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Contents

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Introduction to Cancer Stem Cells

1

Anisur Rahman Khuda-Bukhsh, Asmita Samadder, and Santu Kumar Saha

Abstract

Cancer is a persistent public health-care issue of modern life that poses a global challenge. It comprises several diseases that basically involve abnormal cell growth and have a potential to invade or metastasize to other distant organ systems, spreading the disease to other part(s) of the body. Development of resistance to conventional therapies and disease recurrence are some common phenomena encountered in almost all types of cancer. Understanding "hallmarks of cancer" and "tumor microenvironment" is therefore important for development of successful therapy for cancer. Numerous drugs have been designed and tested for their anticancer efficacy over decades to find out a complete cure for this lethal disease, but without desirable success so far. The concept and role of "stem cell" therapy in oncology research have drawn considerable interest in recent years. Thus, emphasis has been given on proper identification and characterization of the "cancer stem cells" and "other stem cells" for elucidation of the signaling cascades involved in the process of cancer limitation and progression (and resurgence). In the introductory part of this book, an attempt has been made to provide an overall idea on different aspects of cancer stem cells, optimization of rate and type of cell growth, and their associative cure strategy by adopting a well-defined scientific perspective.

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Keywords

Cancer · Stem cell types · Cancer stem cell · Stem cell therapy · Targeted therapy · Therapy resistance

Abbreviations

AML	Acute myeloid leukemia
CSC	Cancer stem cell (CSC)
DNA	Deoxyribonucleic acid
EMT	Epithelial to mesenchymal transition
EPC	Endothelial progenitor cell
FACS	Fluorescence-activated cell sorter
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
MET	Mesenchymal-epithelial transition
MSC	Mesenchymal stem cell
NSC	Neural stem cell
ROS	Reactive oxygen species
SSC	Somatic stem cell
TSG	Tumor suppressor gene
HSCTs	Hematopoietic stem cell transplants

1.1 Cancer and Cancer Stem Cells

Cancer is a global public health challenge, and according to the latest GLOBOCAN report in 2018, approximately 18.1 million new cases and 9.6 million deaths were recorded [1]. Epidemiological studies showed smoking, alcohol, irregular and unhygienic food habits, lifestyle, genetic polymorphism, susceptible alleles, oncogene regulation, chromatin remodeling, and environmental and genotoxic stress to be the major causes of developing cancer. The knowledge of cancer has now extended toward understanding of "tumor microenvironment," and over the years, genomic, epigenomic, transcriptomic, and proteomic databases of around 33 cancer types have also been established [2]. Overall findings of the pan-cancer atlas reflect the six "hallmarks of cancer" [3] and role of ~140 driver genes which are classified into 12 major cancer signaling pathways [4]. Hence, this new era of translational cancer research is focused on early diagnosis and targeted cancer treatment. For targeted therapy, the aim is to employ single molecule or pathway inhibitors with or without conventional treatment.

The conventional treatments for cancer are surgery, chemotherapy, radiotherapy, and hormone therapy. On the initial stage of chemoradiotherapy treatment, tumor shrinkage commonly takes place, but sooner or later tumor growth is reestablished at the original and/or in new sites [5]. Further, when cancer is diagnosed at its advanced stages, most of the conventional therapies fail, and most patients in due course of

treatment develop chemoradiotherapy resistance with the ultimatum toward death [6]. This alarming situation necessitates the immediate attention for understanding the "hidden mechanism" of disease recurrence for better treatment and management of therapy resistance.

Cancer is a heterogeneous disease phenotypically and genotypically controlled. The heterogeneous nature of this disease is evident even within a single patient. It is evident that the intra- and inter-tumor heterogeneity is due to mutational landscapes in the "driver genes." These driver genes are either tumor suppressor genes (TSGs) or oncogenes. The TSGs are functionally involved in transcription regulation, signal transduction, and angiogenesis. In cancer, the TSGs are inactivated due to genetic and epigenetic alterations. The genetic alterations of TSGs include (1) mutation and (2) deletion [7]. The epigenetic inactivating events are (1) methylation, (2) deregulated imprinting, (3) altered splicing, (4) histone modification, and (5) decreased mRNA stability through miRNA or other processes [3, 4, 7]. Therefore, it can be said that "loss-of-function" mutations in TSG contribute to cancer development. Retinoblastoma is a classic example which occurs due to loss of function of Rb-TSG gene.

An oncogene is capable of transforming normal cells into cancerous one, both for cells growing in cell culture in vitro or in animal models in vivo. Oncogenes are said to be derived from their normal cellular counterparts called proto-oncogenes. A classic example is the Ras gene (a proto-oncogene) that encodes for an intracellular signal transduction. The mutant form called the rasD gene (oncogene) is derived from the original Ras. In this way, the encoded mutant protein thus produced is responsible for uncontrolled cell growth [8]. Cellular transformation of a proto-oncogene into an oncogene occurs due to "gain-of-function" mutation by following any of the mechanisms, namely, (1) point mutation, (2) chromosomal translocation and (3) amplification [8].

According to the "Clonal Evolution Model" of cancer development, the driver gene mutations stimulate cell dedifferentiation and phenotypic regression with loss or gain of function, uncontrolled proliferation, and inability to activate cell death pathways. Whereas the "Alternative Model" of cancer development says, every tumor comprises a rare population of cells termed as cancer stem cells (CSCs) or cancer-initiating cells. The CSC hypothesis also says, within the tumor microenvironment, only a subpopulation of cells with self-renewing and tumorigenic properties are responsible for the generation of cancer cells and their hierarchical organization [9].

The CSCs were first identified in acute myeloid leukemia (AML) by Bonnet and Dick [10]. The population of AML-CSCs (~0.1–1% of the overall tumor population) identified with surface marker CD34 + CD38 was found to develop cancer in mice [10]. The CSCs are identical in nature with normal stem cells in respect of their common self-renewal and differentiation properties [10, 11]. The CSCs were also demonstrated to have the role in developing resistance to conventional cancer therapies and may play a role in developing metastasis [12]. Epigenetic reprogramming mechanism can lead to the metabolic and phenotypic changes to convert non-CSC population into CSC to develop therapy resistance [13]. For tumor invasion, the mechanism of epithelial to mesenchymal transition (EMT) can be a

major factor in which epithelial cells lose their original characteristics and gain mesenchymal properties [14]. The EMT has also been suggested to have the ability to induce intravasation, the process by which cancer cells enter the bloodstream for invading healthy tissue. The reverse program of EMT that is called mesenchymal to epithelial transition (MET) can promote new tumor formation [15, 16]. Therefore, understanding these molecular events associated with CSC is very important for targeted cancer therapy [17].

CSCs are a small proportion of cells within a tumor that is self-sufficient to trigger tumorigenesis. These cells have the ability of self-renewal and can produce different lines of cancer cells [18]. In support of the molecular events associated with CSC as mentioned above, the loss of E-cadherin with a concomitant rise of N-cadherin, expression of transcription factors like Snail and Twist and signal proteins VEGF and TGFβ, and overexpression of Sox2, Oct4, and Nanog are the induction factors to initiate EMT, believed to be a major driving force for metastasis [19]. The stemness pathways like Wnt/β-catenin, JAK-STAT, Notch, etc., are abnormally regulated contributing to resistance to apoptosis, progression, and propagation of cancer cells. In addition to maintaining the ends of chromosomes by expressing the hTERT gene, their microenvironment composed of blood vessels and stromal cells supports the multiplication of tumor cells [20, 21]. Further, the potentiality to produce free radical scavengers to scavenge the reactive oxygen species (ROS) and combat oxidative stress is of prime importance for the sustenance of cells by avoiding DNA damage.

Cell surface markers like CD34+ and intracellular markers like aldehyde dehydrogenase 1 have shown a light to detect their presence and distinguish them from normal stem cells [22, 23]. Methods such as DNA barcoding for tracing CSCs using FACS provide an attempt to separate CSCs from the heterogeneous population of cells. Detecting circulating CSCs to determine the recurrence in patients suffering from cancer, transplanting the isolated CSCs into the mouse model, and colony formation assay are other ways to characterize their nature [10]. Researchers have also found strategies to knock down the gene encoding TERT proteins that lead to cell cycle arrest and modified T cells called chimeric antigen receptors for detecting CSCs which direct another way of evoking our immune system to fight infections [24]. To end the deep-rooted cause of progression of cancers, nowadays, clinical trials are underway to target the stemness pathways for long-term outcomes.

In this short introductory section, we will endeavor only to focus briefly on an overall idea about CSCs and how these are different from the normal stem cells.

"Stem cell," as the name indicates, may be defined as the cell characterized by the unique ability of self-renewal for an indefinite period of time. These cells are endowed with the capability to form single cell-derived clonal cell population. These cells can also differentiate into several other cell types. The property of self-renewal in the stem cell pools plays pivotal roles in tissue regeneration and homeostasis [25, 26]. Stem cells can further be categorized as "embryonic stem cells" (ESCs) or "somatic stem cells" (SSCs). The SSCs, also called adult stem cells, are multipotent in nature and bear the potentiality to differentiate into any other cell type of particular lineage. These might include neural stem cells (NSCs), hematopoietic

stem cells (HSCs), mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and many others [27].

Embryonic stem cells (ESCs), just like that of pluripotent cells, can differentiate into many cell types and are thus immensely used as standards for detection of pluripotent cultured cells in vitro with some restriction of usage in scientific studies and clinical trials in human pertaining to ethical considerations [28]. ESCs are now being replaced by *induced pluripotent stem cells* (iPSCs). These iPSCs are reprogrammed adult somatic cells which have enforced expression of pluripotency factors. Embryo destruction is not required for iPSC establishment. iPSCs are like ESCs, except for the fact that they lack immunogenic or ethical limitations, and therefore, they bear the possibility for clinical application more than ESCs [29].

Neural stem cells (NSCs) are a type of stem cells which can self-renew and differentiate into neurons, astrocytes, oligodendrocytes, etc., and express Sox2, nestin, and other classic markers and have been deployed to treat brain, breast, prostate, and lung tumors [30–32].

Mesenchymal stem cells (MSCs), known to be derived from bone marrow, are able to differentiate into mesodermal cells, including bone, cartilage, muscle, stroma, adipose tissue, connective tissue, and tendon. MSCs can be isolated easily, and they are known to propagate in vitro and have huge application in cancer therapy.

Hematopoietic stem cells (HSCs) belong to the most primitive of all the blood lineage cells. They are predominantly found in bone marrow and generally known to produce mature blood cells by proliferation and differentiation of lineage-restricted progenitor cells. HSC transplantation has clinical implication over the last four decades.

Endothelial progenitor cells (EPCs) are primarily concerned with vascular regeneration and thereby have potentiality in cancer therapy by coupling with antitumor drugs or performing transfection or acting with angiogenesis inhibitors [33].

Parthenogenetic stem cells, pluripotent stem cells (PSCs), have now been derived parthenogenetically from activated human oocytes. These cells represent similar characteristics as displayed in the human embryonic stem cells (hESCs) which include the infinite division and in vitro and in vivo modes of differentiation into germ cell lineages [34]. The human parthenogenetic ESCs (pESCs) consisting of homozygous human leukocyte antigen (HLA) are known to strongly increase the degree of matching and significantly increase the histocompatibility among cohorts of cells in human population [35]. The main strategy lies therein is to activate the oocyte artificially without the ample extrusion of second polar body. Further, the events of early recombination in oocyte also results in heterozygous pESC formation.

Now, the question is: how and what properties distinguish the normal stem cells from CSCs? The cellular niche or the surrounding cellular environment helps to maintain the "stemness" property. When a normal stem cell divides to give rise to two daughter cells, a balance is maintained. Among the two daughter cells, one acquires the "self-renewal" property and remains as the stem cell, whereas the other one goes for expansion and differentiation to develop into mature cell. In both cases,

the cells prevent to acquire "tumorigenic" property by sustaining a fine balance of "proliferation inhibition" and "proliferation promotion" [36]. The imbalance may be caused due to mutational "hit" that makes a normal cell to acquire the CSC phenotype. Mutation is a random process, and the frequency to generate a normal stem cell into CSC phenotype varies from cell to cell and organ to organ. It can be said that the greater the number of stem cells, the higher the chances of developing CSC phenotype as well as cancer [37]. Further, as said earlier, the cellular niche or the surrounding cellular environment is also associated with developing the CSC phenotype. Cancer is not just a mass of malignant tumor cells but a complex mix of several components which contribute to its development. This includes the immune cells, cancer-associated fibroblasts, endothelial cells, and blood vessels. These nonmalignant components can comprise up to >50% of the primary or metastatic tumor mass which play a major role as "microenvironment" and in acquiring the CSC phenotype [3]. Some important features of CSCs are their expressivity of the stemness genes, their self-renewal property, and their ability to differentiate and proliferate into other non-stem cancer cells and resist traditional mode of cancer treatment. Non-CSCs in the tumor have been reported to proliferate at a faster rate than that of CSCs but have little tumor-initiating potential [38].

1.2 Identification and Characterization of Cancer Stem Cells

CSCs are cancer cell subpopulation having stem-like properties that can be identified by cell surface markers. The CSCs can be isolated following standard practice from tissues of a patient and cell lines derived from different cancer types. Some of the key features of CSCs for identification, isolation, and characterization can be summarized as follows:

(a) CSC sorting based on biomarkers: CSC subpopulations can be distinctly sorted out from other cancer cells based on their surface markers. Flow cytometric sorting of CSCs is done from the total cancer cell population of a patient's primary tissue as well from cancer cell lines by specific markers, e.g., CD44+, CD133+, Cd117, ALDH1+, Pakt+, Oct4, Sox2, Nanog, ABCG2, ABCC1, Mrp1, Nrf2, BMI 1, etc. The sorted cell populations can be grown in ultralow attachment plates with Matrigel embedded conditions (3D culture condition) for sphere forming assay [39]. In breast cancer of non-responding cases, after neoadjuvant chemotherapy, prevalence of CSCs having CD44+ and CD24−/low has been reported [40]; further, these cells showed CSC renewal and mesenchymal features [41].
(b) Tumor growth study in mice: The flow cytometry-based sorted cell populations can be transplanted in immunodeficient mice (tumor xenograft). The CSCs have the tumorigenic potential and develop tumor when transplanted into immunodeficient mice. The CSCs when they form tumors contain both the tumorigenic and non-tumorigenic cells [38]. In head and neck squamous cell carcinoma (HNSCC), CD44 molecule was first identified as the surface marker of CSC, and

it was also found in only <10% CD44-positive cells with tumorigenic potential but not in the CD44-negative cells [42].

1.3 Cancer Stem Cell Signaling Pathways

Like normal stem cells, CSC follows three major self-renewal pathways, namely, *Hedgehog*, *Wnt*, and *Notch*. Key regulatory genes of these signaling pathways are associated with cancer, and targeting these pathways can be one of the important strategies for cancer therapy [43]. Key regulatory genes of these signaling pathways can be summarized as follows. The *Hedgehog pathway genes* are HHIP, PTCH1, Smo, SuFu, and Gli-1. The *Wnt pathway genes* are DKK1, Wnt, β-catenin, Axin-2, GSK-3β, and APC. The *Notch pathway genes* are Jag1/2, Hey 1, Hes 1, Tace, and presenilin. Increased expression of Gli-1 was observed in HNSCC tumors after developing resistance due to long-term treatment of epidermal growth factor receptor (EGFR) inhibitor [44]. In HNSCC cell line after chemotherapeutic treatment (bortezomib and etoposide), increased frequency of CSC population and overexpression of Wnt signaling proteins DKK1 and AXIN2 were found [45]. Similarly, overexpression of SMO has been recorded in a HNSCC cell line after treatment with cyclopamine [46]. The accumulating data indicates that there is preferential selection of CD44+ CSC populations after treatment with neoadjuvant chemotherapy in HNSCC along with alterations of these self-renewal pathways, particularly Hedgehog and Wnt. Development of chemoresistance in HNSCC might be due to alterations in these CSC pathways. It also seems likely that the prevalence of CD44+ CSCs may be the indicator or biomarker of chemoresistance after neoadjuvant chemotherapy. Increased expression of CD44 might be due to overexpression of Gli/β-catenin, the effector protein of Hedgehog/Wnt pathways [47, 48]. High expression of a Notch signaling ligand DLL4 was reported from HNSCC patients undergoing radio-chemotherapy [49]. Agrawal and his research group have identified mutation in Notch1 mutation in HNSCC patients. Their study further revealed in HNSCC types that Notch1 acted as tumor suppressor rather than oncogene [50].

1.4 Role of Stem Cell and CSC in Developing Disease and Therapy Resistance: Therapeutic Implications and Future Directions

Stem cell therapy is generally based on transplantation of living cells into an organism either to repair a tissue/organ or to restore their optimal functioning which might have been lost completely. Human embryonic stem cells (hESCs) are in use for several cell therapy procedures which accounts for 13% of cases reported so far. However, on the contrary, fetal stem cells (fESC) are used only in 2% of cases. Further, record of usage of umbilical cord stem cells is only 10%, and adult stem cells are in use for treating 75% of cases [51]. Cardiovascular and ischemic

diseases, diabetes, diseases related to liver and hematopoietic organs (more than 25,000 cases of Hematopoietic stem cell transplants (HSCTs)/year and counting), orthopedics, etc., are a few types among myriads of other diseases which are being treated with stem cell transplantation globally [52, 53].

The most common stem cells that are modified by multiple mechanisms for potential use in cancer therapies are NSCs and MSCs which may vary from including the therapeutic enzyme/prodrug system or using a nanoparticle or introducing an oncolytic virus delivery on tumor site. Enzyme/prodrug therapy/suicide gene therapy is one of the promising applications of stem cell against cancer. The NSCs and MSCs can express enzymes which can convert nontoxic prodrugs into cytotoxic products by bioengineering. These modified stem cells, when transplanted into tumor-bearing models, quickly localize to tumor tissues where the exogenous enzyme aided prodrug conversion to cytotoxic molecules ultimately damages the tumor cells [54]. Further, stem cells can overcome the limitations of common cancer therapy and function as in situ drug factories by secreting antitumor agents [55] and through delivery of virus by MSCs toward bio-targets by combining the oncolytic activity with that of the immunoprivileged and tumor-tropic properties of the MSCs [56].

The use of nanoparticles as potent drug delivery systems is now the current trend of treatment of different diseases like diabetes [57–60], cancer [61–65], cardiomyopathy [66, 67], anti-genotoxic [68] and anti-inflammatory [69], based on their bioactive targeted delivery, increased penetration, reduction in drug-dose ratio, sustainable release, faster action, and protection against degradation due to harsh biological environment at administration. However, the efficacy of stem cells as nanoparticle delivery agents has now been a futuristic approach owing to the reduction in unrestricted uptake of different nanoparticles by them, increase in intra-tumor drug distribution, and protecting the drugs from host immunologic reactions [70].

Traditional therapies of cancer cannot eliminate CSCs while they can kill non-stem cancer cells. Chances of relapse of tumors remain usually high when the CSCs which had not been killed during therapeutic processes proliferate and differentiate. Thus, strategies for targeting CSCs may solve several clinical issues of drug resistance and recurrence [71, 72]. Evidences from several studies indicate that the CSCs can develop and maintain different categories of human malignancy which imply great opportunities for assessment of oncologic therapeutic strategies to impart a better life to cancer patients. There exists a minute analysis and comparison between CSC and cells derived from normal tissue. The CSC-targeted therapeutic arsenal often comes across several potential hurdles, like normal stem cell cytotoxicity and acquisition of resistance against the treatment, which need to be addressed to maximize the chances of success [73].

The CSCs have different mechanisms of defense against chemotherapy and radiation. Here, we have highlighted two major mechanisms. CSCs produce antioxidant enzymes to protect against radiation-induced damages. One of the routine treatments of cancer is radiotherapy that produces free radical as a natural byproduct of oxygen metabolism. This oxidative damage causes the damage to DNA to kill

cancer cells. In some studies, increase of resistance to radiotherapy has been accompanied with enhanced DNA repair, less damage to DNA, reduced apoptosis, and increase of angiogenesis [74].

The other mechanism of CSC is through detoxification enzymes which play a role in resistance to chemotherapy. Drug detoxification is done in three stages; in the first stage, detoxification is done through cyto p450, which removes OH^- and free radical $O_2^{\cdot -}$ species. In the second stage, toxins are conjugated using glutathione, glucuronic acid, or sulfate catalyzed by glutathione S-transferase, uridine disulfate, glucuronosyltransferase, and sulfatase. Finally, drug and toxin are also pumped out of the cell through intermembrane channels [74].

Identification of similarities and dissimilarities between normal stem cells, CSCs, non-tumorigenic cells, and normal differentiated cells based on differences in their immunophenotype shall allow the development of CSC-targeted therapeutic strategies which shall definitely impart a relatively low risk toward normal cellular/tissue level cytotoxicity. Evaluating the efficacy of such targeted molecule treatments shall require the advent of modern approaches to determine the CSC frequency and their degree of viability within tumor mass. However, resistance due to clonal selection and tumor microenvironment such as hypoxia might pay hindrance toward the development of the cure and needs utmost care and precautions.

In view of the tremendous importance of CSCs in the management and control of cancer, subsequent chapters of this book have been assigned to deal elaborately and critically with several important aspects, such as types of CSCs, how CSCs can contribute to the development of different types of cancer, isolation and characterization of CSCs, role of other tumor microenvironmental factors in association with CSCs in cancer development, controversies of acceptance for the CSC hypothesis, new strategies or alternative therapies for targeting CSCs for cancer treatment, and some other emerging issues.

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Types of Cancer Stem Cells

2

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Abstract

The discovery of cancer stem cells (CSCs) has revolutionized the field of cancer biology due to the intrinsic role of CSCs in the initiation, progression or relapse of cancers. The identification of different types of CSCs has given a great opportunity to researchers and clinicians, to understand the basic biology of various types

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of CSCs or cancers, and has also added an extra dimension in the development of innovative approaches or therapeutics to specifically target CSCs. The morbidity and mortality caused by various cancers have started to decline rapidly in the past couple of decades. Furthermore, the life expectancy of cancer patients have increased with the invention of modern state-of-the-art technologies, besides rapid advances in the development and preclinical testing of new drugs that target CSCs. Additionally, more insights into the molecular biology of CSCs was made possible, when unique cell markers, which are specific to a particular type of tumor was deciphered. Importantly, the characterization and evaluation of key signalling pathways in CSCs are critical, as emerging evidence indicate that CSCs play a key role in dissemination during cancer metastasis or relapse. In this chapter, we discuss about CSCs that are specific to ovarian, thyroid, melanoma and pancreatic cancers. We also discuss about the key CSC signalling pathways, as understanding them will advance the therapeutic strategies, or evaluation of efficacy of novel CSC-targeting drugs that could be used in the treatment of cancer patients.

Keywords

Cancer stem cells (CSCs) · Cell surface markers · Drug targets · Ovarian cancer stem cells (OCSCs) · Thyroid cancer stem cells (TCSCs) · Melanoma cancer stem cells (MCSCs) · Pancreatic cancer stem cells (PCSCs) · CSC signalling pathway · Hedgehog (Hh) · Notch · Wingless-type (Wnt)/β-catenin · Transforming growth factor-β (TGF-β) · Nuclear factor-κB (NF-κB) · Insulin-like growth factor 1 (IGF1) · Cluster of differentiation (CD)

2.1 Introduction

Cancer stem cells (CSCs) or tumor-initiating cells (TICs) were brought to the limelight after the identification and characterization of a subpopulation of cells, which possess the properties of stem cells in several types of malignant tumors. The comprehensive studies on CSCs gained momentum after the discovery of stem-like cells that can form heterogenous population of cells in the development and progression of cancer [1]. The cancer stem-like cells were initially identified in acute myeloid leukemia (AML), which attracted the attention of many researchers across the world, and later, it was discovered that CSCs/TICs are responsible for the relapse and metastatic potential of various cancers due to their self-renewing capacity [2].

Available reports suggest that CSCs are involved in the initiation and progression of carcinomas; however, the provenance of CSC is equivocal [1]. Since CSCs are responsible for the most lethal events that lead to more cancer mortality such as metastasis and relapse, it is imperative to find the origin of CSCs [3]. There are various theories that have been postulated by scientists during different time points on the origin of CSCs. The origin and the biology of CSCs require better understanding to develop efficient therapeutics, and therefore, several theories including

cell fusion, horizontal gene transfers, genetic instability, and the influences of cellular microenvironment have been hypothesized [4].

Recent studies on CSCs suggest that oncogenic hit or mutation in the stem cells along with the dysfunction of proto-oncogenes, apoptotic factors, and various genes involved in DNA repair during mitosis may result in the formation of CSCs/TICs [5]. The mutation depends on both extrinsic and intrinsic factors including smoking, alcohol and substance abuse, unhealthy lifestyle, and dysfunctional DNA repair machinery due to the epigenetic alterations in the oncogenes and/or tumor suppressors. Current developments in molecular biology have led to the discovery of unique cell surface markers, which can be used in the identification and characterization of different types of tumors [6]. CSCs possess the characteristics of sustained self-renewal and the ability to differentiate into heterogenous population of cells, which promote initiation, progression, and dissemination of carcinomas. Importantly, the isolation of CSCs using cell surface markers has opened the opportunity to categorize the subtypes of different tumors [7]. In this chapter, we discuss the strategy to identify and isolate the CSCs, their cell surface markers, and key CSC signalling pathways in ovarian, thyroid, melanoma and pancreatic cancers.

Among the several cancers, ovarian cancer contributes to most cancer deaths in women, and the 5-year survival rate of patients with locally advanced and metastatic stages is 26% [8]. Though, both extrinsic and intrinsic factors are involved in the initiation of ovarian carcinogenesis, the loss of heterozygosity (LOH) in the breast cancer type 1 or 2 (*BRCA1* or *BRCA2*) gene, and the dysregulation of different intrinsic signalling mechanisms, namely, Wingless type (Wnt)/β-catenin, Hedgehog, Notch, and transforming growth factor-β (TGF-β) pathways, are mostly responsible for the progression of ovarian cancers [9]. The early identification of ovarian CSCs (OCSCs) using a combination of various stem cell markers, namely, cluster of differentiation 133 (CD133), CD117, CD44, CD24 and aldehyde dehydrogenase 1 (ALDH1) or ALDH1 family member A1 (ALDH1A1) could help in the diagnosis or prognosis of cancer, or better treatment outcomes for patients with metastatic ovarian cancers [10, 11].

Thyroid cancer is the most prevalent endocrine cancer that is responsible for high morbidity and mortality among other endocrine-related cancer cases combined. Amid various causative factors, a subpopulation of thyroid CSCs play a pivotal role in the initiation and progression of thyroid malignancy due to the unlimited replication potential of these CSCs [12]. The thyroid CSCs (TCSCs) seize the control of multiple distinctive signalling cascades for their survival and development. In thyroid carcinoma, the thyroid CSCs are used as morphological markers, to define the tissue characteristics of both well-differentiated thyroid tumors like follicular and papillary cancers as well as more aggressive undifferentiated tumors, including anaplastic thyroid tumors. Several biomarkers such as CD13, CD133, epithelial cell adhesion molecule (EpCAM), ALDH, and stage-specific embryonic antigen 1 (SSEA-1) are identified and used as diagnostic markers as they account for poor prognosis in metastatic and chemoresistant thyroid tumors. Aberrant activation of the key signalling such as insulin-like growth factor 1 (IGF1), Hedgehog, Notch, signal transducer and activator of transcription 3 (STAT3) and

Wnt/β-catenin pathways, plays a critical role in the maintenance of thyroid CSCs that promote the initiation and progression of different subtypes of thyroid cancers.

The 5-year survival rate for melanoma is 98% during its localized stages; however, the percentage substantially drops to 64% for regional and 23% for distant metastatic melanoma lesions [13]. Dysregulation of various signalling pathways such as Notch, Hedgehog and bone morphogenetic proteins (BMPs) are involved in the carcinogenesis. These signalling pathways regulate multiple stem cell markers that are used in the identification of melanoma CSCs (MCSCs) such as CD133, and ATP binding cassette (ABC) transporter family members like multidrug resistance 1 (MDR1), ABC subfamily G member 2 (ABCG2) and ABC subfamily B member 5 (ABCB5). CD133 marker is highly expressed during the metastatic dissemination of cancer, whereas ABCG2 and ABCB5 serve as markers of tumor progression [14].

Pancreatic cancer is the fourth common cause of death amongst all the carcinomas. It is an aggressive cancer that is mostly asymptomatic in nature, until the cancer has metastasized to distant sites. The patient survival rate for pancreatic carcinoma is as low as 80%, because most often the cancer is diagnosed only at a very advanced stage in pancreas or metastatic stage in distant organs [15]. Although extensive research on different carcinomas has led to new therapeutics, some improvement in patient survival or treatment outcome, the average survival time of patients with metastatic pancreatic cancer is merely 2–3 months. Recently, the identification of CSC markers such as CD44, CD24 and epithelial-specific antigen (ESA) in pancreatic tumors have led to the identification of pancreatic CSCs (PCSCs) and have increased the chances of early diagnosis. The $CD44^+$, $CD24^+$ and ESA^+ cells have multifold tumorigenic potential in pancreatic cancers as they favour the survival of CSCs and promote the progression of tumors [16]. There are multiple signalling pathways, including Wnt/β-catenin, HH, Notch and phosphoinositide 3-kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (AKT)/mammalian target of rapamycin (mTOR), which are hyperactivated in pancreatic cancers, and they enable the CSCs to self-renew and differentiate into different types of tumour cells [17]. In the subsequent sections, we discuss more about these CSCs, their markers in different carcinomas and the signalling mechanisms that drive the tumor progression and metastasis.

2.2 Ovarian Cancer Stem Cells (OCSCs)

2.2.1 Origins and Markers of OCSCs

Ovarian cancer is one of the deadliest cancers amongst gynecological malignancies, and accounts for most cancer deaths in women globally, because of its high rate of relapse after treatments. It is also one of the widely studied cancers. Ovarian cancer study has lead to the discovery that CSCs, which are a small subpopulation of cells in tumour were responsible for the metastasis, drug resistance, cancer relapse and high mortality rates [18]. CSCs are found in most of the primary ovarian tumors and

ovarian cancer cell lines. The study by Stewart et al. [19] reported that freshly isolated cells from the ovarian cancer ascites express high levels of CSC markers, which represent the CSC population in the malignant ovarian ascites.

There are a diverse number of CSC markers that are distinct to ovarian cancers, which are clinically used to predict the nature and the invasiveness of the tumours. The OCSC markers such as CD24, CD44, CD117, CD133 and ALDH1/ALDH1A1 are used to define the identity and classification of the tumor subtypes, which help in the prognosis, and to determine the clinical outcomes of cancer patients, when using more precise treatment strategies to target the CSCs and tumor [20]. $CD44^+$ and $CD117^+$ cells are isolated from the human ovarian adenocarcinomas, using the cell surface markers CD44 (hyaluronic acid receptor) and stem cell factor receptor (SCFR) also known as CD117 or c-KIT. These subpopulations of cells, i.e., the OCSCs that were isolated from the ovarian adenocarcinomas, upon injection into mice were able to form full blown tumors, due to their tumor-initiating capacity [21].

$CD44^+$ and myeloid differentiation primary response 88 $(MYD88)^+$ cells isolated from the ovarian tumor ascites or the tumor itself, and $EpCAM^+$ and $CD24^+$ cells from the ovarian cancer cell lines manifest the molecular characteristics of CSCs/TICs. Interestingly, $CD24^+$ cells isolated from the tumor samples of the malignant ovarian cancer patients showed enhanced properties of CSCs, i.e, a higher metastatic and cancer relapse potential due to their self-renewing capacity, which doubles the rate of progression of tumours [22]. Furthermore, these cancer patients showed increased resistance to conventional chemotherapy, which resulted in inefficient drug treatments in these malignancies. Importantly, $CD133^+$ and $ALDH1^+$ cells are associated with ovarian carcinogenesis, particularly in the development of tumor during the initial stages, and these markers correlate with poor prognosis of cancers in patients and their overall disease-free survival rates [23].

2.2.2 Identification, Isolation and Characterisation of OCSCs

The OCSCs are extremely plastic in nature due to their distinct gene expression pattern and molecular phenotype. The OCSCs possess the characteristics of normal stem cells, but do not follow established pathways or signalling mechanisms [24]. Hence, CSCs can self-renew which promotes carcinogenesis. Additionally, CSCs can also undergo differentiaton to give rise to different cell types in the tumour, resulting in tumour heterogeneity and increased resistance to therapies. Besides, OCSCs have the capacity to survive and resist hypoxia, and proliferate under nutrient starvation (e.g., without glucose) [25]. Researchers have started to better understand about the role of OCSCs in tumor initiation and progression, after their isolation from the ovarian tumours or ascites using multiparametric flow cytometry [26]. The ascitic fluid of patients with ovarian cancer served as a source for the isolation of OCSCs [27]. The mixture of OCSCs and primary ovarian cancer cells in the stem cell medium was separated on a ficoll density gradient by centrifugation [28]. Then, the OCSCs fraction was purified by fluorescence-activated cell sorting (FACS) using the cell surface markers CD117, CD133, EpCAM, ROR1,

ALDH, SOX2, octamer-binding transcription factor 4 (Oct4), Nanog, MYC, ABCB1 and ABCG2 [29–31]. Additionally, Lin28 and Oct4 were identified as molecular targets, and then used for the isolation of OCSCs [32]. Dye exclusion assays, cell surface antigen identification, tumour sphere assay and clonogenic assays were used for in vitro characterisations of OCSCs [33].

An in-depth knowledge about CSCs surface markers and their unique ability to express in accordance with the tumor types, have opened up the possibilities of targeting specific-tumours with higher precision. The expertise gained from immuno-phenotyping of normal stem cells in tissues or organs has immensely helped in standardizing the optimal protocols in the isolation and identification of CSCs [34]. The highly plastic and multi-potency of OCSCs provide them the ability for chemoresistance against different mono or combination therapies. OCSCs can evade cellular apoptosis mechanisms, and enter the phase of active proliferation, by activating the pathways responsible for maintaining adult stem cell homeostasis [35].

