myc* gene causes T-cell leukemia, exposure to dimethylbenzanthracene causes intestinal cancer, and transplantation of human cancer cells such as B16-F10 melanoma cells causes melanoma in the model (Harfouche et al. 2009).

Pig (*Sus scrofa*)

Animals have extensively been used to learn more about human abnormalities, and they continue to play an important role in cancer research (Wright et al. 2008). Nonmammalian creatures like zebrafish can provide useful information. Zebrafish have the unique benefit of having naturally transparent embryos and larvae, as well as the ability to produce transparent adults (Antinucci and Hindges 2016). This makes it easier to research and track tumor vasculature (Nicoli et al. 2007), spread of cancer cells to other body organ (Wang et al. 2014; Liu et al. 2017), and anti-vasculature (Jing et al. 2018; Landgraf et al. 2018) drug evaluation in vivo. Zebrafish, on the other hand, are extremely different from human in terms of mass, overall survival, and most importantly, environmental influences. Bigger models such as dogs, cats, nonhuman primates, and *Sus scrofa* have been used as research species because they have some parallels to humans. Although dogs and cats grow cancers on their own (MacEwen 1990; Alvarez 2014) and their veterinarian action has supplied precious source for tumor studies, the general public does not accept the utilization of attendant models in properly scientific investigation. Nonhuman primates are also restricted due to tight restrictions imposed and ethical considerations. Pigs, on the other hand, have been domesticated as a source of food for generations. Their human and legally used animals as experimental models under controlled situation raise less problems (Perleberg et al. 2018). *Sus scrofa* are quite suitable animals for biomedical study because they split numerous parallels with human in

terms of mass, size of body organs, structural design, physiology, and pathology (Lunney 2007).

Pigs have traditionally been used to investigate the effects of nourishment, to evaluate new surgical events or advance organ transplant, and to create imaging technologies that may be employed on a human scale in addition to their reasonably long longevity of 12 to 15 years (Hoffe and Holahan 2019). Long-term research can be conducted to evaluate or verify new biological markers, treatments, or imaging choice and track illness development and failure in a single model (Flisikowska et al. 2016). Pigs have identical pharmacokinetic reactions to human beings in pharmacological studies (Myers et al. 2010). Pigs have thus been identified as a valuable animal model for translational drug discovery.

Genetically Modified Pigs

Palmiter and Brinster were the first to use DNA microinjection into the pronuclei of fertilized oocytes to make transgenic rabbits, sheep, and pigs (Hammer et al. 1985). However, only 1–5% of transgenic progeny were produced as a result of this approach (Tian et al. 2018). Porcine oocytes have high lipid content, making it difficult to see the pronuclei. The production of a large number of nongenetically modified models frequently larger than 95% was both ethical and practical. Furthermore, as initially envisioned, DNA microinjection only allows for the inclusion of transgenic genes at random places in the host genetic material. As a result, more competent and adaptable techniques were sought.

Transgenic pigs expressing a *Cas9* transgene that is either inserted at the ROSA26 locus in a Cre-dependent manner (Wang et al. 2017) or universally all through the body have recently been created, inspired by work done in mice (Platt et al. 2014). By delivering single or many guide RNAs with or without Cre-recombinase to somatic cells in vivo, gene editing of somatic cells can be done in vivo. Although in vivo genome editing is still in its infancy, it promises to be a potent tool for modifying the genome in specific organs and cell types at any age. Tumor-supportive genes, for example, can be tweaked in multiple rounds of gene editing to replicate the accumulation of changes that occur as tumor entities move forward (Makohon-Moore and Iacobuzio-Donahue 2016). Somatic modification also allows researchers to study mutations that would otherwise be deadly or detrimental in specific tissues or organs without having to worry about their consequences on the rest of the body. In practice, Cas9-expressing animals can shorten the time it takes to generate and breed new lines with desired mutations, which is a considerable benefit for bigger species.

Porcine Cancer Models

Although the study of porcine cancer biology is still in its early stages, preliminary evidence suggests that pigs can accurately mirror human tumors. In wild-type pigs, spontaneous malignancies develop infrequently, and in humans, they are associated

with increasing age (Watson et al. 2016). Oncogenic transformation of pig cells like that of humans is a rare occurrence that necessitates numerous genetic changes (Schook et al. 2015). The topic of whether duplication of human tumorigenic mutations in pigs has a similar effect on cell transformation and cancer has been a long-standing one. This appears to be the case thus far. When Adam and coworkers overexpressed tumor transgenes into pig primary fibroblast cells, the cells became tumorigenic when autologously transplanted back into the donor animals (Adam et al. 2006). To transform to a completely transmogrified phenotype, Saalfrank and coworkers investigated experimentally the stages of sarcomagenesis in vitro and discovered that porcine mesenchymal stem cells (MSCs) are similar to human MSCs in that they necessitate perturbation of the *p53*, *KRAS*, and *MYC* signaling pathways, as well as spontaneous Rb pathway inactivation and telomerase-independent immortalization steps (Saalfrank et al. 2016). This is in contrast to murine MSCs, which can be changed simply by losing their p53 function (Rubio et al. 2010). These findings imply that porcine and human oncogenesis are fundamentally comparable, yet in vitro culture, randomly integrated overexpressed transgenes, and engraftment of altered cells could all be critiqued as nonphysiological artificial approaches.

Porcine Tumor Xenograft Model

In mice, xenotransplantation of human cancer cells is well established for modeling human tumors. Patient-derived xenograft (PDX) mouse models, which are created either by subcutaneous (s.c.) or orthotopic grafting of tumor samples from human into severe combined immunodeficient (SCID) (Xu et al. 2018) mice, are currently preferred over xenografting of cell lines, which may have lost the original tumor heterogeneity over long periods of culture (Murayama and Gotoh 2019). As a result, the PDX technique is a superior predictor of human tumor activity, and murine PDX models for colon, pancreatic, and breast malignancies have been developed (Puig et al. 2013; Jun et al. 2017).

Researchers have been working to produce immunodeficient pigs which could be used in a similar xenograft system. By removing the thymus and spleen in combination with pharmacological immunosuppression, Nakayama and colleagues were able to create immunodeficient pigs (Itoh et al. 2019). Although efficient, this method is quite intrusive and can only be used to produce a small number of immunodeficient pigs. Germline alteration of genes involved in B- and T-cell development, such as the X-linked interleukin-2 receptor gamma chain gene (*IL2RG*) or the *V(D)J* recombination-activating genes, is a superior alternative (*RAGs*) (Table 2). *IL2RG* disruption has resulted in SCID pigs in several studies (Suzuki et al. 2012; Watanabe et al. 2013). These animals lacked a thymus and had a decrease or reduction of T and NK cells, but they died quickly from diseases such as pneumonia.

Pigs are a relatively novel species for cancer research. Continued advances in physiological, biochemical, immunological, and genetic data will boost their utility in biomedical research, and improved tools for manipulating germline and somatic cells will make developing novel models easier.

Table 2 Major advantages of using some animal models

Animal model	Advantages	References
Mouse	Genetic manipulation is easy	Cheon and Orsulic (2011)
	Cost-effective	
	Requires less time	
	Availability	
	Genome is similar to that of human genome (99%)	
Zebrafish	High fecundity	Hason and Bartůněk (2019)
	Short generation period (approx. 3 months)	
	External fertilization	
	Rapid embryonic development	
Pig	High homology with human genome	Schachtschneider et al. (2015)
	Anatomically, genetically, and physiologically similar to humans	
	Provide relevant information to solve complex disease	

Applications of Animal Models

Xenografts

When it is required to rely on animal model systems that closely mirror tumor growth in a human patient, xenografts are employed to address key concerns in cancer research. In comparison to cells generated in vitro, human xenografts developing in immunodeficient mice are a well-established and valuable model for investigating human tumor biology in a system that better matches the actual tumor. Xenograft studies frequently employ highly passaged cell lines that have been genetically manipulated and artificially cultivated, resulting in clonal selection that may or may not be visible in patients.

The original tumor properties such as diverse histology, clinical biomolecular signature, malignant behaviors and genotypes, tumor architecture, and tumor vasculature are thought to be preserved when xenograft tumor models are created from patient-derived tumor tissue at low passage. Primary tumor xenografts are thought to provide meaningful predictive insights into clinical outcomes when examining the efficacy of innovative cancer therapy, according to this widely held belief.

Genetically Engineered Animal Models

GM animals have indeed been generated and are now being used to supplement or, in some circumstances, replace the standard chronic rodent bioassays used to evaluate risk of cancer (Eastmond et al. 2013). To examine the involvement of

individual genes and signaling pathways in chemical carcinogenesis, a vast variety of GM animals have been utilized and/or are actively being generated.

Genetic modification methods come in use to make mice with mutation in any of its gene. Researchers can now create genetic modifications at specified periods or in specific organs using current technology. Other sorts of models, such as those that make mice sensitive to human contagious diseases such as HIV or CJD, are possible.

Before a new drug can be released into the market, it must undergo extensive testing. The effectiveness of a treatment should be examined in the most similar model to the human problem as possible, which could be a GM animal, generally a mouse that has been created to match the human state. Giving a medicine to a normal mouse may have no impact, but giving it to a mutated mouse may result in a beneficial outcome if the process works.

The development of GM pigs expressing a human gene, which might prevent acute rejection of organs transferred between pigs and people, was one of the first genetic changes of larger animals. When pig tissue is grafted into another species, antibodies in the recipient attack the donated organ, resulting in graft rejection due to the inflammatory reaction. Rejection of the transplant can be avoided by making a change to some of the proteins on cells that induce the body to mount an immunological response, known as complement control proteins.

Zebrafish: Make a Good Animal Model

A fully sequenced genome, facile genome manipulation, high fecundity, short generation period (approximately 3 months), rapid embryonic development (24 hr), and external fertilization are the most favorable traits of zebrafish. Starting with the early stages of embryogenesis, the translucent zebrafish embryo permits researchers to analyze the many stages of development. Furthermore, after 48 hours of fertilization, zebrafish embryos create complete vital organs, such as the heart, gut, and blood arteries. To study human disorders, more than 10,000 mutations in protein-coding genes have been created, as well as multiple transgenic zebrafish lines.

Another significant advantage of zebrafish is the accessibility of various strains. Furthermore, keeping a high number of zebrafish in a limited quantity of laboratory area is quite cost-effective. Although zebrafish are generally simple to handle, particular care must be taken to ensure a balanced diet and proper water quality in order to maximize fish health and growth.

Canine genomes are more comparable to human genomes than rodent genomes. Canines can develop tumors that have clinical, molecular, and histological characteristics that are similar to human cancers. Uva et al. looked at gene expression in human and canine breast cancer samples, as well as normal breast tissue, and discovered that unregulated genes present in human breast cancer were also prevalent in canine breast cancer samples. The *PTEN* gene is also lost in expression in canine breast cancer, according to Ressel et al., and similar conditions can be seen in human breast cancer (Ressel et al. 2009). Nonhuman primates are similar to humans and share many characteristics with them, including physiology, metabolism, immunity, genetics, and many others, making them a good cancer study model.

Major Limitations of Animal Models

Although there are various advantages associated with use of preclinical animal models of cancer, some major limitations for using them are mentioned below:

a) In the scientific literature, animal models have not been validated as a crucial step in biomedical research.
b) Animal research's limits and inability to provide solid forecasts for human clinical trials are becoming better recognized.
c) Because negative outcomes are frequently hidden, animal studies appear to overstate the likelihood that a treatment would be effective by roughly 30% (Sena et al. 2010).
d) Only about a third of highly cited animal research is evaluated later in human trials. Of the one-third that reaches clinical trials, only about 8% of medications successfully complete phase I (Hackam and Redelmeier 2006).
e) Experimental tumors generated in rodents are the most common preclinical instruments for new-agent screening prior to clinical testing. Despite the fact that mice are the most widely used model, they are inadequate models for the bulk of human diseases.
f) Animal models cannot be used to find a cancer cure because of crucial genetic, molecular, immunologic, and cellular differences between humans and mice.
g) Researchers discovered that transcription factor binding locations differed between humans and mice in 41% to 89% of cases in a study of over 4000 genes (Gawrylewski 2007).
h) In many cases, mouse models are used to duplicate certain processes or groups of processes within a disease but not the entire range of physiological changes that occur in humans in disease settings.
i) Poor technique and failure of the models to effectively imitate the human disease situation are likely to blame for the failure to translate from animals to humans. The problem could be embedded in the animal modeling process itself. There are no best-practice standards for animal research, unlike in human clinical trials.
j) It has been suggested that therapeutic drugs be tested not only in rodents but also in higher animal species and that randomization and outcome assessor blinding be carried out. Furthermore, trials including both genders and different age groups of animals should be designed, and all findings, both good and negative, should be published.

Conclusions

In order to equip the increasing demand of different cancer patients across the globe, personalized and precision medicine approaches using various animal models of cancer are gaining momentum for exploiting their potential. Selection of preclinical animal model is a prerequisite for its successful translation into clinic; the benefits and constrains of these models should be accessed cautiously. While doing so,

several key features should be considered including the model should imitate the spectrum of cancer stages in human beings in their initiation, promotion, and/or progression and metastasis besides the route of administration of potential drug or anticancer compound and its underlying mechanism of action, scientific validation of its translational efficacy, and the window of opportunity for the treatment. To improve the clinical outcome, further exploration of preclinical animal model of various deadly cancers is warranted to defeat this fatal disease.

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Alternative Animal Models in Cancer Research

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Abstract

Cancer, a broad word encompassing a range of diseases characterized by the excessive division of abnormal cells, has emerged as a major public health concern around the world, and it became the second biggest cause of death. Animal models are valuable tools in cancer biology by playing vital role in understanding disease and the development of anticancer drugs, despite their limitations in terms of predictive and translational relevance to humans. Cancer models can be classified as naturally occurring or artificially created experimental systems that exhibit similar characteristics to human cancers despite their heterogeneity. One of the most challenging features of cancer evaluation is on deciding the better model representing the given tumor system. Therefore, various investigations on cancer models have been done to better understand cancer invasion, progression, and early diagnosis. These models shed light on the etiology of cancer, its genetic basis, the host–tumor interaction, preclinical investigation of anticancer therapeutics, the role of microenvironment in caner progression, and tumor variability in tumor transformation and metastasis. These models are also valuable for drug development since they can forecast new cancer markers and targeted therapies. Several major tumor animal models, both in vitro and in vivo, and the advancement of animal models in malignant research are comprehensively covered in this work. Although none of the cancer models are ideal, they all have significant shortcomings that limit their application by narrowing the gap between basic cancer research and translational treatment. However, when properly designed and performed, animal models can give invaluable information to our biological knowledge and medicine, as well as the discovery and evolution of advanced drugs.

Keywords

Cancer · Cancer stem cells · IdMOC · PDX

Introduction

Cancer is a universal health concern, and it is the second major cause of mortality. Cancer researchers play an essential role in developing innovative diagnostic and therapeutic approaches. This includes identifying the cancer-causing genes and chemicals, determining how the disease grows and spreads, developing and testing new treatments and tests, and understanding how our immune system can aid in the battle against tumors. In 91 countries, cancer still remains as the leading or second dominant cause of mortality among individuals under the age of 70, according to World Health Organization (WHO) figures from 2015 (Bray et al. 2018). The combined impacts of population aging and population growth are expected to increase the amount of new cancer reports per year from 18.1 million in 2018 to 29.4 million in 2040 (Wild 2019). Approximately 90% of cancer-related deaths are caused by metastasis; yet, many elements of the metastatic cascade are still unknown. New diagnostic technologies and innovative treatment strategies must be developed and researched to minimize the global occurrence of cancer. The experiment on animals serves as a vital link between cell research and clinical trials.

Animal models can aid researcher's better digest the chemical induction, the function of certain genes and their mutations in cancer spread and advancement, and the development and testing of anticancer drugs (Schachtschneider et al. 2017). Maintaining proliferative signaling, withstanding cell death, allowing replicative immortality, avoiding growth suppressors, activation of invasion and metastasis, induction of angiogenesis, immune destruction avoidance, and deregulation of cellular energetics are all hallmarks of cancer's multistep development (Hanahan and Weinberg 2011). To improve cancer detection and medication efficiency earlier, tumor-specific molecular targets must be discovered. As a result, it is highly desired to employ and develop relevant in vitro and in vivo cancer models. As the progress in the medical field such as precision and personalized medicine progress, researchers strive for standardized and personalized tumor models that are comparable to the tumors in human (Xu et al. 2019).

In vitro studies of biochemical pathways in cancer cells are conducted using animal and cancer cell lines originated from humans (Masters 2002). Almost all cancer cell lines in use today are generated from high-grade, high-stage tumors. Well-defined 3D in vitro cancer models mimicking the tumor microenvironment and coculture systems have received attention for a wide range of diagnostic and therapeutic applications by modeling the interactions between tumor cells and the biological matrix (Cekanova et al. 2006; Wang et al. 2014). The use of normal cell lines was carried out by immortalizing them with viral vectors. The use of patient-derived primary cancer cell lines as opposed to generic cell lines has proven to be a viable in vitro strategy for generating cancer therapy regimes on the route to personalized treatment strategies based on an individual's genetic profile (Welte et al. 2013; Rathore et al. 2014). The main limitations of 2D in vitro cell culture in cancer cell lines are selection of phenotypic and genotypic cells during adaptation to in vitro conditions, homogeneous population of cells, accumulation of mutations in cells over time in culture, and isolation of cells from the tumor microenvironment.

Mice, zebrafish, and Drosophila are the most commonly used in vivo models. Small animal models provide significant advantages, including high reproductive ability, less expensive, ease of maintenance, and so on. Many mouse models, such as environmentally induced models, human tumor xenografts in immunocompromised mice, and genetically altered models, have been established to study primary and metastatic cancers. However, visualizing each stage is tough, and obtaining quantitative mechanistic data is typically difficult. However, on the other hand, they are challenging to operate on due to their small size and limited blood supply. Surgery and radiography are challenging to perform on small animals (Mei et al. 2010). Oncology research is heavily reliant on a dependable and representative model framework. Cancer, on the other hand, cannot be classified as a single characterized tumor, but as a complex and highly fluctuating system. As a result, one of the most difficult aspects of cancer analysis is determining the best model to reflect a certain tumor system (Breitenbach and Hoffmann 2018).

Cancer cell lines, 3D model organoids, transwell-based models, spheroid-based models, stem cells, stromal cells, immune cells, extracellular matrix, tumor-microvessel models, organ on a chip, IdMOC, species such as *D. melanogaster*, zebrafish, GEMM, *C. elegans* model, pigs models, PDXs, canine models, chick embryo, and computational cancer models are among the experimental systems used to research human cancer. These models are used to study cancer biochemical and genetic pathways and pathophysiology. Cancer models' combined knowledge aids in a better understanding of the complexity of cancer development (Schachtschneider et al. 2017). This chapter will look at the finest in vitro and in vivo platforms for various applications in tumor biology (Katt et al. 2016).

Primary Cell Line

These cell lines are isolated directly using enzymatic or mechanical methods from the animal or human tissue. It resembles the tissue of origin, so they are similar to the in vivo state and are a good model for testing the effect of drugs and toxic compounds. After isolation, they are grown in artificial environment with appropriate medium having growth factors and essential nutrient. These cell lines are of two types: adherent and suspension type. The adherent cells need surface for attachment, while suspension cells do not require adherence. The most commonly used primary cell lines are the fibroblasts, epithelial cells, keratinocytes, muscle cells, melanocytes, mesenchymal, and hematopoietic stem cells. These cell lines are manipulated chemically or virally using genetic transformation and these cells divide indefinitely to become the immortalized secondary cell lines.

Cancer Cell Lines

For cancer research, animal and mammalian cancer cell lines represent an accessible set of biological models. Cells are taken directly from tumor biopsies and cultured in vitro to generate cancer cell lines. They are simple to grow and promote

direct comparison of experimental outcomes. They have commonly been used to study the molecular mechanism of tumor cell biology. If the culture becomes successful, then good clones of carcinoma cell will emerge out in the culture plate. Cancer cell lines grow autonomously independent of the stromal and extracellular support and exhibit different properties to anchorage independence, proliferate indefinitely, the ability to create high cellular density cultures, high glucose incorporation, low mitogenic growth factor altered cellular shape, and immunocompromised host mice tumorigenicity were all improved. Only Cross contamination should be avoided because verified cancer cell lines maintain most of the genetic features of the malignancy of origin under the right conditions. The Cell line encyclopedia and cancer genome atlas research network has the molecular profiles of all human cancer cell lines.

Cancer Stem Cells

The most common feature of solid human tumors is their heterogeneity among cell types and histological and biological diversity, which is the feature of neoplastic cells. Cancer stem cells are a small group of cancer cells that share characters with normal stem cells, such as self-renewal and cellular differentiation. The strong tumorigenic capacity of cancer stem cells, as well as their phenotypical resistance to medicines and cellular insults such as oxidative stress, has been linked to the high expression of several drug resistance transporter genes (ABC gene family). Cancer stem cell research holds promise for identifying molecular targets and markers that can be used to produce new therapies that target the biological basis of cancer (Kreso and Dick 2014).

Spheroids

Spheroids are group of cells embedded in a 3D matrix or grown in suspension through 3D culture method. Multicellular tumor spheroids or cancer cell spheroids usually represent the avascular tumor nodules or micrometastases (Friedrich et al. 2009). Three steps can be identified in the development of spheroids: (i) upregulated cadherin expression due to dispersed cell aggregate, (ii) cadherin increases on the cell membrane surface, and (ii) the hemophilic cadherin–cadherin contact between nearby cells promotes tighter connections between cells and spheroids. 3D spheroids are widely used to screen out drugs and do studies on tumor growth, invasion, proliferation, angiogenesis, matrix remodeling, and immune interactions. The time taken to culture a spheroid of 400 μm is around 4 days. Spheroids may not be as densely packed as spheroids for short-term culture (<48 h), and they also have low cell–cell and cell–matrix interactions. Various methods like the hanging drop method and microfluidic devices are commonly used to generate spheroids (Mehta et al. 2012). Cancer cells can spontaneously form spheroids in a culture environment, and cell–cell and cell–ECM interactions result in a 3D shape that closely resembles the innate spatial organization and environment of avascular tumors.

Embedded Ex Vivo Tumor Sections

In in vitro conditions, tumor biopsies or resected tumors sections embedded in the extracellular matrix have been used to duplicate the tumor microenvironment (Miller et al. n.d.) and these sections keep tumor cell subpopulation heterogeneity. This model is a valuable tool for solving many complexities of in vivo experiments. This method is commonly used to study growth, chemosensitivity, tumor morphology, and patient-specific therapies. These sections are embedded in collagen type I to imitate the extracellular matrix (Nguyen-Ngoc et al. 2012). Tumor growth and invasion have been studied using embedded tumor sections, drug penetration into the tumor, and drug sensitivity test, and the chemosensitivity of anticancer drugs is screened out by culturing the patient-derived tumors on collagen droplets.

3D Invasion Models

This model system usually focuses on the invasion of cells by seeding clusters or individual cancer cells in an extracellular matrix material, in order to reduce the complexities of the tumor and its microenvironment. Through this approach, we can determine and track the trajectories and speed of individual cell lines, morphology of cells, and invasion through the ECM using the live-cell imaging method. The 3D invasion model can be used to investigate how matrix stiffness, ECM material, hypoxia affect cell adhesion, chemotactic gradients, invasion, and matrix remodeling (Liu et al. 2010).

Avascular Microfluidic Models

This approach is primarily used to understand the migration of cancer cells in the small channels along the chemotactic gradients (Fraley et al. 2010). To encourage migration, these channels can be filled or coated with various types of matrix materials or adhesion proteins. Multiple cell types can be introduced, much like in other in vitro models, and this model helps to determine the cell speed along with the functions of the channel like channel dimension, the presence of obstacles, ECM materials, solute gradient, etc., which is usually found out using live-cell microscopy (Hsu et al. 2011). This model is commonly used to examine the cancer cell attachment to the endothelial monolayer as a precursor to extravasation and it can also be studied with or without antibodies or inhibitors for the adhesion molecules.

Tumor-Microvessel Models

The key point for a tumor growth is the tumor vasculature which provides the growth factors and essential nutrient for the tumor microenvironment and it can be well studied using this model system. Endothelial cells are seeded into ECM scaffolds or self-assemble inside an ECM to make them this model (Bogorad et al. 2015). Blood artery endothelial cells are known to release substances that both promote and inhibit tumor

growth. They play a lead role in metastatic cascade, like an invasion, intravasation, and extravasation. This model system is highly used to study the complex interaction between the tumor vascular tissue and the cancer cells (Butler et al. 2010).

Predefined ECM Scaffold

Subtractive templating methods can be used to construct microvessels in cylindrical morphologies with diameters of 50 μm. Endothelial cells are seeded on the interior surface of a predetermined ECM such as collagen type I or fibrin (Chrobak et al. 2006). The cells on the ECM will self-assemble into a monolayer, which may then be examined for functional features like inflammatory cytokines, vascular mediators, and so on. Endothelial paracrine signaling, tumor-driven angiogenesis, extravasation, and intravasation are some of the tumor–endothelial interactions that can be studied using this method (Buchanan et al. 2014).

Human Organ on a Chip

The most prominent in vitro methods are the organ-on-chip and organoid systems, two initially independent, also interlinked model systems (Allwardt et al. 2020). Organoids are stem cell derived that duplicates the structural and functional units of human. The European Organ-on-Chip Society defined: "An Organ-on-a-Chip (OOAC) is a build-up of Microfluidic-based device, containing living engineered organ substructures in a controlled micro- or nano-environment, that recapitulate one or more aspects of the dynamics, functionality and (patho) physiological response of an organ in vivo, in real-time monitoring mode."

This approach is a microscale imitation of the human body, especially the dynamic mechanical and biochemical functionalities. This system's main purpose is to create human tissue models for disease simulation and medication testing. Using channels, chambers, and membranes, we may examine the flow and behavior of materials and cells in this model. The materials used to create this chip must be optically transparent for the viewing and imaging process and should be made of the suitable materials. Glass and silicone are commonly used for microfluidic devices. Other natural materials like collagen are also used (Sung 2018). Many OOAC like the skin on a chip, liver on a chip, lungs on a chip, intestine on a chip, kidney on a chip, heart on a chip, and multiorgan on a chip are currently available. It is good technology that strongly replaces animal testing and 2D and 3D cell cultures (Wu et al. 2020).

Integrated Discrete Multiple Organ Co-culture

IdMOC stands for Integrated Discrete Multiple Organ Co-culture (IdMOC) technology. In this approach, the primary cells from different organs like the liver, intestine, lungs, etc., are cultured in the same chamber separated by inner wells on the plate but connected by the overlaying medium; it is a well within a well concept. The lack of

various organ interactions seen in a full organism is the fundamental disadvantage of an in vitro system. Different cell types can be co-cultured together and can be simultaneously treated with a test compound using the IdMOC plate. The IdMOC system has applications in drug development, drug metabolism, and toxicity evaluation. By modeling multiple organ cells in a plate, it is very easy to study the pharmacological effects of drugs and their metabolites on target and off-target organs and to study the drug–drug interactions (Li et al. 2004).

Assays Commonly Used in in Vitro Models

A number of tools and procedures have been developed to study the in vitro models, focusing mainly on the mechanism of action of drugs or for toxicity studies. The cytotoxicity, genotoxicity, cell migration, cell proliferation, protein expression, and gene expression using mRNA can be easily studied using the in vitro model system. The cytotoxicity is studied using Trypan blue assay, PI and AO staining, ATP assay, KB assay, and lactate dehydrogenase leakage assay. The genotoxicity is studied through DNA fragmentation, ɤH2AX assay, and other cytogenetic techniques. The cell migration as result of compound/drug treatment can be studied using scratch/wound healing assay, Boyden chamber/transwell migration assay, microfluidic assay, and paper-based invasion assay. The proliferation studies are usually done using assay like MTT assay, BrdU assay, and 3H-thymidine assay. The gene expression can be explored through real-time PCR, microarray, RFLP, and DNA sequencing.

Mouse Models

The mouse genome is quite similar to the human genome; therefore, it can be used to design a variety of biological properties in vivo, such as the occurrence, advancement, and metastasis of human carcinoma cells, role of oncogenes and tumor suppressor genes in cancer progression, and therapeutic response during cancer development. Most research in human tumor genetics relies on mouse models as they have the advantage of convenient feeding, easy gene modification, short gestation times, and cost-effectiveness.

Genetically Induced Cancer Models

Genetically induced cancer models are represented by tumor suppressor genes which are downregulated or tumor genes produced selectively in transgenic mice using three primary methods: DNA construct microinjection, gene-targeted transgene, and retroviral infection. Transgenic mice are used interchangeably with germline genetically engineered mouse models (GEMM) (Tratar et al. 2018). Microinjection of DNA into the fertilized zygotes pronuclei results in genetically modified mice, and

the transgene is incorporated into the genome. Transgenic mice carrying cloned oncogenes (Stewart et al. 1984) and knockout mice lacking tumor suppressor genes (Jacks et al. 1992) have been proven to be effective models of human cancer. Transgenic mice provide a direct way for studying the effects of individual gene gain-of-function on cancer. Second-generation models include those that have had Rb1 and Trp53 targeted deletions, which result in varied cancer development features. Even though these models outperform tumor-transplanted animals, they still fall short of replicating a "carcinogenic genome's" entire expression and mutation patterns. The evolution of clustered regularly interspaced short palindromic repeats (CRISPR) technology has revolutionized the complexity and ease of genome editing. This method enables in vivo genome engineering of mammalian genomes and the quick generation of novel cancer models. The method has already been used to stimulate liver and lung cancer mutation. It is expected to significantly influence the study of human cancer genetics in animal models.

RNA Interference Mouse Model of Cancer

Using short hairpin RNA, the RNAi mouse model can be utilized to silence distinct genes (shRNA) quickly. Transgenic RNAi mice are frequently generated using plasmid-based RNAi (Dickins et al. 2007). Microinjection, electroporation, or viral infection can all be used to deliver a plasmid containing shRNA to ES cells. The ES cells are then implanted directly into pseudopregnant mice after being injected into blastocysts or aggregated with tetraploid embryos. The RNA polymerase II promoter can be used to express tissue-specific short hairpin RNA. The Cre-loxP conditional and Tet-inducible systems can also be utilized to spatiotemporally and reversibly limit gene activity in vivo using the RNAi technique. This transgenic RNAi technique is particularly useful for elevated gene function studies in vivo.

Patient-Derived Xenograft

These are mostly performed on immunocompetent mice and are composed up of living cells, tissues, or organs. Xenografts are employed in cancer research to solve fundamental questions. It is crucial to rely on animal models that accurately mimic tumor growth in human patients (Vandamme 2014). The absence of preclinical cancer models that adequately evaluate clinical trials of key novel compounds in patients has delayed progress in cancer medication research. These restrictions have been overcome in immunocompetent animals such as nude mice, nonobese diabetic (NOD)-SCID gamma mice, severe combined immunodeficiency mice, NOD rag gamma mice, and recombination-activating gene. The transit numbers of xenograft models made out of primary cancerous tissue produced from the patient's malignant cells are kept very minimal. The goal of developing PDX models is to increase preliminary testing and determine molecular tumor biology related to human cancer

as well as how individuals adapt to cancer therapy (Kopetz et al. 2012). PDX models can be used to investigate (i) cancer metastasis and medication blockage, (ii) personalized medicine and treatment, and (iii) preclinical testing and identification of new anticancer drug candidates (Jin et al. 2010). Although subcutaneous implantation is the most commonly used site for tumor implantation in mice, orthotopic implantation transplantation in a homologous organ to the primary tumor could be an option.

Limitations

Mice and humans have inherent species-specific distinctions (Cheon and Orsulic 2011). The mouse has a faster metabolic rate, leading to faster breakdown or clearance of substances, impacting preclinical testing. In contrast to human cells, most mouse cells actively maintain telomeres, predisposing them to become precancerous. Genetic modifications in most human cancer cases are sequential, heterogeneous, and adaptive. These genetic and epigenetic defects in GEMMs may lead to an overestimation of the efficacy of drugs that target the model's particular mutations. The implanted tumor cells may undergo early screening during culture or after transplantation in the animal host in the patient-derived xenograft or cell line-derived xenograft models, resulting in the generation of nonrepresentative clones of the cancer phenotype. The ability of these clones to replicate organ-specific metastatic spread and therapeutic responses is often lost.