Under normal physiological conditions in cells, a fine balance is maintained in the expression of oncogenes versus tumour suppressors or signalling by pro-apoptotic versus anti-apoptotic pathways. But, in ovarian CSCs/TICs, the fine balance between pro-apoptotic versus anti-apoptotic signalling is altered, thereby prolonging the survival of tumour cells or leading to relapse or recurrence of cancers. Notably, OCSCs possess an innate ability to undergo dormancy. The quiescent state increases OCSC's chance of survival and helps to maintain their altered genomes, which establishes their metastatic niche to support ovarian tumour growth. Conversely, OCSCs can reverse their self-induced quiescence or dormancy, which accounts for their metastatic secondary tumours or tumour recurrences and increased mortality rates of ovarian cancer patients [36]. Moreover, OCSCs unlimited differentiation potential and aggressive invasiveness sustain the tumour growth in an extremely stressful alien-metastatic-environment, to establish themselves and their secondary tumours by actively proliferating and also releasing chemokines and growth factors, an indicator of their own better survival [37].

2.2.3 Signalling, Self-Renewal, Metastasis and Differentiation in Ovarian Cancer

Various signalling pathways such as Wnt/β-catenin, HH, Notch, and TGF-β are involved in regulating the self-renewability and maintenance of CSCs/TICs. The inhibition of specific targets in these pathways using specific molecules/inhibitors was shown to be of potential therapeutic value for recurrent malignancies. Dysregulated Notch signalling is highly correlated with poor prognosis of ovarian cancer patients, and in most cases, the Notch signalling molecules are highly expressed in OCSCs. The overexpression of critical Notch signalling molecules, can initiate dysregulation of the pathway and lead to ovarian tumourigenesis. Multiple Notch signalling target genes such as peroxisome proliferator activated receptor gamma (*PPARG*), cyclin D1 (*CCND1*), and runt-

related transcription factor 1 (*RUNX1*) undergo epigenetic modifications, including DNA methylation in most of the high-grade ovarian serous adenocarcinomas [38]. Unlike the knowledge available on signalling pathways in CSCs from other organs/tisues, the core OCSC's signalling is still unclear [39]. Therefore, in this section we have discussed primarily on signalling pathways in ovarian cancer.

Aberrant activation of the Hedgehog signalling pathway is involved in the development of several cancers, including different grades of ovarian cancers. Hedgehog signalling is highly implicated in the regulation and growth of spheroid forming cells/OCSCs, and in the progression of ovarian tumours [40]. Notably, the inhibition of Hedgehog signalling resulted in the suppression of spheroid-forming capacity in ovarian cancer cells. Interestingly, the molecules of HH signalling pathway were shown to cross-talk with Wnt signalling pathway, and upregulate the key WNT molecules such as WNT2B and WNT5A. The target genes of Hedgehog signalling pathway such as leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), CD44, CD133, and other Wnt genes were reported to facilitate the process of tumor growth and progression [41].

The inflammatory cytokine pathway mediated by Nuclear Factor - κB (NF-κB) signalling, is essential for the survival of OCSCs. The inhibition of NF-κB signalling pathway through the tumour necrosis factor-alpha (TNF-α) mediated blockage resulted in apoptotic death of $CD44^+$ ovarian cancer cells. The activation of TGF-β signalling in OCSCs was shown to induce epithelial to mesenchymal transition (EMT) and promotes metastasis [42].

2.2.3.1 Wnt/β-Catenin Signalling Pathway in Ovarian Cancer

Wnt/β-catenin signalling is one of the evolutionarily conserved signalling pathways that regulates the process of embryogenesis, cell proliferation and maintenance of adult stem cell homeostasis [43]. It has wide range of functions, and it is also important for CSCs renewability, as it has to maintain the mesenchymal phenotype in order to differentiate into various other subset of cells [44]. The Wnt/β-catenin canonical pathway has multiple key signalling molecules, and the pathway is activated by binding of Wnt ligand to its receptor for the initiation of the downstream signalling mechanism. The destruction complex in the cytoplasm is comprised of different proteins such as AXIN, adenomatous polyposis coli (APC) and glycogen synthase kinase-3β (GSK-3β), which phosphorylates β-catenin, followed by ubiquitin-mediated proteosomal degradation. During the activation of the pathway, the unphosphorylated β-catenin accumulates in cytoplasm, which then enters the nucleus and binds to the T-cell specific transcription factor/lymphoid enhancer binding factor (TCF/LEF) family of transcription factors to induce its target genes expression [45]. Wnt/β-catenin pathway regulates cancer stemness, invasion and growth, and plays a key role in various cancers [46, 47] including ovarian cancer, as it regulates OCSCs by enhancing their self-renewal capacity and plasticity to differentiate into heterogenous tumor cell types [48]. The Wnt/β-catenin signalling pathway involves several proteins that regulate key cellular events, and the expression of most of these proteins are altered during carcinogenesis [49].

The Wnt/β-catenin signalling is activated when the WNT ligand binds to the Frizzled receptor (FZD) and low-density lipoprotein receptor-related protein 5/6 (LRP5)/LRP6. In most of the malignant ovarian cancer cases, the FZD is overexpressed than in the normal ovary, and the survival rate of cancer patients with FZD-positive tumors was worse [50]. Furthermore, Wnt/β-catenin pathway can be inhibited by various antagonists such as Wnt inhibitory factor 1 (WIF1), Dickkopf family (DKK) proteins and secreted frizzled-related protein (SFRP). Importantly, SFRP5 directly binds to the WNT ligand or FZD receptor and inhibits its action, but the low expression of SFRP5 is associated with chronic aberrant activation of Wnt/β-catenin pathway and progression of aggressive ovarian cancers [51].

The cytoplasmic and nuclear accumulation of β-catenin is considered as the hallmark of Wnt pathway activation, and higher levels of β-catenin are often observed in multiple cancers. Likewise, mutations in the β-catenin (*CTNNB1*) gene is frequently observed in endometrioid ovarian cancers, and overexpression of β-catenin in the nucleus showed significant positive correlation with high-grade serous ovarian cancers [52]. APC, AXIN1 and AXIN2 are the key cytoplasmic proteins that encompass the destruction complex and regulate the Wnt pathway upon phosphorylation. Importantly, mutations in *APC*, *AXIN1* and *AXIN2* genes are observed in multiple cases of ovarian endometrioid adenocarcinoma [53].

2.2.3.2 Notch Signalling Pathway in Ovarian Cancer

Notch signalling was initially discovered in *Drosophila*, and later, it was identified to have roles during embryogenesis and neural development in many species, including mammals [54]. Notch pathway has important function in mammalian cells ranging from cellular proliferation to apoptosis. It is responsible for cell fate determination and differentiation, and is also shown to influence cell division. Notch is a cell-surface transmembrane receptor that transduces its downstream signalling by binding to the ligands such as Delta-like (DLL1, DLL3, DLL4) and Jagged (JAG1, JAG2) on neighboring cells. There are basically four types of Notch receptors that are expressed in humans: NOTCH1, NOTCH2, NOTCH3, and NOTCH4 [55]. The binding of a ligand to its Notch receptor causes a conformational change in the receptor, which induces proteolytic cleavage of the receptor by TNF-α-converting enzyme (TACE) and γ-secretase, followed by release of the Notch intracellular domain (NICD) that leads to the downstream signalling and gene regulation. The Notch signalling regulates several genes of the Hairy/Enhancer of Split (HES) family, and the HES protein is related to YRPW motif-like protein (HEY) family of basic helix-loop-helix (bHLH) transcription factors, such as cyclin D1 and c-MYC that regulate the differentiation and survival of cells.

Recent insights into dysregulation of Notch signalling are highly correlated with many cancers, and genomic alterations in Notch pathway is prevalently seen in ovarian carcinomas [56]. Both *in vitro* and *in vivo* studies on inhibiting this pathway using small molecules and other blockers like γ-secretase inhibitors (GSIs) have demonstrated significant antitumor effects. Notch pathway plays a wide-range of

role in ovarian cancer progression. The dysregulation of this pathway is linked to poor patient survival, and it promotes metastasis and angiogenesis. Notch pathway influences, C-X-C motif chemokine receptor 4 (CXCR4) also called as stromal cell-derived factor-1α receptor (SDF1α), which mediates its signalling to enhance the proliferation and migration of ovarian cancer cells [57]. NOTCH1 and HES1 proteins are overexpressed in most of the ovarian tumours studied. While the overexpression of JAG2, DLL1, Manic Fringe (MFNG) and transducin-like enhancer of split-1 (TSL1) were frequently observed in ovarian adenocarcinomas, the Deltex, Mastermind (MAM), and Radical Fringe (RFNG) were often overexpressed in adenomas [58, 59]. γ-Secretase inhibitors (GSIs) are used to inhibit the Notch signalling pathway, and is therefore, tested in many clinical trials of different cancers, including ovarian cancers (Table 2.1).

2.2.3.3 Hedgehog Signalling Pathway in Ovarian Cancer

Hedgehog signalling pathway contributes to ovarian tumorigenesis along with the Wnt signalling pathway [60]. Aberrant activation of Hedgehog signalling is observed in most of the ovarian carcinomas. In the serous ovarian tumor samples, the Wnt target gene, AXIN2, was significantly expressed along with the overexpression of Hedgehog receptor patched homolog 1/2 (PTCH1/2), ligands such as Indian Hedgehog (IHH), Sonic (SHH) and several transcription factors. These over expression of the various signalling and target components of HH pathway are responsible for the hyperactivation of Hedgehog signalling, which also enhances the apparent cross-talk with the Wnt pathway [60]. Glioma-associated oncogene 1 (GLI1), a transcription factor and patched receptor (PTCH) are the

Table 2.1 Clinical trials using different signalling pathway inhibitors of ovarian cancer

Signalling pathways/inhibitors used	Mode of action	Phase of clinical trial
1. Sonic hedgehog		
Cyclopamine	SMO inhibitor	Phase I
Sonidegib	Inhibits dissemination of metastatic cells	FDA approved
Vismodegib (GDC-0449)	Inhibits the function of SMO	Phase II
2. Notch		
LY900009	Inhibits tumor progression	Phase I
Cediranib maleate	Prevents angiogenesis	Phase I
3. Wnt		
Ipafricept (OMP-54F28)	Acts as a decoy receptor of Wnt ligands	Phase I
4. EpCAM		
Catumaxomab	Decreases tumor development and invasion	Phase III

major molecular components of this crucial pathway. Liao et al. [61] reported that overexpression of GLI1 and PTCH1/PTCH2 proteins in ovarian cancers correlated with poor patient survival. Smoothened (SMO) is an important receptor that regulates Hedgehog signalling. Notably, the treatment of ovarian cancer cells *in vitro* with SMO inhibitor, cyclopamine, resulted in the suppression of cell growth and invasion, but lead to accelerated apoptosis. The altered regulation of Hedgehog signalling contributed to rapid invasiveness and metastasis. Furthermore, Hedgehog signalling along with Notch signalling facilitated tumor growth via neoangiogenesis and epithelial to mesenchymal transition (EMT), and increased the potential for tumor relapse. Studies on Hedgehog signalling have unraveled the basic understanding of this pathway and its role in cancer progression, leading to the identification of specific therapeutic targets, and the development of several inhibitors targeting the pathway for ovarian cancer treatments [62].

2.2.3.4 Transforming Growth Factor (TGF)-β Signalling Pathway in Ovarian Cancer

Hypersignalling of TGF-β plays an essential role in the dissemination of ovarian cancers. TGF-β signalling promotes tumor progression via immune evasion, neoangiogenesis and enhanced EMT [63]. TGF-β superfamily has diverse members, and they play an essential role in the normal physiology of the ovaries, and also aid in the development of follicles. It also mediates important communication between different cell types in the ovary such as the oocyte, granulosa and theca cells [63]. The depletion of forkhead box protein O1/3 (FOXO1/3) and phosphatase and tensin homolog (PTEN) in granulosa cells increased the level of activin, and that resulted in the increased phosphorylation and activation of Sma- and MAD (mothers against decapentaplegic) homolog 2/3 (SMAD2/3), resulting in the active proliferation of granulosa cells and formation of tumors in the ovary. Furthermore, conditional SMAD1 and SMAD5 double or SMAD1, SMAD5, and SMAD8 triple knockout mice developed metastatic granulosa cell tumors, substantiating the involvement of BMP-SMAD1/5/8 signalling in the initiation and formation of ovarian cancers [64]. Interestingly, the inhibition of the ligand-receptor binding using TGF-β receptor type I and II (TGFβRI and TGFβRII) dual inhibitors resulted in decrease in the size of tumors, highlighting the importance of this pathway. The TGFβRII and SMAD signalling are regulated by cancer-associated fibroblasts (CAFs), which promotes the invasiveness of ovarian cancer cells through the activation of NF-κB signalling pathway and other factors such as CD44 and matrix metalloproteinase 9 (MMP9) [65].

2.2.3.5 Nuclear Factor (NF)-κB Signalling Pathway in Ovarian Cancer

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF)-κB, also known as nuclear factor (NF)-κB, is a transcription factor that has been shown to mediate proinflammatory signalling in many cancers [66, 67]. The activation and dysregulation of key molecules in the NF-κB signalling are responsible for the promotion of chemoresistance, immune evasion, metastasis in ovarian cancers, and most importantly, the maintenance of OCSCs. It is reported that hypersignalling of

TGF-β and its cross-talk with NF-κB signalling results in the dissemination and metastasis of ovarian cancers [68]. The role of NF-κB signalling in anti-apoptotic and pro-angiogenic pathway in many different cancers is well-established, and is also shown to be pivotal in ovarian cancer progression. The inflammatory chemokines or cytokines in the tumor microenvironment (TME) activate the canonical NF-κB signalling through the V-Rel avian reticuloendotheliosis viral oncogene homolog A (RELA), also called as NF-κB 65-kilodalton (kDa) subunit (p65) protein [69]. Moreover, increased p65 phosphorylation and pro-tumor macrophage type 2 (M2) infiltration drives the activation of the canonical NF-κB signalling, and thereby induce the advancement of ovarian tumors as observed in the mouse model. Importantly, inhibition of the NF-κB signalling regresses the tumor along with a decrease in the M2 and an increase in the antitumor macrophage type 1 (M1) infiltrations [70]. Importantly notably, the loss of p65 in the xenograft mouse model significantly inhibited spheroid formation, ALDH expression and activity, chemoresistance and tumorigenesis. These studies suggest that canonical NF-κB signalling can be utilized by the OCSCs for their self-renewal, and therefore, the inhibition of ALDH expression can potentially shutdown NF-κB pathway [71]. Therefore, molecules that are involved in the NF-κB pathway can serve as therapeutic targets to develop novel and potent inhibitors/regulators to treat or cure therapy-resistant ovarian cancers.

2.2.4 Ovarian Cancer/OCSC-Specific Therapeutics and Outcomes

Most of the available therapies such as chemotherapy and radiotherapy do not target the OCSCs; rather, they target the differentiated cells among the heterogenous population of ovarian cancer cells. Additionally, surgical debulking of the tumor is only possible with the lower-grade ovarian tumors. In the case of metastatic ovarian cancer, surgical debulking is not an option. It is not possible to get a clear margin of the ovarian cancer, just from morphological observation to excise all of the cancer cells, even with advanced precision surgical instruments and debulking procedure. The conventional therapies are designed to target only the differentiated ovarian cancer cells, but not the OCSCs, because of their tumor niche [72]. The OCSCs are tightly encapsulated and well protected within their TME making it arduous to target OCSCs with conventional therapies and eliminate them to treat or cure ovarian cancer. While the tumor shrinks initially upon treatment with chemo- or/and radiotherapy, the OCSCs that are protected in their niche may revert from their quiescence or dormant state after prolonged chemo- or radiotherapy, which ultimately leads to the development of chemoresistance or radioresistance in ovarian cancer [73].

Cancer relapse is the critical issue in any type of cancer treatment, but its significance could depend on various factors. CSCs are responsible for the cancer relapse in most cancers, including ovarian cancer. Although the 5-year survival rate for ovarian cancer is comparatively high, the survival rate declines dramatically in metastatic and relapsed ovarian cancers [74]. The relapsed ovarian cancers are often chemoresistant because of their earlier drug treatment regimes. Therefore, a

comprehensive understanding of the ovarian CSCs/TICs will throw more light in combating ovarian cancer metastasis and relapse. Hence, targeting the ovarian CSCs/TICs will be a better therapeutic strategy (Fig. 2.1) than the conventional therapies, and therefore, could serve as a novel therapeutic option for the treatment of ovarian cancer patients in the future. In Chap. 16 of this book, "Targeting Therapies for Cancer Stem Cells" are discussed in more detail.

2.2.5 Clinical Trials for Ovarian Cancers

At present, there are numerous therapeutic approaches available for the treatment of ovarian cancer. Apart from the conventional treatments such as chemo- and radiotherapy, immunotherapy is also currently being used in the treatment of ovarian cancer. The conventional therapies do not target the CSCs, and hence, metastasis and cancer relapse are still a major issue in ovarian cancer treatment. Therefore, new strategies are being devised to target various signalling pathways that are involved in ovarian carcinogenesis. The signalling pathways are inhibited using knockout/knockdown strategies, aptamers or small molecule inhibitors. The clinical trials conducted using different inhibitors for ovarian cancer are listed in Table 2.1 [75, 76].

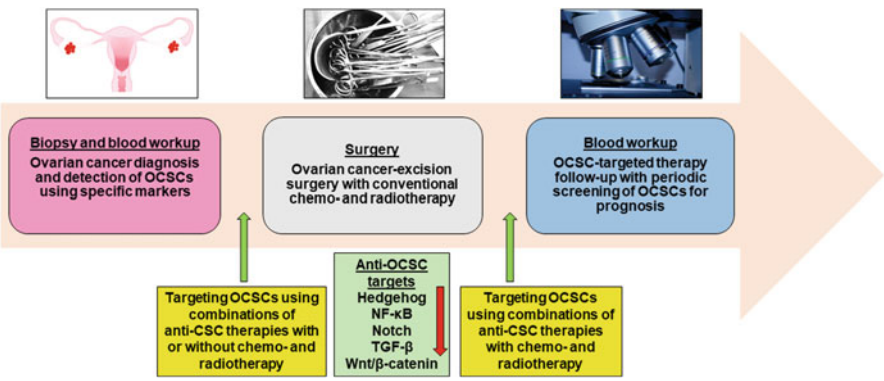

Fig. 2.1 Schematic representation of the strategy for potential combined therapies for diagnosis/prognosis of targeted-treatment of ovarian cancer. Since the discovery of cancer stem cells (CSCs)/ovarian cancer stem cells (OCSCs) and demonstration of their roles in tumorigenesis by scientists, CSCs/OCSCs are now being isolated, characterized, and used by clinicians in the diagnosis/prognosis of different types of cancer, including ovarian cancer. Though, OCSCs are already used in the diagnosis/prognosis, targeting the OCSCs in combination with other conventional therapies for ovarian caner treatment, may provide some advantages. It may likely lower the rate or chance of ovarian cancer relapse and/or resistance, and thereby increase the survival rate of patients

2.3 Thyroid Cancer Stem Cells (TCSCs)

2.3.1 Origins and Markers of TCSCs

Thyroid cancer is the most prevalent endocrine cancer worldwide, and it is classified based on the histopathological characteristics [77]. According to the World Health Organization (WHO) classification, thyroid cancer is diversified into four subtypes, namely, follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), medullary thyroid carcinoma (MTC), and anaplastic thyroid carcinoma (ATC). PTC and FTC are differentiated thyroid cancers (DTC) and are responsible for almost 90–95% of cases among the other subtypes [78]. MTC originates from the parafollicular cells or C cells of the thyroid gland. The prognosis of MTC is comparatively worse than the DTC (PTC and FTC). Moreover, the MTC patients with distant metastases have poor survival. Apart from various environmental carcinogenic factors and contributions from genetics, TCSCs are involved in the active differentiation of heterogenous population of cells due to their mesenchymal properties and facilitate rapid tumor growth and adversity. The role of TCSCs in the progression of thyroid cancer is well documented, and it was shown to be responsible for malignancy, resistance toward various therapies, and cancer relapse [79]. Different theories have been hypothesized, to explain the two different proposed models on the origin of the thyroid CSCs: multistep carcinogenesis model, and fetal cell carcinogenesis model. The theory for the multistep carcinogenesis model postulates that the thyroid cancer cells originate from the thyrocytes, which had undergone epigenetic mutation due to exposure to multiple carcinogenic factors or xenobiotics (Fig. 2.2a). However, the theory for the fetal cell carcinogenesis model states that fetal thyroid cells such as thyroid stem cells, thyroblasts, and prothyrocytes acquire the plasticity and the properties of CSCs due to oncogenic mutations (Fig. 2.2b) [80].

The thyroid CSCs can be identified by their ability to form thyrospheres in an in vitro study and tumors in *in vivo* study. Several biomarkers are used to identify different types of tumor, and they help in better therapeutic treatment [12]. The thyroid CSCs are distinguished from the non-cancer cells based on the different biomarkers such as CD133 (prominin-1), Oct-4, Sox2, Nanog, and ALDH. Further, there are different stemness genes that drive the intrinsic signalling pathways in the maintenance of these thyroid CSCs, which include Notch (HES1, JAG1) and Wnt (MYC, JUN, and FZD5) signalling pathways [81]. Although the identification of these markers has shown a clear outline in the detection of thyroid tumors, these thyroid CSCs acquire their invasive phenotype during the EMT. The thyroid CSCs lose the properties of cellular adhesion and polarity and gain their mesenchymal characteristics that give them the plasticity. The decrease in E-cadherin expression and increased expression of N-cadherin and other markers like Snail, Slug, and zinc finger E-box binding homeobox 1 and 2 (ZEB1, ZEB2) are highly associated with the EMT process [82].

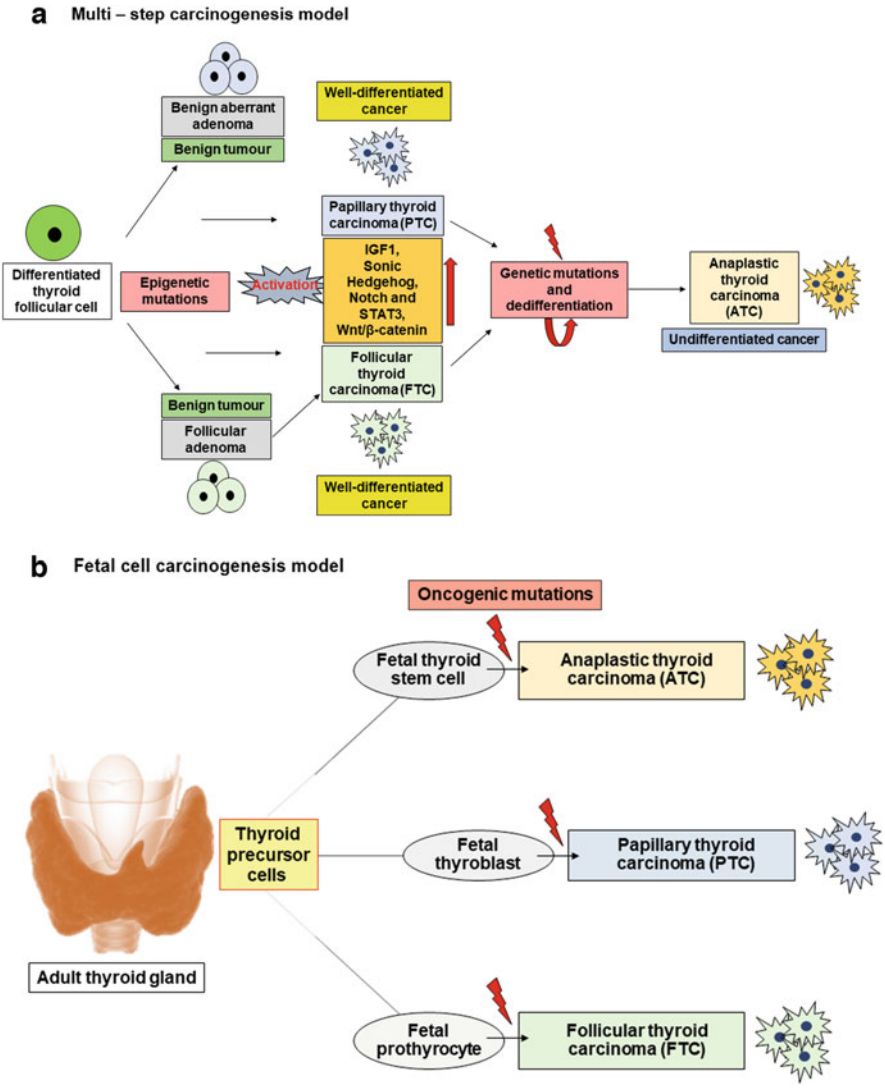

Fig. 2.2 Origin of thyroid cancer stem cells (TCSCs). Different theories have been put forward to explain the two models on the origin of TCSCs, viz., multistep carcinogenesis model and fetal cell carcinogenesis model. (**a**) Multistep carcinogenesis model—To explaind this model, the theory hypothesizes that the differentiated thyroid follicular cell undergoes epigenetic alteration(s) due to various factors leading to the deregulation and activation of diverse key signalling pathways, which actively promote the progression of normal differentiated thyroid cells to thyroid cancer cells. The intial tumour that is formed can be cancerous or benign like a follicular adenoma. However, aberrant activation of key signalling pathways in the initial tumour can induce it to quickly progress and develop into a differentiated carcinoma including the follicular or papillary thyroid carcinoma (FTC/PTC). Furthermore, these differentiated cancers can undergo genetic mutation(s) and dedifferentiation to develop into an aggressive and undifferentiated form of cancer called anaplastic thyroid carcinoma (ATC). (**b**) Fetal cell carcinogenesis model—To explain this model, the theory postulates that there are different types of thyroid gland cells, which regulate the normal physiological processes of an adult thyroid gland. During these processes, the adult thyroid gland gives rise to

2.3.2 Identification, Isolation and Characterisation of TCSCs

Different methods are used in the identification and isolation of the thyroid CSCs among other heterogenous population. Shimamura et al. [83] demonstrated that there are no common CSCs in the eight different thyroid cancer cell lines they studied. Despite that fact, there are various other markers such as CD326 (also called EpCAM), 166, 133, 117, 90, 44, 44v, 24, 15 (also called SSEA-1), 13, and ALDH which are identified as potential candidates to characterize thyroid CSCs, and there are diverse techniques that are involved in the isolation and their characterization [84].

FACS is the most common technique employed in the isolation of a distinct cell type from a heterogenous population of cells using the cell surface markers [85]. In addition to that, Hoechst dye efflux is also used to exclude the side population of the cells (differentiated cells) as these cells are capable of excluding the DNA binding dye Hoechst 33342 using the ABCG2 drug transporter. But this technique has its own limitation due to the toxicity of the dye. The sorted and separated cells from the FACS and Hoechst dye efflux are further subjected to in vitro and in vivo studies, and the cells were able to form spheroids in vitro and tumors when injected into the immunocompromised mice [86]. ALDH plays a key role in developing resistance against chemotherapy and increasing the metastatic potential of the thyroid tumors.

There are specific biomarkers that are expressed at various time points during the cancer progression. MDR1, ATP-binding cassette super-family G member 2 (ABCG2), and multidrug resistance-associated protein 1 (MRP1) are other biomarkers based on which the cells can be isolated and characterized among the therapeutic resistant tumors. Using fine needle aspiration (FNA) biopsy, the tumor cells are analyzed for these biomarkers for the detection of multidrug-resistant tumors [86]. In an *in vitro* study, prolonged culture of HTh74R cell line with low concentrations of anticancer drug, doxorubicin, resulted in the development of resistance in these cells when compared to the parental cell line. Interestingly, the parental cell line showed no significant expression of these multidrug-resistant biomarkers [87]. Among the other markers, CD15, 24, 44, and 133 are widely used in the detection of thyroid CSCs. The CD15/SSEA-1 is used as a marker for the EMT as the SSEA-1^+ cells are predominantly expressed during this transition. The $CD24^+$ and $CD44^+$ cells are responsible for spherogenic/tumorigenic potential, and they were significantly expressed in most of the aggressive thyroid cancer cell lines. $CD133^+$ cells express high levels of intrinsic stemness genes (Oct-4, Sox2, and Nanog) and drug-resistant genes (ABCG2, MDR1, and MRP), which are responsible for the self-renewability and chemoresistance of thyroid CSCs [88].

Fig. 2.2 (continued) precursors of fetal thyroid stem cells, fetal thyroblasts and prothyrocytes. These three types of cells can undergo spontaneous genetic mutations and develop into three different types of thyroid carcinomas. An oncogenic hit on the fetal thyroid stem cells can progress to ATC, and further mutation in the fetal thyroblasts and prothyrocytes can form papillary and follicular thyroid cancers, respectively

2.3.3 Signalling, Self-Renewal, Metastasis and Differentiation of Thyroid Cancer

Multiple signalling mechanisms such as insulin-like growth factor 1 (IGF1), Hedgehog, Notch, and Wnt/β-catenin play a pivotal role in the maintenance of thyroid CSCs, which support the initiation and progression of thyroid carcinomas. These signalling pathways are responsible for the maintenance of normal cellular physiology; however, aberrant activation of these pathways enhances the survival of CSCs and thus the development and progression of tumors [89]. Similar to OCSCs, the core TCSCs signalling pathways are yet to be understood well. Hence, in this section we have discussed mainly on signalling pathways in Thyroid cancer.

2.3.3.1 Insulin-Like Growth Factor (IGF)1 Signalling Pathway in Thyroid Cancer

IGF1 signalling pathway promotes the growth of thyroid cancer due to its mitotic and antiapoptotic properties. IGF1 is an endogenous hormone which is produced by various organs and has predominant role in the metabolism and growth [90]. On the contrary, the elevated levels of IGF1 in the circulation are highly correlated to various kinds of carcinomas including thyroid, breast, and prostate. It is also well-established that IGF1 is indeed involved in the formation of differentiated thyroid cancers. The IGF1 ligands and receptors (IGF1R) are overexpressed in the thyroid cancer cells which leads to the hyperactivation of this pathway [91]. In normal condition, the binding of the ligand to the receptor activates the downstream signalling molecules and stimulates the thyrocyte proliferation. Contrarily, the altered regulation of this IGF1 signalling pathway results in the rapid proliferation of thyrocytes, evading the cellular checkpoints during cell division and forming neoplasm in the thyroid gland. Further, the cross-talk between the IGF1R and thyroid-stimulating hormone (TSH) enhances the activation of IGF axis. In fact, the tumor-initiating potential of TSH is countered by other growth factors, but IGF1 stimulates the pro-tumorigenic effect [92]. Furthermore, the PTC spheres showed increase in IGFIR and IGF1/2 expressions, and interestingly, the activation of IGF1 pathway enhanced the size and number of the spheroids [93].

2.3.3.2 Hedgehog Signalling Pathway in Thyroid Cancer

Hedgehog (HH) signalling contributes to the chemoresistance and radioresistance in several cancers, and this signalling is critical in maintaining the thyroid CSCs [94]. Hedgehog signalling pathway renews the thyroid CSCs through the expression of SNAIL protein, and use of HH inhibitors in ATC cell line resulted in increased sensitivity toward the chemotherapy and radiotherapy due to decreased CSC renewal [95]. Furthermore, the Hedgehog signalling is shown to increase the aggressiveness of thyroid cancers by activating different pathways such as AKT and c-MET through cross-talk mechanism [96].

2.3.3.3 Notch and JAK-STAT3 Pathways in Thyroid Cancer

The Notch signalling's crucial target gene, Hes1, a bHLH transcriptional repressor, is significantly expressed in the thyroid and regulates the expression of sodium/iodide symporter (NIS) [97]. Different levels of Notch signalling genes are expressed in the normal and cancerous thyroid. In thyroid cancer, NOTCH1 expression differs among tumor subtypes. The expression of NOTCH1 was higher in human PTCs (classic and follicular variants, microcarcinomas) when compared with the normal thyroid and peritumoral tissues [98]. However, NOTCH1 expression was decreased in human ATC [99]. Importantly, several components of Notch signalling pathway were upregulated in human PTC as determined by microarray analysis [100]. Furthermore, Yamashita et al. [101] demonstrated an upregulation of NOTCH1 expression in PTC samples derived from human as well as transgenic animals. Interestingly, they showed that treatment of PTC cells with siRNA for NOTCH1 or GSI significantly reduced the cell proliferation and increased the apoptosis.

$CD133^+$ ATC cells showed activation of the Janus Kinase (JAK)-STAT3 pathway, which regulates the process of tissue development and homeostasis. Some functions of JAK-STAT3 pathway are mostly similar to that of Notch signalling except for most critical functions like hematopoiesis, immune development, mammary gland development and lactation, adipogenesis, and sexually dimorphic growth. Shiraiwa et al. [102] demonstrated that treatment of ATC cells with cucurbitacin I, a STAT3 inhibitor, suppressed the thyrosphere-forming ability *in vitro* and tumor growth in nude mice, highlighting this pathway as one of the therapeutic targets.

2.3.3.4 Wnt/β-Catenin Signalling Pathway in Thyroid Cancer

The higher expression of β-catenin in the cytoplasm and nucleus is observed in most of the carcinomas including thyroid carcinomas. The mutation and deregulation of the genes such as APC and AXIN1 and the accumulation of β-catenin in cytoplasm and nucleus are seen in well-differentiated PTC and FTC. Higher incidences of thyroid cancer recurrence and metastasis rates are positively correlated with the constitutive activation of this specific pathway as Wnt/β-catenin pathway supports the survival of thyroid CSCs. Furthermore, continuous activation of the pathway along with the downregulation of E-cadherin has significant positive correlation with increased migration capacity of cancer cells and metastasis in most of the undifferentiated thyroid tumors. Importantly, Wnt/β-catenin pathway plays a key role in thyroid cancer aggressiveness [103]. For additional information on cancer aggressiveness refer to "Chap. 8: CSCs and Tumour Aggressiveness" in this book.

2.3.4 TCSC-Specific Therapeutics and Outcomes

The identification and the isolation of the TCSCs using diverse biological markers specific to thyroid cancer have led to the discovery of numerous therapeutic targets in order to completely regress the tumor and prevent the tumor relapse [104]. From

the above discussed topics, it is obvious that there are multiple signalling cascades and genes that are altered in the process of carcinogenesis. Various kinds of therapeutic approaches including small molecule inhibitors, blockers, aptamers, and conditional knockout strategies are currently used to shut down different pathways that fuel and drive the TCSCs and thereby target the tumors [105]. The current therapies do not directly target the TCSCs as they are tightly encapsulated in the TME. The critical reason for the tumor relapse after the chemo- and radiotherapy is that these therapies fail to target the CSCs in the course of treatment. The CSCs repopulate the heterogenous population of cells, and regrowth of the tumor at the primary site occurs, which further complicates along with resistance toward these therapies [106].

2.3.5 Clinical Trials for Thyroid Cancers

Thyroid cancer is one of the dreadful cancers which accounts for most death among endocrine cancers. The disease progresses rapidly as it is mostly asymptomatic or it starts to present itself only during the locally advanced or metastatic stage, making it arduous to decide on the treatment regime [107]. Tumor resection is one of the common clinical procedures that is done for the early stages of this carcinoma and followed by the conventional chemo- and radiotherapy. But still the patient survival rate declines as there is high chance of thyroid cancer recurrence and chemoresistance due to the TCSCs. Current insights into the nature of CSCs in the tumor development have opened various channels of research in targeting the TCSCs for complete regression of tumor and to curb the chances of recurrence [108]. As discussed previously, multiple signalling cascades and diverse number of genes are involved in the thyroid cancer initiation and progression. Therefore, targeting those crucial genes will eventually shut down the pathway and could be a strategy to develop therapeutics. There are different types of compounds that are being researched in targeting the pathways, and few of them have given promising results in the thyroid cancer cure. The drugs that are tested in the clinical trials are given in Table 2.2 [109–111].

2.4 Melanoma Cancer Stem Cells (MCSCs)

Melanoma is one of the severe subtypes of skin cancer. The discovery of melanoma cancer stem cells (MCSCs) has helped the researchers to have a greater understanding of the underlying signalling mechanisms that lead to the tumorigenic transformation of normal melanocytes into melanoma (Fig. 2.3). MCSCs or melanoma-initiating cells were discovered by Fang group using the sphere-forming assay, in which $CD20^{+}$ cells were isolated from metastatic human melanomas with stem cell-like properties [112]. Later, Schatton et al. [113] reported that the MCSC population expresses ABC transporter ABCB5. The prevailing theory suggests that therapeutic targeting of MCSCs could eliminate tumor progression to melanomas

Table 2.2 Clinical trials using different signalling pathway inhibitors of thyroid cancer

Drug	Type(s) of thyroid cancer	Role of the drug	Phase of clinical trial
1. Axitinib	MTC, DTC	Inhibits VEGF-R, PGDF-R, and c-Kit	Phase II
2. Cabozantinib	MTC	Inhibits VEGF-R, c-Kit, RET, and MET	Phase III
3. Lenvatinib	DTC	Inhibits RET-KIF5B, CCDC6-RET, and NcoA4-RET	Phase III
4. Motesanib	MTC	Targets VEGF receptors and RET	Phase II
5. Sorafenib	DTC, ATC	Targets VEGF receptors, RET, and BRAF	Phase III
6. Imatinib	ATC	Blocks BCR-ABL, PDGF, c-KIT, and RET	Phase II

[114]. Specific-targeting of MCSCs in melanomas is challenging because of the low number of MCSCs with specific markers, intratumoral and intertumoral heterogeneity in MCSCs population, immune evasion, and chemoresistance properties of MCSCs. A proper understanding of the molecular biology, signalling mechanisms, and underlying epigenetic regulation will facilitate the specific targeting of MCSCs and therefore the development of effective melanoma-targeting therapeutics [115].

2.4.1 Origins and Markers of MCSCs

Several biomarkers for MCSCs have been reported, but CD271 (neural crest nerve growth factor receptor) is the most significant marker [116]. Other MCSC markers that are prominent in the 3D culture conditions are Nanog, Sox-10 and Oct-3/4 transcription factors, CD20 and CD133 cell surface receptors, and ALDH1. ABCB1, ABCB5, and ABCG2 were also reported to mark MCSCs in a study that used 3D melanospheres and cell lines [117]. Another important marker for MCSCs is the H3K4 demethylase JARID1B. Musashi-1 (Msi-1), Nestin, CD30, and Tenascin-C are biomarkers that are often associated with highly proliferative JARID1B^{+} melanoma cells [118, 119]. PD-1, VEGFR1, and CXCR6 are some additional surface markers involved in self-renewal, immune evasion, and vascular mimicry [120].

2.4.2 Identification, Isolation and Characterisation of MCSCs

MCSCs are an extremely small sub-population of cells in the melanoma, which show self-renewal, tumour initiation and metastasis or drug resistance. Hence, the isolation and characterisation of MCSCs are essential to understand the melanoma's development, metastasis, relapse, drug resistance and therapeutics. The very few number of MCSCs in a melanoma and limited amount of melanoma tissue biopsy materials available have made the isolation and characterisation of MCSCs as an

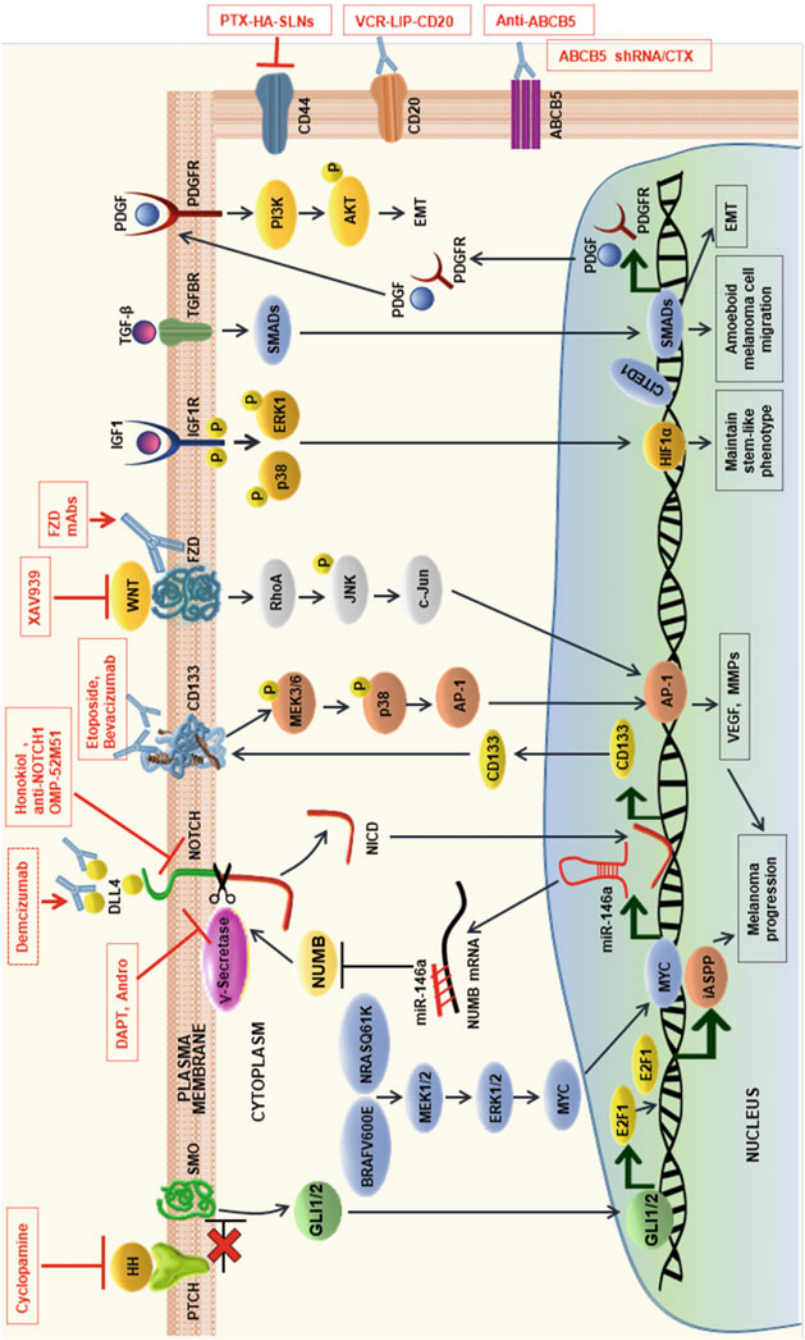

Fig. 2.3 Melanoma cancer stem cells (MCSCs) signalling pathways and possible therapeutic targets. Hedgehog (HH) binds to the receptor Patched (PTCH) and induces GLI1/2 nuclear translocation followed by transcriptional activation of the transcription factor E2F1 expression in melanoma. E2F1 in turn promotes

inhibitor of apoptosis-stimulating protein of p53 (iASPP) expression leading to inhibition of p53-dependent apoptosis and progression of melanoma. Mutations in BRAF (V600E) and NRAS (Q61K) induce miR-146a through MYC, which in turn activates the Notch signalling through the downregulation of NUMB. DLL binds to NOTCH leading to cleavage of NICD by γ-secretase. The NICD undergoes nuclear translocation, followed by transcriptional activation of CD133. Then, CD133 via the p38 MAPK pathway specifically regulates the activator protein-1 (AP-1) DNA binding activity leading to the modulation of several cellular functions that favour the melanoma progression. Wnt via JNK/c-Jun pathway can lead to the activation of AP-1 regulated genes promoting melanoma angiogenesis and metastasis. mAbs targeted against FZD receptor, DLL4, NOTCH, CD133, CD20, or ABCB5 inhibit MCSCs and MCSC-mediated melanoma progression. Small molecule-based inhibition of specific pathways, including Notch [DAPT, Honokiol, and Andrographolide (Andro)], Hedgehog (cyclopamine), and Wnt signalling (XAV939), abolishes MCSCs. Insulin growth factor receptor 1 (IGF1) interacts with its receptor (IGF1R) and stimulates ERK and p38 phosphorylation leading to hypoxia-inducible factor 1α (HIF1α) translocation and binding to DNA, which enhance the maintenance of melanoma stem-like characteristics. Transforming growth factor-β (TGF-β) in conjunction with the SMADs activates the epithelial to mesenchymal transition (EMT) and subsequently induces the migration of melanoma cells. Similar EMT and migratory phenotype is also induced by the platelet-derived growth factor (PDGF)-mediated activation of the PI3K/AKT signalling pathway in melanoma. Monoclonal antibodies can also be specifically used to target melanoma as shown in the figure. DAPT: tert-butyl(2S)-2-[[(2S)-2-[[2-(3,5-difluorophenyl)acetyl]amino]propanoyl]amino]-2-phenylacetate; Andro: 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-napthalenyl]ethylidene]dihydro-4-hydroxy-2(3H)-furanone; Honokiol: 2-(4-hydroxy-3-prop-2-enylphenyl)-4-prop-2-enylphenol); Cyclopamine: (2′R,3S,3′R,3′aS,6′S,6aS,6bS,7′aR,11aS,11bR)-1,2,3,3′a,4,4′,5′,6,6′,6a,6b,7,7′,7′a,8,11,11a,11b-octadecahydro-3′,6′,10,11b-tetramethyl-spiro[9H-benzo[a]fluorene-9,2′(3′H)-furo[3,2-b]pyridin]-3-ol; XAV939: 2-[4-(trifluoromethyl)phenyl]-7,8-dihydro-5h-thiopyrano[4,3-D]pyrimidin-4-ol

extremely challenging task in melanoma research. Therefore, many different techniques have been tried and employed in the isolation and characterisation of MCSCs [121, 122]. In the direct labelling technique, a cocktail of fluorescent-labelled specific-antibodies was used to bind to various cell surface marker antigens such as ABC transporters, CD20, CD133 (prominin-1), CD271 (NGFR/p75 neurotrophin receptor), JARID1B, etc., and then sorted by FACS to highly enrich for MCSCs. Another method employed was the magnetic bead cell-sorting technique, in combination with a unique subtractive or elimination approach. In this technique, an antibody cocktail was used to bind to haematopoietic cells including red blood cells (RBCs), endothelial cells, etc., and their cellular debris, to remove unwanted contaminants from MCSCs, leading to the enrichment (of pure population) of MCSCs for (use in) downstream applications or analysis [123].

2.4.3 Signalling Pathways Regulating MCSCs

Melanoma is the most aggressive and lethal subcategory among skin cancers, and treatment against it continues to remain elusive. The signalling pathways in MCSCs are being intensely investigated to dissect the molecular mechanisms associated with the tumorigenesis of melanomas. MCSCs share common embryonic stem cell pathways similar to that of normal stem cells in regulating self-renewal and differentiation. TGF-β maintains the plasticity of the MCSCs. TGF-β binds to the type II TGF-β receptor (TGFBR2), which then binds to type I TGF-β receptor (TGFBR1) and forms a heterodimeric complex. The activated TGFBR1 phosphorylates the SMAD proteins that result in cascade of events resulting in the activation of TGF-β-responsive genes (Fig. 2.3). Hedgehog (Hh) pathway plays an essential role in the initiation and progression of melanoma [124], and inhibition of this pathway suppresses the self-renewal of $ALDH^+$ MSCS [125]. GLI1/2 is implicated in the regulation of transcription of E2F1, which is critical for MCSC cell proliferation and progression to melanomas [126]. MCSCs show enhanced expression of the Wnt receptor and are often associated with increased metastasis [127].

Notch signalling exerts a key role in MCSC proliferation. Kumar et al. [128] showed an increased Notch1 activation and signalling in the CD133+ MCSC population. Inhibition of Notch signalling pathway with inhibitors of γ-secretase and TNF-α-converting enzyme (TACE) led to the downregulation of NICD2 and Hes, which in turn inhibited the proliferation of MCSCs [129, 130]. Notch4 is responsible for the invasion and metastasis of MCSCs [131]. Several signalling cascades including the TGF-β and PI3K/AKT pathways are reported to induce EMT in MCSCs [132]. The summary of all the key molecular signalling pathways that play essential roles in MCSCs and in tumor progression to melanomas is depicted in Fig. 2.3.

2.4.4 MCSC-Specific Therapeutics and Outcomes

Surgical resection, radio- and chemotherapy, and immunotherapy are the currently available treatment options for melanoma. As melanoma could be lethal, there is an urgent need to develop effective MCSC-specific targeting strategies to treat and cure melanoma patients. One strategy that targets MCSCs (expressing CD133, CD20, ABCB5, CD271, and ALDH1 markers), with monoclonal antibodies, showed some success as they significantly reduced melanoma growth. Another therapy that involved combination treatment with bevacizumab and etoposide was able to significantly induce apoptosis and abolished the sphere-forming ability of $CD133^+$ MCSCs [133, 134]. Additionally, targeting MCSC-specific signalling pathways (Fig. 2.3) with inhibitors such as DAPT (Notch inhibitor), cyclopamine (Hh signalling inhibitor) and XAV939 (Wnt signalling inhibitor) could serve as alternative strategies to treat melanoma patients [135, 136].

2.4.5 Clinical Trials for Melanomas

The drugs that are in clinical trials for melanomas are (1) XmAb20717 (Phase I), (2) entrectinib (Phase I/II), (3) encorafenib (Phase I/II), (4) binimetinib (Phase I), (5) DCC-2618 (Phase I), (6) LGK974 (Phase I), (7) Nivolumab (Phase II), etc. Although there have been some advancements in the last few years in the FDA-approved therapies (dacarbazine, cobimetinib, pembrolizumab, vemurafenib, tipifarnib, etc.) for melanomas, more effective and further major advancements in immunotherapies to treat melanomas are anticipated in the coming years. The reader can refer to the recent articles on this topic for additional information [137, 138].

2.5 Pancreatic Cancer Stem Cells (PCSCs)

Pancreatic cancer stem cells (PCSCs) were identified by Li group using the xenograft mouse model [16]. Like normal stem cells, the PCSCs display the potential to undergo self-renewal and differentiation. The PCSCs represent only a very small fraction of the tumor (0.2–0.8% of the pancreatic cancer cell population), and were distinguishable from the bulk tumor population based on a unique cell surface marker signature ($CD44^+CD24^+ESA^+$) [139]. In a parallel study, Hermann group identified $CD133^+$ as an additional cell surface marker for PCSCs and also showed that CD133 had 14% overlap with pancreatic cancer cells expressing $CD44^+CD24^+ESA^+$ [140]. Although PCSCs constitute a small portion of the tumor, they mostly contribute to the invasive and metastatic potentials of pancreatic cancers, and resistance to conventional cancer therapies or cancer relapse after treatment.

2.5.1 Origins and Markers of PCSCs

To evaluate the efficacy of PCSCs in tumor initiation and growth, PCSCs were either flow or magnetically sorted, and then, injected into immunocompromised mice [141]. CD133, CD44 and CD24 are the most widely accepted markers of PCSCs. There are also other markers such as OCT4, sex-determining region Y-box 2 (SOX-2), tyrosine-protein kinase KIT (c-KIT) or CD117, homeobox protein NANOG, ATP binding cassette (ABC) subfamily B member 1 (ABCB1), ABC subfamily G member 2 (ABCG2), CD3, integrin α6β4, claudin-7, epithelial-specific antigen (ESA), Nodal/Activin, doublecortin and CaM kinase-like 1 (DCLK1), nestin, C-X-C motif chemokine receptor type 4 (CXCR4) and leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) [142]. Additionally, ALDH1 is reported to mark the stemness of PCSCs, and $ALDH^+$ cells are reported to have enhanced tumorigenic potential than $ALDH^-$ cells. Furthermore, $CD133^+/CXCR4^+$ PCSCs have been shown to exhibit enhanced invasiveness [143]. Although many different markers of PCSCs have been discovered so far, still there is a lack of one or a group of unique markers to detect all of the PCSCs in a tumor. However, $CD24^+/CD44^+/ESA^+/CD133^+/CXCR4^+$ and $ALDH1^{high}$ are the most widely accepted markers that are used to enrich the PCSCs population "from the bulk pancreatic cancer".

2.5.2 Identification, Isolation and Characterisation of PCSCs

To characterize PCSCs, they were first isolated from patient tumour tissue samples by incubating with dissociation buffer consisting of type IV collagenase and dispase, and then, the digested mixture containing tumour cells was subjected to centrifugal separation on a ficoll density gradient [144]. Subsequently, PCSCs were enriched from the gradient cell fraction using several PCSC markers (e.g., ABCB1, CD24, CD44, CD133, CXCR4, DCLK1, EpCAM and OCT4) by FACS [145]. The morphology of PCSCs expressing the cell surface markers such as CD44 and CD24 is different from pancreatic cancer and was used for characterisation of PCSCs [146]. In addition to these protein markers, the pancreatic cancer stemness-specific miRNA markers (miR-17-92, miR-335, miR-1181 and miR-1246) were used for additional characterisation of PCSCs [141].

2.5.3 Signalling Pathways Regulating PCSCs

Studies on the signalling pathways and their complex interactions in normal stem cells have provided a framework, to understand the molecular biology of the cellular signalling mechanisms in PCSCs. Some of the well-studied signalling mechanisms that regulate the maintenance of PCSCs population include the Wnt/β-catenin, Hedgehog, NF-κB, Notch, and PI3K/AKT/mTOR pathways

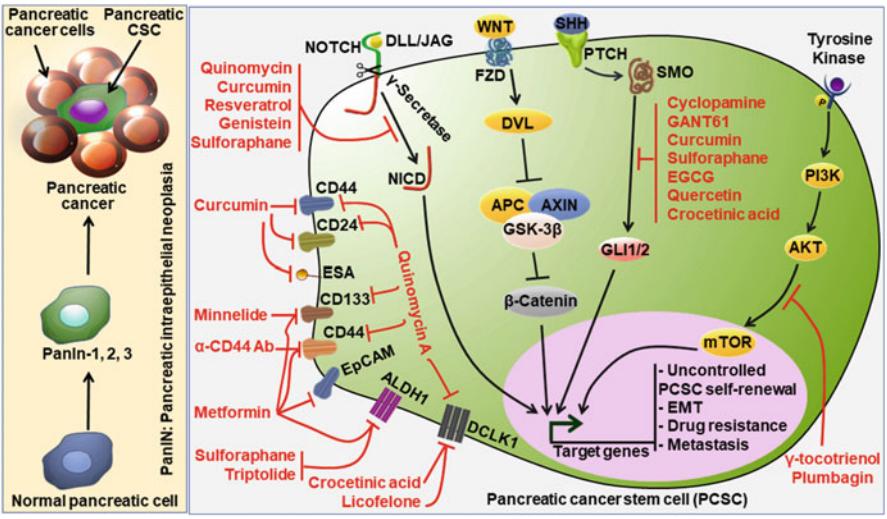

Fig. 2.4 Schematic representation shows the stepwise progression of normal cells to pancreatic intraepithelial neoplasia (PanIN) and to pancreatic tumor, and the pathways regulating PCSCs. Left figure: A model showing progression of pancreatic cancer. Stepwise progression of normal epithelial cells to different grades of PanIN. PanIN-1 is the lowest grade, and it can extends to the highest grade PanIN-3 lesion, which is characterized by a loss of cell polarity, extension of cells into the duct lumen and considerable nuclear aberrations. Right figure: Potential signalling pathways that drive PCSCs, and its targetable cell surface markers are depicted in the schematic figure. A list of inhibitors (shown in red) play a specific role in targeting different pathways associated with PCSC signalling and are potential therapeutics

[146]. A list of generally used compounds that inhibit different signalling pathways in PCSCs are depicted in Fig. 2.4.

2.5.3.1 Wnt/β-Catenin Signalling Pathway in PCSCs

Wnt/β-catenin signalling pathway plays an important role in the pancreatic tumorigenesis and therapeutic resistance. Wnt signalling pathways can either be β-catenin dependent (i.e., canonical) or β-catenin independent (i.e., noncanonical). Abnormal activation of the canonical Wnt/β-catenin signalling contributes to the pancreatic adenocarcinoma [148]. When WNT ligands are available, they bind to the Wnt-receptor complex. The Wnt-receptor complex is comprised of a seven-transmembrane receptor of the Frizzled (FZD) family and a single-pass low-density lipoprotein receptor-related protein 5/6 (LRP5/6). The WNT ligand-receptor complex then interacts with the destruction complex, which contains AXIN, APC and GSK-3β, leading to the repression of β-catenin activity, while the AXIN-binding molecule Dishevelled (DVL) represses β-catenin phosphorylation (Fig. 2.4). This causes β-catenin accumulation and nuclear translocation, followed by binding to TCF/LEF family of transcription factors, and activation of target genes that can lead to tumorigenesis (Fig. 2.4). However, in the absence of Wnt ligands, β-catenin gets phosphorylated at serine (Ser) and threonine (Thr) amino acid residues

by casein kinase 1 (CK1) and GSK3β, which form a complex with APC and axin, leading to the destruction of β-catenin [149]. The expressions of Wnt genes are upregulated in pancreatic tumor cells. Hence, more studies are warranted to develop novel Wnt pathway inhibiting molecules that could be used to attenuate the proliferation of PCSCs and target pancreatic cancers.

2.5.3.2 Hedgehog Signalling Pathway in PCSCs

Hedgehog (HH) pathway plays a critical role in the maintenance of human PCSCs. Aberrant activation of Hh signalling is reported in pancreatic cancer [149]. There are three ligands for HH signalling, namely, Indian (I), Desert (D), and Sonic (S) HH. These ligands bind to the receptors called Patched (PTCH1 and 2). Binding of HH to its receptor inhibits the activation of a seven-transmembrane protein named Smoothened (SMO), which in turn activates the GLI protein to translocate into the nucleus (Fig. 2.4). Lee et al. [150] reported that the $CD44^{+}CD4^{+}ESA^{+}$ PCSCs showed a 46-fold increased expression of SHH transcripts compared to the normal pancreatic epithelial cells or the $CD44^{-}CD24^{-}ESA^{-}$ pancreatic cancer cells. Moreover, inhibition of HH ligands with cyclopamine (Hh antagonist) leads to the inhibition of metastasis (Fig. 2.4). Similarly, treatment with inhibitor IPI-269609 (not indicated in Fig. 2.4) was reported to decrease the metastasis of human pancreatic adenocarcinoma cell lines [151].

2.5.3.3 Notch Signalling Pathway in PCSCs

Notch signalling is a critical determinant in the regulation of cell fate determination via cell-cell interaction, stem cell maintenance and differentiation. Hyperactivation of the notch pathway resulted in enhanced self-renewal of the CSCs in pancreatic adenocarcinoma [152]. Notch signalling is turned-on, when any of the four NOTCH (NOTCH1–4) membrane anchored receptor proteins interact with any of the five canonical transmembrane ligand family members, Delta-like (DLL1, DLL3, DLL4) and Jagged-1 (JAG1, JAG2). The interaction between the NOTCH receptor and the ligand induces conformational changes leading to proteolytic cleavages of NOTCH receptors by TNF-α-converting enzyme (TACE), a member of a disintegrin and metalloprotease domain (ADAM) family of metalloproteases (not shown in Fig. 2.4) and γ-secretase (Fig. 2.4). The cleavage releases the intracellular domain of NOTCH (NICD), followed by nuclear translocation and subsequent activation of the target genes; Hey and Hes heterodimerize with the DNA-binding protein CSL (RBP-Jk) and co-activators. Inhibition of the Notch pathway in pancreatic ductal adenocarcinoma (PDAC) cells led to a significant decrease in the percentage of PCSCs and the overall tumor formation. A significant challenge in developing an efficient NOTCH inhibitor-based strategy for cancer treatment is to develop a CSC-specific targeted inhibition of NOTCH without perturbing the signalling and physiology of the normal somatic stem cell population [152].

2.5.3.4 PI3K/AKT/mTOR and Other Signalling Pathways in PCSCs

Phosphoinositide 3-kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (AKT)/mammalian target of rapamycin (mTOR) signalling pathway (Fig. 2.4) play an important role in PCSC proliferation. $CD133^{high}$ PCSCs showed elevated levels of mTOR signalling; however, the inhibition of mTOR signalling using rapamycin (not shown in Fig. 2.4) inhibited the PCSC proliferation [153].

In addition, to the embryonic signalling networks mentioned above, there are different other signalling pathways involved in autophagy, interleukin 8 (IL8/CXCR1), forkhead box protein M1 (FOXM1) signalling, Nodal/Activin, and the K-RAS/c-Jun-NH2-kinase (JNK) signalling pathways that are reported to be involved in the regulation of PCSCs function. However, future investigations are required to clarify and explore the interactions between these pathways in PCSCs to better understand the significance of complex signalling networks during pancreatic tumorigenesis [154].

2.5.4 PCSC-Specific Therapeutics and Outcomes

Strategies involving targeting PCSCs were shown to involve interruption of critical stem cell survival and functioning pathways [154]. Targeting pancreatic stem cell niche by changing the TME to overcome drug resistance or targeting cell surface markers or disruption of the supportive vascular niche could sensitize CSCs to the effects of conventional cytotoxic radio- or chemotherapy and/or potentiate the effects of other CSC-targeted therapies. Numerous nonclassical drugs or inhibitors or other molecules indicated in Fig. 2.4 have been shown to inhibit the various signalling pathway components or other stemness-related proteins in PCSCs at varying degrees.

2.5.5 Clinical Trials for Pancreatic Cancers

In addition to many chemotherapeutics that are currently used to treat pancreatic cancer, there are several recently developed new drugs, which are being tested for pancreatic cancer treatment. These drugs that are being tested for safety and efficacy will have to undergo different phases of various trials, before it is approved by the U. S. Food and Drug Administration (FDA) or other similar agencies in different countries, before it is available commercially to health care providers to treat pancreatic cancer patients. Some of the promising drug candidates being tested for pancreatic cancer are (1) Gemcitabine/nab-paclitaxel (Phase II), (2) GVAX pancreas vaccine (with Cyclophosphamide) +/− nivolumab and urelumab (Phase I & II), (3) nal-IRI/5-FU/LV and oxaliplatin (Phase II), (4) Pembrolizumab (Phase II), (5) Niraparib (Phase II), etc. [155, 156]. Furthermore, novel nucleic drugs that could potentially be used as a monotherapy or combination therapy to target pancreatic cancer are also being developed with the help of next generation sequencing technology based genetic screening or diagnosis.

2.6 Conclusions

Cancer is a complex disease in which alteration of genetic, epigenetic or inflammatory factors can contribute to the process of carcinogenesis. Impaired DNA repair mechanisms or suppressed immune system or activation of oncogenes or inactivation of tumour suppressor genes can initiate or promote the growth of cancer. CSCs possess the characteristic of stemness, which provides them the self-renewal capacity and high plasticity. Hence, CSCs exhibit tumour aggressiveness, and after metastasis from the primary tumour, CSCs can initiate tumours at distant secondary sites in organs/tissues and also cause cancer relapse post-treatment. CSCs can differentiate into different types of tumour cells and this contributes to tumour heterogeneity. Furthermore, CSC's role in metastatic dissemination, tumourigenesis and tumour relapse is attributed to the hyperactivation of key signalling pathways such as Hedgehog, Notch, TGF-β and Wnt/β-catenin. CSCs utilize these signalling pathways for their self-renewal and survival, and hence, these pathways are critical for CSCs. Importantly, these hyperactivated CSC signalling pathways are shown to alter the expression and function of crucial genes, including the oncogenes and tumour suppressor genes, and thus, favour tumourigenesis. The discovery of the CSC markers and more focused research on them over the past two decade has enhanced our understanding about the CSC types. Nonetheless, emerging evidence indicates that CSCs are well nurtured and protected within their TME, making CSC-specific targeted therapy to eliminate CSCs, an arduous task in many different cancer treatments. Hence, improvements in CSC-specific stem cell markers or signalling pathways targeting therapy or other innovative approaches, followed by rigorous experimental and clinical studies are vital for the development of future cancer treatments to improve the life expectancy of the cancer patients or cure cancers.

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Isolation and Characterization of Cancer Stem Cells (CSCs)

3

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Abstract

The tumour-initiating cancer stem cells (CSCs) are neoplastic cells which produce self-renewable and heterogeneous population of pluripotent stem cells. The exploration on stationary and coursing CSCs because of protection from regular treatments and powerlessness in complete annihilation of malignant growth is basic for creating novel helpful systems for a progressively successful decrease in the danger of tumour metastasis and disease repeat. This chapter incorporates data about various strategies for discovery and separation, side population, cell markers and establishment of CSC culture, as well as attributes of CSCs, for example, tumorigenicity, and pathways related with self-restoration and the ability of the histological tumour recovery in different malignant growths.

Keywords

Cancer stem cells · Cellular markers · Self-renewal · Side population · Tumorigenicity

3.1 Introduction

Malignant growth of cancer stem cells (CSCs) has been recommended as the primary cause of cancer. The hypothesis of the presence of CSCs in a tumour populace was first proposed by Bonnet et al. In leukaemia, they distinguished a small cloning cell populace with comparative attributes to the immature microorganisms of the circulatory system. CSCs are motile tumour cells with

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moderate cell cycles, have the capacity to self-renew (boundless cell division and upkeep of the undifferentiated cell pool), and have separate tumour prognosis (tumorigenicity). These cells make up a couple of level of the malignant cells inside a tumour, regularly 0.01–1.0% [1]. Breast, colon, ovarian, lung, and head and neck carcinomas and also few other tumours contain CSCs [2]. CSCs are responsible for protecting the body against tumour growth after chemotherapy and also tumour entry into vessels and neighbouring organs. The origin of CSCs stays a disputable issue so far [3]. Three primary speculations have been raised about the starting point of CSCs: the arrangement of typical undifferentiated stem cells, transition of grown-up but undeveloped cells into pluripotent malignant cells through an epithelial-to-mesenchymal transition [4]. Considering the significant highlights of CSCs, they extraordinarily tranquilize obstruction, have obtrusive and metastatic capacity and tumorigenicity and are self-renewable. CSCs are segregated depending on properties that separate these cells from different cells in tumour mass chiefly from two sources: malignant growth cell lines and tumour tissues. The grown malignant cell lines are widely applied for CSC separation and isolation [5, 6]. As a result of culture adjustment and hereditary modifications occurring through subculturing of the cells, the biological features of primary CSCs are not reflected under hyperoxic conditions [7]. Applying powerful systems for producing such essential cell lines from tumour tissues may provide important methods to advance the investigations [6, 8–18]. With advancement in the available biomarkers, it is easy to target the CSC-associated pathways using this cell line with diverse drugs [9, 19]. Various strategies are utilized to separate these malignant stem cells. Some of them are set up dependent on the utilization of cell surface markers. Isolation and separation of these CSCs utilizing putative surface markers have been a need of research in cancer. Other strategies rely for the most part upon the functional aspects of CSCs [10–14]. This chapter will provide current approaches for isolation and identification of CSCs.

3.2 Features of Malignant Stem Cells

CSCs are vigorous cells which may have procured attributes like their typical tissue. The ABC transporter outflow with increased telomerase and glutathione synthetase is a prominent feature of typical CSCs that is copied to their offspring [20–25] that consider cell endurance and multiplication even after introduction to anticancer therapeutics. For instance, certain gastrointestinal disease cell lines show expanded protection from oxidative pressure by means of communications among CD44 and cell surface cysteine–glutamate trade transporters which bring about expanded combination of diminished glutathione, a key particle included in the balance of responsive oxygen species [15]. The CSCs are involved in metabolic reprogramming and also [16, 26] rapid repair of DNA damage [17, 27], with enhanced ABC transporters involved in drug excretion [28] of chemotherapeutic agents and also other anticancer drugs. Even the small populace of these cells, bypass anticancer treatment and stay undetected even when there is a tumour mass relapse leading to the recoverywhole tumor [29, 30]. Kurtova et al. discovered that

apoptosis was activated in healthy cells via certain chemotherapeutic drugs discharging prostaglandin E2, instigating the dormant keratin 14+ CSCs in bladder for multiplication [31]. Overcoming the hyper-aggressive nature of CSC, found after incomplete therapy, is the need of the hour for development of novel therapeutics.