Furthermore, changes in immune systems between mice and humans may influence the host's response to a growing malignancy. The human immune system has been reconstituted in mice to "humanize" them. Tissue engineering and regenerative medicine approaches are used in the humanized mouse model (HMM) to create humanized microenvironments within the animal. Once the humanized immune system is in situ, graft versus host disease will trigger selection against murine cell subsets, and the human-like hematopoietic system will respond to human cancer cells. Despite the fact that this may result in immature cell lineages that do not fully replicate the human immune system (Shultz et al. 2012), the HMM approach is still the global standard for producing preclinical mouse models. Future advancements will focus on overcoming these technical and biological limits by creating increasingly complex models.

Zebrafish Models

Over many years, more than 200 species of fish have been utilized for tumor studies (Slatick and Harshbarger 2001). Zebrafish (*Daniorerio*), initially known as *Brachydaniorerio*, is a tiny freshwater fish originally seen in the tropical regions such as Ganges river and its tributaries in northern parts of India (Tavares and Lopes n.d.). Even though Zebrafish emerged as a scientific model system in recent times, it

became a remarkable and widely used nonmammalian vertebrate model in numerous human malignancies, especially in recent cancer research. Being a vertebrate, it has relevance varying from conserved pathways in signaling and mechanisms in transcriptional regulations, to organogenesis, differentiation of tissues, to cellular transformation and tumor initiation. Zebrafish came into the limelight as a versatile model organism because of its beneficiary features over other species such as (i) high fecundity (100–200 embryos per clutch) providing statistical significance, (ii) cost-effective husbandry, (iii) external fertilization with the formation of the translucent embryo that helps in the screening of embryo and stages of organogenesis based on phenotype, (iv) rapid development of the embryo with a small generation time of about 3 months, and (v) the transparency of embryo and larvae that help to envisage the tumor development, dissemination, and progression at its initial stages in vivo, which gives an advantage over the murine model (White et al. 2013). With the development of transparent mutant strains like Casper and Crystal paved the way for in vivo imaging of all development stages, including adults (White et al. 2008), (vi) availability of a sophisticated and more accessible genomic tool for genome manipulation and embryology, (vii) availability of transgenic lines like Tg(fli1:EGFP) and Tg(flk1:EGFP) with green fluorescently tagged circulatory system and Tg(gata1:DsRed) having blood cells in red fluorescence to visualize formation and localization in vivo (Choi et al. 2007) and xenotransplanted Zebrafish, which helps to reveal the different aspects of carcinogenesis by demonstrating xenograft assays (Rudzinska-Radecka et al. 2021; Xiao et al. 2020), and (viii) comparison of genome of Zebrafish and humans revealed that Zebrafish has about 70% orthologue of at least one copy of human genes and 80% orthology with disease-causing human genes (Howe et al. 2013), and (ix) drug assays and screening can be done by dissolving in water as they can absorb molecules from water (Dang et al. 2016). Zebrafish genome has homology with significant players of human cancer-related pathways in addition to its similarity in histology, etiology of mutations in genes (*tp53*, *pten*, etc.), the transcriptome, and copy number alterations (CNAs) (White et al. 2013; Kumari et al. 2018) and high fecundity and fast development of the embryo allow for its utilization in studying the mechanisms of occurrence and treatment and development of potential drugs and therapies for cancers and other human disorders to hasten preclinical large scale whole animal screening of drugs (Letrado et al. 2018).

Cancer models can be developed by different approaches such as chemical carcinogenesis, forward and reverse genetic screens, transgenesis, and xenotransplantations. There are several strategies used to regulate genes that control cancer: firstly, through the use of tissue-specific promoters for spatial regulations (Park et al. 2020); secondly, for cell-specific expression, GAL/UAS system utilization (Liu and Leach 2011); thirdly, recombination using the Cre-Lox mechanism to exchange or remove intervening sequence with oncogenes (Seok et al. 2010); fourthly, inducing heat shock promoter activity (Shoji and Sato-Maeda 2008) or the Tet-ON system enhances temporal expression of oncogenes (Ju et al. 2015); and the fifth strategy is ligand-associated activation of oncogenes (Yan et al. 2019a).

Zebrafish as Genetic Models of Cancer

In the past few years, several sophisticated techniques and approaches have been developed for manipulation or introduction of genes into the genome of Zebrafish, resulting in the formation of fish with loss of function phenotype or mutated genes of human cancer. Forward genetic screens help to discover novel genes for tumor suppression or genes concerned with tumorigenesis by inducing cancer by the suspected test molecules and compounds or chemicals. *Tumor suppressor 53* (*tp53M214K*) *is* one of the first mutations identified in the Zebrafish cancer model induced by an alkylating agent N-ethyl-N-nitrosourea (ENU) (Beckwith et al. 2000). *Other mutated genes identified causing tumors found in Zebrafish are* dnaaf1 (lrcc50), bmpr1bb (alk6b), mybl2b (bmyb), espl1 (separase), llgl2 (lgl2), myh11 induced by ENU and proteins that make up ribosome (rps8, rps15a, rpl7, rpl35, rpl36, rpl36a, rpl13, rpl23a, rps7, rps18, and rps29), nf2a, and over-expression of fgf8 by retroviral insertions (Raby et al. 2020). Reverse genetic screens alter the known cancer-related genes in humans for studying the phenotypic changes caused. Arbitrary development of mutations can lead to rare people carrying mutations of inhibition in a desired gene, especially a gene associated with human cancers. For screening reverse genetics, TILLING or Targeting Induced Local Lesions IN Genomes is widely employed. Herein, mutations are achieved in Zebrafish by point mutagens such as ENU, and individual genomes of F1 progenies are screened for altered genes by recognizing DNA heteroduplexes. Once the desired mutation is identified, cryopreserved sperms of the specific individual are in vitro fertilized for recovering mutant lines. Some mutant lines for tumor suppressor genes identified by TILLING are tp53, ptena, ptenb, apc, mlh1, msh2, msh6, and brca2 (Raby et al. 2020). Insights from genetically engineered Zebrafish models reveal the tumorigenesis mechanisms at an early stage with phenotypic changes. The various tools for genetic approaches are genome editing technologies by site-specific endonucleases such as Transcription Activator-Like Effector Nucleases, Zinc-Finger Nucleases, and CRISPR/Cas9 (Li et al. 2021; Doroftei et al. 2021; Carmona-Aldana et al. 2021). The mutated genes targeted by ZFN include nf1a, nf1b, tet2, and by TALEN are rb1, cdkn2a/b, tp53del/del, irx1a. spred1, tp53, pten a/b, cdkn2a, ptch1, atrx, suz12a, suz12b, dact2, and twist3 were targeted by CRISPR/Cas 9 (Raby et al. 2020).

Zebrafish as Transplantation Model

Allograft or xenograft cell transplantation can be carried out for developing a Zebrafish transplantation model. The transplanted cells are engrafted onto the embryo until the development of their adaptive immune system (7dpf). To overcome transplantation rejection in the later stages, sublethal irradiation of recipient immune cells is done. Another method is to introduce the cells to already immune-compromised fishes. Outcrossing transparent mutant line Casper with immunodeficient lines has been

developed for allograft and xenograft transplantation cancer applications (Yan et al. 2019b). A newly generated *foxn1/Casper* mutant allows the nonconditioned engraftment of numerous cell types and in vivo visualization and characterization of transplanted cells (Lv et al. 2020). Standard immune-compromised lines are rag2E450fs, jak3P369fs, prkdcD3612fs, and zap70y442. Allograft transplantation cancer models using primary cell lines and Zebrafish melanoma cell line ZMEL are widely used for cancer pathogenesis and repression (Hason and Bartůněk 2019). Xenotransplantation of patient-derived tumor cells (PDX) into zebrafish embryos and larvae permits personalized cancer therapeutic strategies. It helps to study the change in gene expressions, tumor dissemination, and behavior of the introduced human cells (Kirchberger et al. 2017).

Zebrafish for Neoplasia Studies

Neoplasia can be benign or malignant. Zebrafish has its utilization in several types of neoplasia such as cutaneous, muscular, adipocytes, vascular, intestinal, hepatic, exocrine pancreatic, hematopoietic, lymphoid, neural, neuroendocrine neoplasia, and gonadal neoplasia. The focus of most of the studies on cutaneous neoplasia is melanocyte-procured tumors incident by the expression of oncogenes of human in melanocytes (Shive 2013). Several models for benign nevus and cutaneous melanoma are available. Like mammals, the origin of pigmented cells is from neural crest cells earmarked during embryogenesis. Only a few nonmelanoma models are available. One such model was developed in adults by treating with ENU, resulting in a 100% incidence of sessile or pedunculated epidermal papillomas. Although benign nevus is not transplantable into sublethally irradiated recipients, Zebrafish melanomas are transplantable. For the development of rhabdomyosarcoma and liposarcoma models, a transgenic approach is performed. Ras Map pathway or PI(3)-Akt pathways are modified to form transgenic fishes to which neoplasia is induced. Ectopic expression of the Zebrafish recombination activating gene-2 (rag2) promoter with human transgene in mesenchymal progenitor cell or early embryo can also result in rhabdomyosarcoma and liposarcoma models. RAG2 is expressed in lymphoid cells. Haploinsufficiency for *pten* resulted in hemangiosarcoma in Zebrafish. In Zebrafish, mutation in sex cells for heterozygous condition in apc (adenomatous polyposis coli), the gene of tumor suppression, leads to tumors in intestine, kidney, and pancreas, whose phenotype of vulnerability is close to the human familial adenomatous polyposis syndrome associated with inheritable mutations of APC. This can be induced by carcinogens or gene manipulations. Neoplasia of neural origin is not uncommon in Zebrafish. Neuroectodermal tumors occur occasionally in them either by genetic defects or exposure to carcinogens or induced in transgenic fishes (Peterson et al. 2013). There are few reproductive neoplasia models in Zebrafish. Female models are quite rare, but testicular tumors are found at different frequencies in both transgenic and mutant models (Shive 2013).

Limitations of Using Zebrafish Cancer Model

Some organs like the breast, prostate, and lungs are absent and possess less complex structured organs. The conservation of molecular interactions between zebrafish and transplanted human cells is unclear in patient-derived xenograft (PDX). In addition to that, for PDX, the temperature should be raised to 32–35 °C from their physiological temperature of about 28 °C. Teleost-specific gene duplication is a drawback in genetic manipulations. In Tumor Micro Environment (TME) studies, under-developed adaptive immunity in larval stages is a drawback (Kirchberger et al. 2017).

Future Perspective

One of the future perspectives of Zebrafish utilization is an investigation of the tumor microenvironment (TME) and its reconstructions as it can mimic the human microenvironment of the tumor. Attribute such as adaptive immunity and immune ME needs to be addressed. Nevertheless, having regenerative potential, spinal cord injury leads to enormous loss of cells of innate immunity (Tsarouchas et al. 2018). Being transparent in developmental stages and availability of mutant strains (Casper and Zebrafish with vascular-specific fluorescence) with real-time imaging techniques, investigations on tumor features such as cell migrations, morphology changes, neovascularization, and drug screening are possible. Switching between migratory phenotypes and microenvironmental parameters can be applied to further tumor research (Stuelten et al. 2018). Applications of Zebrafish patient avatars and future implications are reviewed by Fazio et al. 2020.

Drosophila Melanogaster

By unveiling the action mechanisms of cancer-related proteins, *Drosophila melanogaster* has achieved substantial advances in deciphering the underpinnings of cancer *biology.* The significant advantage of using this organism is that it can be bred and evaluated in a small laboratory with limited time, space, and funds due to its small size and simple requirements. Humans have 23 pairs of chromosomes, but *D. melanogaster* has only four. The ease with which drosophila genes could be mapped to examine genetic transmission was one of the reasons for using in genetic studies. Proteins that cause cancer in humans have more than 50% orthologs in drosophila, indicating that their genomes have similarities. In flies, most signaling pathways that induce cellular proliferation and invasion in mammals have a conserved role, allowing them to be modulated in tumor biology models (Millburn et al. 2016). Drosophila can also help researchers better understand organotypic malignancies. Organ cells and functional units are usually substantially preserved at the biochemical and structural levels, despite evident variations at the macroscopic level. Researchers were able to develop lung, blood, prostate, gut, thyroid, and brain models based on the most prevalent genetic lesions found in real cancers

because to this conservation. ***Drosophila*** *melanogaster* cancer models are developed by employing ethyl methane sulfonate to induce mutations in larvae (EMS). The outer proliferative center and central brains of larvae possess tumorous tissue. The larval brain is removed and inserted into mature female flies' abdominal region, from which it remains to proliferate. The host's ovaries are dissected after a few days, and immunofluorescence is used to examine for tagged tumor cells that have spread. Genetically engineered *D. melanogaster* strains are important platform in the development of medicinal drugs and for drug testing. In Drosophila, asymmetric division, instability in genome, metabolism, irregular expression of genes, and centrosome malfunction all played a significant role in tumor initiation and progression.

Recent advancements in experimental approaches now provide unparalleled opportunities to investigate the developmental aspects of cancer-causing genes. The impacts of ectopic expression of gene can be readily examined in the flies because oncogenes are frequently either abnormally active (Ras) or overexpressed (cyclin D), which is essential for researching the etiology of cancers. Fly researchers can examine the science of overexpression of gene without harming the animal by ectopically expressing the desired gene using a particular promoter.

Drosophila is a valuable model system for studying cancer causes because it has potent genomic tools that allow clones, or regions of cells, to be created that include numerous mutations inside tissues of phenotypically normal cells. To generate genetically complex cell clones, MARCM or Mosaic Analysis with a Repressible Cell Marker system (Lee and Luo 1999) integrates Flipase/FRT driven mitotic recombination between a mutant and a nonmutant chromosome with the Gal4-UAS system for tissue-specific overexpression of genes or dsRNAis. The MARCM approach can be used to generate clones of GFP-tagged cells that are homozygous mutant in a gene while also overexpressing one or more other desired transgenes or dsRNAi. The fly system has to play exceptional role in the future of cancer research because of its adaptability in addressing a wide spectrum of cancer biology associated topics and the evidence of its clear connection to carcinogenesis in mammals.

Caenorhabditis Elegans

Caenorhabditis elegans are nonparasitic, free-living soil nematodes mainly existing as hermaphrodites, although occasionally males develop at an incidence rate of 0.1%. It is one of the smallest multicellular organisms that emerged as an alternative nonmammalian cancer model. Many attributes contribute to their attention as a model system. It can be maintained easily in huge numbers in vitro by culturing in agar plates or liquid medium providing *Escherichia coli* bacteria to feed on. Under favorable circumstances, *C. elegans* have a life expectancy of 2–3 weeks and a short generation time of three and a half days at 20 °C. A wild-type hermaphroditic *C. elegans* has a fecundity of roughly 300 progeny by fertilization by oneself and more than a thousand young ones when fecundated by a male. Noninvasive optical techniques can be employed as it is transparent at all stages. This makes it easy to

track and localize numerous biological activities and molecules, which helps to investigate tumorigenesis and the therapeutic potential of anticancer drugs (Kyriakakis et al. 2014). When comparing the genome of *C. elegans* with mammals, 60–80% homology can be elucidated in several biological processes. Quite a large number of human genes and players involved in cancer-related pathways are preserved in *C. elegans*. Some of the gene families in this pathway, such as pRb and p53, are single member in their family, which makes the study much easier (Harris et al. 2004). It is the first metazoan to be sequenced its genome. Forward and reverse genetics are well-established with conventional and modern genetical approaches (Leung et al. 2008).

De-regulation of the cell cycle results in cancer. Many of the cell cycle genes in *C. elegans* are orthologous to humans, with conserved function (van den Heuvel 2005). Same as that of human cancer, variations in cyclin D, cyclin-dependent kinase 4, and p16INK4A result in high mutation frequency than other cell cycle regulating genes (Sherr 1996). Mammalian orthologues for miR-125 (lin-4), Cip/Kip family (cki-1, cki-2), FoxB1 (lin-31), Retinoblastoma, p107, p130 (lin-35), etc., lead to hyperplasia in most of the tissues like VPC, hypodermis, intestine, sex myoblast (Kirienko et al. 2010). The main effectors of apoptotic pathways in *C. elegans* are conserved in mammals. Loss of function or mutation in any effectors can fail apoptosis and lead to hyper-proliferation and cancer. DRE-1 is the analog of FBXO10, the human protein, and its mutation or reduced expression in humans diffuses large B-cell lymphomas (Chiorazzi et al. 2013). Mutagenesis is the driving force that leads to cancer. ***Caenorhabditis*** *elegans* are an ideal model system for experiments to evaluate mutational impressions linked with deficiency in DNA repair and genotoxins exposure such as UV-B and gamma rays and alkylating agents, or in the combination of both. Wild-type and defective mutants such as DNA repair and DNA damage sensing mutants are developed and accomplished for the studies (Meier et al. 2020). This model organism can demonstrate most of the hallmarks of cancer. The absence of a circulatory system is compensated with the proteins pivotal for angiogenesis, like the PDGF/VEGF-like factor (PVF-1), which is identified in *C. elegans* and, therefore, can stimulate angiogenesis in vertebrate assay models.