3.3 Identification of CSCs Through Specific Biomarkers

No specific or sensitive biomarkers have been developed to detect CSCs. Table 3.1 shows the CSCs and their corresponding markers in various cancers.

3.4 Surface Markers for CSC Isolation

The CSCs are characterized by few proteins outflow. Diverse cell surface markers act as important contender for CSCs identification and targeting in various diseases. The articulation designs of these markers and their degree of articulations fluctuate among various tumor mass; in any case, no unmistakable markers have been presented. In malignant growth investigate; the markers of CSC subset such as proteins are most of the time used to construct a profile for separating the CSC populace from the mixed population of cells [51]. These markers generally have a place with the characterization of layer proteins [52]. For recognizing and disconnecting CSCs, choice of proper CSC surface markers is the principal need. At that point, these markers are utilized to detach potential CSCs by fluorescence-initiated cell arranging (FACS) or attractive enacted cell arranging (MACS) procedures [53]. Several surface markers, for example, prominin-1 (otherwise called

Table 3.1 CSCs and their corresponding markers in various cancers

Cancer class	Markers	References
Leukaemia/lymphoma	$CD34^+$, $CD47^+$, $CD96^+$, $CD25^+$, CCL-1^+, $CD38^-$	[5, 32–36]
Head and neck squamous cell carcinoma	$CD44^+$, $BMI1^+$, $CD24^+$, $CD133^+$	[37–39]
Glioblastoma	$CD133^+$, $CD49f^+$, JAM-A, $HER2^+$, $EGFRvIII^+$	[40–44]
Lung	$CD44^+$, $CD133^+$	[45, 46]
Breast	$CD44^+CD24^{-/low}$ and $ALDH1^+$, $CD133^+$, $CD61^+$	[44–52]
Ovarian	$CD44^+$, $CD117^+$	[53]
Pancreas	$CD44^+$, CD24, ESA^+	[54, 55]
Gastric	$CD44^+$, $CD133^+$, $ABCB1^+$, $ABCG2^+$	[56, 57]
Colorectal	$CD44^+$, $CD133^+$, $CD166^+$, $CD24^+$	[58–61]
Prostate	$CD44^+$, $CD133^+$, $ALDH^+$	[62–64]
Bladder	$CD44^+$, $CD90^+$, $CD49f^+$	[65–67]
Melanoma	$CD20^+$, $CD271^+$, $ABCB5^+$	[68–70]

CD133), CD24, CD16, CD13, CD44, CD38, CD34, epithelial-explicit antigen (EpCAM/ESA), CD20, CD176 and CD66c alone or in combination, have been used for sorting CSCs distribution in various malignancies [54]. Huge numbers of the surface receptors applied for CSC arranging have been distinguished observationally which were recognized on ordinary undifferentiated cells (SCs, for example, embryonic stem cells ESCs) and adult stem cells (ASCs) [54]. Additionally, separation of the tumor tissue into a solitary suspension of cells may prompt disability of antigen on the surface and reduced productivity of CSC detachment [55]. Besides, the cells may become unavailable upon protein treatment and in the wake of arranging forms. Cell arranging itself has affirmed to be wrong technique in which 1–3% of tumorigenic cells debasing the non tumorigenic populace [56]. One of the powerful techniques is the way towards creating mono suspension of cells from tumour tissues followed by immunizer based stream cytometric measure. In correlation with different techniques, separation dependent on cell markers is more explicit than the side population (SP) measure and the development of spheroids, be that as it may, there are a few disadvantages including predetermined number of detached cells and conceivable harm of surface markers during test handling utilizing protein lysing compounds. Complex, tedious and costly handling alongside low feasibility of confined cells is an inconveniences of surface marker–-subordinate separation of CSCs, which is a limiting factor for utilization of this technique [57–63]. The utilization of CSC markers is famous in the guess of disease. As, most of the inadequately separated tumors have the most noteworthy weight of breast CSCs, in their malignant growth [61]. Additionally, in colon malignant growth, CD133 articulation is a marker of poor visualization which is connected to metastasis of the liver [64–66]. Raised CD133, an antagonistic marker of prognosis in the cancer of pancreas, is related with lymph hub attack [67, 68]. CD133 articulation is related with poor clinical result in ovarian disease [69]. Additionally, CD44 over articulation was connected with poor prognosis in pancreatic cancer patients [71–73].

3.4.1 Magnetic-Activated Surface Antigen-Based Cell Sorting (MACS)

MACS technique permits the enrichment and isolation of stem cells without staining. Cells that are labelled and conjugated to magnetic nanomaterials with antibodies are transmitted upon a section of highly compatible magnetic field. All through this procedure, cells the antigen expressing cells are attracted to the magnetic dots and stay attached to the column, yet the various negative cells for the antigen will fall off the column [74]. Consequently, the cells of interest will be eluted from the column from varied populations of the cells. This method relies on mono parameter separation requiring cell suspension. However MACS is a simple strategy in CSC disengagement with the ability of isolating Small populace of the tumour mass cells [75–77].

3.4.2 Fluorescence-Activated Cell Sorting (FACS)

FACS is another cell confinement technique which can separate the cells utilizing fluorescent labelled markers focusing on specific surface proteins or intracellular surface markers through immune staining. This strategy permits a cellular sample or particles in suspension to be isolated through a limited fluid stream. As the sample goes through a laser, it takes into account the recognition of granularity, size and fluorescent properties of individual cells/particles [78]. For the most part, FACS partition utilizes fluorochromes straightforwardly conjugated with either essential or optional antibodies with various emanation wavelengths. FACS commonly requires more cells, as a huge division of the sample might be lost. In many cases, the total number of CSCs available for a biopsy test or in the patient blood might be exceedingly low. Therefore, an increasingly effective procedure to advance CSCs from an uncommon example is required. FACS is multiparametric strategy contrasted with MACS. Despite the fact that MACS is less difficult and requires less complicated instrument than FACS, it is mono-parametric and cannot isolate cells by means of numerous markers at the same time [79, 80]. Additionally, the adequacy of CSC disengagement by FACS is respectably satisfactory.

3.5 Functional Assays to Identify CSCs

CSCs have some natural properties, including self-reestablishment, tranquillity, and uneven cell division, slow multiplication phenotype, high ABC transporter articulation, aldehyde dehydrogenase 1 (ALDH1) action, and diminished mitochondrial action in many malignant growths. These useful highlights are utilized to allow productive CSC separation and create recognizable proof methods as depicted in Fig. 3.1.

3.5.1 Spheroid Formation Assay

Screening cancer medication in vitro is usually done via 2D cell culture model which cannot duplicate the 3D microenvironment of the tumour in the human body, for example, cellular interactions and other matrix interactions between cell and the extracellular matrix (ECM), tissue-explicit engineering and various other signs that are fundamental for tissue explicit capacities. These issues have been overcome through copying of tumour tissue properties over 3D tissue cultures. The metabolic spherical culture status has undergone developmental changes in the layers of the culture, namely, inward layer with hypoxia and acidic conditions with necrotic cells, intermediate layer that included quiet cells, and outer layer with high multiplication cells due to excess supply of oxygen and other nutrients [81]. It is demonstrated that generation and testimony of matrix proteins are higher in spheroids when contrasted with two-dimensional culture [68, 82, 83]. The capability of spheroid coculturing of malignant growth cells with various kinds of cells in tumour expanded cell-to-cell

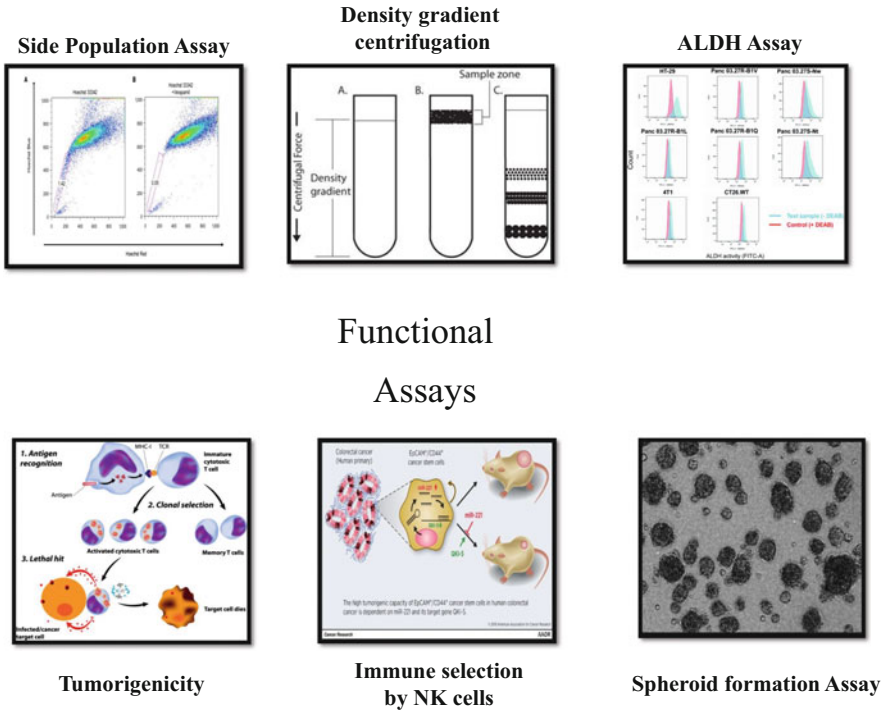

Fig. 3.1 Functional assays to identify CSCs

association through cell–cell connection and development factors [69, 70]. These attributes make spheroid development test perhaps the best contender for assessment of cutting edge anticancer medications in decades ago. Four principal circular malignant growth models dependent on culture techniques have been created: tumour spheroids with multiple cells, tumour circles, tissue-determined tumour circles and organotypic multicellular spheroid models. Multicellular spheroids are characterized as the whole of mono diseased cellular spheroids or coculturing of malignant cells with other cell types, which is referred to as multitypic spheroids or embedded cells developed in frameworks in a 3D culture. Circle size contingent upon cell type and circle age techniques can be fluctuated between under 100 µm and 3 mm in width with an enhanced size of 200–500 µm in distance across. Single cell suspension culture is used for creating spheroids within the sight of fetal bovine serum (FBS) and with no external matrix protein [84]. At present, a few multicellular tumor spheroid (MCTS) strategies are accessible for creation of spheroid, including hanging drop, spinner cups, fluid overlay and cellulose-based microparticles. In spheroid tumour examination, the tumour tissue sample to be tested is separated to mono cell suspension through compound or mechanical power. From the mono cell suspension the platelets are removed for further processing. After this, the

suspension of cells is cultured in media without serum which is enhanced by including epidermal growth factor (EGF) for development of thecells and basic fibroblast growth factor (b-FGF) in less amount. All these conditions are essential for the growth of cells as clonal round groups [48, 49, 85]. Despite the fact that tumour sphere science is not notable, it has been indicated that tumorsphere model does not completely imitate in vivo tumour structure and microenvironment. Age of organotypic multi-cell spheroids rather than tissue-determined tumour circles (TDTSs) does not have disassociation step. Tumour tissue in the wake of cutting into pieces with 0.3–0.8 mm width is refined in tissue culture cups covered with 0.75% agar in moulded media enhanced with 10% FBS and superfluous amino acids. It has been indicated that after cryopreservation of glioma spheroids, their histological attributes stayed steady and just minor genotypic and phenotypic changes were seen subsequent to defrosting. Recognizable proof of stem and ancestor cells from strong tumour presents remarkable difficulties to getting a functional single cell suspension. Since immature microorganisms are regularly rather uncommon populace of a strong tumour, it is basic to upgrade every confinement venture to augment result [71–75].

3.5.2 Clonogenic Assay

Colony formation assay is a quantitative strategy in vitro to assess the self-restoration limit of a cell in a colony of at least 50 cells through clonal development [76]. This assay is a strategy for recognizable proof of CSCs which is broadly used to assess adherent cells in a two-dimensional culture [77]. To assess clonogenic capacity, the colonies derived from CSCs are plated as single cell in a soft agar which is incubated for 21 days and are stained with crystal violet or nitro-blue tetrazolium (NTB). The total number of colonies obtained is isolated and determined with colonies got from the non-CSC portion. The colonies derived from CSCs have a larger size than the colonies derived from the non-CSCs. Many technical changes can affect clonogenic assay, namely, the autoclaved medium must contain diluted cells when the temperature is cool enough not to kill cells, yet at the same time warm adequate to be filled wells; appropriate dilution is hazardous to affirm that every colony results from a solitary cell; additionally, the toxicity of agar to the cells must be considered or it may impact the result. In spite of the fact that the component by which CSCs explicitly structure clonal circles is commonly obscure, the investigation is made on a serum-free suspension for estimating the self-renewal populace of tumour cells [78–81].

3.5.3 Tumorigenicity

Tumorigenicity is the most effective and popular gold standard method for identification of therapeutically responsive CSCs in biology [75–77]. Limiting dilution assay (LDA) is the best tumorigenicity strategy that is ordinarily utilized for

assessment of dynamic CSC recurrence [82, 86]. In this test, the valuable extreme limiting dilution analysis (ELDA) programming is utilized; it is conceivable to register subpopulations with 0–100% reactions [83]. In any case, the aftereffects of this strategy are influenced by the cellular quantity, the cell carrier, the site and time of incubation and implantation [86]. High-throughput screening cannot be utilized in this technique [87–90]. In rats, it is hard to examine the microenvironment impacts on the CSC work since the vasculature is the controller of human CSC development [83, 86, 87].

3.5.4 Lipophilic Dye Retaining Method

PKH26 and PKH6 are lipophilic fluorescent cell membrane connecting dyes which after cell division separate similar daughter cells. Holding a fact that a moderate cell division can proficiently hold the dye, there is an instantaneous dilution in the dye from the membrane of rapidly dividing cells. The dye retaining labelling technique via PKH26 has been utilized to distinguish CSCs. These labels remain to the CSCs because of their longer asymmetric divisional period to form daughter cells. The osteosarcoma and breast cancer CSCs are segregated utilizing this procedure [91, 92]. Bromodeoxyuridine (BrdU) labelling depends on a similar label maintenance approach. When contrasted with differentiated cells, CSCs hold more BrdU than the dividing cells [93]. Carboxyfluorescein succinimidyl ester (CFSE) dye has also been utilized to follow the cell division recurrence in few solid tumours. Current examinations have discovered that CFSE labelling can be utilized for recognizing and confining moderate dividing cells from glioblastomas [93–99]. The cerebral tumour pathology can be successfully studied through these dyes because of their retention property [96–98].

3.5.5 Activity of Aldehyde Dehydrogenases (ALDHs)

These isoenzymes of cytosolic origin are engaged in oxidation of intracellular aldehyde [100]. ALDH isoforms in human have 19 different types. ALDH1 catalyses the conversion of retinol to retinoic acid during malignant transformation, and it also affects the proliferation and differentiation of these malignant stem cells [101–103]. ALDH1 enzyme additionally actuates Wnt/β-catenin movement through activation of Akt signalling pathway. CD44 expression is related with ALDH1 which, on the other hand, protects normal cells from chemotherapy due to overexpression of ALDH1. From the outset of cells, the immature stem cells can be separated using ALDH1 for regenerative medicine with potential applications. The proteins from CSCs act as reliable markers in various solid tumours due to the movement of cytosolic ALDH1. Cell populace with high ALDH1 levels can be distinguished by ALDEFLUOR assay or utilizing FACS examination [104]. Investigation of the ALDH1-positive cells utilizing these two strategies showed expanded spherical arrangement ability, self-reestablishment properties, tumorigenicity and

high expression of stemness qualities contrasted with negative ALDH1 cells [105]. The reaction product of ALDEFLUOR assay collects in undifferentiated cells that are associated with ALDH movement [106–109]. The response allows viable cells to incorporate changing of ALDH substrate, BAAA (BODIPY-aminoacetaldehyde), into the fluorescent product BAA (BODIPY-amino acetic acid derivation). Significant levels of ALDH1 inside the cells become splendidly fluorescent and recognized by flow cytometry [110]. The ALDH1 specific substrate is reacted upon by ALDEFLUOR forming an immune reaction specific for the recognition of CSCs. The ALDEFLUOR test discovers epithelial tumour CSCs [111–113]. The other impediment utilizing this assay is that the osteosarcoma ALDH1-positive cells are enriched in the sphere formation with an obtrusive population of cells under investigation [93, 103]. Poor prognosis of various tumours is associated with increased expression of ALDH1 [83].

3.5.6 Side Population Assay

The response incorporates changing of a substrate into fluorescent product which is held in viable cells. Brightly fluorescent high ALDH-containing cells are distinguished by flow cytometry or improved by cell arranging for more purification [110]. The substrate for ALDH1 reaction is provided by ALDEFLUOR reagent to which an ALDH1 specific antibody can be utilized to recognize CSCs in clinical examples [111]. ALDH1 may not be an appropriate CSC marker for all tumour types, for example, liver and pancreas [112, 113]. The other impediment utilizing this measure has been exhibited in bone cancer ALDH1-positive cells enhanced in the circle framing division and related with a progressively intrusive populace [32–34]. The absence of a tumour-explicit phenotype has made trouble in the recognition of CSCs [84, 93]. SP segregation is a promising strategy for recognizing undifferentiated cells and various malignant growths. Mouse bone marrow cells for side population assay were measured using FACS. This strategy distinguishes CSCs by efflux of organic DNA binding dyes such as Hoechst 33342 and Rh123. This is achieved by ABC transporters and multidrug resistance (MDR) mechanism situated inside the cell. Cells inside this population were designated “SPs” due to their area in the flow cytometry peak plot. The outflow of ABC multidrug efflux transporters in stem cells is raised as a vital defensive component against cytotoxic substances. Fundamental individuals from this family are ABCB1 (Multidrug Resistance-1, MDR1), ABCC1, ABCF2, ABCB2, ABCC7, ABCG2 and ABCA5, which are upregulated in various tumours. The SP cells have some striking highlights that contrast them from other cell populace. They can initiate tumour formation at a high recurrence with the capacity to experience deviated division to build detachment amount in both SP part and non-SP portion. These cells have clonogenic limit, tumorigenicity, multipotency and chemoresistance [14]. Recognizable proof and confinement of stem cells through SP assay have superior goals than ordinary immunostaining measure with antibodies against ABC transporters that gives the exact discovery of uncommon SP portions ($<0.5\%$ of the absolute cell populace)

inside heterogeneous examples. In addition, utilitarian portrayal of the cells in vitro and in vivo utilizing different DNA-restricting dyes is troublesome; however, the SP test being performed on practical cells gives simpler and dependable strategy to describe the cell populace [103].

3.5.6.1 Hoechst 33342 Dye

The stem cell isolation relies on the Hoechst SP assay strategy. The fluorescent Hoechst dye binds to AT-rich areas of the nucleic acids particularly to the minor groove of DNA. The cell plasma membrane can also be accessed via Hoechst staining of the living cells [73]. Upon excitation, Hoechst at 405 nm transmits a blue signal that is gathered with a 450/40 nm band and passes through filter with excitation as red fluorescence at 610/20 nm filter. The SP can be characterized as the number of cellular population with negative for both Hoechst blue and red [59]. As indicated by an examination of STA-ET-1 cell line of Ewing sarcoma, there is a relationship between the grouping of Hoechst colour and also the distinguishing ability to disconnect the undifferentiated cells [48].

3.5.6.2 Rhodamine 123 (Rh123)

Rh123 is a mitochondrial dye which is utilized in SP measure that stains activated cells with higher resolution than the other cells. The force of fluorescence of Rh123 shows the activation state as well as the multidrug efflux pump activity related with the mass of mitochondria. Expanding efflux of Rh123 prompts the lesser dye accumulation inside cells [93]. Lesser cytotoxicity and excitation at 488 nm is effective for practical use of Rh123. A double colour efflux procedure utilizing Hoechst 33342 and Rh123 might be appropriate to separate cells with the high action of hematopoietic undifferentiated cell. Point mutation carrying cells at the R482 position are more resistant to chemotherapeutics with elevated Rh123 efflux which limits their utilization by these cells [103].

3.5.6.3 Other Fluorescent Dyes

SP examination can also be done with other fluorescence colours, for example, dye cycle violet (DCV), a cell-porous and DNA-binding dye which is excited by a violet laser at a wavelength of 395–410 nm. Contrasted with the violet laser which is accessible on most flow cytometry instruments, UV lasers are not accessible. Accordingly, this dye alternates other organic dyes like Hoechst used in SP assay [80–84]. Indoline colour ZMB793 is another dye with fluorescent nature, which is energized at 488 nm and emitted at wavelengths of 600 nm and longer. The limiting factor is that this dye cannot be utilized in certain higher instruments apart from flow cytometer [100–104].

3.5.7 Identification of CSCs Through Centrifugation

Gradient centrifugation using density as a factor is a strategy of partition for different types of cells and confinement of physical properties dependent on CSCs. This

strategy utilizes distinctive density gradient media which will influence the viability based detachment of cells [112]. For an hepatocellular carcinoma (HCC) model, at the end of centrifugation depending on the thickness of the cells, four portions with assorted properties and levels of biomarker articulation were obtained utilizing percoll consistent gradient centrifugation. Among the acquired portions the presence of malignant stem cells was confirmed, the part which has higher nuclear to cytoplasm proportion and furthermore high expression levels of SC markers. There are comparable outcomes for the segregation of stem cells utilizing this strategy. For instance, progenitor bone marrow cells from are more confined to percoll gradient using this strategy [75–79].

3.6 Controversies of Cancer Stem Cells

As depicted above, developing proof has been collected to support the CSC model. In many cases, there is still debate with respect to CSCs. It is as yet vague how CSCs are created. It has been hypothesized that typical undifferentiated cells in different tissues are malignantly changed by various factors, for example, hereditary and epigenetic transformations [49, 53]. It isn't comprehended whether tumour movement driving hereditary occasions aggregates just in CSCs. Although most strong tumours show broad genomic instability, there is no data with respect to genomic soundness in CSCs. An ongoing report recommends transformation among CSCs and non-CSCs [73]. These authors showed the likelihood that the dedifferentiation of malignant cells brings about the generation of CSCs. This versatility may represent the present irregularities observed in the CSC model.

Another irregularity generally seen is that CSCs are constantly uncommon; this view depends on the first information on acute myeloid leukemia (AML) stem cells [106]. Nonetheless, the mice microenvironment of mice is inappropriate for human malignant cells growth causing tumour relapse, limiting the life expectancy to 2 years. A few examinations have announced intends to conquer the underlying issue of xeno-transplantation. In the HCC mouse model, CD133 + CD45− subpopulation caused cancer in naked mice strain [67]. Different animal model has to be developed for further examination and elucidation of malignant stem cells.

3.7 Concluding Remarks

These stem cells are evident in cancer biology for causing tumour genesis. CSC chosen through chemical screening methods from established cell lines is useful for performing and analyzing in vitro experiments. Since the mechanisms of action of CSCs on patients remain unexplored, future studies are required to investigate them through clinical specimens. Although the contributions of CSCs to cancer development remain unclear, the conventional chemotherapies are not able to eliminate CSCs. Targeting CSCs may pave a new way in drug development for treating metastasis and recurrence.

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Lung and Prostate Cancer Stem Cells

4

Sudeep Bose, Valentina Sain, Sartaj Khurana, and Rajat Gupta

Abstract

The most prominent cause of deaths due to cancer is lung cancer that typically includes the failure of treatment, reoccurrence of cancer, and dispersion that is only possible due to the existence of cancer stem cells (CSCs). The current development in translational and molecular investigation on lung cancer postulates the unique data and detailed comprehension of lung cancer biology and various treatment approaches. Targeting lung CSCs with detailed focus on specific markers of lung CSCs may give a conception to eliminate lung cancer without reoccurrence and may finally improve long-lasting clinical outcome. Prostate cancer (PCa) is the most prevalent type of cancer and the major cause of mortality in males around the globe. It is a heterogenous condition attributed to instability of genome and mechanisms related to epigenetics resulting in cellular differentiation. The previous decade has seen evidences that have clearly revealed the critical role of PCa stem cells (PCSCs) in PCa. Metastasis, till date, remains a big challenge in the treatment of these cancer types due to limited survival advantage of the second-generation drugs as observed in sufferers. Molecular mechanisms reveal that mutations in tumor suppressors together with oncogenic activation are capable of inducing a major mechanism termed as partial epithelial–mesenchymal transition (EMT), which provides plasticity to cancer stem cells (CSCs) and eventually contributes to metastasis. Thus, a clearer

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understanding of fundamental stem cell mechanisms pointing toward the various signaling pathways that regulate the fate of cell during development is crucial to improve stem cell-based regenerative medicine and anticancer strategies for both PCa and lung cancer.

In this chapter, we encapsulate our present understanding of normal stem/progenitor cells of prostate and lung cancer that highlight the recent progress that has been made on CSCs and discuss the properties and hallmarks of biology of prostate and lung CSCs and their involvement in resistance to therapy, tumor progression, and metastases.

Keywords

Prostate cancer · Lung cancer · Cancer stem cells · Metastasis · Cancer stem cell markers · Signaling pathways · EMT · Drug resistance

4.1 Introduction

Cancer is one of the leading causes of mortality worldwide. The most life-threatening among all cancer types is lung cancer, which is responsible for 20% of cancer-related deaths globally with unfavorable diagnosis mostly due to late stage disease presentation. This cancer is now contemplated as a pandemic which is accountable for highest mortality rate among all the types of cancers, i.e., one in four cancer deaths with major social and financial consequences [1]. This cancer has been categorized into two groups based on its pathological features: (a) small cell lung cancer (SCLC) found in 20% of all types of lung cancers and (b) non-small cell lung cancer (NSCLC) found in about 80% of lung cancers [1]. NSCLC is found to be very lethal which is again categorized into large cell carcinoma, adenocarcinoma, and squamous cell carcinoma that revealed the 5-year survival rate of about 17.8% only, and high incidence of new cases is diagnosed annually with low survival rate [2]. Today, it is a well-known fact that the continuous buildup of multiple alteration in genes of normal cells leads to malignant phenotypes, and also various theories have been anticipated to elucidate the origin of cancer. Cancer stem cells (CSC) are considered as a seed of cancer that exhibit high tumorigenic potential, expression of specific markers and genes, resistance to chemotherapy, and high migration and invasion characteristics and also share some specific characteristics of normal stem cells like differentiation capability (asymmetric cell division) and self-renewal and utilization of common signaling pathways. Based on aforesaid descriptions of the CSC population, CSCs becomes a leading reason of disease reversion and sows the barriers in all the present-day used curative approaches involving surgery and chemo-, radio-, and targeted therapy for lung cancer management.

Another major type of cancer is prostate cancer (PCa) which has been consistent in holding the banner for the most prevalent cancer identified in males and also the second important malignancy of cancer-associated morbidity in the United States and Europe [3]. The prostate gland comprises three essential types of cells: luminal (secretory), basal, and neuroendocrine cells with each type identifiable by a

characteristic expression of markers. Moreover, there is a tiny group of intermediary cells that express both basal and luminal cell markers [4]. These cell types possess diverse characteristics. Most of the luminal epithelial cells have been shown to manifest the androgen receptor (AR), secrete prostate-specific antigen (PSA) along with prostatic acid phosphatase (PAP), and need AR signaling for sustenance. Basal epithelial cells are AR negative and therefore not subtle to castration [5]. In the luminal epithelial layer, there are neuroendocrine cells which are rare cells and are distributed in prostatic glands constituting less than 1% of prostatic epithelium.

High-grade prostatic intraepithelial neoplasia (HGPIN) develops PCa that progresses to locally invasive carcinoma and then to metastatic cancer [6]. Early-stage PCa cells are primarily composed of differentiated glandular structures positive for androgen receptor (AR) and prostate-specific antigen (PSA); however, undifferentiated or poorly differentiated cells are largely negative for PSA and AR expression. Also, a distinct cell subpopulation, i.e., PSA+ AR+, PSA+ AR−, PSA− AR+, and PSA− AR−, has been found to be well documented [7].

4.1.1 Features of Cancer Stem Cells

Characterization and identification of human CSCs are based on numerous characteristics: capability to (a) differentiate, (b) self-renew, and (c) form secondary or tertiary tumors when these CSCs are transferred to immunodeficient rodent [8]. Figure 4.1 represents the different properties of cancer stem cells. Like normal cells, tumors are made up of diverse populations that are dissimilar in relation to phenotypic and morphological profiles that also include differentiation and proliferation capabilities [9]. Inside the tumor, the development of every cell differs

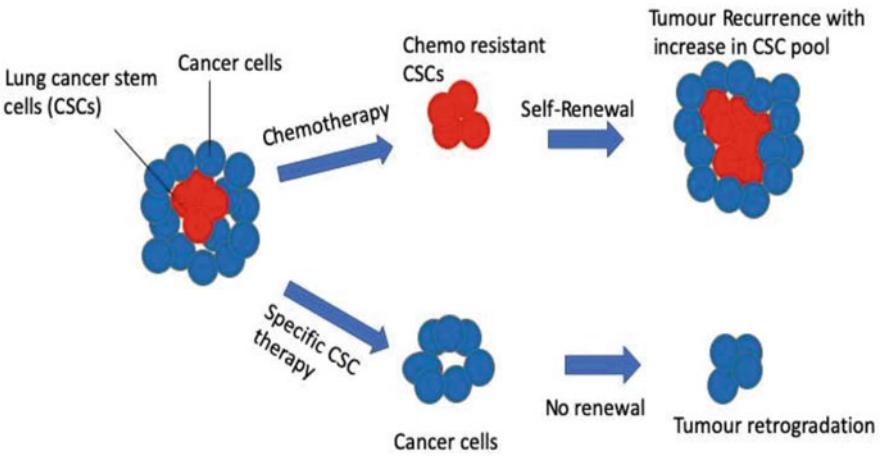

Fig. 4.1 Cancer stem cells (CSCs) properties. CSCs (red) can dedifferentiate and selfrenew within tumours to form CSCs pool again and non-tumorigenic cancer cells (blue) have restricted proliferative ability. With the tumour growth, CSCs can either have reduced benign growth or dispersed malignancies and are chemotherapy resistant that leads to cancer relapse

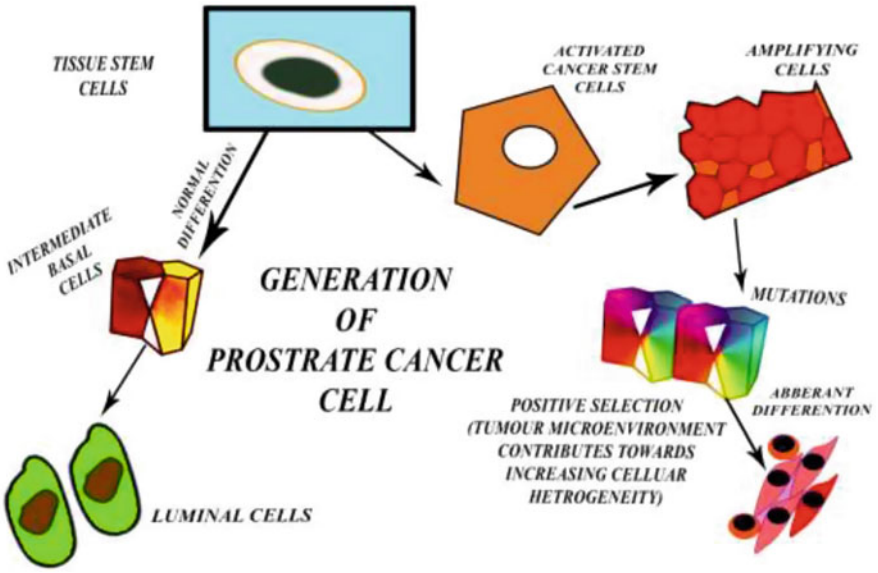

Fig. 4.2 The figure depicts developmental differences between luminal cells and cancer stem cells. Tissue stem cells undergo regular differentiation into luminal cells. Stem cells are transformed into cancer stem cells for further self-renewal to evade various tumor suppressor pathways. Mutations in various genes leads to irregular characteristics. The cells thereafter undergo de-differentiation and enter a state of rapid division

independently; for example, some cells are categorized into the cycling or non-cycling tumor cells, and some cells may be in dormant state [10]. Therefore, the targeting of specific CSCs becomes a challenging task due to the diversity of tumor heterogeneity that urges to look for exclusive lung markers that can recognize the CSC population only in lung cancers.

Lung cancer is the most complicated type of cancer due to the production of different histological and genotypic tumors within the same tissue by lung CSCs which needs to be explored further. The proposed origin of CSCs of lung cancer includes several places like from airway stem cells, basal/mucous secretary bronchial progenitor cells, neuroendocrine progenitor cells, and bronchiole alveolar progenitor cells, which consequently resulted into the formation of region-specific lung cancers or cancer subtypes having specific CSCs [11].

Similarly, PCa displays heterogeneity similar to other types of cancer. Out of the different cell types, a little cell population is critical in progression and PCa formation, and therefore, it results in the development of the heterogeneous PCa cell mass. This distinctive population of cell is defined as PCa stem cells (PCSCs). Normal stem cells that are found in the basal layer of prostate gland give rise to prostate cancer stem cells as shown in Fig. 4.2. In the usual condition, the second population of cells is generated by the stem cells which then differentiate into mature secretory cells [12]. In previous literature, it has been suggested that during the

process of carcinogenesis, the normal stem cells go through mutations to form metastasis—initiating cancer stem cells and highly tumorigenic cells [13]. However, out of the number of basal subtype cells found in the prostate, determining which basal subtype cell contains the chief stem cell niche in the prostate of an adult is still elusive. A study by Goldstein et al. [14] reported that a certain panel of specific markers functionally discriminate between the two diverse subpopulations of basal cells. The elevated levels of Trop2 were expressed only in basal cells, and they possessed stem cell type features in the human and murine prostate. The expression of CD133 (also known as Prominin-1) is the characteristic of stem-cell populations in the adult human prostate [15]. Similarly, Wang et al. identified a small subset of luminal cells that survive castration (thus called CARNs for castration-resistant Nkx3.1-expressing cells), which possess self-renewal properties in vivo and redevelop a prostate in renal grafts [16].