Limitations

Despite several advantages, there are a few limitations to using *C. elegans*. Firstly, they are evolutionarily distant from humans; hence, the considerable number of genes and many well developed organs or tissues, such as brain, a defined fat cell, other internal organs, and blood are lacking. Secondly, the small size of *C. elegans* makes it difficult for experimentation, especially in examinations on biochemistry, microarrays, chromatin immunoprecipitation, which utilizes entire worm extracts which contains worms of mixed-stage or similar growth stage. Thirdly, after several generations, adaptive mutations can be obtained in both the wild-types and mutant strains. This causes a lack of knowledge of its genotype (Johnson 2003).

Pig Cancer Model

Pigs are more accurate predictors of human therapeutic treatments than rodents (Meurens et al. 2012; Schook et al. 2016). Pigs are an excellent model for studying cancer because they are anatomically, physiologically, metabolically, and genetically comparable to humans. Leukemia, lymphoma, soft tissue sarcoma (STS), pancreatic ductal adenocarcinoma (PDAC), HCC, and other hematological cancers have all been successfully modeled using it. Aside from the high degree of similarity between the pig and human genomes, the porcine genome also possesses highly conserved epigenetic control, as evidenced by pigs and humans having similar genome-wide DNA methylation patterns (Schachtschneider et al. 2017). Because of its inducible nature, the Oncopig cancer model is ideal for discovering potential biomarkers. Porcine models for cancer research in vivo include the APC1311 pig variety of familial adenomatous polyposis, a heterozygous TP53 deletion type of spontaneous osteosarcomas, and a chemically precipitated porcine HCC model (Schook et al. 2016).

Chick Embryo

The chick embryo is an unparalleled model system to study cancer biology in vivo by overcoming several limitations. The chorioallantoic membrane, a well-vascularized extra-embryonic tissue positioned beneath the eggshell, is easily accessible and has been widely utilized for the molecular analysis of cancer and pathways of oncogenesis, including viral oncogenesis, carcinogenesis, tumor xenografting, tumor angiogenesis, and cancer metastasis. Human cell lines such as HeLa, BLM, SW480, PC3, HT1080 are successfully cultured in CAM (Kain et al. 2014). Xenografting can be done either *in ovo*, which can be conventionally processed, or *ex ovo*, making it easy for analysis in situ, such as using direct observation through intravital imaging. An advantage of xenografting is that chick embryo is naturally immunodeficient. CAM models are mainly utilized for studying the cell motility and the associated role of MMPs and scaffold and stromal proteins for metastasis (Zijlstra et al. 2008). Another application of CAM in cancer study is tumor angiogenesis. Tumors can grow well on the surface of CAM. Behaviors of vasculature and factors controlling vascular growth are identified and monitored using *in ovo* and *ex ovo* CAM models (Zijlstra et al. 2002). Advances in imaging technologies, the discovery of novel contrast, and imaging agents that can selectively label developing vessels make it easy to visualize angiogenesis, tumor growth, and dissemination with optical clarity. By utilizing the different targeting strategies, CAM finds its application in drugs screening, designing novel biomaterials and carrier molecules.

Computational Cancer Models

Computer-based modeling linked to tumor treatment and physiology is referred to as a "computational cancer model" (Barbolosi et al. 2016). To detect, track, and anticipate cancer growth, computer-based research has been widely used. 3D

microscopic pictures, tumors, and tissue can all be visualized using computational simulation models. Numerical or computational cancer models, in contrast to in vitro cellular cancer models, are frequently coupled with algorithms and other computational software packages, resulting in a lack of comparability and reproducibility. Computational and mathematical models have been used to study cancer's evolution. New biomarkers in signaling networks and prospective targets for anticancer therapy can be discovered using computational approaches. Image processing and interpretation are also aided by computational systems in cancer research and therapy. Recently, computed tomography image analysis has been presented as a way for examining specific cancer responses (Ogilvie et al. 2017). A comprehensive computational model enables the experimental inquiry to be modified, reducing expenses, and, most importantly, boosting the translational value of the supplied data. Computational models allow for a better understanding of molecular changes in disease-related pathways, as well as a more efficient prescreening process for selecting crucial candidates. It has the potential to increase our understanding of disease progression and treatment response.

Limitation of Animal Models in Cancer Research

Cancer researchers' ultimate goal is to transform scientific insights into clinical applications. Primary research should start at "the bench," progress via preclinical animal investigations, and finally, prove therapeutic efficacy in human clinical trials. Despite the fact that animal models continue to execute an crucial role in evaluating the efficacy and safety of new cancer therapies, genetic, biochemical, and physiological constraints frequently restrict their effectiveness (Mak et al. 2014). Despite successful preclinical research, 85 percent of early clinical trials for new medications fail, and only half of those make it to phase III approved for clinical use (Ledford 2011). The trials for cancer medicines account for the majority of failures (Arrowsmith 2011); furthermore, less than one out of every five cancer clinical studies makes it to the peer-reviewed literature due to negative results (Curt and Chabner 2008). As a result, promising preclinical animal investigations that demand significant time and financial resources rarely result in effective treatments.

Conclusion

Animal models are useful tool for analyzing the tool invasion, development, and diagnosis of human diseases and exploring the principles of disease prevention and treatment, and they continue to bring new ideas for cancer research. Every model's ultimate purpose is to provide outcomes that improve patient well-being. Combining in vitro, in vivo, and computational models in preclinical testing improves the cost-effectiveness of drug authorization and development. Cancer cell lines have a significant proliferation rate and are easier to control than pigs, with a longer generation period and more significant living requirements. Organoids are a unique cancer model

for studying tumor heterogeneity and microenvironment. Oncology research relies heavily on mouse models of human cancer. PDXs are easy to use, reproducible, and reasonably priced. When compared to GEMMs, PDXs have a shortcoming in that they do not mimic the genetics and histology of human tissue tumors due to a lack of a complete immune system. Due to its genomic similarities to humans and all other cancer models, Drosophila has explained the molecular basis of carcinogenesis, but structural and physiological differences with humans have limited its uses. Oncopig, which displays genetic, epigenomic, and chromosomal similarities with humans, has overcome this shortcoming. Zebrafish provide several advantages over traditional cell lines. Zebrafish have been utilized in the laboratory for various purposes because they can provide precise results for a variety of phenotypes, but they still have a lot of drawbacks. Zebrafish could be a perfect model for researching new biomarkers in the future if the right techniques and approaches are applied. Computational models address inadequacies in in vitro and in vivo models. Mechanistic computer models are constructed based on the patient's omics data to investigate pharmacological effectiveness and affects. The existing computational model, on the other hand, fails to capture the complexity of a developing human tumor. In the future, we may be able to combine various construction approaches with gene-editing technology to create a model which is more appropriate for cancer research.

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Preclinical Models in Colorectal Cancer Drug Discovery

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Contents

Abstract

Currently, colorectal cancer (CRC) remains one of the most common causes of cancer-related death worldwide despite advances in medical therapies of CRC. Therefore, appropriate animal models of CRC are urgently needed to help understand the pathophysiological mechanism of CRC, develop new drugs, and evaluate the efficacy of new drugs at the preclinical level. At present, CRC animal models mainly include carcinogen-induced models, transplant models, and transgenic animal models. The selection of suitable modeling animals and corresponding modeling methods is the key for the success of CRC drug discovery, depending on factors such as experimental purpose, experimental cycle, and experimental technology. Here, this chapter reviews the pros and cons and research progress of different animal models in CRC, providing ideas and methods for further research on CRC drug discovery.

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Keywords

Colorectal cancer · Animal models · Carcinogen-induced models · Xenograft · Gene-modified models · Drug discovery

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive tract with a global estimate of 1.9 million new cases and 935,000 deaths in 2020. According to global cancer statistics in 2020, the incidence and mortality of CRC ranked the third and second, respectively, among the most common malignancies worldwide (Sung et al. 2021). The pathophysiological mechanisms of CRC are complex, and animal models need to mimic the pathological characteristics and course of CRC as closely as possible, because candidate drugs for clinical trials are derived from drugs with potential for clinical application in preclinical trials. However, only approximately 5% of these drugs have been reported to show clinical efficacy in phase III clinical trials (Kola and Landis 2004). In addition, new cancer drug development is a long-cycle and high-cost process (varies from 944 million US dollars to 4.54 billion US dollars) (Schlander et al. 2021). Therefore, selecting appropriate preclinical animal models of CRC can truly reflect the effect of drugs on CRC and then reduce the chances of failure in clinical trials of new drugs and the total cost of drug research and development, which plays a crucial role in CRC drug discovery. Currently, animal models of CRC encompass a wide spectrum and mainly include the following: (1) carcinogen-induced models: in experimental conditions, animals in the study were exposed to a wide range of carcinogens to induce tumors; (2) transplant models: CRC cells or tissues are implanted into laboratory animals to produce tumors; and (3) transgenic animal models: knockout or insertion of specific genes using technologies such as transgenes, gene targeting, and conditional gene targeting, thereby inducing tumors in animals. The research on transgenic animal models is developing rapidly. This chapter reviews the available animal models of CRC and common routes of forming CRC models (Fig. 1) to recapitulate the application, advantages, and limitations of various models in CRC drug discovery and development (Table 1).

Carcinogen-Induced Models

At present, the most commonly used tumor inducers for CRC are 1,2-dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM) because these two chemical reagents are characterized by cheap and excellent carcinogenic effects (Ulger et al. 2013; Vinothkumar et al. 2014). DMH is the metabolic precursor of MAM, which has no direct carcinogenic effect. While AOM, a metabolite of DMH in liver, has direct and indirect carcinogenic effect by alkylating DNA and thus resulting in base pairing errors (Neufert et al. 2007). BALB/C mice were subcutaneously

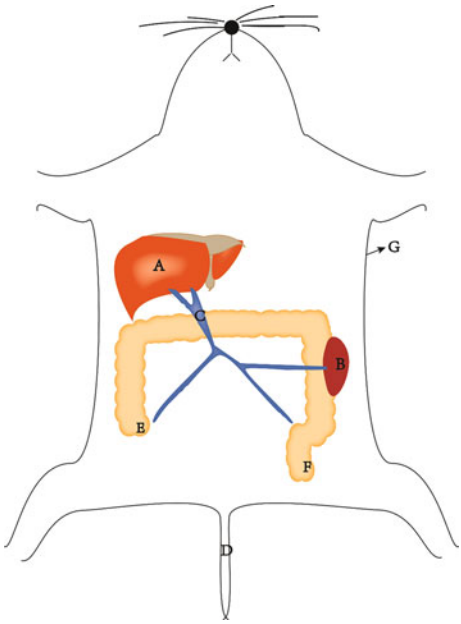

Fig. 1 Schematic of the common routes of forming colorectal cancer models. Ectopic xenografts include the following routes: subcutaneous xenograft models (**g**), liver metastasis models [liver inoculation (**a**), splenic injection (**b**), and portal vein injection (**c**)], and tail vein injection (**d**) for lung metastasis models. The sites of orthotopic xenografts include the cecum (**e**) and rectum (**f**)

injected with 1,2-dimethylhydrazine for 10 weeks, and Marion et al. showed that 60% of the animals developed tumors within 20–24 weeks (Lenoir et al. 2016). Venkatachalam et al. (Venkatachalam et al. 2016) injected DMH subcutaneously into rats for 15 weeks. The experiment found that rats treated with DMH showed an increase in the development of aberrant crypt foci, tumor formation, and multiplicity.

In addition to the above two carcinogenic drugs, the addition of the exogenous chemical inflammatory agent DSS on the basis of AOM-induced mutations, the AOM/DSS model, is also a widely used chemical induction model for CRC. DSS can induce chronic inflammatory bowel disease in animals, and further development leads to CRC (Neufert et al. 2007; Clapper et al. 2007). Lauren C et al. (Chartier et al. 2018) induced colitis-related colon cancer in C57BL/6 mice through the combined application of AOM and DSS. Another study also confirmed that intraperitoneal injection of AOM combined with DSS feeding can establish a dynamic whole process from ulcerative colitis to tumor development in mice (Lippert et al. 2017).

Carcinogen induction models have many advantages, such as high reproducibility, simple implementation, and low cost. In addition, this model is also able to induce the development of CRC on mice with different genetic backgrounds, which differs from xenograft and genetically modified models that must be implemented on genetically homogeneous mice. However, tumors induced by carcinogen-induced models are less prone to metastasis, and the AOM/DSS model is mainly used to induce animal models of CRC associated with inflammatory bowel disease rather than sporadic CRC.

Table 1 Summary of advantages and disadvantages of different CRC modeling methods

Animal models	Advantages	Disadvantages
Carcinogen-induced models	1. Easy to establish and low cost 2. Most strains of mice can be induced to form tumors	1. Long induction time and high animal mortality 2. Suitable for intestinal inflammation-related CRC rather than sporadic CRC
Ectopic xenografts	1. Simple implementation and rapid tumor onset 2. Monitor tumor growth in real time (subcutaneous xenograft models) 3. High rate of liver metastasis (direct liver inoculation, splenic injection, and portal vein injection) and lung metastasis (tail vein injection)	1. Subcutaneous xenograft models: different microenvironment is different; low metastasis rate 2. Immunodeficient animals are required
Orthotopic xenografts	1. Similar to the tumor microenvironment of CRC 2. Closer to the development of CRC tumors	Complex procedure (require surgery)
Patient-derived CRC xenograft models	–	–
Cell line-derived xenograft	1. Maintains most of the biological characteristics of the primary tumor 2. High tumorigenicity and short experimental cycle 3. A large amount of available experimental data	1. Requires the use of more costly immunodeficient animals 2. Difficult to mimic the stromal heterogeneity and histological properties of CRC
Patient-derived tumor xenograft	1. Preserves the stromal heterogeneity and histological properties of CRC patients 2. More objective and comprehensive reflection of the development of tumors and the drug efficacy	1. High cost 2. Technically challenging to perform 3. Low rate of forming tumors
Transgenic animal models	–	–
APC	Simulate the development process of human FAP and sporadic CRC	1. Most tumors are in the small intestine 2. Low rate of metastasis
Msh2	Mimic deficiency of MMR, suitable for HNPPC-related studies	1. Prone to lymphoma 2. Low rate of metastasis

Transplant Models

The transplant models can be categorized to ectopic xenografts model and orthotopic xenograft model, based on the transplantation site. The most common model is the murine xenograft, and other models, including patient-derived xenografts and

orthotopic transplant models, are becoming more widely used in CRC research. The tumor cell lines of CRC xenograft models mainly include human cell lines (HCT116 and HT29) and murine cell line (CT26).

Ectopic Xenografts

Ectopic xenograft model is the simplest method for xenograft model. Among them, the subcutaneous xenograft model established by injecting CRC cells or tissues into subcutaneous tissue of mice is one of the most widely used for anticancer drug discovery, due to the simplicity of implant tumor technology and the ability to monitor tumor growth in real time with the naked eye (Talmadge et al. 2007). Animals in this model mostly use classical nude mice (lacking T lymphocytes) and mice with severe combined immunodeficiency (lacking both T and B lymphocytes) (Blunt et al. 1996). For instance, Jiang et al. successfully established a colon cancer model by subcutaneous injection of CT26 colon cancer cells into BALB/c mice (Jiang et al. 2014). The establishment of subcutaneous xenograft models is easy to perform, and the transplanted tumors are easy to observe and measure. Therefore, this model has significant advantages in monitoring tumor progression and evaluating therapeutic effects of drug on tumor progression. However, there are some limitations in subcutaneous xenograft models: first, tumor cells or tissues are inoculated into subcutaneous tissue of animals in this model, and the subcutaneous microenvironment is significantly different from that of the colorectal microenvironment, which makes it difficult for the model to metastasize. Second, the use of immunodeficient animal models also ignores the effect of the immune system on tumor development. Finally, if the animal model was established by transplanting a cell line of CRC, then this model does not mimic the histological features of human tumors. And these factors mentioned above may lead to disappointing results: despite efficacy in animal models, many anticancer drugs fail in clinical trials.

Subcutaneous xenograft models are in general considered difficult to mimic CRC metastasis as described above; however, the primary cause of CRC-related death is metastases to distant organs such as the liver and lung. Therefore, the establishment of a CRC metastasis model is critical to the development of new drugs. Liver metastasis models for CRC mainly include direct liver inoculation, splenic injection, and portal vein injection. Nude mice and mice are the dominant model animals to study the metastasis of CRC, and it is necessary to select cell lines with high metastasis potential, such as CT26, HCT-116, etc. Direct liver inoculation refers to the injection of tumor cells directly into the liver parenchyma, which is the most direct method to study the progression of liver metastasis of CRC (Kuo et al. 1995). The experimental model of liver metastasis also could be established by injecting cancer cells into portal vein. This method has been widely used in the study of CRC liver metastasis because of its simplicity, high rate of liver metastasis, and rapid development (de Jong et al. 2009). However, the CRC liver metastasis model induced by portal vein injection does not conform to the rule of CRC metastasis and growth in clinical practice. While the CRC liver metastasis model induced by

injection of CRC cells into the spleen can mimic the route and process of CRC cells blood to the liver with the high success rate of modeling. And the phenotype of this model is closer to the clinical characteristics of postoperative liver metastasis of CRC with the characteristics of simple modeling (Morimoto-Tomita et al. 2005; Nguyen et al. 2009). The lung metastasis models of CRC usually were established by intravenously injecting tumor cells via the tail vein (Song et al. 2020; Hou et al. 2020); it is often used to study the mechanism of pulmonary metastasis or screen for anticancer drugs to inhibit pulmonary metastasis in CRC.

In conclusion, ectopic xenograft models have high tumor formation rate with short experimental cycle, and these models are widely used in drug discovery of CRC. If investigators are only interested in studying the proliferation of CRC, they can use the subcutaneous xenograft models, which is the most commonly used in vivo model. If investigators want to study the metastasis of CRC, they need to construct metastasis models by injecting tumor cells into the caudal vein or spleen. However, the tumors formed in the ectopic xenograft model are not naturally formed tumors, so it is difficult to mimic the situation of orthotopic growth of human colorectal tumors and the characteristics of tumor invasion and metastasis.

Orthotopic Xenografts and Patient-Derived CRC Xenografts

Orthotopic xenograft models of CRC are established by implanting CRC cells or tissue directly into the cecum or rectum of animals. The advantage of this model is that the pathological and physiological characteristics of the tumor in this model are similar to that of human CRC, which makes up for the deficiency of subcutaneous transplantation and spleen injection. Hite et al. (Hite et al. 2018) established a model simulating CRC primary tumor growth and metastasis of the liver and lung by injecting luciferase-labeled HT29 tumor cells into the rectum of NOD/SCID mice. And Liao et al. (Liao and Hung 2017) successfully constructed an orthotopic xenografts model of CRC by inoculating HCT116 and HT29 cells into the subserosa of the cecum. But orthotopic xenografts also have the disadvantages of complicated operation, easy death of model animals, and difficult observation of tumor growth.

At present, patient-derived CRC xenograft models are mainly divided into two types: one is to inoculate patient-derived CRC cell lines into immunodeficient mice, called cell line-derived xenograft (CDX) model, and the other is to inoculate CRC tissue blocks derived from patients into immunodeficient mice, called patient-derived tumor xenograft (PDTX) model. Because human-derived CRC lines used to establish CDX models have the advantages of easy access, good tumorigenic effect, detailed validation data (a large number of cell functional and pharmacodynamic data are available for reference), and simple operation, CDX is widely used in various types of laboratories. But during the passage of human-derived cell lines, their tumor cytological characteristics, expression profile levels, and tumor heterogeneity are quite different from those of original tumors, and they are likely to fail to meet the demands for evaluation of medication effects. In contrast, the PDTX model can better maintain the biological characteristics of the primary tumor and genetic

characteristics and tumor heterogeneity similar to those of CRC patients, which can play an irreplaceable role in the precision treatment study of clinical tumors (Hidalgo et al. 2014; Goto 2020). The PDTX model has incomparable advantages in predicting drug efficacy, and it can be used not only for drug research and development in CRC but also for personalized treatment of CRC patients (Siolas and Hannon 2013; Rizzo et al. 2021; Ramzy et al. 2020). For example, after surgical resection of colorectal tumors in CRC patients, colorectal tumors cut from patients were implanted into mice. The mice were then treated with a variety of chemotherapeutic agents, and the therapeutic effects of the chemotherapeutic agents were evaluated to determine which chemotherapy drugs the patients treated could obtain the best therapeutic effect. Although the application prospect of PDTX is promising, there are still shortcomings in the success rate, timeliness, and cost control of PDTX model establishment. In addition, the PDTX model also requires the use of immunodeficient animals, which ignores the effect of the immune system on tumors, which leads to inconsistent results of drug treatment effects in preclinical studies with those in clinical trials.

Transgenic Animal Models

In recent years, genetically engineered CRC mouse models have become popular due to the popularity of transgenic technologies. These models directly edit genes related to CRC with high specificity and have unique advantages in the study of mechanism of CRC development and target drugs. This chapter will introduce some of most common genetically engineered CRC mouse models.

Adenomatous polyposis coli (APC) is an important tumor suppressor gene in the Wnt pathway, and mutations in APC will lead to dysregulated growth of intestinal epithelial cells, which plays an important role in the development of CRC (Zheng et al. 2012). ApcMin is a mouse strain selected by ENU mutagenesis. The allelic mutation of APC gene encoding leucine at codon 850 (TTG) causes multiple intestinal adenomas, so it is called Min (multiple intestinal neoplasia) mouse (Su et al. 1992). The ApcMin mouse models are considered to be a classical animal model to study the development of intestinal tumor. Based on this model, several types of APC transgenic mouse models can be developed for CRC stud. For example, Robanus et al. (Robanus-Maandag et al. 2010) constructed the APC15lox/+ transgenic mouse model on the basis of the APC mouse model, which has a long survival time and leads to the formation of tumors in the large intestine. It can mimic the development of human familial adenomatous polyposis (FAP) and sporadic CRC. DNA mismatch repair (MMR) gene is a DNA mismatch repair system, and mutations in any of its related genes will cause defects in cellular mismatch repair function, resulting in DNA replication errors or microsatellite instability, which in turn induces human hereditary nonpolyposis colorectal cancer (HNPPC). For example, knockout of the Msh2 gene (MMR-related gene) in mice, whose lymphocytes undergo neoplasia, also predisposes to tumors in the gastrointestinal tract (Reitmair et al. 1996; de Wind et al. 1995). This closely resembles

patient characteristics of human MMR gene mutations and can be used as a perfect model to study HNPCC. Other transgenic CRC mouse models, such as Mlh1 knockout mice (Pussila et al. 2013) and Villin-KrasG12D transgenic mice (Rustgi 2013), have also been successfully applied in CRC-related studies.

Conclusion

Animal models of CRC play a key role in the study of CRC pathogenesis and drug discovery; however, no one model can recapitulate all the characteristics of human CRC. Current CRC models can only mimic part of the characteristics of human CRC, and it is essential to select appropriate animal models to address CRC-specific research problems based on the advantages and disadvantages of various models in combination with experimental purposes. In conclusion, it is necessary to establish better CRC animal models to guide the study of CRC molecular mechanism, physiology and pathology, and drug discovery.

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Clinical Utility of Noncoding RNAs as Systemic Biomarkers in Animal Models

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Contents

Abstract

Noncoding RNAs are the RNAs that do not encode for proteins, yet play a vital role in various cellular processes. Compelling cancer research evidence has revealed the function of ncRNAs either as oncogenes or agonizing as tumor suppressors via participating in various cancer hallmarks including cell proliferation, evasion, metastasis, angiogenesis, and dissemination. Both in vitro and

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in vivo studies have indicated the significant role of ncRNAs as potential biomarkers for scrutinizing cancer progression, early diagnosis, relapse, or response to the therapy. The focus on animal model study promotes specific understanding of ncRNA's role in cancer development and prognosis due to their anatomical, genetic, and physiological similarities with human subjects. Besides, the study has highlighted various genetic as well as epigenetic alterations in noncoding regions of DNA. Precisely, among various ncRNAs, the ubiquitous nature of microRNAs and long noncoding RNAs is majorly found in both physiological and pathological stages at cancer progression. However, the exact mechanism underlying ncRNAs activities is yet delusional. This chapter discusses the role of various ncRNA studies in the animal model and identifies their exact role in tumor pathogenesis. The chapter highlights the role of various ncRNAs in distinct cancer types along with their involvement in distinct cellular processes. It also discusses the therapeutic potential of ncRNAs in combatting the problem of chemoresistance and radioresistance.

Keywords

Noncoding RNAs · ncRNAs · miRNAs · lncRNAs · Animal model · In vivo · Biomarker(s)

Introduction

Cancer is serious public health concern that incurs significant challenges in the development of effective diagnosis and therapeutic measures. The World Health Organization (WHO) has marked cancer as progressive chronic disease that accounts for approximately ten million deaths in 2020 (WHO 2020). The most common cancer deaths were reported due to lung, breast, liver, stomach, colon, and rectum cancers. Although the precise cause of cancer remains undefined to date, external causes are described to be physical, chemical, and biological carcinogens, while the internal cause could be due to genetic factors (WHO 2020). Moreover, late diagnosis exaggerates cancer development which further increases the mortality rate and limits recovery rate (Li et al. 2021). Due to such disparity in disease condition and severity, there is a need for the development of new diagnostic methods as well as treatment tools that can efficiently lower global cancer incidences. Research on the animal model is a valuable tool for conducting an elaborate investigation on cancer diagnosis, prognosis, anticancer therapeutics, and measures for cancer prevention. Besides, accumulating and assimilation of data obtained from the animal model can enable researchers to develop insights related to underlying external and internal factors responsible for tumorigeneses, malignant transformation, and cancer progression. Furthermore, the use of the animal model in cancer research can aid in understanding the factors including the genetic causes of cancer development, involvement of specific genes, and distinct mutation (Li et al. 2021). To enhance

the understanding of these factors, untranslated transcripts called ncRNAs have shown promising role as biomarkers.

Noncoding RNAs (ncRNAs) are potential multifunctional regulators involved in various biological processes, and progressive studies have unraveled its association between aberrant ncRNA and cancer. ncRNAs can be categorized as short (19–31 nucleotides), medium (20–200 nucleotides), and long (>200 nucleotides) on basis of their nucleotide length (Agostini et al. 2020). ncRNA majorly transcribed by RNA polymerase II possesses mutual characteristics with messenger RNA (mRNA). The cap structure is present at 5′ end, and poly(A) tail at 3′ end is regulated by canonical promoter and transcription factors (Agostini et al. 2020). Besides, ncRNAs at both transcriptional and posttranscriptional stages have a role in regulating gene expression in humans. The accumulating evidence reported that ncRNAs including microRNA (miRNA), small interfering RNA (siRNAs), short hairpin RNA (shRNA), circular RNA (circRNA), and long noncoding RNA (lncRNA) are dysregulated at various stages of cancer. Additionally, miRNAs, lncRNAs, and circRNAs are widely explored in terms of clinical relevance against cancer due to their diverse functionality (Wang et al. 2019b). Besides, the clinical application of ncRNAs is already under elaborative evaluation as a biomarker for patient's diagnosis, survival, metastasis development prediction, and response to distinct therapy. This reflects that ncRNAs have multifold functionality in distinct steps of cancer progression. Moreover, ncRNA regulation in animal models can mimic the human system and thus can provide insight related to its involvement in distinct cancer types and stages. The purpose of this book chapter is to highlight the clinical utility of ncRNAs as systemic biomarkers in animal models.

Noncoding RNAs: Potential Biomarker in Cancer

ncRNAs are present ubiquitously, and their dysregulation has vital role in oncogenesis and cancer development. Recent studies have caught attention for several ncRNAs as potential biomarkers in different cancer research. Besides, several cellular processes such as chromatin alteration, transcriptional changes, and alteration in post-transcriptional modification have shown the involvement of ncRNAs in its regulation. Among all, the ncRNA, miRNA, and lncRNA have caught significant attention as a biomarker and presented their involvement in the cellular process including proliferation, differentiation, stress response, DNA modification, and apoptosis. For instance, a wide number of ncRNAs including miR-138, miR-375, miR-133, etc. have been reported to have been associated with esophageal squamous cell carcinoma (ESCC) (Sugihara et al. 2015). Figure 1 depicts an elaborative overview of specific ncRNAs involved in distinct cellular pathways in various cancer types. Interestingly, some of the miRNAs including miR-423 were found to be upregulated due to smoking in ESCC. This indicates that upregulation of certain ncRNAs in presence of risk factors can be a probable indication for cancer development. Additionally, overexpression of miR-31 and miR-21 was reported to

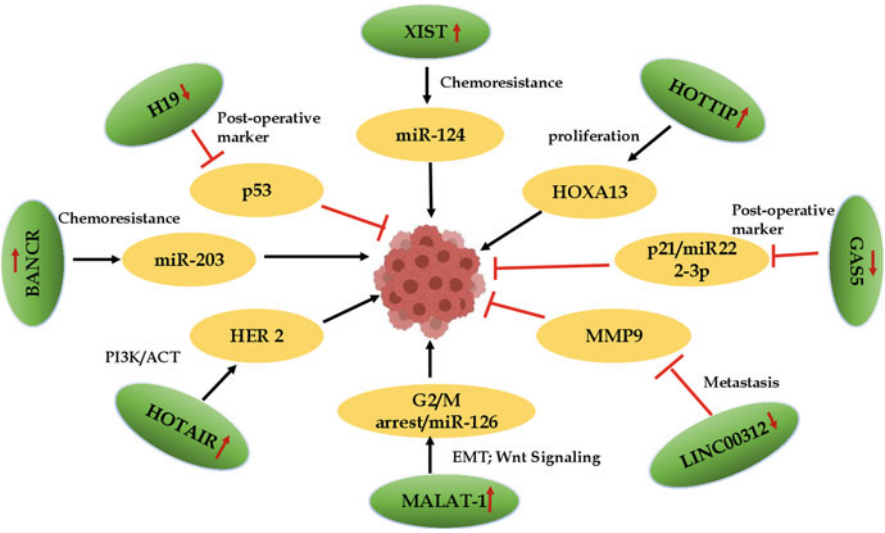

Fig. 1 ncRNAs as potential biomarker in cancer diagnosis and treatment studied in animal model

contribute to inflammation thereby furthering the development of ESCC in the rat model (Sugihara et al. 2015).

Similarly, lncRNAs including GAS5, ZFAS1, MLLT4 antisense RNA 1, PVT1, and RP11-119F7 have been reported to be potential biomarkers in small cell lung cancer, thyroid, gastric, and colorectal cancer (Sun et al. 2015; Lai et al. 2017; Tan et al. 2017; Fan et al. 2018; Han et al. 2019). This suggests that both small ncRNA and lncRNA can act as potential biomarkers in the area of cancer research for the identification of potential risk factors, cancer diagnosis, and prognosis. Besides, early detection of the risk factor biomarker can potentially contribute to prevention measures in the path of cancer development. Further, the chapter discusses the role of distinct ncRNA biomarkers in animal studies and reported on different types of cancer.

Role of ncRNAs in Various Cancer Types

Lung Cancer (LC)

LC is reported to be responsible for the highest mortality rate due to cancer globally. ncRNAs play key roles in animal models of lung cancer including angiogenesis. Therefore, multifold role in regulating multiple cellular processes and ncRNAs can serve as effective diagnostic and/or prognostic targets. For instance, popular lncRNA, namely metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), is reported to be overexpressed in several cancer including lung, breast, liver cancer, and so on. Further, knockdown of MALAT-1 in murine models of breast cancer led to differentiation of primary tumors eventually reducing metastasis (Arun et al. 2016;

Wu et al. 2020) (Table 1). Moreover, as corroborated by Liu et al., restoring miR-126 expression downregulates VEGF levels in mice models of lung cancer, suggesting that miR-126 could be a promising therapeutic target (Liu et al. 2009). Further, miR-21 deletion in in vivo models displayed a protective effect against tumor formation by targeting Ras/MEK/ERK pathway and inhibiting apoptosis (Hatley et al. 2010). Literature also cites that targeting miRNAs such as miR-128 and miR-31 involved in regulating VEGF-C and KRAS could inhibit tumor progression in lung cancer (Hu et al. 2014; Edmonds et al. 2016). Other mouse model-based studies suggest that miRNAs such as miR-34a and let-7 have been reported to be therapeutically relevant in targeted therapy for lung cancer (Trang et al. 2010, 2011). Moreover, Hosono et al. (2017) reported an oncogenic role of lncRNA THOR in both in vitro and in vivo models by demonstrating that targeting THOR alters tumor growth. In another study, lncRNA PTTG3P and its target FOXM1 were observed to be potential therapeutic targets for lung adenocarcinoma (Huang et al. 2020). In another crucial study on lncRNAs, Deng et al. (2015) found that depletion of HOTTIP lncRNA inhibited tumor growth in a murine lung cancer model along with arresting the cell cycle at the G0/G1 phase (Deng et al. 2015).