In the current scenario, the debate is: which subpopulation of the cells represents the real PCa cell of origin? Approximately 95% of histopathology data reveals that untreated primary PCa (i.e., adenocarcinoma) is confined to luminal AR and PSA expressing cells predominantly and basal-like cells are rare [4]. So normal prostate luminal cells may serve as a driving force for oncogenic transformation for PCa. Shen's group demonstrated that PTEN-deleted prostate cells in a mouse model give rise to high-quality PIN and carcinoma after castration [16]. Pooled together, these studies indicate that prostate luminal cells are the cells of origin for PCa. Interestingly, a current study showed that fibroblast cells associated with cancer expressing integrin $\alpha 2\beta 1$ derived from prostate basal cells of human regenerate tumor grafts [17]. All these evidences suggest that both human and mouse prostate basal cells can also serve as cells of origin for PCa. Figure 4.2 depicts the generation of prostate cancer stem cells.

4.1.2 Origin and Biology of CSC

Many theories have been suggested to elucidate the origin and exact function of CSCs in cancer including horizontal gene transfer, cell fusion, cell microenvironment and mutations, autoreactive T-cells, etc. [18].

4.1.2.1 Cell Fusion Theory

Cell fusion theory describes that the CSCs are formed by the fusion between tumor cell and bone marrow-derived cells (BMDC). These BMDCs arise from tissue distress with chronic inflammation. These consequential hybrid cells generate cells which are radiotherapy resistant and with upgraded cell repair mechanisms. This concept was supported by outcomes of an animal model study in vivo [19].

4.1.2.2 Horizontal Gene Transfer (HGT) Theory

HGT theory revolves around the fact that DNA outside the cell is capable of flowing in the eukaryotes until it finds its appropriate recipient cell. CSCs can add more

genetic alterations that powered up the cancer through environmental carcinogens, inheritance, errors in DNA replication, and resistance through HGT [20].

4.1.2.3 Cell Microenvironment

Cell microenvironment comprises the extracellular matrix (ECM), neighboring cells, hormones, and varied forces due to the movement of the host that affect the cell environments and performance directly or indirectly [21]. The control on microenvironment of stem cells plays an important role in keeping the agility of the cell. Any faults in this control could be responsible for dedifferentiation of stem cells, thus causing cancer. According to some reports, inflammatory microenvironments help in developing precancerous lesion growth and tumorigenesis. Microenvironments of tumor hold such factors that can indirectly assist the tumor heterogeneity and chemotherapy resistance [22].

4.1.2.4 Autoreactive T-Cells

Autoreactive T-Cells may develop CSCs if these T-cells manage to evade the weak immune system of host [23]. This concept gives us new insights in cancer treatment where we should concentrate on augmenting the immune system instead of diminishing it.

4.2 Cancer Stem Cells in Lung Cancer

The role of CSC in lung biology is still less studied; some of the CSC markers related with resistance to anticancer therapies have been studied, which include CD133, aldehyde dehydrogenase 1 (ALDH1), CD44, CD117, CD87, and side population (Hoechst negative). The variability of CSC phenotype and relapse of markers on cell surface due to intertumoral heterogeneity and plasticity becomes a great obstacle in identification of novel lung CSC markers.

4.2.1 Different Methods of Identification of CSCs

The isolation and identification of CSCs can be done by using functional experiment like side population (SP) assay and by CSC surface marker expression.

4.2.2 Side Population (SP) Assay

In SP analysis, the cells are distinguished on the basis of cellular differential potential to outpour a DNA-binding dye (fluorescent Hoechst) through the ATP-binding cassette (ABC) transporters [24]. The SP cells taken from cell lines of lung cancer exhibit boosted invasive ability compared to the non-SP cells, are more tumorigenic, show high expression of ABCG2 and other ABC transporters, are

resistant to multiple chemotherapeutic drugs, and present the self-renewal feature with the capability to produce floating spheres with high proliferative potential [25].

4.2.3 Surface Marker Expression

Only limited lung CSC markers have been authenticated till now, although extensive studies have reported identification of numerous CSCs that vary from other cells in the tumor. Most CSCs exhibit various markers at the same time, and using only one marker to define particular CSC is not promising as seen in Table 4.1.

4.2.3.1 CD133

CD133 is a glycoprotein of cell surface and is made up of two large glycosylated extracellular loops and five transmembrane domains. It is considered an important stem cell marker of nervous system and the hematopoietic system. CD133+ cells showed better capability of drug resistance, tumor initiation, and self-renewal. In other study by Chen et al., it was established that in CD133+ cells derived from lung cancer, expression of Oct-4 maintained the cancer stem-like features. Oct-4 manifestations are generally seen in pluripotent and totipotent stem cells of pre-gastrulation embryos. It signifies that Oct-4 shows a critical role in keeping cancer stem-like and chemo, radioresistant features in CD133+ cells derived from lung cancer [26].

4.2.3.2 CD90

GPI-anchored glycoprotein CD90 (Thy-1) expression is primarily seen in leukocytes and also in the cell-matrix and cell-cell connections. In one experimental study by Yan et al., CD90 was used as a marker for probing the lung CSCs and established sturdier proliferation and self-renewal capabilities and high level of expression in cell lines [27].

Table 4.1 List of markers used for identification of CSC in various tumour

Markers	Tumour
CD133 (prominin-1)	Lung, colon and brain
CD133+ ESA	Lung
CD44 (membrane-bound glycoprotein)	Lung
Aldehyde dehydrogenases	Lung, liver, leukemia, breast, pancreas and colon cancers
CD90	Lung
CD87(uPAR)	Lung
Side population	Lung
CD166+ EpCAM+ and CD166+ CD44+	Lung

4.2.3.3 CD44

CD44 is another membrane-bound glycoprotein and is a CSC marker initially suggested for colorectal cancer that plays imperative roles in cell adhesion, modulation of cell-matrix interaction and cell migration and shows association with various signaling pathways that explain its involvement in cancer initiation and enhancement [28, 29]. Furthermore, it has been stated that CD44 augments cancer cell invasiveness and multidrug resistance. It is also found that CD44+ functions like a tumor initiator marker in cells of lung cancer when examined both in vivo and in vitro [30].

4.2.3.4 CD166

CD166 is a CSC marker for lung cancer in addition to other solid tumor in which this marker is extensively studied, but CD166 expression and its role in lung cancer are not much studied. One comprehensive study by Zhang et al. showed the function of CD166 as a marker in lung CSCs and its potential for the determination of CSCs in NSCLC [31]. CD166 shows high self-renewal potential and high in vivo tumor-initiating capability as compared to CD44+ and CD133+ isolated from the same cells. Therefore, the marker CD166 is contemplated as the sturdiest CSC marker in determination of lung cancer.

4.3 What Is Stem Cell Niche?

Several studies suggest the presence of microenvironments that are capable of supporting CSCs known as the CSC niche. This tumor niche generates the signals that are responsible for survival, self-renewal, ability to invade tissues, and the metastasis of CSCs. Interestingly, attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy analysis by Günnur Güler et al. demonstrated that the lipid composition and dynamics of prostate CSCs are different as compared to other cell types such as differences in their major cellular macromolecules, including protein content and abundance of nucleic acids (DNA/RNA), altered nucleic acid conformation, and carbohydrate composition [32]. Collins et al. [33] identified prostate CSCs derived from primary human PCa such as $CD44^{+/}$ $\alpha_2\beta_1$ ^{hi}CD133+. Reports also suggest that PCSCs that originated from primary human PCa express the cancer resistance protein ABCG2 of breast. PCSCs have also been spotted in cell lines of PCa with the help of cell surface markers from epithelial cells of immortalized human prostate and xenograft tumors and demonstrated upregulations in stemness genes, including OCT3/4, BMI1, and β–catenin.

A recent study by Mateo et al. [34] found out that the invasiveness of a cancer stem cell is determined by a subpopulation of non-CSC resulting in a significant increase in tumorigenicity and metastasis of cancer stem cells. Thus, the subpopulations of cancer cell can start networking with other normal cells which are existing in the environment of tumor and assisting with them for benefits.

4.3.1 Signal Transduction Guided Lung Cancer Stem Cell Activity

Hedgehog pathway (HH), Notch pathway, and Wnt pathway that control differentiation and proliferation during the process of embryogenesis are also related with the CSC self-renewal.

4.3.1.1 Hedgehog (HH) Pathway

The Hedgehog (HH) signaling pathway helps in controlling homeostasis, morphogenesis, and repair of stem cells in the human body. In one study, it was found that this pathway was triggered in both small cell lung cancer and non-small cell lung cancer [2]. HH pathway can intensify the chemo-resistance, thus triggering chemotherapy breakdowns in lung cancers. HH pathway with continuous accumulation of mutations gives way in progression and activation of tumorigenic path and CSC ultimately commanding the development of cancer [35]. The three ligands that take part in HH pathway are Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH) that have numerous temporal and spatial manifestation levels along with their role as mitogens and stimulate differentiation and cell division [36]. The key ligand receptor in this pathway is the Patched receptor that is expressed around nearby origin place of the HH signals and represses the action of other transmembrane protein known as Smoothened (Smo) when HH signals are not present there [37]. On interaction with any of the three ligands to the Patched receptor, accumulation of Smo occurs that stimulates the GLI family transcriptional factors which will then go inside the nucleus and finally trigger the HH target genes as represented in Fig. 4.3.

4.3.1.2 Wnt Signaling Pathway

The pathway of Wnt signaling is very complicated in mammalian cells in which the ligands of Wnt bind to a receptor complex on a cell surface triggering the Dishevelled family protein (Dsh) phosphorylation. This, in turn, stimulates GSK-3 or glycogen synthase kinase 3 and CK1 or casein kinase 1, which helps in deprivation and buildup of β-catenin molecules in the cytoplasm from where a certain amount of β-catenin is capable of moving inside the nucleus and starts interacting with the transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family transcription factors to stimulate the expression of specific gene. It is demonstrated in some earlier reports that Wnt1 and Wnt2 are overexpressed in the primary tumors and NSCLC cell lines [38].

4.3.1.3 Notch Signaling Pathway

An evolutionarily conserved Notch signaling pathway plays distinct roles in normal tissue development and homeostasis. This pathway includes four receptors (Notch1–4) and five ligands, JAG1, JAG2, DLL1, DLL3, and DLL4 in humans. Several evidences suggested the Notch pathway is linked to cancer in some way which includes that triggering mutations in Notch1 can cause T-cell leukemia and various factors involved in this pathway are related to the advancement and metastasis of solid tumors [39].

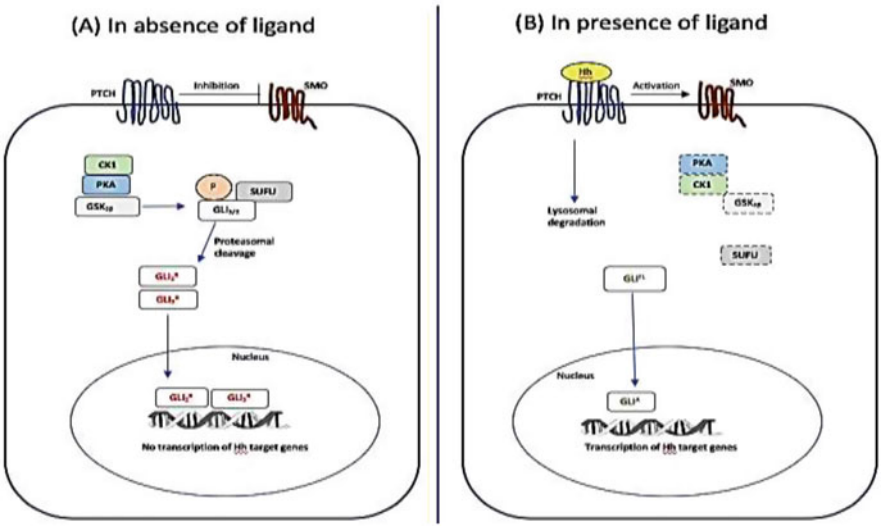

Fig. 4.3 Hedgehog (Hh) signalling pathway. (**a**) Without ligand, patch receptor inhibits SMO accumulation and allows the phosphorylation of GLI2 and GLI3 by PKA, CK1 and GSK3β that generates the binding site for E3 ubiquitin ligase β-TrCP and finally generates the repressor form ($GLI_{3/2}^{R}$) which go inside the nucleus and starts inhibiting the transcription of HH target genes and (**b**) With ligand, patch receptor relives the SMO repression and allows the accumulation of GLIFL (full length form) and activation of signalling cascade and GLIA (active form) inside the nucleus induce transcription of HH target genes

4.3.2 Signal Transduction Guided PCSC Activity

Evidences of signal transduction pathways involved in regulation of PCSC activity are emerging. For example, Nanog which is a homeodomain transcription factor is vital for PCSC activity and tumorigenicity. Related studies reveal that miR-34a negatively regulates CD44+ and tumorigenic and metastatic PCSCs [40]. PCSC stemness might also be controlled by the NF-κB and PTEN/PI3K/AKT pathways [41]. Thus, these regulators of PCSCs reported earlier may all become remedial targets or tools for the treatment of PCa, especially in the perspective of castrate-resistant prostate cancer (CRPC).

4.3.2.1 Metabolic Reprogramming of PCSC

Emerging evidence on PCa reveals that metabolic reprogramming in PCa stem cells is one of the hallmarks of PCa progression. In prostate tumors, cancer-associated fibroblasts (CAFs) are responsible for inducing reprogramming of common metabolism in stroma and tumor cells. This all comprises a shift in metabolism of CAFs toward glycolysis with accelerated manifestation of glucose transporter GLUT1 along with added secretion and production of lactic acid. In turn, CAF-generated lactate is involved in stimulating biogenesis in mitochondria and aerobic metabolism in PCa cells, also known as the reverse Warburg effect, and is related with a decline

in expression levels of GLUT1 transporter and stimulation of lactate upload [42]. Various evidences propose that CAFs induce EMT and stemness in PCa through the upregulation of inflammation gene signature in PCSCs, and chronic inflammation plays a key role in the development of aggressive PCa [43].

4.3.2.2 Hallmarks of PCSC Metabolism

Reprogramming of cellular energy metabolism is vital for tumor initiation, progression, and resistance to therapy. Compared to healthy epithelial cells, PCa cells have a high level of citrate oxidation followed by oxidative phosphorylation (OXPHOS), controlled by AR signaling [44, 45]. In order to meet high energetic demands, rapidly proliferating tumor cells also follow Warburg effect. Moreover, mutations in mitochondrial DNA and tumor suppressor genes like PTEN and TP53 result in a metabolic shift from OXPHOS to aerobic glycolysis [46]. This metabolic reprogramming and maintenance of embryonic stem cells and PCSCs require enhanced MYC expression [47]. Recent findings shed light on the fact that targeting MYC has been seen to inhibit PCSC maintenance and tumorigenicity [48]. Figure 4.4 represents the hallmarks of PCSC metabolism.

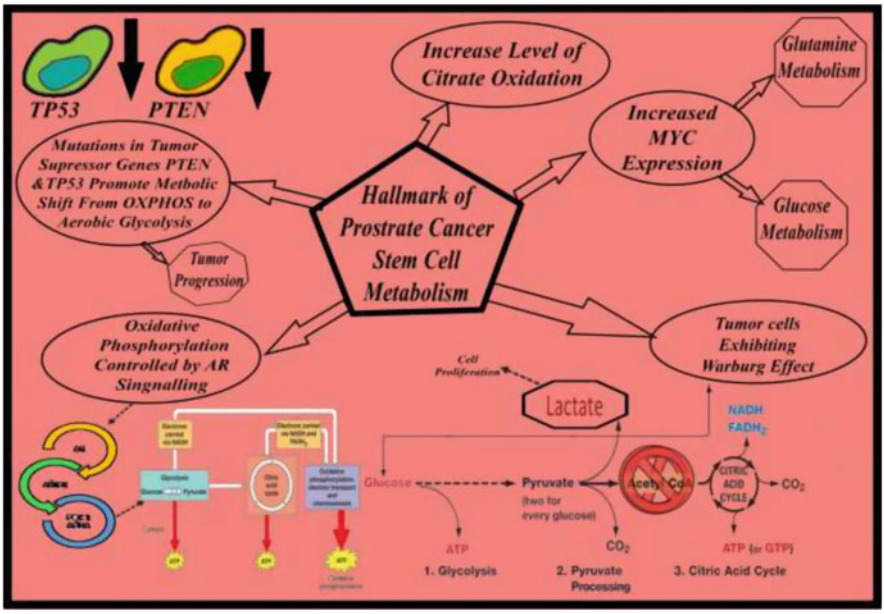

Fig. 4.4 Detailed description of how reprogramming of cellular energy metabolism play crucial role in tumor initiation and progression. Citrate oxidation and AR signaling regulates oxidative phosphorylation in the mitochondria. Tumor cells exhibit Warburg effect to, promote cell proliferation. Moreover, mutations in mitochondrial DNA and tumor suppressor genes like PTEN and TP53 causes metabolic shift, further enhancing tumor progression. MYC over-expression promotes glucose transporter GLUT1, promoting production of Lactate and also controls Glutamine metabolism produces malate, and converts to pyruvate and further to lactate

4.3.2.3 PCSC and Tumor Microenvironment

Epithelial-mesenchymal transition (EMT) is a phenomenon of interchanging from the characteristics of epithelial cell to mesenchymal phenotype which is more migratory that is related with thrashing of the epithelial markers (e.g., E-cadherin) and addition of mesenchymal signatures (e.g., vimentin, fibronectin, N-cadherin). EMT is a crucial process for embryogenesis and wound healing; it is newly documented as one of the drivers of metastases and tumor progression [49]. Increasing evidences corroborate that EMT plays a crucial role in the PCSC regulation, metastatic ability, and therapy resistance of PCa cells [50].

4.3.2.4 Prostate Cancer Bone Metastasis

Most of the sufferers of advanced PCa develop bone metastases. The development of bone metastases occurs when the bone microenvironment releases a wide range of cytokines and growth factors that bind to the prostate tumor cell receptors and are responsible for regulating their growth and survival [51]. In turn, bone PCa cells can generate pro-osteolytic factors like IL-1, IL-6, parathyroid hormone-related protein (PTHrP), and PSA that activate the osteoclast formation and stimulate bone matrix resorption [52]. Metastasis is held accountable for more than 90% of cancer-associated mortality and continues to be a great deal in cancer research. Stromal cell-derived factor-1 (SDF-1) and chemokine receptor (CXCR4) directs PCa metastasis to the bone. In hTERT-immortalized human prostate CD133+ epithelial cells, cells displayed stemness accompanied by the increase in expression of CXCR4.

Recent pieces of evidence pooled together demonstrate the CSC's role in general and specifically PCSCs as "seeds" of metastasis, in part via the SDF-1/CXCR4 axis. Interestingly, elevated levels of SDF-1 are observed in bone marrow, liver, lung, and lymph nodes which are the common organs of metastasis.

4.3.2.5 Role of PCSCs in Castrate-Resistant Prostate Cancer (CRPC)

CRPC represents one of the major clinical challenges, but the underlying mechanism of its origin remains elusive. PCSCs may throw some light on CRPC development. The emergence of CRPC mainly involves AR and AR signaling and increased AR-independent and survival pathways [53]. PCa stem cells are resistant to radiotherapy, chemotherapy, and hormonotherapy. Therefore, the cancer reoccurrence may be due to killing of preferential and more differentiated cells while leaving the undifferentiated cancer stem cells. Various mechanisms responsible for the CRPC development have been explained; many of them are centered on the regulation of AR signaling. Thus, directing toward the dysregulation of AR signaling in PCa cells has been among the chief interests in PCa research.

The main reason behind cell survival post androgen deprivation therapy but eventually leading to tumor relapse is elusive as well as intriguing. Tumor relapse along with metastatic potential has been associated with epithelial-to-mesenchymal transition (EMT) phenotype. Furthermore, EMT phenotype is associated with application of androgen deprivation therapy [54].

The discovery of a more effective and promising therapy for advanced PCa capable of targeting CSCs is the need of the hour. Interestingly, metformin, a

common orally administered drug used in the treatment of type 2 diabetes, has been established to possess anticancer effects as well. Metformin acts by reducing the production of ATP in the mitochondria by oxidative phosphorylation, which is the major source of in CSC energy Liu et al. [48].

4.4 Challenges and Perspectives

The CSC idea has been drawing a lot of interest, and CSCs have been examined in different tumor systems, including PCa and lung cancer. One of the strategies in increasing the cancer therapeutics is to overcome the lung CSCs as it is related to poor diagnosis. The CSC heterogeneity has now become a great challenge in recognizing conspicuous CSC subpopulation in lung as it comprises complex structure with various morphologies that function differently and are responsible in balancing the fluid in lung, in facilitating gas exchange, in detoxifying foreign materials, and stimulation of inflammatory responses due to damage [55]. Furthermore, we cannot always rely on available surface markers to identify CSCs because some markers may not be precise in affecting the CSC, and this is revealed in one study in which single marker of CD133+ and CD133− displayed similar CSC features like self-renewal, differentiation, colony formation, and invasion. In addition, heterogeneity of the cells and involvement of various genomic pathways also cause a challenge in targeting lung CSCs. Therefore, our present exploration is in struggling phase in sighting selective approach to constrain the CSCs and their characteristics because CSCs share a comparable feature with the normal stem cells, where targeting the CSCs might also influence the normal stem cells that can be noxious to human health.

Similarly, a thorough understanding of the functional and phenotypic properties along with the PCSC molecular regulators would help us to better apprehend the mechanisms and etiology responsible for the development of PCa. Majority of the evidences of PCSCs that have been reported so far are a result of studies on xenograft models, long-term cultured cell lines, or murine PCa models. For instance, in various well-characterized xenograft models, specific PCa cell subpopulations have been stated that are augmented in the activity of PCSC including PSA−/lo, CD44 + α2β1 +, and CD44+ cells. Till now, less reports are available on whether different patient's tumors may have distinct PCSCs and whether the human primary PCa also ports tumorigenic SC-like cancer cells. In PCa treatment, CRPC signifies one of the major challenging stages. Current studies have provided evidences that some subpopulations of PCSC may express low levels of AR and intrinsically be resistant to castration, although PCSCs that are castration resistant have not yet been revealed in samples of primary human PCa. The expansion of such cells may promote development of CRPC that is quite feasible. Consequently, these PCa cells that are castration resistant may signify potential cellular targets for development of novel drug. To detect castration-resistant PCSCs in patient tumors at different clinical stages, there is a need for scientific research with improved tumor-reconstitution

protocols and model systems, and the accomplishment of which will aid the future therapeutic development and benefit the patients with PCa.

4.5 Conclusions

Due to chemodrug resistance and relapse of cancer, it becomes very complicated to treat lung cancer with an ease. Therefore, it is a crucial requisite to know the basics of CSC development and maintenance so that appropriate step can be taken against cancer progression, tumorigenicity, and chemotherapeutic resistance before it would be too late. Consequently, CSCs are now becoming an important clinical target in cancer therapeutics in modern time. Although, it is not an easy assignment to target CSCs and get rid of any type of cancers forever. We have to bear in mind the fact that every type of tumor involvesdistinct types of stem cell which is controlled by various molecular based pathways, and it is much more complicated in case of lung cancer due to the existence ofdifferences in the manifestations of the markers in the lung cancer subtypes. Therefore, this chapter focusses on understanding the origin, CSC properties, markers of lung CSC, role of signaling pathways, and the novel therapeuticapproaches which all conclude that a detailed knowledge of basics in cancer biology and gene expression of these stem cells is needed with respect to targetedtherapy in combination with conventional therapy, ultimately boost the efficacy.

Similarly, recent pieces of evidence support a better understanding of the role of PCa stem cells in tumorigenesis. Although the stem cell therapy has unraveled mysteries of cancer cell heterogeneity within tumor mass, a thorough knowledge of the properties and characteristics of PCa cells is warranted to provide new insights into the origin of PCa. However, there still is an urgent requirement for the identification of exclusive markers for cancer stem cell in order to distinguish the normal stem cells from the cancer stem cells. But the most crucial requirement in the forthcoming years would be the development of novel and efficient stem cell-directed drugs and reduction of the threat of relapse.

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A Differential Role of miRNAs in Regulation of Breast Cancer Stem Cells

5

Shreetama Bandyopadhayaya and Chandi C. Mandal

Abstract

Breast cancer is one of the most frequently occurring cancers in women worldwide. Enormous evidences emphasized that tumorigenesis is steered by a subpopulation of tumor cells known as cancer stem cells (CSCs). These CSCs play a pivotal role in cancer cell growth and metastasis. They show resistance to therapies and are also responsible for tumor recurrence. Substantial studies revealed a crucial role of microRNAs (miRNAs) in modulation of tumorigenic potential. This chapter emphasizes mainly on those miRNAs which modulate the stemness property of breast cancer stem cells (BCSCs). miRNAs are a class small non-coding single-stranded RNAs (~20–24 nucleotides) which usually bind to 3′UTR of target mRNAs. This binding eventually inhibits protein synthesis by repressing translation and/or decaying the target mRNAs. This chapter elaborately discusses the various miRNAs (e.g., miR-200c, miR-34c, miR-214, miR-21, etc.) which not only act as either oncomirs or tumor suppressors but also regulate stemness property along with epithelial-mesenchymal transition, invasion, and metastasis. This study also enlightens the involvement of various crucial signalling pathways (e.g., Notch, Wnt, and PI3K-Akt) in miRNA-mediated regulation of BCSCs. Thus, expression profile of a specific miRNA or a set of specific miRNAs could be used as a diagnosis and/or prognosis marker for breast cancer. Moreover, targeting these specific miRNAs (e.g., miR-200c, miR-34c, miR-21, etc.) either by antagomir or mimic miRNA seems to be a promising therapeutic strategy for breast cancer treatment.

Keywords

miRNA · Breast cancer · Breast cancer stem cells · Stemness · Therapeutics

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5.1 Introduction

Tumors are heterogeneous in nature, and they exhibit stem cell-like properties. The ability of self-renewal is a hallmark of stem cells. The stem cells divide asymmetrically, and thus, out of two of the daughter stem cells, only one becomes capable of differentiation. There are numerous factors which govern these complex mechanisms including various transcription factors, epigenetic alterations, and other associated hormones. Stem cells are broadly classified into two types: the embryonic stem cells (ESCs), which are present in the early onset of development, and the somatic or adult stem cells, which persist all throughout. The ESCs are pluripotent in nature, thereby enabling the cells to differentiate into all three germ layers. On the other hand, the somatic stem cells are multipotent in nature and have the capability to divide into specific cell types, originating from specific tissue or organ [1]. There is a concept that initiation of cancer occurs due to a certain type of stem cells called cancer stem cells (CSCs). CSCs are malignant in nature, and they are highly resistant to drugs, thereby facilitating the tumor progression and metastasis [2]. Recent advances highlight that stem cells possess distinct miRNA profiles. These miRNAs have a major role in the reprogramming of cells, maintenance of pluripotency, and self-renewal and in many more aspects contributing to the regulation of stem cells. The miRNA profiles vary largely when considered in CSCs as compared to the normal non-tumorigenic stem cells. Some miRNAs are upregulated, while some are downregulated in the normal stem cells. This chapter throws light into the role of the miRNAs in affecting the human breast cancer stem cells (BCSCs). It focuses on the dysregulation of the miRNAs in human BCSCs and how it targets the genes disrupting different signalling pathways associated with it. Thus, the expression levels of certain miRNAs seem to be used as a potential biomarker for cancer prognosis and diagnosis. This book chapter not only summarizes the stem cell markers of breast cancer stem cells (BCSCs) but also highlights those miRNAs which can modulate the stemness property.

5.2 Breast Cancer Stem Cells and Their Biomarkers

Identification of CSCs is a very important and basic requirement to study the characteristics of CSCs and also a difficult task to distinguish it from the rest of the heterogenous tumor population. The CSC population constitutes a very limited proportion of the total tumor cell population, and hence, it becomes very challenging to mark them specifically. Therefore, keeping in mind about the fundamental characteristics of the CSCs, scientists have developed various in vitro and in vivo assays to recognize the CSCs. In order to identify the BCSCs, there are various techniques which include the aldehyde dehydrogenase assay, specific cell surface markers, side population dye exclusion, and culture of tumorsphere and label-retention assays like PKH staining. For the recognition of BCSCs in vivo, serial transplantation assays are useful to assess the differentiation and self-renewal capacity. It is reported that when the dye exclusion assay is performed, some populations

Table 5.1 Different markers of breast cancer stem cells

Stem cell markers	References
CD44	[7, 18–24]
CD24	[7, 18–24]
ALDH1	[7, 18–22, 24]
CD133/Prominin1	[20, 23–25]
ITGA6	[19]
CD49f	[20, 21, 23, 24]
CD90	[20]
SOX2	[21]
CD29	[23]
CD34	[23]
CD61	[24]
PROCR	[26, 27]
ESA	[26]

CD cluster of differentiation, *ALDH* aldehyde dehydrogenase, *PROCR* protein C receptor, *ESA* epithelial specific antigen

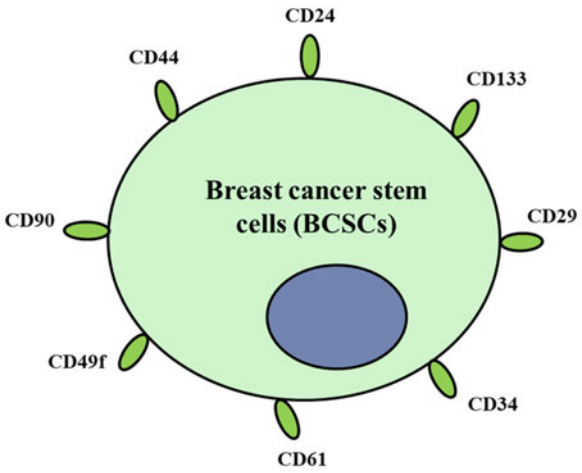

Fig. 5.1 Different cell surface breast cancer stem cell (BCSC) markers

of cells have the capability to efflux dyes like Rhodamine or Hoechst because these populations of cells feature high expression of the ATP-binding cassette transporter proteins like ABCG2/BCRP1. Now, this dye exclusion activity exhibited by a small population of stem cells (also known as the side population, SP fraction) can be quantified with the help of flow cytometry [3]. These SP populations were then studied in detail and were found out to have stem cell-like property, predicted to be present in different human breast cancer types [4–6]. There are several cell surface markers which are present in the BCSCs, and they can be easily identified by flow cytometry. The most common among the different cell surface markers elaborated in Table 5.1 and Fig. 5.1 are CD24 and CD44 [7]. These cell surface antigens seem to be used extensively to identify and/or isolate the BCSCs from the tumorigenic

population [7]. A study conducted in 2010 reported that other than the CD24−/CD44+ and CD24+/CD44+ cell population, another population of tumor cells consisting of CD44+ CD49f $_{hi}$ CD133 $_{hi}$ showed high level of tumorigenicity and stemness property in vivo [8]. Other cell surface markers like CD29 and CD49f together with CD24 or EpCAM show more specificity in identifying the mammary stem cells [9, 10]. The aldehyde dehydrogenase (ALDH) activity is another reliable way to detect the BCSCs from the bulk tumor population. ALDH is an enzyme involved in the intracellular oxidation of aldehydes playing a crucial role in the differentiation of stem cells following the retinoic acid metabolism pathway [11]. Aldefluor assay measures the ALDH activity for various types of cancer, breast cancer being one of its eminent types [12]. Tumorsphere assays are another way of detecting the BCSCs setting up gold standards. This assay is performed by plating the cells in either petri dishes or flasks in serum-free media consisting of B27, insulin, hydrocortisone, epidermal growth factor (EGF), and fibroblast growth factor (FGF) under low attachment criteria. Those cells only grow in the media which have the potential to self-renew and hence form tumorspheres and therefore can be identified as the BCSCs [13]. Another well-established procedure to determine the BCSCs is the cell membrane label-retaining assay [14]. A PKH fluorescent dye is used in this assay which binds to the lipid bilayer of the cell membranes, and it is determined that the stem cells have the potential to retain this PKH dye which is further detected by flow cytometry [15, 16]. The PKH26 dye stains both the normal and malignant mammary epithelial cells, but later in the mammosphere culture, the $PKH26_{high}$ cell populations when sorted determine the stem cell-like properties [14, 17].

5.3 Biogenesis of miRNAs

MicroRNAs are described as a class of conserved non-coding regulatory RNAs of 20–24 nucleotides which are expressed in both animals and plants and are known to regulate various biological processes [28]. miRNAs recruit the RNA-induced silencing complex (RISC) to its appropriately related target sites and regulate the mRNA stability and production of proteins. Mainly the miRNAs play a role in the post-transcriptional gene expression and its regulation. The regulation of these mRNA by a well-organized process where the miRNAs recognize the target sites of the mRNA which are located on the 3′ untranslated region (3′UTR) and base pairing occurs between 2 and 8 bps of the miRNA, called the seed sequence and the cognate mRNA sequence [29, 30]. The change in the expression of miRNAs also contributes to the rise of various human diseases, one of the crucial diseases being cancer [1]. miRNAs initially exist as primary transcripts (pri-miRNAs) that are long and are produced in the nucleus by either of the RNA polymerases, which are RNA polymerases II and III. A hairpin structure of the pri-miRNA is formed in most mammalian miRNAs. This hairpin structure is later identified by the RNase III processing complex formed by Drosha which is an RNase III enzyme and the other being an important factor of Drosha, Dgr8, finally giving rise to the formation of pre-miRNA hairpin inside the

nucleus [31]. For this whole molecular event to be carried out, specific proteins regulate every specific miRNA such as p53, SMAD, hnRNPA1, and Lin28 [29]. The pre-miRNA hairpin is transported to the cytoplasm by a nuclear RNA-export factor, exportin 5 (Exp5) which is further cleaved by Dicer, another RNase III enzyme producing a short double-stranded duplex of 22–24 nucleotides [32]. A RISC complex is then formed along with the Argonaute proteins. The RISC complex charged with specific miRNA targets the appropriate mRNA by the miRNA-seed sequences and inhibits translation and mRNA degradation [29].