Growing literature has established that ncRNAs could be promising candidates for LC therapeutics and provide novel insight unfolding the cause underlying LC pathogenesis.

Breast Cancer (BC)

BC is among the highly diagnosed cancers in females with leading mortality rate in metastatic form. Treatment relapse and distant metastasis pose great challenges in BC treatment. A nuclear-enriched lncRNA transcript named MALAT1 has shown elevated expression among the wide range of cancer types including BC. Contrastingly, studies conducted via MALAT1 knockout mice demonstrated normal development, viability, and growth of the organism which could be due to redundancy. Yet, further studies using genetic knockout and antisense oligonucleotide (ASO) knockdown of MALAT1 in in vitro and *in vivo* models revealed impaired cell migration, significant reduction in metastasis, and tumor progression (Arun and Spector 2019). While another study is conducted on xenograft model by insertion of lacZ and polyadenylation sequence, 69 base pairs downstream of MALAT1 transcriptional start site showed targeted inactivation. This supports the notion of MALAT1 involvement in promoting BC metastasis (Kim et al. 2018). Mechanistically, MALAT1 was found to have a role in binding and inactivating the pro-metastatic transcription factor TEAD resulting in blocking TEAD's association with its co-activator YAP and target gene promoter (Kim et al. 2018). Although the role of MALAT1 is contrasting in the distinct study, its role in tumor metastasis is prominent in BC and other cancer types shown in an animal model which makes it a potential metastatic biomarker.

lncRNA CCAT2 is reported to be overexpressed in BC and thus promotes tumor growth via Wnt signaling pathway. The in vivo silencing of CCAT2 revealed the

Table 1 Potential ncRNAs as biomarker studied in the animal model

S.No.	ncRNAs	Cancer type	Target	Function	Reference
1.	MALAT-1	Lung, breast, pancreatic	miR-126; VEGF levels	Oncogenic	Arun et al. (2016); Kim et al. (2018); Zeng et al. (2018); Li et al. (2019b); Arun and Spector (2019); Wu et al. (2020)
2.	miR-21	Lung	Ras/MEK/ERK pathway	Oncogenic	Hatley et al. (2010)
3.	miR-128	Lung	VEGF-C and KRAS	Oncogenic	Hu et al. 2014; Edmonds et al. (2016)
4.	miR-31	Lung	VEGF-C and KRAS	Oncogenic	Hu et al. 2014; Edmonds et al. (2016)
5.	miR-34a	Lung	Kras activation	Anti-oncogenic	Trang et al. (2010); Trang et al. (2011)
6.	let-7	Lung	Kras activation	Anti-oncogenic	Trang et al. (2010, 2011)
7.	lncTHOR (ENSG00000226856)	Lung	mRNA stabilization via IGF2BP1 interaction	Oncogenic	Hosono et al. (2017)
8.	lncRNA PTTG3P	Lung	FOXM1	Oncogenic	Huang et al. (2020)
9.	HOTTIP	Lung	HOXA13	Oncogenic	Deng et al. (2015); Li et al. (2019a)
10.	lncRNA GAS5	Breast; pancreatic; thyroid	miR-222-3p; p21	Anti-oncogenic	Han et al. (2016); Zeng et al. (2018); Xia et al. (2018); Li et al. (2019b)
11.	H19	Breast; pancreatic	p53	Anti-oncogenic	Han et al. (2016); Liu et al. (2014); Zhai et al. (2015); Li et al. (2019b)
12.	lncRNA CCAT2	Breast	Wnt pathway	Oncogenic	Cai et al. (2015); Hu et al. (2018)
13.	LINC00346	Pancreatic	miR-188-3p, miR-505-5p and miR-1224-3p	Anti-oncogenic	Shi et al. (2019)

(continued)

Table 1 (continued)

S.No.	ncRNAs	Cancer type	Target	Function	Reference
14.	HOTAIR	Breast; pancreatic; AML; leukemia	HER 2; HOXA5	Oncogenic	Su et al. 2014; Cerk et al. 2016; Jiao et al. 2015; Liu et al. 2014; Zhai et al. (2015); Wang et al. (2019a)
15.	ENST00000480739	Pancreatic	HIF-1α; OS-9	Anti-oncogenic	Li et al. (2019a)
16.	lncRNA XIST	Colorectal	miR-124	Oncogenic	Zhu et al. (2018)
17.	lncRNA CPS1-IT1	Colorectal	EMT pathway, HIF-1α, and autophagy	Anti-oncogenic	Zhang et al. (2017, 2018)
18.	lnc BRAF-activated noncoding RNA (BANCR)	Colorectal	miR-203	Oncogenic	Ma et al. (2018)
19.	lncRNA HOXA AS	Leukemia	miR-520c-3p	Oncogenic	Dong et al. 2018
20.	SNHG16	Leukemia	has-miR-124-3p	Oncogenic	Yang et al. (2019)
21.	lncRNA UCA1	Leukemia	miR-296-3p	Oncogenic	Li et al. 2020
22.	lncRNA DANCR	Leukemia	LSCs	Oncogenic	Bill et al. (2019)
23.	lncRNA LINC00467	Leukemia	microRNA-339	Oncogenic	Li et al. 2020
24.	lncRNA CASC9	Thyroid	miR-488-3p	Oncogenic	Chen et al. 2020
25.	NEAT1	Thyroid	miRNA-214	Oncogenic	Li et al. (2016)
26.	LINC00312	Thyroid	MMP9	Anti-oncogenic	Min et al. 2018
27.	FOXD2-AS1	Thyroid	miR-185-5p	Oncogenic	Li et al. (2019a)
28.	LINC00152	Thyroid	miR-497	Oncogenic	Sun et al. (2019)
29.	ZFAS1	Thyroid	miR-329, miR-150, miR-484	Oncogenic	Han et al. (2019)
30.	BCLAT1	Thyroid	Lymph Node Metastasis (LNM)	Downregulated	Liao et al. 2018

biological function of the ncRNA in tumor growth suppression (Cai et al. 2015; Hu et al. 2018).

Clinical studies assessing surgical effects on patients with BC have reported the involvement of ncRNAs. Han et al. (2016) tested plasma samples of BC patients at preoperative and postoperative stages that revealed the role of lncRNAs GAS5 and H19. Both GAS5 and H19 were observed to be substantially downregulated in the

plasma of postoperative patients. This infers that expression study of specific biomarkers could potentially aid in early diagnosis of specific cancer type at an early stage as well as the success of the surgery. Further, the study on the animal model has shown the involvement of four lncRNA clusters in BC prognosis. lncRNA HOX antisense intergenic RNA (HOTAIR) was found to be upregulated in HER-2-enriched subgroup (cluster II), while lncRNA HOTAIRM1 overexpression was found in the basal-like subgroup (cluster I). Moreover, the expression of estragon receptor was found with lncRNA in cluster III and cluster IV (Su et al. 2014; Cerk et al. 2016). Hence, different ncRNAs possess both therapeutic potential and diagnostic potential and thus can act as a potential biomarker.

Pancreatic Cancer (PC)

Pancreatic ductal adenocarcinoma (PDAC) is another malignant cancer with bad prognosis. Studies have reported that PDAC is often characterized by the desmoplastic reaction which further creates dense microenvironment and results in hypoxic conditions, inducing epithelial-to-mesenchymal transition (EMT) which facilitates invasion and metastasis (Li et al. 2016). Recent in vitro and in vivo studies have observed the role of several lncRNAs that can potentially act on competing endogenous RNA or 'RNA sponges' interacting with miRNA in such a manner that it sequesters these molecules and results in the reduction of their regulatory effect on mRNAs (Li et al. 2017b). Further, the studies have confirmed the overexpression of LINC00346 facilitating PC. The knockdown of LINC00346 via the use of specific shRNA showed decreased cell proliferation and colony formation in pancreatic cells (Shi et al. 2019). Cell cycle distribution revealed significant decrease of cells in S-phase and an increase in G2/M phase. Moreover, p21 expression and Chk1 phosphorylation increased in cells that showed LINC00346 depletion. Additionally, the study established the relation between miRNAs and lncRNAs in the biological process. Through in silico analysis, the study identified many miRNAs including miR-505-5p, miR-1224-3p, and miR-188-3p to be putative targets of LINC00346. A similar result in the animal study indicated that LINC00346 overexpression augmented pancreatic xenograft growth (Shi et al. 2019). Thus, the study demonstrated the crucial role of LINC00346 in PC.

Further, MALAT-1 has been reported to be upregulated in PC stem cells which further promote the proliferation of pancreatic stem cells. The study demonstrated the formation of anchorage-independent spheroid formation in nude mice xenograft models, thereby confirming the role of MALAT-1 in enhancing tumorigenicity (Li et al. 2019a). This indicates that the identification of MALAT-1 as a biomarker can significantly enable the early detection of PC (Zeng et al. 2018; Li et al. 2019a). Interestingly, the role of other oncogenic lncRNAs like HOTAIR and H19 has been studied in PC (Jiao et al. 2015; Liu et al. 2014; Zhai et al. 2015). In contrast, GAS5 acts as tumor suppressor lncRNA that has displayed its role in the prevention of p21 mutation and delay of cancer progress (Zeng et al. 2018; Li et al. 2019a). LncRNA ENST00000480739 levels were reported to be significantly decreased in PC, thus

preventing tumor invasion and metastasis via targeting HIF-1α through upregulation of OS-9 (Sun et al. 2014). Thus, it can be concluded that some lncRNAs possess therapeutic potential for suppression of tumor invasion and metastasis, while others could be used as diagnostic markers for early cancer detection.

Colorectal Cancer (CRC)

CRC is a malignant neoplasm that occurs in the gastrointestinal tract and has shown incidences of high mortality and morbidity. Researchers have highly focused on mRNAs for understanding the cause of underlying tumorigenesis, but some recent studies have revealed the role of ncRNAs in the regulation of physiological conditions like cell proliferation, apoptosis, migration, and tumor development. Literature suggests that X-inactive specific transcript (XIST) can regulate doxorubicin (DOX) resistance initiated through the regulation of miR-124 at the SGK1 axis. XIST knockdown during xenograft study performed on BALB mice demonstrated its potential in the chemosensitivity of CRC cells by elevating the expression of miR-124, which resulted in the suppression of CRC cells (Zhu et al. 2018). Another lncRNA CPS1 intronic transcript 1 (CPS1-IT1) has been studied to suppress the progression, invasion, migration, and proliferation capability of cells in CRC (Zhang et al. 2017, 2018). Further, the in vitro study revealed the exact role of CPS1-IT1 in suppressing the EMT pathway and metastasis by inhibition of hypoxia-induced autophagy via inactivation of HIF-1α. Furthermore, the role of CPS1-IT1 on EMT, HIF-1α, and autophagy was confirmed in vivo xenograft that showed significantly reduced protein expression of HIF-1α and LC3-II in CRC specimen. In contrast, lncRNA BRAF-activated noncoding RNA (BANCR) has a tumorigenic potential as well as a potential of adriamycin resistance (Ma et al. 2018). Specifically, BANCR acts as molecular target sponging miR-203 and sequestering away from human chromosomal segregation 1-like (CSE1L) leading to its upregulation. CSE1L also known as exportin-2 maps on chromosome 20q13 is overexpressed in CC. The current study showed positive correlation of BANCR in colorectal cancer progression and chemoresistance, while the knockdown of CSE1L enhances Adriamycin sensitivity, thus resulting in increased apoptosis along with substantial loss in proliferation and invasion capability (Ma et al. 2018). Thus, it can be inferred that ncRNAs can act as potential biomarkers in CRC diagnosis and management.

Leukemia

Malignancy of the body's blood-forming tissues is regarded as leukemia. Acute myeloid leukemia (AML) is among the complex form of leukemia showing high recurrence rate and shows the major problem of chemoresistance. Recently, lncRNA called HOXA cluster antisense RNA 2 has pivotal role in various tumorigenic features including cell survival, invasion, and proliferation. Besides, HOXA-AS is shown to be significantly overexpressed in the case of multidrug resistance and

chemoresistance among AML patients, thus suppressing the effect of Adriamycin drug. HOXA-AS knockdown has inhibited the ability of cell proliferation and increased apoptosis in in vitro studies while decreasing the tumor volume in vivo (Dong et al. 2018). Research has indicated that the presence of lncRNA can be directly linked to the expression of tumor cells. Similarly, SNHG16 is one such lncRNA, whose interaction with hsa-miR-124-3p axis and epigenetic deactivation can trigger functionally implied changes in the ALL cells. Besides, downregulation of SNHG16 revealed limited proliferation in the oncogenic leukemia cells (Yang et al. 2019). Upon analysis of the samples collected from the AML patients, HOTAIR was another lncRNA whose concentration was notably spiked. Silencing of this lncRNA and simultaneously overexpressing the HOXA5 caused a drastic decline in the proliferation of the cells involved in the malignancy, while the apoptosis factor was comparatively increased in the in vivo samples tested for the same (Wang et al. 2019a). Another study showed the knockdown of DANCR lncRNA in leukemia stem cells (LSCs) improved the outcomes in AML patients by reducing the reconditioning of LSCs. Further, the downregulation or knockdown of the gene in the murine model made them dormant that replicated in AML patients. Hence, the LSC-specific lncRNA sequence can be treated as a potential therapeutic source (Bill et al. 2019). Research conducted on lncRNA LINC00467 conveyed that its expression is elevated in various tumors. It also elucidated that knocking down of this lncRNA results in downregulation of the SKI target gene while upregulating the microRNA-339 that results in the inhibition of the proliferation of various AML types, hence showcasing an active role in the pathogenesis of oncogenes in AML patients (Lu et al. 2021). lncRNA UCA1 has been actively identified in various malignancies. It was found to be upregulated in the AML. Upon deactivation of this lncRNA in the AML patients, it led to a decrease in the rate of cell proliferation and also showed signs of apoptosis induction as indicated through the MTT and flow cytometry assays. Its effect was also tied to the overexpression of the Myc target of the miR-296-3p inhibition showing anti-AML effect. lncRNA UCA1 knockdown also showed positive inhibition of tumorigenesis in *in vivo* models (Li et al. 2020). Thus, major involvement of lncRNAs among leukemia patients can potentially be exploited in improving the prognosis of the patient already suffering from the disease and also help in early diagnosis.