5.3.1 miRNA and Stem Cells

Recent findings have thrown light on the fact that miRNA plays a role in the regulation of stem cell functions, and this had led to the identification of different stem cell-specific miRNAs [33]. Depending on the functions of the miRNA on translation inhibition, miRNAs have been noted to play a significant role in regulating the fate of stem cells and various other functions associated with it. Distinct pattern of expression of miRNA is seen in embryonic stem cells (ESCs). It is reported that around 60,000 copies/cell or more miRNAs are expressed in the ESCs [1]. Out of these, the most abundantly expressed miRNAs in ESCs are miR-290-295, miR-302, miR-17-92, miR-106b-25, and miR-106a-363 which cover almost 70% of the total miRNAs expressed in the ESCs [1]. Lin-4 and let-7, the key regulators that are responsible for the proper maintenance of the developmental lineage, are known to be the founder of the miRNA family and are the first source of evidence depicting the essential role of miRNAs in the developmental processes [29]. The fact that miRNAs play a role in the stem cell regulation was first found out when a knockout experiment was performed where Dicer and DGCR8, the two most crucial genes responsible for the mature miRNA generation, were knocked out, as the ablated Dicer$^{-/-}$ or Dgcr8$^{-/-}$ ESCs showed abnormal differentiation of stem cells [2, 29, 34]. It was noted that the Dgcr8$^{-/-}$ ESCs did not differentiate into the germ layers when experimented on mouse models and remained arrested in the G1 phase with a downregulation of the differentiation and proliferation markers. Another evidence that proves the involvement of miRNA in stem cell regulation is the expression level of the self-renewal genes that include SOX2, OCT4, and NANOG which generally have low expression during the differentiation but in Dgcr8$^{-/-}$ ESCs are found to be highly expressed [2]. The genes OCT4, SOX2, cMYC, and KLF4 are the four major transcription factors, also known as Yamanaka factors, facilitating reprogramming and also are primarily responsible for the production of iPSCs (induced pluripotent stem cells) [35]. miRNA is noted to play a role in both self-renewal and differentiation which eventually leads to the identification of cell fate; for example, in self-renewing human ESCs, there is a low expression of miR-145, but its expression increases during the differentiation process [29]. Stem cell reprogramming is governed by the miRNAs. It mainly is associated with the regulation of the reprogramming efficiency of the iPSCs. The miR-290 and miR-302 family incites the reprogramming of the iPSCs by its overexpression. Even the

human orthologs of the miR-290 and miR-302 family referred to as miR-372 are also reported to increase the reprogramming efficiency of stem cells [34]. miRNAs are potent enough to reprogram the somatic cells into iPSCs directly, for instance, the cluster of miR-302 reprograms the human skin cancer cells into a pluripotent cell [34].

5.3.2 miRNA and Cancer Stem Cells

A subgroup of cancer cells that possess the potential of self-renewal, promote the growth of cancer cells, and help in metastasis and the recurrence of tumor, thereby making the cancer cells resistant against drugs, are known as cancer stem cells (CSCs). The microRNAs are known to be dysregulated in many human cancers, and recent advances suggest that the miRNAs have gained a potency of stem cell-like properties and are also showing a wide association in the reprogramming and regulation of CSCs during tumorigenesis. Studies suggest the well-known fact that the cancer cells are known to express miRNAs, and it has been reported that if cancer cells are cultured under hypoxic condition, they express an elevated level of ESC with more miRNAs than it does in normal conditions [36]. This gives further insight to the researchers to keep a check on the miRNAs and use them as a therapeutic tool to target the CSCs, thereby leading to the reduction of tumorigenesis. miRNAs not only act as oncogenes which promote rapid cell proliferation but also are responsible for controlling growth acting as tumor suppressors [37]. For example, miR-21 acts as a tumor promoter, whereas miR-200c blocked cell proliferation, and miR-214 showed a dual role in tumorigenesis [38]. Some statistical evidences and miRNA profiling have suggested that there are many miRNAs which are present in the nearby chromosomal breakpoints, some genomic regions associated with cancer, and some fragile sites where mutations/deletions can occur. Therefore, miRNAs are believed to be very closely associated with an important role in the generation of CSCs [39]. The miRNAs that are playing a role in the process of differentiation can perform their role in two ways. First, it can subdue the state of self-renewal directly, or it can suppress the pluripotency markers like Nanog, POU5f1, commonly known as Oct4, and Klf4 which are responsible for the maintenance of pluripotency of the ESCs. Second, it can stabilize the cell fate of the differentiated cells by aiming at the transcripts regulated by the transcription factors of pluripotency which include Nanog, Oct4, Tcf3, and Sox2 [39]. A miRNA associated with tumorigenesis, miR-17-92 polycistron, regulates the expression of c-Myc and speeds up the tumorigenic process in different types of cancer such as prostate, stomach, colon, lung, pancreatic, and breast cancer. Some clusters of miRNAs such as miR-290, miR-302/367, and miR-371 alter the cell cycle in the human ESCs and thereby inhibit the transition from the state of self-renewal to the differentiated state. The miR-302 family reprograms the human skin carcinoma cells into the pluripotent ESC-like state [36, 39]. In hepatocellular carcinoma, miR-371-373 cluster is found to be upregulated, while in breast cancer, the miR-371-373 and the C19MC clusters are reported to target CD44 and become very aggressive promoting the tumor metastasis

and invasion [36]. The tumor growth of human pancreatic cancer cells with the CSC biomarkers CD44+ and CD133+ was reported to be suppressed when the miR-34 was restored; the tumor growth was inhibited both in vitro and in vivo. These pancreatic CSCs lack the expression of miR-34 normally, and therefore, the expression of Bcl-2 and Notch, the genes targeting the p53 tumor suppressor, becomes high as it is reported that miR-34 regulates the Bcl-2 and Notch target genes and further activates caspase-3 and brings on apoptosis [36, 37, 39].

The miR-130b is found to be elevated in the liver cancer stem cells that are CD133+, while the overexpression of miR-130b enhances the tumorigenic potential and heightens the resistance to chemotherapeutic drugs [36]. The tumor suppressive role of miRNA was evidenced when the E2F3 level was reduced on the artificial elevation of miR-34 in neuroblastoma inhibiting the cell proliferation, depicting a tumor suppressive role of miR-34 [37]. Studies conducted in 2013 suggested that there are 43 miRNAs whose differential expression targets different genes like p53, Notch, ErbB1, TGF-β, and Wnt which are involved in different crucial signalling pathways of the stem cells, thereby regulating cell death, cell proliferation, and development and functioning of cancer and stem cells, especially in glioblastoma [39].

The ESC-enriched miRNA plays a significant role in the CSC functioning, but it also shows an inhibitory effect on CSCs by suppressing the pluripotency. The members of the let-7 family possess a tumor suppressor role; it targets K-Ras and c-Myc and represses their expression in different CSCs such as breast, lung, head, neck, and liver. Let-7 regulates the breast CSCs, when it is overexpressed; it reduces the stemness property of the CSCs and upregulates their chemosensitivity and decreases the proliferation, tumor formation, and metastatic potential [36]. The miR-200 family plays an important role in the induction of iPSCs and is found to be downregulated in CSCs isolated from ovaries, lung, head and neck, pancreas, breast, and liver cancer stem cells. miR-200 majorly activates the MET (mesenchymal to epithelial transition) by targeting the mesenchymal markers, thereby downregulating the expression of EMT (epithelial to mesenchymal transition) markers [36]. Recent advances have pointed that there are similar properties shared between the CSCs and the cells undergoing EMT [40]. Notch1 inhibits miR-200b and miR-200c and enhances the induction of EMT with the constant expression of the CSC markers in the case of pancreatic cancer cells. Considering breast cancer and ovarian cancer, miR-200 family also restrains migration, metastasis, and invasiveness in these cancer types as well [36].

5.3.3 miRNAs and Breast Cancer Stem Cells

Among all the types of cancer, breast cancer is reported to be the most prevalent cancer to be diagnosed, and it is one of the leading causes of cancer death worldwide. The human breast tumors are heterogeneous in nature; hence, they possess different histological patterns and can be classified broadly into various types and subtypes based on their different gene expression profiles, and this heterogeneity of the breast

tumors can be explained well in the cancer stem cell models [41]. The existence of stem cell-like property in human solid tumors is difficult to find; however, the breast cancer stem cells are the cancer stem cells which were found first from the solid human tumors [42]. Breast cancer stem cells possess certain characteristics which include differentiation, self-renewal capacity, capability to metastasize and show tumorigenic potential, and resistance to chemotherapies [43]. Al-Hajj et al. in 2003 were the first to specifically describe the breast cancer stem cells (BCSCs) [41]. Isolation of the cancerous subset of the human breast tumors led to the identification of certain cell surface markers: CD44+, CD24, and ESA (epithelial specific antigen) [41, 43, 44]. This was the first strong evidence evicting the fact that there is an existence of the CSCs in breast cancer, and it proposed that only a small proportion of the breast cancer cells possessing a CD44+, CD24, and ESA expression are capable of generating new tumors [43, 44]. Another cell surface marker known as aldehyde dehydrogenase 1(ALDH1) was later found out in 2007 by Ginestier et al. which is later claimed to be a characteristic for breast CSCs along with the other above-mentioned crucial cell surface markers [43, 45]. Other than these prominent breast cancer stem cell markers, there are many other markers associated in the identification of the BCSCs, and they are listed in Table 5.1. Few researchers established a cell culture system in vitro where they cultured the non-adherent human mammary epithelial cells and they discovered that under these conditions, the cells which possessed the stem cell-like properties were only capable to survive. These cells which survived proliferate to form mammospheres, which are defined as the multicellular structures that possess both cells having properties of stem cells and progenitor cells [41]. Later, researchers claimed that some markers which are used to identify the breast cancer-initiating cells in vitro do not complement with the in vivo system [46, 47].

Hence, in order to determine the specific markers of BCSCs, it was found that miRNAs play a role in breast cancer progression by changing their stemness property, further leading to tumor formation, differentiation, metastasis, self-renewal, and resistance to chemotherapy, and they can be targeted to treat breast cancer. Since cancer cells are heterogeneously found in a tumor and the miRNA expression in the tumorigenic population is also differential between the cancer stem cells (CSCs) and the non-tumorigenic cancer cells, the CSCs are found in minor proportion in the human breast cancer population. When the breast CSCs were isolated from the breast cancer patients surgically, there were different types of miRNAs which got identified. The major families of miRNAs involved in human breast cancer with their prominent roles in BCSC regulated are listed in Table 5.2. The most critically occurring miRNAs, some of which are upregulated while some are downregulated in breast cancers, are described below.

5.3.3.1 Let-7 Family

The expression of miRNAs in BCSCs was identified in 2007, and the expression profiles of the miRNAs were compared between the differentiated BCSCs and the self-renewing BCSCs that were obtained from the breast cancer samples derived from the primary breast tumors. The researchers injected the breast cancer cell line

Table 5.2 miRNAs involved in BCSC formation, self-renewal, and differentiation

S. no.	miRNAs	Target gene(s)/ transcription factors	Signalling pathways	Role	References
1.	miR-200	ZEB1 and ZEB2	Notch	Inhibits the BCSC growth and tumor progression	[71–74]
2.	miR-200c/ 141	ZEB1, BMI1	Wnt/β-catenin	Inhibits the BCSC phenotype	[74–77]
3.	miR-140	SOX9, ALDH1	Targets the phenotypic markers of BCSCs	Reduces BCSC formation	[78]
4.	miR-34a	p53, Notch1, ALDH1, CD44	Notch and the phenotypic markers of BCSCs	Suppresses BCSC formation	[79–82]
5.	miR-205	Ligand jagged1, Hairy and enhancer of split-1, Notch1, Notch2, CD44, ALDH1	Notch and the phenotypic markers of BCSCs	Inhibits the BCSC phenotypes and stemness	[83–85]
6.	miR-7	STAT3, SETDB1	STAT	Decreases BCSC formation	[86]
7.	miR-29	KLF4, SPIN1	Wnt/β-catenin	Inhibits the reprogramming and maintenance of BCSCs	[87, 88]
8.	miR-34c	Notch 4	Notch	Inhibits BCSC formation	[89]
9.	miR-93	STAT3, JNK1, HMGA2, SOX4, EZH1	STAT	Depletion of BCSCs	[90]
10.	miR-99a	Rapamycin (mTOR), HIF1	PI3K/AKT	Reduces the self-renewal capacity of BCSCs	[91]
11.	Let-7	E2F2, c-Myc, KRAS	Targets directly	Inhibits proliferation of BCSCs	[92]
12.	miR-33b	SALL4, Twist1, HMGA2	Not specifically known	Inhibits stemness of BCSCs	[93]
13.	miR-16	Wip1	DNA damage signalling	Inhibits proliferation and differentiation of BCSCs	[94]
14.	miR-600	SCD1	Wnt/β-catenin	Inhibits stemness of BCSC	[95]

(continued)

Table 5.2 (continued)

S. no.	miRNAs	Target gene(s)/ transcription factors	Signalling pathways	Role	References
15.	miR-128	BMI1, ABCC5, SNAIL, CSF1, KLF4, LIN28A, NANOG	TGF-β, STAT, PI3K/ AKT	Inhibits the clonogenicity and tumorigenicity of BCSCs	[63, 96]
16.	miR-181	ATM	TGF-β	Increases the BCSC phenotype	[97]
17.	miR-183	SNAI2, SMAD4, β-catenin, and BMI1	Wnt/β-catenin	Activates EMT and the self-renewal in BCSCs	[61, 98, 99]
18.	miR-142	APC	Wnt/β-catenin	Promotes BCSC proliferation	[100]
19.	miR-495	E-cadherin, REDD1, JAM-A	TGF-β	Promotes invasion and metastasis to maintain BCSC properties	[101–103]
20.	miR-214	Ezh2, p53, TFAP2, PTEN, BIM, and β-catenin	Wnt/β-catenin	Enhances cell differentiation, stemness, apoptosis, and invasion of BCSCs	[38, 55]
21.	miR-221	Ataxin-1	Targets the phenotypic markers of BCSCs	Stimulates the stemness of BCSCs	[104]
22.	miR-125a	LIFR	Hippo	Targets the phenotypic markers of BCSCs	[105]
23.	miR-146a	KLF8, NUMB, CD44, and ALDH1	Notch	Increases BCSC traits	[106]
24.	miR-21	HIF1-α	AKT, ERK1/ 2, TGF-β	Induces BCSCs	[107]
25.	miR-221/222	ZEB1	PI3K/AKT	Causes BCSC formation	[59, 108]
26.	miR-106b-25	SMAD7	TGF-β	Promotes tumor initiation	[109]

ZEB Zinc finger E-box-binding homeobox 1, *TGF-β* transforming growth factor-β, *SOX* sex-determining region Y-box, *ALDH1* aldehyde dehydrogenase 1, *STAT* signal transducer and activator of transcription, *SETDB1* SET domain bifurcated 1, *KLF* Krüppel-like factor, *LIFR* leukemia inhibitory factor receptor, *JNK* Janus kinase 1, *HMGA2* high mobility group AT-hook 2, *EZH1* Enhancer of zeste 1 polycomb repressive complex 2 subunit, *SALL4* Sal-like protein 4, *TWIST1* Twist-related protein 1, *REDD1* DNA damage-inducible transcript 4 protein, *WIP1* wild-type p53-induced phosphatase 1, *BMI1* B cell-specific Moloney murine leukemia virus integration site *1, SCD* Stearoyl-CoA desaturase, *ATM* Ataxia telangiectasia mutated, *ABCC5* ATP-binding cassette subfamily C member 5

SKBR3 possessing BCSCs into NOD/SCID mice and treated them with chemotherapeutic agents. When they isolated the tumor cells from the mice, they found that the tumors contained a high level of CD44+/CD24 and the cells had a mammosphere forming capability as well [48]. With the study of the miRNAs, they found that the miRNA let-7 is to be the most promising miRNA, since it was downregulated in the tumor-initiating population of cells compared to the cells having a self-renewing capacity. Let-7 that acts as a tumor suppressor targeting the oncogene RAS was first identified in *C. elegans*, which involved in the regulation of the larval stage to adult stage of *C. elegans* [42]. An increased expression of let-7 is noted in the cells differentiating to non-tumorigenic cancer cells. The tumor suppressive potential of let-7 is well identified, as when it was introduced lentivirally, it resulted in diminished mammosphere formation, less proliferation, and less stem cell differentiation, and it also reduced the tumorigenic and metastatic potential of NOD/SCID mice in vivo [41]. So, let-7 being a tumor suppressor has been reported to be downregulated in many cancers, and its restoration helps in inhibiting cancer, thereby establishing it as a potential molecular marker and a therapeutic target for BCSC. Lin-28 protein, a member of the let-7 family, blocks the generation of let-7, thereby increasing the chances of tumorigenicity in breast cancer cells. This Lin-28 targets the let-7 via STAT3 signal transducer and activator of transcription factor 3 pathway, and let-7 further targets HMGA2 which enhances the expression of mesenchymal markers, thereby causing EMT. Therefore, it can be concluded that this let-7 miRNA is involved in the regulation of self-renewal and differentiation of breast cancer cells [41].

5.3.3.2 miR-200 Family

miRNAs also occur in the genome in the form of clusters and then are later transcribed into multi-cistronic primary transcript. The genes of miRNA occur in the form of clusters as well, usually having two to three miRNA genes in a cluster, but there are larger clusters of genes also present in some, for example, clusters of miR-17-92 and miR-106a-363 consisting of six members [42]. miR-17-92 cluster was the first polycistronic miRNA cluster which was reported to play a role in tumorigenesis [49]. The most extensively studied miRNA cluster in the human genome is miR-200. miR-200 family is the most conserved family of miRNAs of the animal kingdom [50]. There are five members in the family of miR-200: miR-200a, miR-200b, miR-200c, miR-141, and miR-429 [41, 42]. Based on the different gene clusters, the family of miRNAs can be subdivided into different locations on two different chromosomes: miR-200b/miR-200a/miR-429 is located on chromosome 1 in human and on chromosome 4 in mouse, while on chromosome 12 in human and chromosome 6 in mouse lies the miR-200c/miR-141 gene cluster [41, 42]. It is reported that the miR-200 family has a close association in the maintenance and regulation of BCSCs. When a comparison was made between the tumorigenic and non-tumorigenic population of human breast cancer cells, it was found that out of the different miRNA expression levels, the miRNA clusters that remained downregulated were miRNA-200c-141, miR-200b-200a-429, and miR-183-96-182, thereby suggesting their role in the self-renewal and differentiation

of cancer stem cells. In fact, miR-200c was also reported to suppress the clonogenicity of BCSCs in vitro and inhibited the formation of mammary ducts and tumorigenic potential of BCSCs, suggesting that the downregulation of miR-200c has a role in cancer stem cell regulation [41]. The expression of miR-200 which is depicted in mammospheres is reported to be regulated epigenetically, and it is predicted that when miR-200 is re-expressed in those stem cells which have downregulated expression of miR-200, it led to the introduction of non-stem cell-like phenotype and the cells also became capable of performing mesenchymal-to-epithelial transition (MET) [51].

miR-214

miR-214 is evinced to have an important role in proliferation, stemness, metastasis, invasion, and apoptosis. miR-214, present in the human chromosome 1, is reported to be either upregulated or downregulated in human tumors [38, 42]. However, in human breast CSCs, miR-214 is found to be upregulated, especially in the luminal A and triple-negative types. The ablation of miR-214 in mice though is found fertile and viable but hampers proper cardiac function and leads to cardiac failure [42]. miR-214 has a varying role in different types of cancer, for example, in ovary cancer, it enhances the CSC property by targeting the Nanog expression [52] while in hepatocellular carcinoma, it represses the stem cell-like properties [53]. In breast cancer, it was documented that a low level of miR-214 increases the expression of oncogenic EZH2, which is a component responsible for the catalysis of PRC2, a causative of breast cancer malignancy [54]. MiR-214 downregulates p53 and causes and increases the invasion in breast cancer [55].

miR-221-222 Cluster

The cluster of miR-221-222 comprises miR-221 and miR-222 and is found on chromosome Xp11 in human [56]. The seed sequence of miR-221 and miR-222 is the same, and they behave as both oncogenes and tumor suppressors in different human tumors, for example, as oncogenes in human epithelial tumors and as tumor suppressor in erythroleukemia. The miR-221-222 cluster is found to be upregulated in many cancer types like human breast CSCs and pancreas and glioblastoma cells [42, 57, 58]. miR-221-222 inhibits PTEN and helps in the formation of BCSCs [59]. So, this suggests that miR-221-222 has a significant role in regulating stemness, cell cycle progression, migration, and apoptosis.

5.3.3.3 miR-30 Family

In the mammospheres, alongside the let-7 family, it is detected that the miR-30 family is downregulated in the tumor-initiating human BCSCs. In the miR-30 family, miR-30e particularly plays an important role. It is shown that the downregulation of miR-30e leads to the enhancement of the self-renewal capacity not only in breast cancer but also in lung cancer. Other studies conducted on breast cancer suggest a significant role of miR-30 in both adhered and non-adherent mammospheres. miR-30a plays an effective role in the regulation of the growth of non-attachment mammospheres [60]. It is noted that the overexpression of miR-30a

led to the reduction of the mammosphere formation ability while when inhibited, it increased the number of mammospheres. These evidences from literatures suggest that miR-30 family plays an important role in cell proliferation and apoptosis of BCSCs [41].

miR-142

The expression of miR-142 is detected largely in the hematopoietic lineages playing a crucial role in hematopoiesis; however, it is found to be expressed highly in human BCSCs and not detectable in normal stem cell population. The miR-142 follows the canonical Wnt signalling pathway targeting the APC, and it leads to the further activation of miR-150 which is also simultaneously upregulated in human breast CSCs [42].

miR-16

Another family of miRNA responsible for the maintenance of the proliferation, differentiation, and stemness potential of mammary CSCs is the miR-16 family. A low level of miR-16 is detected in human breast cancer as depicted by the number of mammospheres formed. miR-16 is regulated by the Wip1 (wild-type p53-induced phosphatase 1) oncogene, thereby leading to the increase of the Wip1 protein in the mammospheres. Overexpression of miR-16 leads to the repression of cell proliferation in MCF-7 human breast cancer cell line [41].

miR-183

The miR-183 cluster of miRNAs is reported to be upregulated incessantly in different types of cancer, though mainly it plays a role in the maturation of sensory organs [61]. However, literature reports that there is a downregulation of miR-183 cluster in human BCSCs which suggests that this downregulation is responsible to maintain the stem cell property of cancer cells [42]. This miR-183 cluster consists of miRNA-183,-96,-182 bearing a homology in their sequence and is mainly located on chromosome 7 in human and on chromosome 6 in mouse [62]. The suppression of miR-183 cluster in the human BCSCs drives the EMT activation and self-renewal property targeting the Wnt signalling [42].

miR-34c

miR-34c is basically a tumor suppressor, and it is reported to have a reduced expression in human breast cancer cell lines such as MCF-7 and SK-third which are enriched for BCSCs. Hypermethylation occurs in the promoter sites of BCSCs which leads to the downregulation of miR-34c, thereby leading to the increment of EMT and stemness property of the breast cancer cells. In fact, when this miR-34c is expressed in the BCSCs ectopically, it leads to the inhibition of EMT and stemness property, and also the mammosphere formation was also reduced. Migration was also reported to be hindered, which further strengthened the idea that miR-34c can be a positive target for BCSCs [41].

miR-181

The level of miR-181 in different breast cancer cell lines such as MDA361, MCF-7, and BT474 was found to be high in the mammospheres of tumor origin rather than those which are non-tumorigenic in origin. miR-181 targets the TGF-β and plays a crucial role in the regulation of BCSC and helps in the formation of mammospheres [41].

miR-128

In BCSCs, both isolated from breast cancer patients and the breast cancer cell lines like SK-third and MCF-7, the level of miR-128 is reported to be reduced, and this reduction of miR-128 increases the expression of its target genes, Bmi-1 and ABCC5 (ATP-binding cassette subfamily C member 5) [41]. In fact, it is noted that when miR-128 is expressed ectopically, it leads to the decrease in the Bmi-1 and ABCC5 levels in BCSCs, thereby depicting the therapeutic potential of miRNA. There are many literatures in support of this fact that forced or ectopic expression of miR-128 leads to the reduction of tumor growth and induces apoptosis in vivo and decreases the mammosphere size in in vitro cell culture model, respectively [41, 63].

miR-495

The upregulation and/or downregulation of different miRNAs are involved in the BCSC regulation, it was reported that miR-495 is upregulated, thereby predicting a significant role of its involvement in BCSC regulation. To predict its role, literatures suggest that the overexpression of miR-495 led to the enhanced colony formation in vitro and also reduced the tumor forming potential in vivo in human BCSCs. The upregulation of miR-495 targets those genes which downregulates the genes involved in EMT like E-cadherin and REDD1 (short for regulated in development and DNA damage responses) and maintains the stemness property in breast cancer [41].

5.4 Signalling Pathways Involved in miRNA-Mediated Regulation of BCSCs

Till now, the crucial role of various miRNAs associated with BCSCs was discussed where in some cases, the miRNAs enhanced the tumorigenic potential while in some cases, they acted as a tumor suppressor. Now, the target genes and their signalling pathways which are regulated by various miRNAs which are discussed below.

5.4.1 Notch Signalling Pathway

The Notch signalling pathway plays a significant role in self-renewal and apoptosis of BCSCs. It is well involved in the cell fate regulation in the development of mammary gland and shows a strong association with tumor initiation and proliferation. The Notch receptors bind to different Notch ligands like the Delta-like

1 (DLL1), Delta-like 3 (DLL3), and Delta-like 4 (DLL4), Jagged1 (JAG1), and Jagged2 (JAG2) and activate the Notch signalling. After the activation, the intracellular domain of Notch localizes in the nucleus of the cell, thereby activating its target gene expression which includes the cell cycle progression genes like cyclin D1 and p21 [64]. It is reported that if the Notch pathway is inhibited, it reduces the number of BCSCs significantly and also the metastasis of breast cancer to brain [65]. Literatures report that miR-34a helps in the downregulation of the Notch1 receptor and inhibits the stemness property in breast cancer as noticed in MCF-7 cells. miR-200 inhibits the Notch signalling by means of targeting the Notch ligands like JAG1 and other important co-activators, MamI2 and MamI3 [42, 65]. miR-9 and miR-34c expression when induced leads to the suppression of the Notch signalling, and it helps in the reduction of the metastatic potential of the triple-negative breast cancer cells (TNBC) [66]. Upregulation of miR-146a in human BCSC is also associated with the suppression of the Notch signalling, thereby proving the fact that Notch signalling is one of the most essential signalling pathways involved in the regulation of human BCSC [42].

5.4.2 WNT Signalling Pathway

Wnt signalling (canonical and noncanonical) plays a crucial regulatory role in the cell proliferation, differentiation, migration, adhesion, and the renewal of stem cells [67]. The development of the mammary glands is governed by the Wnt signalling pathway, and simultaneously, it also plays a role in the regulation of the differentiation of stem cells. It also plays a role in stabilization of the quantity of the BCSCs [68]. There are several miRNAs which are involved in targeting the Wnt signalling pathway and disrupt its signalling by targeting APC such as miRNAs like miR-125, miR-135, miR-129, miR-27, miR-663, miR-142, let-7, miR-155, and miR-106b. When TNBC is taken into consideration, it is reported that miR-29b expression is correlated negatively to the BCSC potential and it inhibits the proliferation, stemness, and invasion of the TNBC cells by downregulating the Wnt signalling pathway [42]. The miR-29 targets Dikkopf-1 (Dkk1), secreted frizzled-related protein 2 (sFRP2), and Kremen2, which are the negative regulators of Wnt signalling [65]. Let-7, another important family of miRNAs, has a downregulated expression in the BCSCs and targets the Ras oncogene and MYC proto-oncogene, thereby having a feedback effect on LIN28 gene expression, which is known to be the downstream effector gene in the Wnt pathway, hence affecting the self-renewal process [65]. The human breast CSC also expresses miR-142, and it targets APC and it activates the canonical Wnt signalling pathway [42]. Another miRNA, miR-150, though specifically expressed in the mature lymphocytes [69], is also identified to be expressed highly in the breast CSCs. The upregulation of miR-142 is linked with the higher expression of miR-150 as the Wnt signalling is activated by the upregulation of miR-142 which results in the miR-150 expression [42]. The differentiation of BCSCs is enhanced by another family of miRNA, miR-600, involving the modification of Wnt pathway proteins, further inhibiting the Wnt signalling [70]. miR-146

has been predicted to be one of the promising diagnostic markers of human breast CSCs following the Wnt pathway which is stabilized by miR-146a by the help of Snail and β-catenin [42]. miR-200 family which is downregulated in the human BCSCs also functions as the suppressor of the Wnt signalling pathway by targeting β-catenin [42].

5.4.3 PI3K/AKT Signalling Pathway

miR-221/222 as mentioned earlier is one of the miRNAs responsible for the increase of BCSCs. It suppresses the PTEN (phosphatase and tensin homolog) protein and thereby inhibits the phosphorylation of AKT and thus enhances the cell stemness. miR-99a and miR-30a are also responsible for targeting the PTEN-PI3K/AKT signalling pathway in BCSCs [65]. Another miRNA, miR-595, promoted the breast cancer progression following the PI3K/AKT signalling pathway [43]. So, these miRNAs can be believed to be the potential target of the PTEN-PI3K/AKT signalling pathway involved in the human BCSCs (Fig. 5.2).

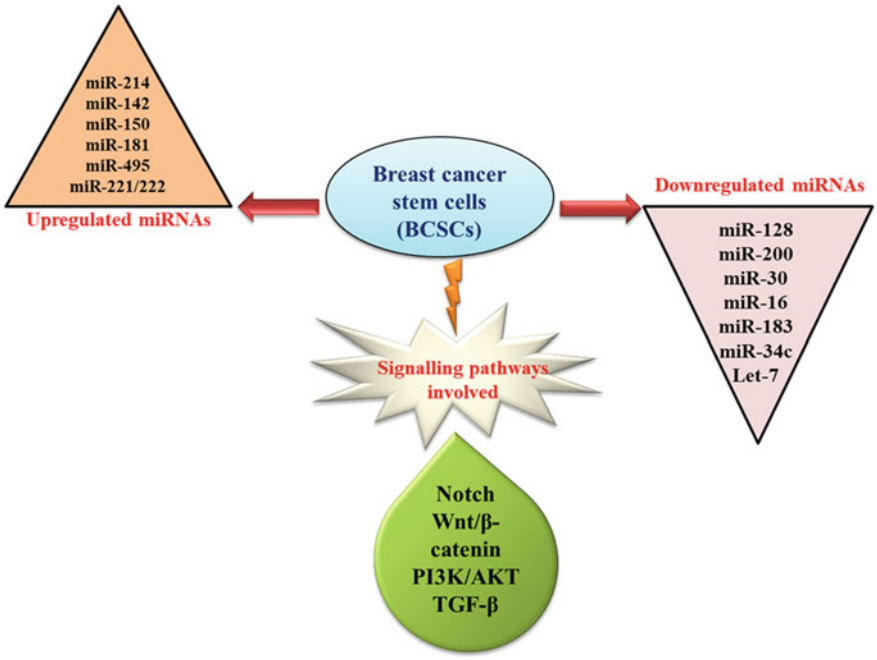

Fig. 5.2 Various miRNAs and their signalling pathways involved in the regulation of breast cancer stem cell (BCSC) markers

5.5 miRNAs as Cancer Therapeutics

As discussed previously in this chapter, that the miRNAs are a potential therapeutic target in treating cancer, it is noted that there are numerous small RNA-based drugs which have undergone clinical trials, one of which is fomivirsen. Fomivirsen, an example of an RNA-based FDA-approved drug, was used to treat cytomegalovirus retinitis [110]. Similarly, MRX34, a drug designed to mimic the tumor suppressor miRNA, miR-34, was discovered [111]. Mimic miR which stimulates the miR and/or the antagomir is a new measure of immense potential to fight cancer. There are certain chemically available molecules, for example, 2′-O-methoxyethyl oligonucleotides, peptide nucleic acids, and locked nucleic acids, which protect the in vivo action of the mimic miR [111]. In vivo delivery of the miRNAs seems to be a difficult task, and therefore, liposomes and synthetic polymers such as nanoparticles made up of biodegradable polymers of chitosan and polylactate-co-glycolate came into use [112]. Another method of miR delivery is the use of dendrimers, which are very toxic delivery vehicles having positive charge, thus enabling cell lysis and disability [38]. Exosome-mediated delivery of miRNA has been evidenced to be more effective than the other methods of delivery. miRNA let-7a is successfully delivered to breast cancer expressing EGFR via exosomes with GE11 peptide or EGF [113]. Exosomal delivery of miRNA lessens the chances of immunogenic responses in patients and is highly efficient. Though there is progress in the field of miRNA therapeutics, still there is an enormous scope for improvement. For better prognosis of breast cancer, the CSC-targeting therapy needs to be more polished, and it should be seen that these drugs are particularly targeting the BCSCs rather than the normal stem cells. This budding miRNA therapeutics approach to treat cancer will lead to immense positive effect in eradicating cancer progression and metastasis in the near future.