Thyroid Cancer (TC)

Papillary thyroid carcinoma (PTC) is a frequently occurring endocrinal cancer, with a comparatively higher incidence of occurrence in women as compared to men. Previously conducted studies have highlighted the role of epigenetic and genetic alterations in the initiation and progression of the PTC (Min et al. 2018; Chen et al. 2020). The lncRNA cancer susceptibility candidate 9 (CASC9) is studied to promote the proliferation of PTC through sponging of miR-488-3p hiked in cases of PTC (Chen et al. 2020). In the mice, xenograft model knockdown of CASC9 showed its negative association with miR-488-3p and positive association with ADAM9

expression, followed by activation of EGFR-Akt signaling. It is inferred that lncCASC9 aid in the promotion of the malignant phenotype of PTC through modulation of miR-488-3p/ADAM9 pathway and hence can be used as potential biomarker (Chen et al. 2020). Another nuclear-enriched abundant transcript 1 (NEAT1) in TC cells has shown overexpression and thus acts as potential oncogenic biomarker. The knockout of NEAT1 in the xenograft model resulted in impairment of the malignant process of TPC-1 and expression of attenuated β-catenin. The study revealed downregulation of miR214 in TPC; however, the involvement of β-catenin in NEAT1 induced increase of TPC is yet elusive which further requires investigation (Li et al. 2016). LINC00312 is another newly discovered intergenic long noncoding RNA that is a novel biomarker for metastasis. Their upregulation and downregulation have a clear inverse relationship with the expression of MMP9 that has significant role in the ECM degradation (Min et al. 2018). It can also be seen that the overexpression of the LINC00312 can effectively inhibit the proliferation and inhibition through the PI3K/Akt signaling pathway. In another study conducted, upregulation of FOXD2-AS1 was a clear indication in the PTC cells. Knockdown of the FOXD2-AS1 leads to suppression of proliferation, and invasion of PTC calls in vitro and suppression of the PTC tumor in the in vivo models (Li et al. 2019a). BCLAT1 serves as a negative prognostic factor in cancer prognosis. In PTC, BCLAT1 acts as tumor suppressor and thus can be exploited as a novel prognosis biomarker (Liao et al. 2018). LINC00152 was seen to be significantly upregulated in in vivo PTC tissue studies. Its knockdown notably inhibited the proliferation, colony formation, and migration and impaired the tumor progression in vivo (Sun et al. 2019). Besides, LINC00152 agonizes *endogenous* RNA to the miR-497 sponge in which it downregulates the BDNF neutrophic factor that acts as an oncogene in PTC. lncRNA Gas5 has also been proven to operate as a sink for miR-222-3p to repression tumor in PTC cells, therefore altering the expression of PTEN (Xia et al. 2018). This study also elucidated upregulation in PTC cells as compared to the surrounding non-tumor tissues. Hence, its inhibition leads to reduced translocation and incursion in metastasis of tumor cells. Upregulated ZFAS1 serves as a clear indication for clinicopathological conditions and also showcases itself as a root cause for poor prognosis in thyroid carcinomas (Han et al. 2019). Hence, the study highlighted how inhibition of ZFAS1 can positively strut proliferation of the cells in the tumor. This indicates that ncRNAs can be exploited differentially for diagnostic, prognostic, and treatment purposes based on their intensity at distinct stages. Further, collective effort is made to present major ncRNAs discussed in the above study in the tabular format while highlighting their role in specific cancer types and their regulatory function (Table 1).

Role of ncRNA in Chemotherapy and Radiotherapy Sensitivity

The optimized treatment in various types of cancer requires removal of the entire tumor and other appropriate measures for preventing secondary cancer formation. However, in many cases, curative surgery might be challenging due to the absence of

specific symptoms at an earlier stage and the lack of early detection. In such situations, chemotherapy and radiotherapy play crucial roles in prolonging the overall survival of the patient. Unfortunately, the biological features of cancer progression at times lead to chemo and radioresistance. Studies have correlated the role of ncRNA with inducing chemosensitivity. For instance, knockdown of HOTTIP in PC has shown controlled cell proliferation and migration and increased apoptosis (Li et al. 2015, 2019a). Other studies showed loop correlation between HOTTIP and Hoxa13 as a transcription factor. Furthermore, control of Hoxa13 expression showed a significant reduction of HOTTIP in pancreatic cells (Li et al. 2015, 2019a).

Similarly, blocking HOTAIR expression showed reduction in multidrug resistance-associated protein 1 (MRP-1) via targeting the Pi3K/ACT signaling, thereby improving imatinib sensitivity in CML (Wang et al. 2017). Additionally, blockade of estrogen receptors (ER) in tamoxifen-resistant BC tissues restored the expression of HOTAIR as corroborated by Xue et al. (2016). Circular RNAs such as hsa_circ_0081143 have been studied recently in chemoresistance. Knockdown of hsa_circ_0081143 in the animal model showed enhanced cisplatin sensitivity in gastric cancer via targeting the miR-646/CDK6 pathway (Xue et al. 2019). Hsa_circ_0081143 was also seen to regulate tyrosine kinase inhibitor resistance in non-small cell lung cancer through miR-1183/PDPK1 pathway (Zhou et al. 2019).

Apart from chemoresistance, ncRNAs showed a promising role in enhancing radiosensitivity, thus potentially overcoming the problem of radioresistance. For instance, blocking of lncRNA LINP1 in BC cells increases the sensitivity to radiotherapy (Zhang et al. 2016). Also, silencing MALAT-1 sensitizes the nasopharyngeal carcinoma cells by modulating the miR-1/SLUG pathway to radiotherapy (Jin et al. 2016However, the findings have not been prominently reported yet in animal models; yet the in vitro data indicates the potency of ncRNAs in enhanced chemo and radiosensitivity.

Thus, these findings suggest the importance of ncRNAs in designing strategies aimed at improving radiosensitivity and chemosensitivity.

Challenges of ncRNA Study in Animal Model

The ubiquitous nature of ncRNAs has made it an important target as biomarker in cancer diagnostic and treatment. However, the study of ncRNA in animal models is continuously subject to certain challenges. For instance, miRNAs bind to their targets present on mRNAs via the mechanism of imperfect base pairing with miRNA response elements (MREs). The interaction between miRNAs with their target gene present in the animal system might involve 5'-UTRs that potentially suppress their expression. For instance, miR378a-5p targeting 3'UTR has shown tremendous decrease in expression of CYP2E1 protein production, thus indicating an inhibitory effect in translational repression (Ning et al. 2019). Furthermore, the involvement of miRNA-induced silencing complex (miRISC) between miRNAs and mammalian gene due to activation of AGO protein suppresses its expression. Retrospectively,

the perfect hybridization between miRNA-MRE complex induces cleavage of target mRNA via functionalization of AGO protein. However, imperfect pairing results in the prevention of cleavage mediated by RNA interference (Ning et al. 2019).

Moreover, the cross-taking interaction among various ncRNAs including lncRNAs, miRNAs, and so on is facilitated via both exogenous stimuli (environmental factor) and endogenous stimuli (hormonal factor) that modulates drug safety and efficacy (Ning et al. 2019). The interaction between distinct exogenous and endogenous stimuli, epigenetic components, and genetic makeup affects the final expression of ncRNAs and associated enzymatic activities. Several instances might come across including genetic polymorphism, DNA methylation, and so on which interfere with ncRNAs expression. Thus, it can be inferred that although ncRNAs studied in animal models can act as potential biomarker development, its study on animal models possesses some critical challenges which require consideration.

Conclusion and Future Perspective

The role of ncRNA is ubiquitous and thus has been reported in several biological processes. The chapter has highlighted the specific role of distinct ncRNAs that are involved in specific cancer types via targeting several pathways. Precisely, the study focused on the outcome found in vivo as it closely replicates the human system. It is observed that ncRNAs have potential advantages in cancer modalities ranging from the potential application as biomarkers in early cancer detection to acting as a potential target in increasing chemosensitivity of the drugs. Interestingly, it is observed ncRNA can have differential roles with oncogenic as well as antioncogenic potential. This is because various ncRNAs including MALAT1, HOTTIP, HOTAIR, BANCR, etc. have oncogenic potential as they were found upregulated in various cancers and are independent predictors of disease-specific survival. Further, the downregulation or knocking out of such ncRNAs can potentially show improved cancer prognosis and therefore can also be stated to possess therapeutic potential. On the other hand, ncRNAs including H19, GAS5, and LINC00312 are found to be downregulated in different cancer types and thus have been exploited in animal models for reduction of tumor size and volume. In such instances, ncRNAs have therapeutic potential that can be induced for control of tumor progression by arresting cell proliferation, metastatic potential, and angiogenesis along with correcting cell cycle checkpoints.

Compelling evidence from research highlights the function of ncRNAs as either oncogenes or tumor suppressors through their participation in various cancer hallmarks such as cell proliferation, metastasis, angiogenesis, evasion, and dysregulated expression during cancer onset. Hence, in the future, clinical investigation of ncRNAs for exploring their role as potential biomarkers for early diagnosis, metastasis development, survival chances, and therapy response can lead to advancement in cancer therapeutics. Thus, future research in cancer focusing on exploring the role of ncRNAs in distinct cancer and stage can lay a prominent path in cancer therapeutics.

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Applications of Nanomedicine in Animal Models of Cancer

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Contents

Abstract

Cancer is one of the foremost causes of death worldwide, and advanced therapeutics are desperately required. The progress of new nanocarriers and nanomaterials has resulted in significant advancements in cancer drug delivery. Most nanocarrier uses have had the primary goal of protecting the drug from rapid

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degradation systemic distribution and allowing it to reach therapeutic concentrations at tumor sites, while avoiding drug delivery to regular locations as much as possible to limit unwanted effects. Scientists now have new techniques for treating and imaging cancer thanks to breakthroughs in nanotechnology. This approach has enabled nanoscale devices to be merged with numerous functional molecules simultaneously, such as antibodies, tumor-specific ligands, imaging probes, and cancer treatments. Because structural and physical factors such as size, load, shape, and surface features affect drug biodistribution, pharmacokinetics, internalization, and security, rational nanoparticle design is crucial. Because traditional cancer therapies have limitations, various nanotechnologies for more effective and safer cancer treatment, also known as cancer nanomedicine, have been developed and applied. This chapter discusses cancer advancements, challenges, and opportunities in animal models for cancer management with nanomedicine.

Keywords

Nanomedicine · Nanoparticles · Nanocarriers · Cancer nanomedicine · Nanoscale devices · Cancer nanomedicine in animal models

Introduction

Worldwide, cancer has a high mortality rate, and its prevalence is rising. Common treatments for cancer include radiation, chemotherapy, and surgery. Anticancer therapies frequently encounter problems such as nonspecific antitumor agent systemic distribution, insufficient medicine levels getting the tumor, and a partial capacity of monitoring therapeutic response. Poor delivery of drugs at the specific site causes medication multiresistance, which is a significant adverse effect (Hausman, 2019). Cancer nanotechnology provides answers to various current barriers in cancer therapies by employing a broad and diverse range of nanoparticles with designs derived from chemistry, engineering, and medical sectors for targeted therapy and molecular imaging. Nanoparticles have tiny diameters ranging from 5 to 200 nm, allowing them to interact in novel ways with biological systems. Nanoparticles can self-assemble and maintain stability and specificity due to their material composition, critical for biocompatibility, and drug encapsulation. Recent advances in cancer nanotechnology pave the way for personalized oncology, in which patients' molecular profiles are used to tailor diagnosis and therapy (Roy and Saikia, 2016).

Nanoparticles as Drug Delivery Systems and Its Categories

Polymers, metals, and ceramics are just some of the materials used to make nanoparticles. Depending on the production processes and materials employed, these particles can come in various sizes and shapes, each unique characteristic.

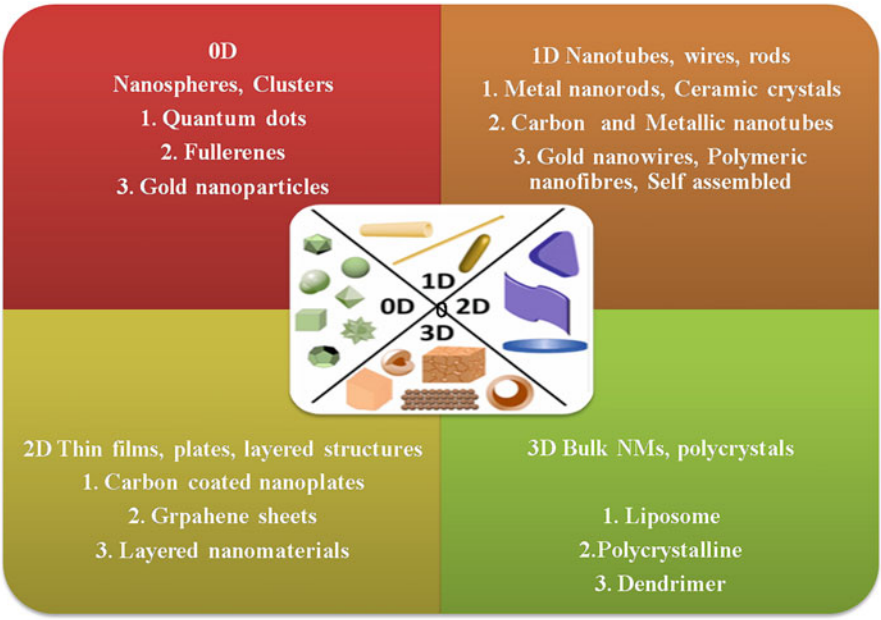

Fig. 1 Schematic representation of various types of nanomaterials

Liposome- and other lipid-based carrier-based drug delivery solutions exist in various phases of development, including lipid-drug complexes and lipid emulsions, polymer microspheres, polymer-drug conjugates, micelles, and other product target ligand (Allen and Cullis, 2004). The different types of nanomaterials are shown in Fig. 1.

Multifunctional Nanoparticles for Tumor Imaging

The reappearance of the carcinoma and examine therapeutic responses in clinical oncology are screened using radiological examinations. Different types of nanoparticles which have multifunctional properties are displayed in Fig. 2. Traditional tumor imaging techniques, including MRI (magnetic resonance image) and CT (computed tomography), are primarily concerned with describing the tissue morphological characteristics, organs, and tumors, such as extent, anatomic location, and tumor size, at various spatial contrast and resolution levels (Burcu et al. 2013). Even though, advancements in sophisticated equipment for imaging and its modalities with a spatial resolution that utilizes nontargeted contrast agents, such as CT and MRI, facilities to afford precise and valuable disease details and have limited sensitivity, which is increasingly recognized as a barrier to earlier diagnosis and treatment response monitoring (Atri, 2006).

Fig. 2 Multifunctions of nanoparticles

Zebrafish Cancer Models

Danio rerio, zebrafish, a tropical fish that lives on freshwater, has long been used in medical and scientific research as an animal model. It belongs to the family Cyprinidae and class Actinopterygii and thrives primarily in India's Ganges River. With the simplicity of zebrafish compared to the mouse model and ease of genetic manipulation, George Streisinger employed the fish as a study model for the first time in the 1970s (Liu and Leach, 2011). However, after two scientists utilized zebrafish to establish two vast mutant lineages in the 1990s, the usage of zebrafish expanded. This model offers several essential qualities and benefits that make it a good choice for biology and medical research (Brown et al. 2017). Low cost, fast growth, embryonic accessibility, optical transparency, genetic manipulability, and, most prominently, genetic and physiological similarities to humans and a comparable brain, digestive tract, immune system, and circulatory system are among those the model's significant advantages.

Furthermore, human genes (70%) are functionally comparable to zebrafish. Furthermore, adult zebrafish may be cultivated in small freshwater ponds, requiring

less area due to their small size. Furthermore, zebrafish investigations could be carried out at any developmental stage, including larval (3–29 depths) and adult stages (2 years – 90 dpf). Furthermore, the model of zebrafish optical transparency has significant advantages for therapeutic applications of nanoscale drug delivery systems (Astell and Sieger, 2020). The zebrafish embryo's transparency allows researchers to see interior processes such as tumor or organ formation, vascular expansion, and nanomaterial distribution. Until 60 h after fertilization (FPH), zebrafish embryos are translucent when the coloring process begins. 1-Phenyl 2-thiourea (PTU) is used to treat the embryos, which suppress the development of pigmentation or Casper, the transgenic models, which lack colors in their skin.

Furthermore, few animal model embryos develop faster than zebrafish; the screening time is less, as is the time between significant changes in behavior and morphology of model. Because of the model's simplicity to be constructed, which may be achieved by xenotransplantation, genetic engineering, and chemical exposures, cancer is one of the most studied ailments employing this model. In zebrafish, overexpression of the Myc gene, for example, causes T-cell leukemia. However, in the model, exposure to dimethyl benzanthracene causes cancer in the intestine, and tumor cell transplantation such as B16-F10 melanoma cells causes skin cancer (Fazio et al. 2020). In addition, many methodologies are utilized to establish genetically, xenograft, and pharmacological zebrafish models to explore changes in biological pathways and functions of a gene in cancer development and to test new anticarcinogenic medications.

Xenograft Models

One method for modeling cancer that involves implanting malignant cells into an animal model is xenografted zebrafish embryos. While the adaptive immune response of zebrafish has not so far evolved, cancer cell transplantation is possible at about 2 dpf (Shi et al. 2020). As a result, utilizing 2 dpf embryos could permit cancer cells to spread, metastasize, and survive without using immunosuppressive drugs. Melanoma, leukemia, colorectal, kidney, breast, ovarian, prostate, pancreatic, oral, and lung cancer tumors have been implanted to develop cancer xenograft zebrafish models (van Weerden and Romijn, 2000).

Genetic Models

Because they are generated in numerous ways, mainly forward or reverse genetic procedures, zebrafish mutant and transgenic models are vital and potent disease and cancer research models (White et al. 2013). Forward genetic techniques like ethylnitrosourea are used and reverse genetic techniques like CRISPRs (clustered regularly interspaced short palindromic repeats) and the TAL-like (Transcription activator-like) effector nucleus. Furthermore, in zebrafish cancer models, significant conservation of oncogenes like NRASQ61K (neuroblastoma Ras viral oncogene

homolog) and BRAFV600E and microinjection of human tissue-specific promoters result in tumors similar to human malignancies. The functions of specific knockdown or knockout genes of interest were briefly explained utilizing reverse genetic techniques. Furthermore, for the past two decades, the mouse model has been the only vertebrate model that has utilized reverse genetic approaches. In vitro, homologous recombination in embryonic stem cells was used to develop the methods for these approaches. There have been attempts to develop zebrafish embryonic stem cell lines. No homologous recombination-based embryonic stem cell knockdown procedure for zebrafish has yet been created.

Another reverse genetic approach is another early microinjection of antisense morpholino oligonucleotides into the blastomeres or yolks of zebrafish embryos. When a morpholino is injected into a target gene, it prevents that gene from being translated. Genetic procedures, namely, random mutagenesis, target-selected mutagenesis, and genetic screening, were employed in conjunction with genetic screening and mapping to hunt for specific genes' roles, products, and mutations (Xie et al. 2015). To target-selected mutagenesis in the genome of zebrafish embryos, researchers have used various techniques, including chemical mutagens like N-ethyl-N-nitrosourea, to target locally produced lesions in genomes (ENU). These procedures result in sudden mutational changes in ribosomal protein or particular tumor suppressor gene, namely, p53; APC (adenomatous polyposis coli) and NF2 (neurofibromatosis type 2) in a high quantity of stable mutant zebrafish lines have taken form (Wienholds et al. 2002). Due to wild-type p53, DNA-binding domain mutation may impulsively generate malignant peripheral nerve sheath tumors in the mutant zebrafish model line tp53M214K at 8.5 months and 16.5 months with a 28 percent occurrence. Furthermore, by microinjecting DNA constructs into early zebrafish embryos with numerous tissue-specific promoters and systems to achieve spatial and temporal control of the expression of some transgenes, the GAL4-UAS, Cre-LoxP, Tol2 transposon, and LexPR binary systems were used to create several transgenic zebrafish cancer models (Ota and Kawahara, 2014).

Chemical Models

Chemical carcinogens were one of the first ways utilized in the zebrafish animal model to produce cancer mutations for tumor formation or developmental abnormalities. The carcinogenic materials that are liquefied or suspended in the fish's swimming water and the animal is subjected to long-term exposure. According to specific research, the zebrafish animal model is the most responsive to several carcinogens (Mizgireuv et al. 2004). This animal model has a smaller diversity of neoplasm mutations and kinds than other fish species. Exposing zebrafish embryos to N-methyl-N-nitrosoguanidine (MNNG) and dimethylbenzanthracene (DMBA), for example, has been shown to cause a variety of tumors in the animals, including rhabdomyosarcomas, hemangiomas, seminomas, leiomyosarcomas, chondromas, and hemangiosarcomas, and especially, N-N-nitrosodiethylamine (DEN) has been shown to induce liver and pancreatic carcinomas, whereas N-nitrosodimethylamine

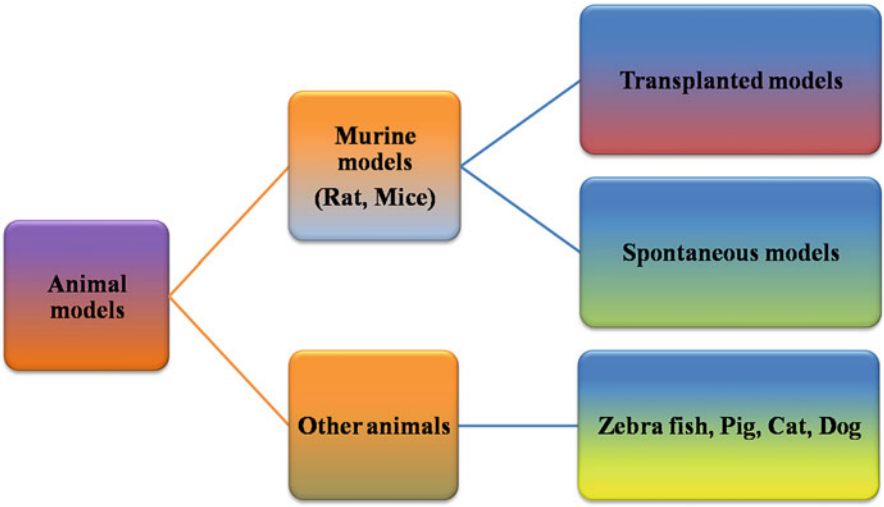

Fig. 3 Several animal models for cancer

(NDMA) primarily induces cancers in the liver (Kemp, 2015). Furthermore, when given to mutant fish with a cancer gene, some of these chemical carcinogens increased the fish's susceptibility to tumor formation as compared to wild-type fish. Chemical carcinogens benefit from making specific tumors accessible to research because they can be easily induced. Figure 3 showed the various types of animal models available for cancer.

Breast Cancer In Vitro Models for the Development of Nanomedicine

A different model system is required for each stage of nanomedicine research, from 2D cell culture analysis through in vivo testing in small and big animal models. Cost-effectiveness, difficulty, and ethical considerations must all be "weighed" against the potential for producing meaningful data when selecting appropriate model systems for each level. Immortalized cell lines are cost-effective and straightforward tumor models. Breast cancer cell lines retain the essential genetic mutations related to the tumor subtype despite their alterations during formation and continuous culture, justifying their usage in cancer models. Even though there are many human breast cancer cell lines available for study, studies frequently employ the MCF7 (luminal A subtype) and MDA-MB-231 (TNBC – triple-negative breast cancer subtype) cell lines, as well as the mouse 4 T1 (TNBC subtype) cell line. Nanomedicines can be tested in typical cell culture methods for toxicity, subcellular localization, absorption, modes of action, and influence on specific biological processes (Aaliyari-Serej et al. 2020). Dye exclusion, calorimetric, fluorometric, and luminometric tests are among the cell viability assays used to investigate the toxicity of nanomedicine,

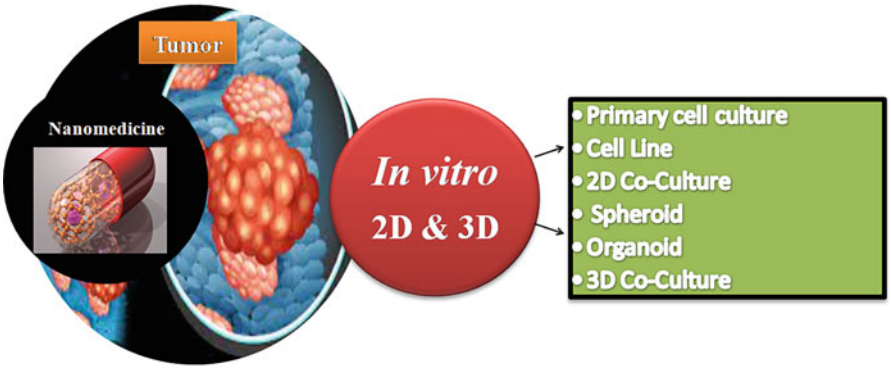

Fig. 4 In vitro models for breast cancer

though the calorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is the mainly used cell viability assay. The in vitro models of breast cancer are depicted in Fig. 4.

Fluorescence microscopy, confocal imaging, and flow cytometry have all been used to examine the uptake of nanomedicine and succeeding subcellular localization in cell lines. A nanomedicine mechanism of action can be established by examining protein or RNA levels or utilizing functional tests to predict in vivo effects. Migration and invasion tests are among them. Traditional 2D cell cultures, although their relevance, lack the crucial tumor features needed to predict nanomedicine treatment response accurately. The disadvantages of homogeneous monolayer cultures are the lack of a 3D structure and poor inter-tumor and intra-tumor heterogeneity representation. These constraints are being addressed by the continual development of novel cell cultures and methodologies like advanced 3D cell cultures and, cell co-cultures, patient-derived cells. Combining tactics to improve complexity (e.g., 3D cell co-culture) can result in a more accurate disease model (Vohra et al. 2013). The following sections will provide a comprehensive overview of relevant methodologies and their implications for evaluating nanomedicines.

Tumor Growth and Metastasis Assessment

Animal models are now required for new approaches to visualize the multiply and disturbance of drug testing, microenvironment of the tumor, and therapeutic responses. As a result, the Zebrafish animal model is one of the finest for studying malignancies because tumor engraftment and development are incredibly fast, with readouts taking days rather than weeks like other models. Furthermore, xenografted zebrafish could absorb small mass compounds straight from the surrounding water, making them ideal drug testing models (Vargas-Patron et al. 2019).

Numerous approaches have been developed to quantify and assess the proliferation of human tumor cells xenografted in zebrafish embryos. Using a fluorescent

microscope and image analysis software, fluorescently tagged cancer cells, for example, might be monitored in embryos from the day of injection, generally at 48 or 72 depths until the endpoint day. Enzymatically dissociate the embryos at the endpoint, time them into a single cell suspension, count the number of fluorescent cells in the suspension, divide that number by the 24-hour cell number to get the fold increase in cell number, or use flow cytometry after enzymatically digesting the embryos to determine tumor cell proliferation.

The cells would be labeled with a fluorescent protein or stained with a chemical dyestuff to see if the cancer cells had spread throughout the zebrafish embryo's body. The cancer cell metastasis inside the embryo body would be imaged 2 days after the injection. The number of metastatic cells in these fish could then be measured using confocal microscopy and software like Fiji. Augustine and colleagues, for example, employed cancer cells that expressed the green fluorescent protein (GFP) to detect and quantify moving cancer cells in ovo (Teng et al. 2013). Furthermore, by identifying a cancer-specific gene or a human housekeeping gene, qPCR might be utilized to track tumor growth.

Prospective Nanotechnologies to Advance Cancer Therapy

As emerging nanotechnologies strive to enhance PK/PD (pharmacokinetic-pharmacodynamic), effectiveness and selectivity, many preclinical investigations are underway to develop triggered drug release and multimodal therapies that are highly selective for malignant cells. A targeted pharmaceutical release can reduce the lowest effective dose, while improving patient quality of life and decreasing overall toxicity. Therapeutics can be produced to give maximum efficacy with the least amount of damage when technology advances to employ specialized delivery (Kopeckova et al. 2019). Certain targeted medicines may be tumor-specific; however, they have clinical limits due to the characteristics or distribution of pharmacokinetics and pharmacokinetics. The apoptosis-inducing ligand linked to tumor necrosis factor (TNF-related apoptosis-inducing ligand – TRAIL) is an excellent anticancer drug because of its effectiveness and selectivity against cancer cells, while leaving healthy cells alone. The apoptosis-inducing ligand linked to tumor necrosis factor (TRAIL) is an excellent anticancer drug.

On the other hand, the off-targeted TRAIL cannot go beyond the preclinical stage due to its short half-life and rapid renal clearance. In xenograft breast cancer and orthotopic pancreatic models, a unique creation of a TRAIL-active trainer ferritin nanocage (TRAIL-ATNC) has a 16-fold longer serum half-life while sustaining anticancer efficacy in vivo. The nanoformulation might improve any treatment's PK/PD characteristics, allowing the medication to be reused. The loading ability of extremely hydrophobic PTX (pneumothorax) was recently increased by lipid tail modifications of cationic liposomes, which is advantageous for creating liposomal PTX delivery to decrease side effects and costs (Chaturvedi et al. 2019). The newly synthesized DLinTAP with two linoleoyl tails had a lower loading capacity than

lipid tails with one loyal (DOPC/DOTAP), suggesting that even slight adjustments to nanoformulation can drastically enhance drug delivery systems.

Cancer Diagnostics on the Nanoscale

"One ounce of prevention is worth one pound of cure," as the saying goes; in the case of cancer treatments, this can be worth a metric ton of cure. Developing cancer pharmaceutical products is an expensive undertaking. Developing an effective drug can cost billions of dollars, and most candidate drugs fail clinical trials. Hundreds of compounds in advanced stages of research are added to the oncology pipeline, yet the FDA (Food and Drug Administration) has only authorized 50 new small-molecule anticancer treatments between 2015 and 2020. As noted above, the occurrence of drug resistance requires the growth of new therapies, thereby increasing costs. By identifying cancer at an early stage, saving patient expenditures, and extending longevity, a particular diagnostic test, on the other hand, can be eternally gratifying and effective.

Since 90% of cancer deaths are due to metastases, early detection has an extraordinary impact on healing success and overall survival statistics. Even the average cost of cancer diagnosis therapy at an advanced stage vs the early stage is considerably higher. The benefits of early recognition and regular screening are many, especially since some cancers present symptoms only at a late stage (Hammond 2016). Screening methods may also be used to assess and optimize each patient's treatment. Although technology has advanced in many areas, there is still a need for routine screening methods that effectively detect cancer in its early stages without overdiagnosis. Nanomaterials can meet this demand by improving the sensitivity and accuracy of cancer biomarker detection because of their unique magnetic, optical, chemical, mechanical, and physical capabilities.

Role of Nanomedicine in Cancer Therapy

In cancer therapy gold nanoparticles are used because of their immense biocompatibility as well as drug loading capacity (Mukherjee et al. 2017). Gold nanoparticles are accessible for surface modifications, easy synthesis, stability, mobility, etc. (Xia et al. 2009; Niikura et al. 2013). In drug delivery nanoparticles should be small. Smaller nanoparticles can easily pass through the physiological barriers as well as large enough to carry a therapeutic bioactive compound to the site of action (De Jong et al. 2008; Song et al. 2013). Gold nanoparticles have the ability to slow down the transport of bioactive molecules by disturbing the cytoskeleton and cell surface structure. This creates impact in the Akt pathway (Pan et al. 2014). Gold nanoparticles provide therapeutic treatment via degradation, by enhancing solubility and extending bloodstream circulation time. It simultaneously performs target transporting and releasing therapeutic effects in controlled manner (Emeje et al. 2012). In animal model, study for the gold nanoparticles went without any mortality or

toxicity. In an animal model, the study for the gold nanoparticles went without any mortality or toxicity and this result was provided by investigating the animal model tissue morphology, behaviour of the animal, serum biochemistry and haematological and histopathological analysis (Lasagna-Reeves et al. 2010). The gold nanoparticles modify the intracellular signalling by suppressing the mitogen-activated protein kinase pathway, slowing down the metastasis through the interference with EMT (epithelial-mesenchymal transition). Through this proceeding heparin-binding growth factor plays a vital role. This process is significant for metastatic potential of tumor cell (Arvizo et al. 2013). In antiangiogenic cancer therapy, silica and silicate-based nanoparticles were used. Intracellular reactive oxygen species is produced by mesoporous silica nanoparticles. This activates the p53 tumor suppressor pathway (Setyawati and Leong, 2017). Mesoporous silica nanoparticles' (MSNs) properties are uniform pore size, large surface area, pore volume, and easy to produce large scale (Bouchoucha et al. 2016; Farjadian et al. 2019). Food and Drug Administration (FDA) this features provide excellent drug encapsulation such as high efficiency and good delivery without any complication in preparation of aqueous solution methods such as solgel "chimie douce" (Croissant et al. 2018). The Food and Drug Administration reported silica-based materials are safe and MSNs help to create nanomedicine platforms for drug delivery and cancer chemotherapy (Li et al. 2019). Copper nanoparticle inhibits the human umbilical vein endothelial cell (HUVEC) proliferation and cell migration. Copper nanoparticles can cause significant cell cycle arrest in a concentration-dependent manner (Song et al. 2014). Silver nanoparticles show the antiangiogenic properties. It inhibits the vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation as well as formation of capillary-like tube and cell migration.

Conclusion

There are numerous specific examples of how oncology clinical trials in cancer-infected animals have allowed researchers to obtain essential data that has helped them make clinical trial decisions for future cancer research. Proof-of-concept data from preclinical studies may be used to support further study. Dosing choices and explaining tumor-targeting effectiveness and medication safety are based on pharmacokinetics and data. Contacts with searchable databases of veterinary clinical trials and veterinary specialty groups active in research can provide access to such resources. It is hoped that as knowledge of these models expands, researchers will utilize naturally existing companion animal models of human disease to speed up biomedical research and clinical application of discoveries. Advancements in nanomedicine offer new opportunities to improve the anticancer arsenal. Nanoparticles, both target and nontarget, are presently in preclinical and clinical stages, demonstrating the impact of administration systems. More research in nanomedicine will broaden the remedial window of drugs while reducing side effects, which will improve patient outcomes.

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