5.6 miRNA Resistance to Chemotherapy

The study of miRNAs and their strong association with the CSCs discussed so far thus throws light into the fact that they can be a potential therapeutic target helpful to diagnose cancer. Targeting the miRNAs can help put an end to the CSC self-renewal capacity and anti-apoptosis, thereby essentially improving the resistance against tumorigenesis. The major obstacle in the treatment of breast cancer is its resistance to chemotherapy, and the main responsible factor is the BCSCs. miRNAs play a crucial role in the regulation of the BCSCs; therefore, they generate chemoresistance against breast cancer. For example, the downregulation of BMI1 in breast cancer cells leads to the reduction of the CD44+/CD24- cells in the BCSC population by means of miR-200c. This induces apoptosis and also increases the sensitivity of breast cancer cells to 5-fluorouracil [114]. The proliferation of BCSCs is suppressed by the chemosensitivity to paclitaxel (PTX), an anticancer chemotherapeutic drug with the overexpression of miR-34a, targeting the Notch pathway [43]. MiR-125b which induces the activation of the Akt signalling enhances the sensitivity toward

letrozole and facilitates the letrozole resistance. Chemotherapeutic resistance toward doxorubicin which leads to enhanced cell viability and anti-apoptosis is governed by the downregulation of miR-128 [43, 65]. Similarly, the overexpression of miR-16 creates resistance to doxorubicin in MCF-7 cells [43, 65]. The BCSCs exhibit a high expression of ALDH1, and based on this idea, when a cohort of breast cancer samples were treated with paclitaxel and epirubicin-based chemotherapy sequentially, it was found out that those expressing high ALDH1 expression showed low response pathologically and resistance to chemotherapy [115]. The BCSCs are believed to be the essential drivers of breast cancer progression and invasion, while the miRNAs are the critical regulators of BCSCs. Out of the variety of miRNAs, some behave as onco-miRNAs such as miR-146a, miR-125, miR-526b, miR-106b-25, miR-888, and miR-22, while some as tumor suppressive miRNAs (anti-onco-miRNAs) such as miR-99a, miR-200, miR-34, miR-140, miR-16, miR-7, and miR-93. Those miRNAs behaving as oncogenes can be inhibited, while those behaving as tumor suppressors can be enhanced to inhibit breast cancer [43].

5.7 Future Direction

At present, scientists have paid their attention to miRNAs for their use as diagnosis and prognosis markers for cancer. Targeting CSCs is still a huge challenging task to the cancer researchers. Thus, the researchers are now inclined to focus on those miRNAs which especially regulate the function of CSCs. It is more important to identify those miRNAs that are dysregulated in CSCs and also play a vital role in cancer metastasis, therapy resistance, and tumor recurrence. For example, miRNAs like miR-200c, miR-34c, miR-214, and miR-21 regulate the stemness property, metastasis, and therapy resistance. Some miRNAs like miR-214 show dual nature in tumorigenesis depending on tissue types. Similarly, miR-200c prevents EMT of cancer cells; however, the role of this miRNA in cell proliferation is also context dependent. Thus, we should identify a set of miRNAs which are dysregulated in CSCs, instead of a particular one or two miRNAs. The change of a set of miRNAs profile will definitely give a better prediction for diagnosis and/or prognosis of the diseases.

Based on this miRNA profile and their functional activity, a set of specific miRNAs can be targeted together by antagomir and/or mimic miRNA for enhancing the anticancer potential. In addition, specific drug which targets the key signalling of CSCs may be combined with antagomir/mimic miRNA to improve further treatment efficacy.

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Skin Stem Cells in Cancer

6

Monica Piccione and Rosa Di Liddo

Abstract

Stem cells (SCs) are responsible for maintaining and regenerating tissues and show unique defining characteristics, including self-renewal, asymmetrical cell division, low proliferation rate, and clonogenic potential. Niches of epidermal SCs have been identified in the bulge of hair follicles, the basal layer of the interfollicular epidermis, and the base of sebaceous glands. Accumulating evidence suggests that multipotent bulge cells generate hair follicles under physiological conditions and regenerate the epidermis and sebaceous glands in response to skin injury. In contrast, SCs of the interfollicular epidermis and sebaceous glands are lineage specific and generate their respective tissues without recruiting SCs from the bulge compartment. Cancer stem cells (CSCs) represent a class of tumor cells exhibiting stem cell-like properties and ability to initiate tumors. They are derived from SCs or from non-stem cells that acquire self-renewal potential. Likely SCs, CSCs express regulatory factors of self-renewal, such as SOX2, MYC, and OCT4, and some common "stemness" pathways, such as Wnt signaling. In contrast, they could not be multipotent and lead to single lineage tumors, such as squamous cell carcinoma (SCC) (epidermal lineage), various follicular tumor types (hair follicle lineage), and sebaceous gland tumors (sebaceous lineage). Currently, several studies on CSC biology have been performed to develop new targeted therapies for patients with skin tumors with poor prognoses.

Keywords

Skin · Stem cells · Skin cancers · Melanoma skin cancer · Non-melanoma skin cancer · Interfollicular epidermis · Cancer stem cells

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6.1 Introduction

In the last decade, excellent and pioneering studies have been performed to characterize the role of epidermal and dermal stem cells (SCs) in melanoma and non-melanoma skin cancers (NMSCs), including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). The epidermis is a stratified squamous epithelium that is continuously replenished by heterogeneous populations of tissue-resident stem cells in *follicular region* (HF), which is the epidermis enriched of pilosebaceous units, and *interfollicular compartment* (IFE) [1]. HF and IFE have been demonstrated to be differently involved into tissue homeostasis or restoration of wounded skin. HF bulge stem cells give rise to transit-amplifying (TA) cells, that are committed progenitors that remain undifferentiated, subsequently dividing to expand the progenitor cell population while sparing continued SC division. These multipotent slow-cycling and label-retaining cells (LRCs) contribute to the anagen phase of the hair growth and to the repair/regeneration of damages. In contrast, IFE exerts an active role during routine epidermal cell renewal but not under injury [2]. A balance of proliferation and differentiation between stem and progenitors guarantees a normal epidermal cell turnover every 2–4 weeks [3]. The disruption of it is reported as a hallmark of skin cancer.

6.2 Epidermal Stem Cells (ESCs)

ESCs are present in the hair follicle bulge, the basal layer of interfollicular epidermis, and the base of sebaceous glands [4]. Like stem cells of other tissues, they play a central role in homeostasis and wound repair, while under pathological conditions, they exert a key role in tumor initiation. According to the most universally accepted criteria, keratinocyte stem cells are slow or rarely cycling and showing self-renewal potential, proliferative activity, and ability to preserve skin integrity. Based on long-term labeling of skin cells with a DNA precursor such as [^{3}H]Thymidine or bromodeoxyuridine (BrdU), the slow-cycling stem cells have been identified as label-retaining cells (LRCs) [1]. According to Watt and co-workers [5], stem cells within interfollicular epidermis express integrin β1, have high clonogenic potentiality, and are more vulnerable to environmental insults than basal cells present at the bottom of the deep rete ridges [6]. In contrast, Kaur and co-workers [7] report that α6 integrin is more specific for keratinocyte basal cells and is a useful marker to purify stem cells from transit-amplifying cells (TAC), which rapidly amplify the pool of differentiated cells produced at each stem cell division [3]. Currently, it is not known whether each TAC is committed to differentiate along one specific lineage or various lineages. Under normal conditions, SCs from interfollicular epidermis and sebaceous glands are lineage specific and generate their respective tissues without recruiting bulge stem cells. Besides generating hair follicles, the follicular SCs from hair bulge regenerate the epidermis and sebaceous glands in response to skin injury [4]. A requirement for the survival of stem cells is that the quiescent state is

strictly controlled by signaling cascades, including sonic hedgehog (SHH) and Wnt pathway.

Under resting conditions, SHH ligand binds to its membrane-spanning receptor, called Patched (Ptch), inhibiting Smoothened (Smo), a transmembrane protein. SHH is an important regulator of HF bulge stem cells and dermal papilla [8]. Mutations in human Ptch are implicated in the basal cell nevus syndrome, which is a dominant autosomal condition characterized by a complex set of developmental defects and high incidence of basal cell carcinomas [9].

Wnt ligands are secreted glycoproteins that bind to Frizzled receptors for triggering the displacement of GSK-3β from the APC/Axin/GSK-3β complex, leading to β-catenin stabilization, Rac1 nuclear translocation, and recruitment of LEF/TCF DNA-binding factors [10]. Wnt signaling controls the commitment of HF bulge cells in mouse to maintain the stem state or permit entry into a differentiation pathway. Notch and epidermal growth factor receptor (EGFR) signaling contribute to the development of IF epidermis. In particular, Notch signaling is active within epidermal basal layer and regulates the differentiation process of keratinocytes [11]. Several miRNAs, which are small (~19–24 nucleotides) noncoding RNAs, are demonstrated to exert specific functions in skin. For instance, miR-203 is expressed at high levels only in the suprabasal epidermis or the inner root sheath of the HF, but not in progenitor/stem cells of HF epidermis, in both HF bulge and HF matrix [12]. Using transgenic mice, it has been demonstrated that the primary role of miR-203 in the epidermal basal layer is related to the differentiation of the transit-amplifying cells or to limit their proliferative life span. Interestingly, miR-203 has been shown to repress the expression of tumor protein p63 (p63), a putative stem cell marker of epidermal keratinocytes, promoting cell cycle arrest during the transition of cells to suprabasal layers [12] through the transcriptional regulation of Dicer and miR-130b [13]. Moreover, knocking down miR-125b in transgenic mice results into the alteration of stem cell differentiation provoking enlarged sebaceous glands, thickened epidermis, and lacking of hair coat [14]. Potential targets of miR-125b include vitamin D receptor (VDR) [14].

6.2.1 Hair Follicle Stem Cells

The hair follicle is a structure that projects down into the dermis and undergoes intermittent cycles of growth, regression, and quiescence [15]. During each cycle of growth, the follicle is generated from stem cells that are located in the hair bulge, which is a not well recognizable structure in human. The human hair follicle cycle takes about a decade and expresses CD200 (cluster of differentiation 200), a type I membrane glycoprotein containing two immunoglobulin domains [16]. In contrast, keratin 15 (K15) is a reliable marker in mouse bulge cells, but not in human [17]. Two different bulge K15-positive stem cell subpopulations have been identified in human epidermis: CD200+/CD34−/K15bri (basal), forming larger clonal growth colonies, and CD200+/CD34−/K15dim (suprabasal) [18]. CD34 (cluster of differentiation 34), a single-pass transmembrane sialomucin protein, has

been not identified in human bulge tissue [19], but only in murine follicles, wherein CD34+ cells are label retaining and clonogenic [20]. Keratin 19 (K19) and K15 promoter are also detected in mouse LRCs [21]. The region between the bulge and the IF epidermis in murine epidermis also contains putative stem cells that are MTS24+/Lrig1+/α6dim cells and highly clonogenic [22]. Sca-1+ cells are present in the infundibulum [23] and repopulate the IF epidermis. Lrig1+ cells are quiescent in the junctional zone [24] and are demonstrated in reconstitution assays to give rise to all epidermal cell lineages [24].

6.2.2 Sebaceous Gland (SG) Stem Cells

SG is an important structure of epidermis that is produced in mouse from stem cells located at HF above the CD34+/K15+ bulge region and expressing LGR6 [25]. This population is multipotent and is able to replace epidermis (HF and IF) and SG structures in mouse [25]. However, they are not label retaining and can renew the sebaceous gland and sebaceous gland stem cells Blimp-1 positive [26]. The bulge area also contains melanocyte stem cells and stem cells with neural crest properties [27].

6.2.3 Other Epidermal Stem Cells

Bone marrow-derived stem cells (BMSCs) are found in the epidermis during epidermal regeneration. Several studies suggest that BMSCs are able to derive in vivo from both dermal and epidermal cells [28] and, only under ex vivo settings, fuse with keratinocytes [29].

6.3 Skin Cancer Stem Cells

Cancer stem cells (CSCs) are tumor cells exhibiting self-renewal potential, stem cell-like properties, and abilities to initiate tumors. To date, CSCs from skin are hypothesized to originate from immature compartments [30] or from reprogrammed differentiated cells [31]. Genic alterations in hair follicle bulge potentially promote the development of tumors representing lineages of epidermis, hair follicles, and sebaceous glands, while lineage-committed mutant stem cells generate only tumors from that lineage. For instance, interfollicular SCs typically generate squamous cell carcinomas (SCCs), while transit-amplifying cells of the hair follicles and mutant sebaceous gland SCs promote sebaceous tumors and cause hair follicle tumors [32]. The fate of CSCs is reported to be strictly controlled by stromal niche signals and cell-cell interactions with immune cells, cancer-associated fibroblasts, and endothelial cells [33]. The niche has been recently defined as a stromal template that acts not only to maintain the “stemness” grade of skin [34] but also for the epithelial organ maintenance and regeneration through the simultaneous release of

proliferative and differentiative cues [35]. Hedgehog signaling pathway is known to be active in stromal niches and specifies the expression of secreted chemical signals. Notably, mutations leading to cell-autonomous activation of the Hedgehog pathway in primary tumor cells drive the development of basal cell carcinoma (BCC) [36]. In squamous cell carcinoma (SCC), distinct CSC populations coexist, and their tumor initiation and metastatic potential may be uncoupled. Cancer cells become hyperproliferative [37] due to the stimulation of niche factors, such as transforming growth factor-β (TGFβ) [38] and vascular endothelial growth factor (VEGF) [39], or due to an upregulated expression of SOX2 [40] or mutations in *Kras* and *Smad4* genes [41]. White et al. [41] demonstrated that CSCs are rare in primary SCCs, but their number dramatically increases in metastatic SCCs or tumors showing epithelial to mesenchymal transition. Depending on tumor type, cancer stem cells express putative cell surface markers that can be either shared with or distinct from normal stem cells (Table 6.1), such as CD34, a cell surface marker of bulge SCs or SCCs [53].

Table 6.1 Skin stem cell markers

Structural proteins	Epidermal stem cells (basal layer)		Epidermal stem cells (hair follicle bulge)		Mesenchymal stem cells (dermis)	
K5	+	Fuchs [42]	+	Bose and Shenoy [43]	–	
K14	+	Fuchs [42]	+	Bose and Shenoy [43]	–	
K15	–		+	Bose and Shenoy [43]	+	Forni et al. [44]
E-cadherin	+	Jamora et al. [45]	+	Jamora et al. [45]	–	
LGR5	–		+	Kretzschmar and Watt [46]	–	
LGR6	–		+	Snippert et al. [25]	–	
CD29	+	Martin et al. [47]	+	Inoue et al. [18]	–	
CD34	+	Kretzschmar and Watt [46]	+	Jang et al. [48]	+	Wong et al. [49]
CD49f	+	Yang et al. [50]	+	Jang et al. [48]	–	
CD44	–		–		+	Klimczak and Kozlowska [51]
CD73	–		–		+	Klimczak and Kozlowska [51]
CD90	–		–		+	Forni et al. [44]
CD105	–		–		+	Forni et al. [44]
CD117	–		+	Jang et al. [48]	+	Kang et al. [52]
CD200	–		+	Jang et al. [48]	–	
CD271	–		+	Inoue et al. [18]	+	Klimczak and Kozlowska [51]

6.3.1 Skin Cancer Niches

The epidermis renewal under physiological conditions is commonly attributed to a single type of stem cells. When external insults, genic mutations, impairment of immune system, and skin barrier occur (Fig. 6.1), the proliferation and differentiation processes could be affected and skin cancers are developed. Both BCC and SCC are reported to arise from a self-renewing cancer-initiating cell (CIC), a stem or progenitor cell that, showing innate self-renewal, is primed by minor genetic alterations for transformation into a self-renewing cancer SC (CSC).

Besides HF and IFE SCs, more differentiated cell populations can undergo malignant transformation with a "progenitor-like" signature. In the epidermis, benign tumors, rather than carcinomas, readily form in response to oncogenic mutations arising in committed cells. Malignant conversion of skin papillomas originating from differentiated cells to carcinomas is a rare event that is characterized by the altered expression of markers, such as TGF-β, Keratin-13, and α6β4 integrin [54]. Benign tumors showing high risk of malignant conversion are primarily derived from cells located within HF, although the nature of CICs remains the major determinant of malignant potential. Research findings have reported the implication of *Grainyhead-like 3* (*Grhl3*) gene in the formation of SCC from differentiated cells. Grhl3 is a transcription factor that is involved in the expression of structural proteins and lipid metabolizing enzymes (e.g., Transglutaminase 1; TGase1) related to epidermal barrier formation and terminal differentiation [55]. Grhl3 deficiency has been demonstrated in mice to cause the loss of TGase1 expression, epidermal acidification, altered organization of stratum corneum, pup dehydration, and death immediately after birth [56]. Moreover, the induction of epithelial-to-mesenchymal transition (EMT) is also observed in differentiated suprabasal epithelial cells. Interestingly, the regression of epidermal barrier does

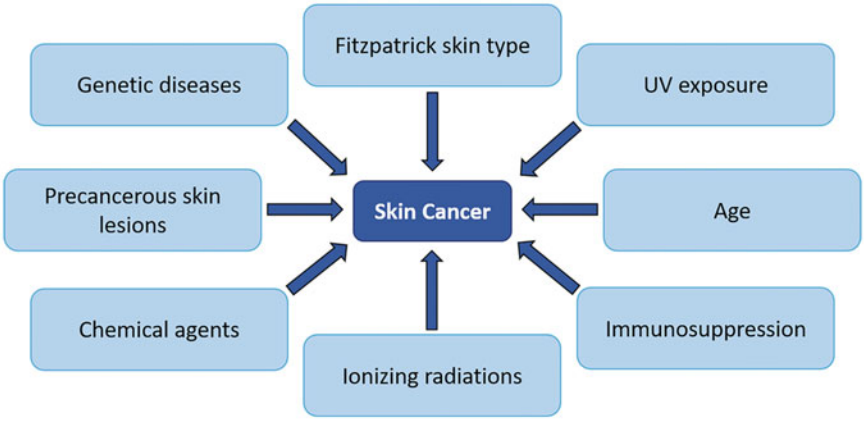

Fig. 6.1 Inherited and environmental factors related to the onset of melanoma and non-melanoma skin cancer

not occur in adults when Grhl3 is deleted, suggesting that, although Grhl3 is essential for barrier establishment, it is not required for maintaining epithelium integrity [57].

6.3.2 Plasticity and Heterogeneity of Skin Cancer Cells

CSCs display functional heterogeneity and ability to rapidly respond to environmental cues acquiring specific genic and immunophenotypic profile. Because of their "dynamic stemness," SCC tumors demonstrate to contain multiple CSC populations with a proliferative (Side Population/SP) or migratory phenotype (CD34+/CD49f+). Although both SP and CD34+/CD49f+ populations are tumorigenic, only tumors originated from SP cells undergo EMT and metastasize [41]. A substantial amount of literature recognizes among CSCs a great heterogeneity for the expression of CD44 glycoprotein, metabolites, and growth factors impacting on the effectiveness of antitumoral drugs. In the past few years, the development of non-melanoma skin cancers has been reported as dependent on a "bottom-up" and "top-down" mechanism of tumorigenesis. The "bottom-up" model involves a cancer stem cell arising in the basal epidermis, which houses progenitor cells contributing to wound healing and normal cell turnover of overlying epidermal layers. The "top-down" concept involves a more differentiated cell that undergoes genetic modifications and dedifferentiates to CICs. BCC derives from pluripotent keratinocytes presumably located in the basal layer of the hair follicles, while SCC seems to originate from keratinocytes possibly located in the suprabasal layers of the epidermis. UV radiation suppresses the immune response in the skin, starting the malignant transformation of epidermal cells. Upon light exposure, keratinocytes upregulate the expression of RANK ligand, a receptor activator of NF-kB ligand, that, in turn, induces the proliferation of immunosuppressor T-cells (Tregs). UV light is known to induce DNA breaks in exposed cells. Most DNA breaks or local DNA damages are repaired by the *p53* tumor suppressor gene, which is regarded as the "guardian of the genome." When DNA damage is not restored, keratinocytes undergo apoptosis activated by p53 protein and BCL2 family proteins. Under pathological conditions, some individuals show a lower DNA repair capacity and thus develop precancerous skin lesions leading to malignant transformation. Unfortunately, *p53* gene could be itself mutated by UV irradiation promoting an uncontrolled cell proliferation and loss of apoptosis in mutated cells. Loss-of-function mutation of p53 has been detected in about 56% of BCC and in >90% of SCC [58].

6.3.3 Molecular Profiling of Skin Cancer Stem Cells

According to cancer stem cell theory, tumor stem cells are slow cycling and not impacted by anticancer agents [59]. Mutations in rarely dividing long-lived stem cells lead to the accumulation of genetic changes that overcome cell control and lead to cancer growth. To date, the molecular profile of tumor stem cells is still lacking

although accumulating body of evidence suggests that the tumor-initiating cells express CD133, and the cancer stem cells located along the dermal/tumor interface are integrin positive and CD34bright/dim [60]. Other protein markers identify putative cancer stem cells, such as Aldehyde dehydrogenase (ALDH) in melanoma cells [61] and CD44 (hyaluronic acid receptor) in squamous cell cancer stem cells [62]. Negative expression of CD24 is observed in postmitotic human keratinocytes [63]. The marker protein profile observed in BCC is consistent with a hair follicle origin of the tumors [64]. Moreover, the level of LGR5, an HF stem cell marker, is increased in most BCCs [65] (Table 6.2).

Table 6.2 Relevant markers in melanoma and non-melanoma cancer stem cells (CSCs)

	Melanoma stem cells (Me-SCs)		Basal cell carcinoma stem cells (BCC-SCs)		Squamous cell carcinoma stem cells (SCC-SCs)	
Molecular markers	NANOG	Perego et al. [66]	K14	Peterson et al. [67]	TGFβ	Schober and Fuchs [68]
	OCT3	Perego et al. [66]	K15	Sellheyer [64]	MYC	Jian et al. [69]
	OCT4	Perego et al. [66]	K17	Peterson et al. [67]	OCT4	Kim et al. [70]
	CD20	Lang et al. [71]	K19	Al-Garf et al. [72]	CD34	Trempus et al. [20]
	CD133	Roudi et al. [61]	CD29	Sellheyer [64]	CD44	Lapouge et al. [73]
	CD271	Civenni et al. [74]	CD200	Peterson et al. [67]	CD49f	White et al. [41]
	Wnt	Katoh [75]	P63	Al-Garf et al. [72]	CD133	Olivero et al. [76]
	Notch	Venkatesh et al. [77]	LGR5	Jang et al. [48]	CD200	Stumpfova et al. [78]
	SHH	Kumar et al. [79]	LGR6	Zhang et al. [14]	Wnt	Jian et al. [69]
	ALDH1	Roudi et al. [61]	SHH	Callahan et al. [80]	SOX2	Boumahdi et al. [81]
	SOX2	Santini et al. [82]	PDGF	Sellheyer [64]		
			SOX9	Sellheyer [64]		
			Tenascin-C	Sellheyer [64]		
			Bmi-1	Sellheyer [64]		
			Follistatin	Sellheyer [64]		

6.4 Skin Cancers and Immunosuppression

Immunosuppression exerts a pivotal role in skin carcinogenesis [83]. NMSCs are frequently infiltrated by immune cells, but the immune system often shows to be unable to eradicate the tumor. In SCC, the local tumor-specific immunity is compromised by the downregulation of E-selectin, recruited regulatory T cells, and malfunctioning intratumoral myeloid dendritic cells. In BCC, the absence or downregulation of MHC-I and the presence of regulatory T cells are observed. Due to the heterogeneous expression of class I HLA proteins in SCC, immunosuppression increases the SCC risk 65-fold, while BCC risk only tenfold [84]. Yesantharao et al. [85] proposed SCC cancer cells have an abnormal membrane expression of HLA-G protein in immunosuppressed patients allowing tumoral cells to negatively regulate Natural Killer- and T lymphocyte-mediated destruction and cytotoxic response, as already demonstrated in other cancers (melanoma, breast, colon, lung, and renal). In addition, suppressive effects on skin immunity have been reported by UV irradiation. Indeed, it has been reported that UV radiation-induced photolesions such as cyclobutane pyrimidine dimers (CPDs) affect immune system, inhibiting mast cells, cytotoxic T cells, and memory T cells and activating regulatory B lymphocytes, T lymphocytes, and natural killer cells [86]. Furthermore, molecules with immunosuppressive properties such as IL-10, prostaglandins, platelet-activating factor, and ROS are also stimulated. In addition, UV radiation also affects Langerhans cells (LC), lowering the number of LC in the skin promoting LC migration to the draining lymph nodes [87].

6.5 Concluding Remarks

Skin tumors are the most common malignancy worldwide. Current therapies based on chemotherapeutic agents, or surgical methods, are not specific and limited by high economic burden and uncontrolled side effects. To date, the univocal molecular signature of the cell of origin (Table 6.2) is the main goal for the development of targeted therapies for more effective treatment of melanoma and non-melanoma skin cancers.

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Ocular Cancer Stem Cells: Advances in Therapeutic Interventions

7

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Abstract

Cancers affecting the eye are rare and can be either initiated in the eye, primary intraocular cancers, or invaded into the eye as a malignant tumor started elsewhere, secondary intraocular cancers. Melanoma and non-Hodgkin lymphoma in adults and retinoblastoma and medulloepithelioma in children are the most common intraocular cancers. Similar to other cancers, cancer stem cells are reported among retinoblastoma, lymphoma, and melanomas that can be malignant even though are very rare in occurrence. Here, we explore the cancers of the eye and cancer stem cells with the perspective of advanced therapeutic applications for vision and globe salvage.

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Keywords

Cancer stem cells · Ocular tumors · Eye cancer metastasis · Retinoblastoma · Ocular melanoma

7.1 Introduction

Despite the current advancements in cancer treatment, the recurrence and metastasis of tumor at site or at distant site is prevalent. The available treatment options besides eliminating the cancer cells also target the normal healthy cells, resulting in the tissue damage and recurrence of the tumor due to residual cancer cells. Similar to the stem cell population, existence of cancer stem cell (CSC) subpopulation initiating the tumor and driving its proliferation are widely reported. Like normal stem cells, CSCs have the ability to self-renew, preserve the undifferentiated stem cells, and regulate their quantity, generate a range of tumor cells at different stages of differentiation. Within the tumors, the CSC can be characterized using specific markers and differentiated from those of normal stem cell population. These CSC subpopulations exhibit similar pluripotency and proliferation characteristics mimicking normal stem cells [1]. The current approach to chemotherapy demands a strategy inclined toward targeting specifically the CSC population to prevent the recurrence of the tumor. The CSC differentiates into different tumor components through the stemness pathways that control many important biological processes. In CSC these stemness pathways are not strictly regulated resulting in differentiation of various tumor components [2, 3].

This book chapter outlines ocular stem cells and cancer stem cells emphasizing on marker characterization with genetic mutations affecting cancer stem cells, their regulation via various signaling pathways, and resistance to chemotherapy.

7.2 Stem Cells

Stem cells are ascribed for their extensive self-renewal, differentiation, and clonally regeneration properties within tissues they inhabit [4, 5]. Stem cells undergo repeated divisions producing undifferentiated stem cell and differentiated progenitor cells with not all stem cells having infinite self-renewal potential (see [6]). Like stem cells from trabecular meshwork, orbital and sclera whose regeneration potential is not completely understood experimentally. On the other hand, limbal, corneal, conjunctival, and retinal stem cells have been exploited for their application in regeneration and treating degenerative disorders in animal and human clinical trials [1]. Limbal stem cells are widely recognized for their repair and regeneration of cornea-related diseases [7].

The repair and regeneration process of stem cells involves replenishing the lost cells with healthy regenerated cells. The injury caused at the site reduces stem cell population resulting in many diseases associated to its deficiency [8]. Stem cell population involves many intricate interactions of cytokines and growth factors regulating modulation of fibroblasts and epithelial cells.

7.3 Cancer Stem Cells (CSCs)

A tumor is an abnormal indefinite growing mass of cells. The rapid indefinite proliferating ability of cancer stem cells through accumulation of mutations leads to tumor development [9]. CSCs exhibit similarities of normal stem cells with regard to ability to proliferate, self-renew, and trigger epithelial to mesenchymal transition, giving rise to differentiated cells. Similar to normal cells, CSCs also undergo aberrant differentiation due to continuous accumulation of mutation leading to heterogeneity of cells. The heterogeneity also arises through the clonal origin with diverse phenotypic expression of tumorigenic cells. The phenotypically variable expression of tumorigenic cells with CSCs to possess indefinite as well as limited or no proliferative potential explains the self-renewal and differentiation properties, respectively, as retained by the normal stem cells [10]. The occurrence of such combined subpopulation of CSCs in a tumor makes tumor targeting difficult and thus leaving the subpopulations of CSC's undisturbed during chemotherapy sessions. Thus the CSC subpopulation retained would maintain and reinitiate the tumor growth exhibiting metastasis to distant areas and attaining resistance to chemotherapy [11] (Table 7.1).

7.4 The Types of Eye Cancer

In this chapter, the types of ocular cancer shall be broadly categorized into the following:

1. Eyelid tumors.
2. Conjunctival tumors.
3. Corneal tumors.
4. Orbital tumors.
5. Intraocular tumors.

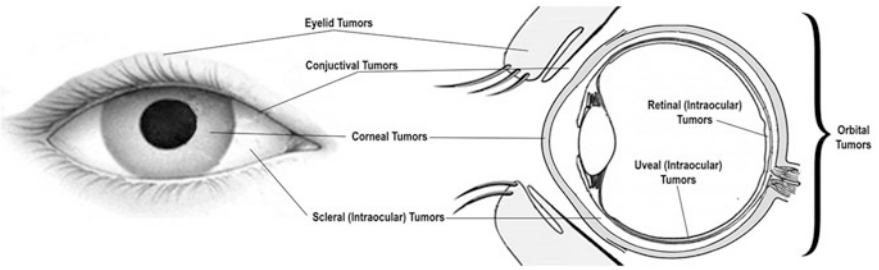

Table 7.1 Markers of ocular stem cell and cancer stem cell of the eye

Ocular cancers (name the cancer and CSC associated)	Stem cell markers	Differentiation markers
Cornea (limbus and stroma)	Limbus: ABCG2 (ATP binding cassette subfamily G member 2) [12], α-enolase, cytokeratin (CK) 19, Musashi-1, vimentin [13]	CK 3/12, connexin 43, and involucrin [14]
	Stroma: ABCG2, Bmi 1, CD166, C-kit, Pax6, Six2, and notch 1	Upon differentiation, stromal stem cells expressed keratocan, ALDH3A1, CXADR, PTDGS, and PDK4 [15]
Conjunctiva	CK19 positive CK3 AND CK12 negative [16]	
Iris	Expression for nestin, Msi, Pax6, Chx10, rho, Otx2, and Olig2 [17]	
Ciliary body	Expresses neuronal/retinal markers nestin, Chx10, Pax6, Sox2, Lhx2, Dach1, and Six3 [18]	
Trabecular meshwork	Expresses mesenchymal cell-associated markers CD73, CD90, and CD105 [19] and stem cell markers ABCG2, notch 1, OCT-3/4, AnkG, and MUC1. AQP1, CHI3L1, and TIMP3 have been differentiation markers [20]	
Retina	Retinal pigment epithelium (RPE)-derived positive markers include nestin, notch 1, CHX2, Map2, CRALBP, tyrosinase, and tyrosine-related protein 1 and 2 [21]	
Choroid	Expressing markers Sca-1, CD90.2, CD44, CD105, CD73, ABCG2, six 2, notch 1, and Pax 6 [22]	
Sclera	Expresses ABCG2, Six2, PAX6, and notch 1 [22]	
Orbit	Epithelial cell markers CD34 and zonal occludin-1 and differentiation markers CK3 and CK19 [23]	
Eye lid (sebaceous gland carcinoma)	ALDH1, CD133, CD44, ABCG2 (cytoplasmic marker) Sox4, Sox9, and slug (nuclear marker) [24]	

7.4.1 Eyelid Tumors

Eyelid tumors are of various types and are the most common type of ocular-related cancers [25]. Most of the neoplasms that originate in the eyelids (between 65% and 85%) are reported benign in nature [25–28]. Eyelid tumors affect all population demographics across the world. Eyelid tumor is a very broad category containing multiple different types of cancers which can be divided on the basis of tissue/cell of origin and as benign or malignant [26]. Some of the benign epithelial tumors are squamous papilloma, seborrheic keratosis, inverted follicular keratosis, etc. Basal cell carcinoma and squamous cell carcinoma are some of the malignant-type epithelial tumors [29]. Many other types of tumors exist which are categorized as eyelid tumors too.

7.4.1.1 Metastasis of Eyelid Tumors

Metastasis of eyelid tumors is very rare. Different reports have studied the occurrence of these tumors and traced it to the point of origin. Most of these reports agree that the most common primary site is breast cancer [30, 31] but list other sites which too cause eyelid metastasis like the lungs [30], gastrointestinal tract, and kidneys [32]. These metastatic eyelid tumors clinically appear as cutaneous nodules and swellings. It is also noted that the upper and lower eyelid may be equally affected [33]. Eyelid metastasis seems to have problems of diagnosis associated with them such as cases where the eyelid tumor became symptomatic before the primary breast tumor was even detected [34] as reported by Ian Hood et al. and issues of misdiagnosis as a chalazion as reported by both J. Kanitakis et al. and G. W. Weinstein et al. [30, 35].

On the other hand, the spread of eyelid cancer has been researched extensively too. Retrospective studies have shown that basal cell carcinoma has very low chances to metastasize with less than 0.5% of cases showing spread [36]. The metastasis is as high as 24% in squamous cell carcinoma of the eyelid or periocular skin with very high incidence of local recurrence (35%), moderate number of regional nodal metastasis (24%), and very few distant metastasis (6%) [37].

7.4.2 Conjunctival Tumors

A thin membrane which covers the eye and the inner layer of the eyelid is called the conjunctiva. Tumors which grow on this membrane are called the conjunctival tumors. The most common of these diseases are squamous carcinoma, malignant melanoma, and lymphoma [38]. These tumors occur in older individuals who have long exposure to sunlight due to outdoor activities [39]. Important factors which seem to play a vital role in the development of this type of tumor include ultraviolet radiation exposure, vitamin A deficiency, ocular injury, exposure to petroleum products, and chronic HIV, HPV, or hepatitis B infection [40].

Conjunctival
<1%
Orbital
9%
Corneal
<1%
Intraocular
2%
Eyelid
89%
Eyelid
Intraocular
Corneal
Orbital
Conjunctival
Metastases
3%
Recurrences
14%
Deaths
11%
Cured
72%
Cured
Deaths
Recurrences
Metastases
Percentages of Cancer
18.00%
16.00%
14.00%
12.00%
10.00%
8.00%
6.00%
4.00%
2.00%
0.00%
15.39%
12.94%
9.91%
0.20%
Breast Cancer
Lung Cancer
Prostate Cancer
Eye Cancer
Cancer percentages compared to total number of cancers
0.18%
0.16%
0.14%
0.12%
0.10%
0.08%
0.06%
0.04%
0.02%
0.00%
0.17%
0.02%
0.004%
0.0006%
Eyelid
Orbital
Intraocular
Corneal and Conjunctival

7.4.3 Corneal Tumors

The sclera and cornea are important barriers which prevent spread of neoplasms to other parts of the body. It is very rare for corneal tumors to arise [41, 42]. Even among the corneal tumor types, epithelial tumors are more common than corneal stromal tumors [43]. These tumors, though rare, are of two types, the congenital and the acquired lesions. Acquired lesions are further subdivided based on the origin of the mass like epithelial, vascular, fibrous, neural, etc. [44]. Exposure to ultraviolet radiation causes growth of carcinoma in the eyelids and neoplasms [45, 46] on the corneal region and is considered the primary cause for acquired lesions.

7.4.3.1 Metastasis of Conjunctival and Corneal Tumors

Metastatic tumors rarely occur in the conjunctiva [43]. Primary sites which cause metastatic conjunctival tumors are again mainly the breast and lung cancer appearing over a very wide window ranging from 8 to 100 months [47]. In cases of advanced stage of organ metastasis, conjunctival masses appear. A study carried out by C. L. Shields showed that the conjunctival primary-acquired melanosis or nevus has lower risk of death and metastasis than de novo melanoma [48].

Conjunctival malignant melanoma is a fatal tumor with recurrence rates at 35%, metastasis reported in 25% patients, and nearly 15% deaths [49]. The same study used the Kaplan-Meier survival estimates, and at 10- and 15-year follow-ups, recurrence rose to 51% and 65%, respectively. The patients who did develop metastasis showed growth in the facial lymph nodes, lungs, brain, and liver. Other research which studied the conjunctival squamous cell carcinoma reported deep corneal invasion, intraocular extensions, and orbital invasions [50]. Hence conjunctival tumors show aggressive capabilities to metastasize.

Very few reports mention any form of corneal metastasis at all. Most studies, which do analyze corneal tumors, mention clearly that no evidence of metastasis ever surfaced in the patients.

7.4.4 Orbital Tumors

Orbital tumors are rare, and metastatic orbital tumors can spread from a variety of different sites like the breast, lung, melanoma [51], etc. Jerry A. Shields et al. have shown that reports which study the orbital tumors are biased toward the interest of the reviewer. For example, neural tumors like meningioma and optic pathway glioma will appear under neurosurgical study. On the other hand, reports from otolaryngology will include mucocele, paranasal sinus neoplasms, and other secondary lesions [52]. Even orbital bone cancer, which constitute between 0.5% and 2.0% of total orbital cancer, is studied under orbital tumors [53]. In this chapter, we shall focus mainly on those tumors which affect the stem cell present in the orbit.

Orbital tumors are a heterogeneous group of neoplasms [54, 55] including cystic lesions, neural tumors, histiocytic tumors, bone and cartilage tumors, etc. and hence

require diagnosis based on clinical analysis, imaging, and other studies before the suitable treatment is administered.

7.4.4.1 Metastasis of Orbital Tumors

Orbital metastasis occurs mainly due to primary growth in the breasts, lungs, gastrointestinal tract, and prostate [30, 56, 57] with nearly 45% of orbital tumor cases presenting signs of systemic cancer. The orbital tumor conditions in children are shown to be very different by a case study done by Daniel M. Albert et al. They found that children with orbital tumor metastasis were either suffering from neuroblastoma or Ewing sarcoma which affects bones and had no occurrence of intraocular metastasis from a solid tumor [58]. The findings were very different in adults, who frequently had cases of intraocular metastasis [59, 60].

The metastasis of orbital tumors is well documented by multiple studies. Robert A. Goldberg et al. found that in around 25% patients, the onset of ocular cancer is the manifestation of systemic disease and displacement of the eyeball due to change in its volume (enophthalmos) was frequently seen in patients [51]. A study done by Gunalp et al. showed that the average detection time for secondary site was shortest for lung cancer (2 months) and longest for breast cancer (34 months) making follow-up checks extremely important for these patients [61]. In senior adult population, orbital tumors were malignant in up to 65% of the cases with 25% developing systemic problems.

7.4.5 Intraocular Tumors

Intraocular melanoma is a malignant form of cancer which happens in the tissues of the eye. These occur in the wall of the eye. The wall comprises of three parts, the sclera (outer layer), the uvea (middle layer), and the retina (inner layer).

The uveal tract is divided into three parts too, the iris, ciliary body, and choroid. Iris melanoma is generally a small neoplasm and hardly ever spreads. These are very rare with occurrences as low as 3% of all uveal melanomas, and reports show that an elevated intraocular pressure influences the iris melanomas [62]. The ciliary body gives rise to neoplasms which are larger and more capable of spreading, while the choroidal neoplasms are the largest and most likely to spread [63, 64].

Retinal neoplasms are of different types. The most common of these is retinoblastoma, an aggressive childhood affliction with occurrence 1 per 15,000 to 20,000 children [64, 65]. Other retinal cancers exist like vasoproliferative retinal tumor which typically manifests at ages 20 to 25 [66] and retinal hemangioblastoma which is usually detected between ages 40 and 60 [67]. Exposure to sunlight and ultraviolet rays seem to be the primary reason for the cause of these intraocular malignant melanomas [68].

7.4.5.1 Metastasis of Intraocular Tumors

Intraocular tumors usually arise in the uveal tract and the choroid due to their high vascularity, making uveal and choroidal neoplasms the most common malignancy in

adults. Patients with this kind of posterior choroidal metastasis have low life expectancy, but over the last few decades life expectancy has progressively improved [59, 60]. Primary cancers which lead to choroidal metastasis are mainly breast (40–47%) and lung (21–29%) cancer [59, 69, 70] which cover two third of reported cases. The remaining one third of patients shows no sign of primary cancer at the time of diagnosis [70].

As for spread of intraocular tumors, 45% of patients develop metastasis in the liver, often many years later. Most cases show the liver growth within 5 years, but frequently the cases arise 20 years after the initial diagnosis [71]. The progress of this metastasis is rapid, and hence this remains the most common cause for death in patients with uveal melanoma [60, 71]. In cases of retinoblastoma, if the patient shows optic nerve invasion, then metastasis is expected. If invasion is beyond the lamina cribrosa layer, there is a far greater risk of metastasis [72, 73].

7.5 Regulation of Stem Cells and Cancer Stem Cells

Normal stem cells and cancer stem cells have the self-renewal capability with many common classical pathways regulating the stem cell and cancer stem cell development [10]. Signaling pathways such as Notch, Sonic hedgehog, and Wnt associated with tumor regulation and development are also associated with normal stem cell regulation [74]. These signaling pathways when dysregulated result in tumorigenesis. The CSC attracts the normal stem cells through cytokine secretion, further enhancing the cancer cell metastatic movement and risk of tumor formation [75].

Signaling pathway	Normal stem cell/progenitor cells—pathway regulated	Cancer stem cells—pathway dysregulated
Wnt	Development of epidermal and other tissue	Epidermal tumors
Sonic hedgehog	Neural development	Basal cell carcinoma
Notch	Neural development	

7.6 Conclusions

Compared to other cancers in the rest of the organs, cancers related to the eye are at lower percentage, and the metastasis both intraocular and spread to other organs is at lower percentage. While melanomas constitute melanomas among eye cancers, other cancers such as retinoblastoma, eye lid cancers, and choroidal cancers are also frequently observed. The presence of cancer-specific stem cells among eye cancers as of now reported is very few except for the retinoblastoma, melanoma, and squamous carcinoma. While common markers CD44, CD133, and SOX2 and other cancer stem cell-specific markers are reported, further studies are needed to

propose the aggression of tumors and the ambivalence of the currently established markers in tumor progression in eye cancers.

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Cancer Stem Cells and Tumour Aggressiveness

8

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Abstract

Tumours are groups of cells, consisting of heterogeneous types of cells that exhibit abnormal cellular characteristics and behaviours. The molecular characteristics of tumour cells can be used to classify the tumour types. In a tumour, the complexity of the population of cell types involved and their diverse gene expression patterns, contribute significantly to tumour heterogeneity, growth, metastasis and aggressiveness. Cancer stem cells (CSCs) are a small population of cells in a tumour that are highly plastic in nature and possess self-renewing capacity. The CSCs can differentiate into different cell types, and play crucial roles in tumour initiation, growth and progression. CSCs drive metastasis, therapeutic resistance and recurrence of cancers, and thus act as the key regulators of tumour aggressiveness. The CSCs trigger the epithelial to mesenchymal transition (EMT) of cells in the tumour, which leads to increased invasiveness of

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these cells. These unique subpopulations of cells can communicate with their tumour microenvironment (TME) or niche, and stimulate their niche to secrete several intrinsic factors, which triggers neoangiogenesis to promote metastasis. The multipotent and tumour-initiating abilities of CSCs stimulate or alter various signalling networks to cause extravasation of primary cancer cells that result in cancer metastasis. Consequently, the CSCs promote tumour aggressiveness, which can lead to relapse of cancers after various treatments, and thus, pose critical problems in designing novel therapeutics to specifically target and eliminate CSCs. Therefore, CSCs and tumour aggressiveness still remain as one of the major challenges in curing cancer, despite recent advancements in therapeutic approaches to treat various cancers. Here, we discuss the key roles of CSCs in the regulation of EMT, metastasis, cancer metabolism and critical signalling pathways that influences tumour aggressiveness.

Keywords

Cancer stem cells · Self-renewal · Tumour initiation · Epithelial to mesenchymal transition · Invasiveness · Angiogenesis · Metastasis · Tumour aggressiveness

8.1 Introduction

Cancer is a major lethal and universal ailment that kills millions of people every year throughout the world. In cancer, there is alteration of cell and tissue architecture [1], and if cancer is not treated at an early stage, it spreads from the primary site of tumour to various organs, which acts as its secondary site [2, 3]. This progression by which cancer cells disseminate to other parts of the body is called metastasis. There are various factors and processes involved in metastasis.

Cancer cells can spread to different parts of the body through various steps such as by simple invasion into neighbouring normal tissues or migration via the lymphatic or blood vessels from the primary site of tumour to distant secondary sites (i.e. different organs or tissues) for colonization. The formation of new blood vessels (neoangiogenesis) provides additional blood supply for more nutrients and growth factors, which would help in tumour growth or metastasis [4, 5]. The tumour is complex in nature as it contains a heterogeneous population of cells encapsulated inside the tumour that is surrounded by blood vessels, extracellular matrix (ECM), fibroblasts, immune cells, etc., which forms a distinct tumour microenvironment (TME), called niche [6]. The emergence of the existence of the cancer stem cells (CSCs) and recent evidence have widely opened a whole new approach in the understanding of this heterogeneous disease. The initiation and the development of the tumour are driven by these CSCs, the specialised cell subtypes, which are multipotent in nature and also possess self-renewing capacity [7]. CSCs are responsible for the differentiation and migration of the cancer cells along with the tumorigenicity. The CSC subpopulations are protected inside their niche, which acquires the necessary growth factors, and derive energy in the form of ATP from the

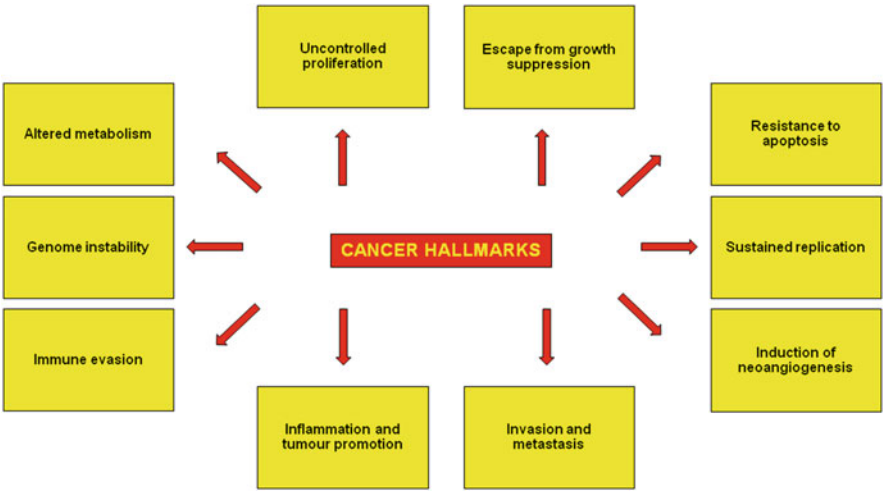

Fig. 8.1 Hallmarks of cancer. This illustration explains the unique characteristics of cancer during its initiation and growth. An extensive study on the hallmarks of cancer have been performed in the past decade, and has led to a better understanding of various steps in oncogenesis

glycolytic pathway and oxidative phosphorylation (OXPHOS) depending on their necessity.

In most cancer types, the unifying process that accounts for increased mortality is metastasis. Metastasis is a multi-step process. It involves different phases of cells, and it initially starts with mutation at a molecular level by an oncogenic hit due to genetic or environmental cause [4]. Recent insights in the field of cancer biology highlight the complex nature of this biological process, and helped in organising the intricacy based on defined principles called cancer hallmarks [8]. The hallmarks of cancer are depicted in Fig. 8.1.

Cancer cells rarely metastasise to skeletal muscle, and specific factors encourage comprehensive muscle wasting, which results in a condition known as cachexia, wherein the zinc gets hoarded in skeletal muscles through abnormal upregulation of the metal ion transporter ZRT- and IRT-like protein 14 (ZIP14). This phenomenon is the critical mediator of metastatic cancer-induced muscle wasting [9]. Epithelial to mesenchymal transition (EMT) is a process by which the CSCs obtain their mesenchymal phenotype, which favours the progression and aggressiveness of the tumours, resulting in metastasis.

Most of the tumours are comprised of normal cells along with other different cell types which help in driving the phenotypic heterogeneity and malignancy of cancer. An important part of the cancer cells and the so-called metastasis inducers are characterised by CSCs [10]. In the tumour mass, these cells are capable of self-renewal and differentiation. CSCs are also responsible for the metastatic properties of cancer cells [11]. The CSCs secrete cytokines which are important in preparing

the tumour microenvironment (TME) and employing the myeloid cells to strengthen the cancer progression. Cancer-associated fibroblasts (CAFs) and activated tumour-associated macrophages (TAMs) release high amounts of matrix metalloproteinases (MMPs), growth factors and cytokines to withstand angiogenesis and to encourage the CSC invasion [12–14].

The cytokines, chemokines and growth factors secreted in the TME boost the migration capability of cancer cells and enhance the angiogenesis [15]. Also, CSCs can easily escape from the immune system [16] by altering the protein expression of intrinsic molecules including programmed cell death ligand 1 (PD-L1) [17], which further ensures the formation of the immunosuppressive microenvironment [18]. Also, most importantly, the cell surface markers of CSCs, like CD44 and CD133, are very vital molecules that confer the specificity in CSCs' binding and targeting properties on the other tumour cells [19]. CSCs share genetic and epigenetic intricacy along with other cancer cells but adapt to survival challenges which is a vital property of these cells in escaping the cancer chemotherapy. Moreover, with their slow cell proliferation rate, when compared to other cancer cells, CSCs are believed to gain survival tactics, while the fast-growing cancer cells are destroyed by the various therapeutic treatments [20].

CSCs possess the ability to hijack the intrinsic signalling pathways such as Wnt/β-catenin, Hedgehog and Notch pathways [21]. These pathways are the key regulators of the adult stem cell homeostasis, and the CSCs impede the normal mechanism of the pathway and exploit its function over maintaining its own plasticity [22]. Interestingly, these subpopulations of cells evade apoptosis and take control of the immune system by inhibiting the function of the immune cells. Although it is potentially possible to treat cancers in its early stages, the metastasis and the cancer relapse are still the major issues even with all the cutting-edge state-of-the-art technology. CSCs undergo dormancy after they form a colony at a distant site, and are well protected inside their TME. Hence, the current therapies that target only actively proliferating cancer cells are unable to target and eliminate CSCs [23]. The tumour has heterogeneous populations of cells, and therefore, the treatment regimes fail to target CSCs. Eventually, the tumour regresses responding to treatment, but it relapses with high metastatic ability along with resistance towards the therapies, which leads to the high mortality rate in cancer patients. CSCs are resistant to many therapies because of their chemoresistance, radioresistance and immunosuppressive properties. Numerous laboratories and pharmaceutical companies are currently focusing on developing therapies to specifically target CSCs. Significant ways aimed at eliminating CSCs would impact ominously on cancer treatment, by countering metastasis and cancer relapse [24]. This chapter discusses some of the important roles of CSCs in modulating the metastatic potential, metabolic alterations and key signalling pathways and thus, tumour aggressiveness.

8.2 Cellular Behaviour and Niche

Aberrant activation in the cell signalling networks along with the production of different essential growth factors are necessary for the initiation of tumour. Furthermore, synthesis of angiogenic proteins by the tumour itself decides the fate of aggressiveness of the tumour [25]. Not all subtypes of tumours are highly metastatic in nature. The behaviour of the cells depends on the habitat where the tumour exists. The niche provides an enriched environment with the supply of all the intrinsic factors to the TME that drives the progression of the tumour in its growth and adversity [26]. The principal characteristics of the CSCs along with its phenotypic plasticity are well protected within the niche against the classical immune system and therefore promote the metastasis. Stem cell markers are functionally specific proteins that help in the process of identification of different tumour subtypes. There are various stem cell markers such as CD44 [27], CD133 [28], CD49f [29], ALDH [30], etc., which have specific roles in the development of different tumours. The characteristics of these stem cell markers are represented in Table 8.1.

In the primary carcinoma, the lack of oxygen in tumour cells increases the production of reactive oxygen species (ROS) due to impaired vascularisation inside the TME. The cellular stress due to hypoxia and the increased ROS levels initiate the CSC's stress signalling pathway to boost the cancer cell survival and also, to maintain its stemness [31]. The mesenchymal stem cells (MSCs) along with the CSCs produce intrinsic chemokines, cytokines and other angiogenic growth factors to initiate neoangiogenesis, thereby, directing more blood supply to the tumour and increasing the plasticity of CSCs. Furthermore, it compromises the immune system by suppressing the cytotoxic functions of the immune cells [32].

In the normal cellular physiology, the MSCs establish a normal stem cell niche with various stemness factors to support stem cell survival. But the oncogenic hit transforms the normal stem cell niche to a CSC niche that triggers the initiation of the tumour. In the TME, the CSCs can activate the EMT pathway in the adjacent normal

Table 8.1 Stem cell markers, tumour types and their characteristics

Stem cell markers	Tumour types	Characteristics
CD44	Breast, pancreas, prostate, head and neck	Found in the CSCs; glycoprotein that has role in inflammation, tumour progression and metastasis
CD133	Brain, prostate and colon	Five transmembrane domain cell-surface glycoprotein that helps in disease progression
CD49f	Breast and colon	Acts as an inflammation sensor to regulate differentiation, adhesion and migration
ALDH	Haematopoietic and breast	Maintenance and differentiation of stem cells; promotes chemoresistance and survival mechanisms in CSCs

The table enlists the key stem cell markers and their unique expression in different types of tumours. Each stem cell marker possesses specific characteristics, which enables the isolation and analysis of CSCs. Moreover, this helps to understand the role of these stem cell markers in CSCs and tumour progression

tissue and transform them into tumour cells to invade further and to grow at a new site with a separate niche to support the CSCs at that distant site [33]. Also, the primary CSCs can control the potential metastatic sites by releasing exosomes to facilitate the arrival of invading tumour cells. The exosomes along with other necessary growth factors prepare the metastatic niche and favour CSCs to establish a new colony at a distant site through the process of extravasation [34].

8.3 Conjunction Between CSCs and Tumour Aggressiveness

CSCs share the common characteristics of normal stem cells such as self-renewability and multi-lineage differentiation that have the capacity to drive tumour growth. The role of CSCs starts early during tumour initiation and sustains throughout the progression of the carcinoma. CSCs play a critical role in the metastasis and tumour relapse due to its high plasticity. The CSCs utilise various signalling pathways to evade immune cells, and also impede immune cell function against cancer cells. However, during normal metabolism, the systemic immune cells target cancer cells exhibiting high levels of cellular stress and ROS production [35].

In most of the currently available cancer therapies, the treatment regime is designed to usually target the whole organ for radiotherapy or the whole body for chemotherapy. But in advanced treatment programme such as the proton beam therapy, the tumour is targeted specifically [36]. But even with this treatment, the normal tissues are affected in an insignificant manner, whereas in other treatments, the damage done by the therapy is far worse causing overall deterioration of the body. Since the present therapies follow a holistic approach in their treatment regime and the CSCs are encapsulated and well protected within their niche, it becomes difficult to target the CSCs [37]. On the contrary, when the treatment kills cancer cells and the tumour shrinks eventually, the CSCs may go to a dormant state, and develop resistance towards that drug. In a few years, the tumour may relapse with enhanced resistance towards that drug treatment and exhibits aggressive metastatic potential.

CSCs play an integral role in oncogenesis by promoting tumour initiation, invasion and metastasis. CSCs initiate cancer due to their stemness which is acquired due to accumulation of genetic or epigenetic alterations and oxidative stress. CSCs establish their communication network with the TME, which favour their survival and migration, and also promote angiogenesis for metastasis [38].

8.4 Impact of Metastatic Potential on Tumour Aggressiveness

Metastatic dissemination of cancer cells is the ability of the malignant cells to initiate the invasion from the primary site to form a colony at a distant site. This invasiveness of the cancer cells involves various cascades of events such as intravasation, EMT and hijacking of different signalling pathways. These events enable the CSCs to gain their self-renewing property, which under normal physiological condition is

primarily used for the maintenance of adult stem cell homeostasis, wherein the epithelial cells acquire mesenchymal characteristics [39]. While the tumour develops, it is intrinsic for the tumour cells to sustain growth and function in the hypoxic environment with the recruitment of the cellular components and modulation of their extracellular matrix.

The tumour acquires its vasculature through angiogenic factors and retains its blood supply for the growth of tumour. The tumour cells invade the normal tissues, and upon reaching the blood vessels, the tumour cells infiltrate through the endothelial barrier of the blood vessel, and enter into the circulation by the process called intravasation. After intravasation, the blood vessels in the tumour establish connections between the tumour and other organs, thereby providing a route for the circulating tumour cells (CTCs) to extravasate into the circulation for metastasis [40] (Fig. 8.2). CTCs possess epithelial properties, and cannot escape the microenvironment until they gain mesenchymal properties. EMT is one of the key events necessary for the extravasation of the CTCs into the vascular system. While the CTCs in circulation adhere and secure themselves in the adjacent organs, the entire process of tumour development at primary tumour sites are driven by CSCs, and the release of the CTCs is an active process that supports metastasis to distant secondary sites. Also, the CSCs prepare the metastatic niche at distant secondary sites with the help of necessary growth factors, before the arrival of the CTCs and its development to form secondary tumours [41].

The growth of the tumour after colony formation at the distant site is one of the crucial events in the process of metastasis. But in most cases, the CTCs after adhering at a distant organ enter a state of dormancy (Fig. 8.2). Metastatic dormancy is one of the major reasons for the tumour to relapse after cancer therapy [42]. Cancer treatments are often developed to target the tumour at the primary site. Though, the treatment kills most of the tumour cells and the tumour regress, it does not kill all the CSCs in the tumour. Few of the CSCs at the primary tumour often escape death from the treatment or enter a dormant state. Also, some tumour containing CSCs can often escape even from a successful precision surgery. The dormant tumour cells at a distant site or CSCs at a primary site can remain resilient towards a treatment regime, and can relapse to a full-blown tumour again in a few years. In relapsed cancers, the metastatic cells are dominant and are more fatal with mesenchymal properties, and their aggressiveness is doubled due to their acquired resistance to the therapies [43].

8.5 Factors Influencing Tumour Aggressiveness

The CSCs are influenced by various components such as hormones, enzymes, cytokines and growth factors (Fig. 8.3). Several studies have shown that hormones hasten the process of oncogenesis in different tumour conditions. The hormones, oestrogen and progesterone influence the growth of breast cancer cells by binding to their specific receptors in the cells. These hormones increase cancer cell division and rapidly promote tumour growth in a very short period of time,

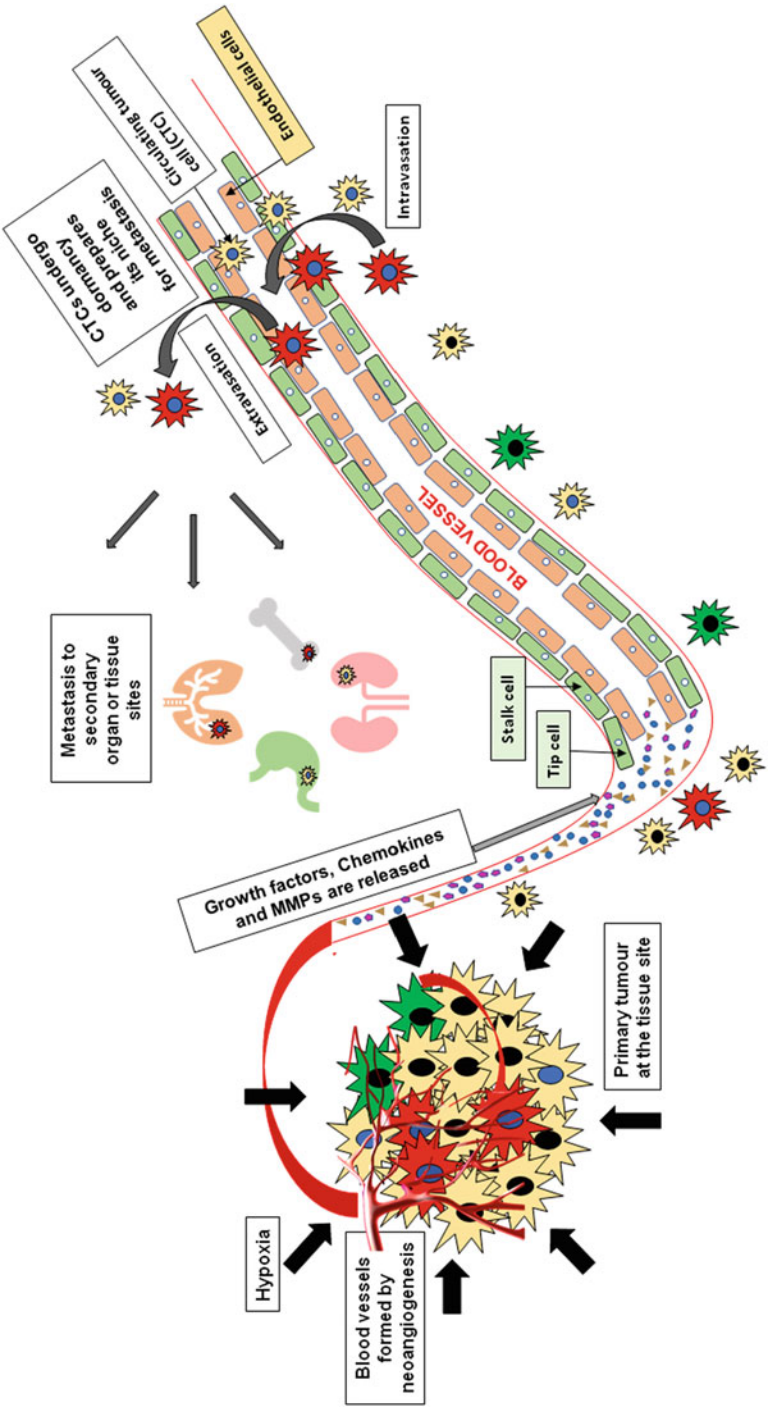

Fig. 8.2 CSCs promote neoangiogenesis and metastasis. The active proliferation of cancer cells at the site of tumour creates cellular stress due to the hypoxia and lack of necessary nutrients. This hypoxia condition results in the release of different growth factors, chemokines, cytokines and angiogenic factors resulting in the formation of new vasculature in the tumour to supply nutrients required for the tumour growth. The cancer cells start to invade the normal tissue and reach near the blood vessels. Intravasation is a crucial event in the dissemination of cancer, and it requires the cancer cells to enter the circulation to form a colony at a different secondary site. The circulating tumour cells (CTCs) travel in the circulation and extravasate at the distant tissue and undergo dormancy, preparing their metastatic niche at the secondary site. Later, they undergo accelerated proliferation and form a colony which quickly progresses to a full-blown tumour at the secondary organ site. Cancer cells are represented with serrated margins. Red coloured cells with serrated margins are CSCs. Green and yellow coloured cells with serrated margins are different tumour cells, which are represented to show the tumour heterogeneity. The blue or black coloured nucleus represents heterogeneity in the cancer genomes. *MMPs* matrix metalloproteinases, *CTCs* circulating tumour cells

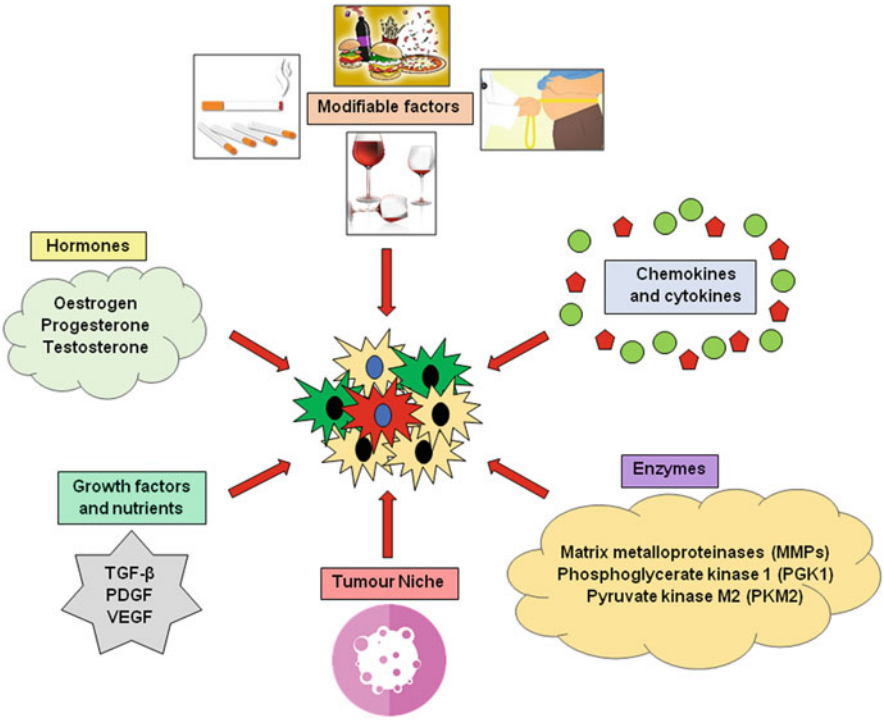

Fig. 8.3 Various factors that influence tumour aggressiveness. Several factors influence the initiation and progression of tumour. These include both intrinsic and extrinsic factors that drive tumour growth and aggressiveness. The extrinsic (modifiable) factors include smoking, consumption of alcohol, unhealthy diet and lifestyle, which can be modified. The intrinsic factors include enzymes, hormones, growth factors, nutrients, chemokines, cytokines and tumour niche, which contribute to the rapid progression and dissemination of the tumour. Cancer cells are represented with serrated margins. Red coloured cell with serrated margins is CSC. Green and yellow coloured cells with serrated margins are different tumour cells, which are represented to show the tumour heterogeneity. The blue or black coloured nucleus represents heterogeneity in the cancer genomes. *TGF-β* transforming growth factor-beta, *PDGF* platelet-derived growth factor, *VEGF* vascular endothelial growth factor, *MMPs* matrix metalloproteinases, *PGK1* phosphoglycerate kinase 1, *PKM2* pyruvate kinase M2

making it one of the aggressive forms of tumour. Furthermore, hormones such as testosterone, oestrogen and progesterone enhance drug resistance and metastasis of hormone-dependent tumours [44].

Along with hormones, growth factors, cytokines and chemokines play a crucial role in the progression of tumours. The TME contains different types of cells that undergo hypoxia and cellular stress as the tumour grows, and so the tumour itself releases the necessary growth factors such as transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), etc., in order to form new vasculatures (neoangiogenesis) for the tumour to supply blood and nutrients [45]. Additionally, there are few enzymes such as matrix

metalloproteinases (MMPs), phosphoglycerate kinase 1 (PGK1) and pyruvate kinase M2 (PKM2), which affect the tumour cell behaviour and accelerate their progression by breakdown of the extracellular matrix (ECM), and fuelling the tumour cell metabolism to aid the invasion of cancer cells, by rapidly promoting peritoneal dissemination to the distant sites [46].

The crucial factor that is involved in tumour progression and development is the hypoxia caused due to increased proliferation, and thus, elevated cellular stress. The uncontrolled cell division creates an unfavourable environment inside the TME, which eventually leads to poor nourishment of cells. This initiates the need for excess growth factors and nutrients to cells, and therefore, the tumour itself secretes necessary angiogenic factors to construct new vasculatures (neoangiogenesis) inside the tumour, which enables blood supply to the tumour. Also, alteration in tumour metabolism is a key factor that determines the tumour growth and aggressiveness [47].

The CSCs control the switch between using the glycolytic and OXPHOS pathways to extract ATP. It is also based on the niche, which partly chooses the pathway for the ATP production. During the tumour initiation, the CSCs use the glycolytic pathway, but they switch to the OXPHOS pathway during tumour progression. Metastasis requires elevated energy expenditure as cells have to migrate to a distant site, and so, it depends on both the pathways [48]. Also, in few carcinomas, hormones act as critical factors, which double the invasiveness of tumour. Although several molecular mechanisms play vital roles in the initiation and progression of tumour, the environmental carcinogens influence the growth of tumour in the form of DNA damage due to higher ROS production and increased cellular stress. More importantly, modifiable risk factors including smoking, alcohol consumption, eating junk food and unhealthy lifestyle (e.g. obesity) increase the risk of cancers (Fig. 8.3) as these factors can also promote metastasis [49].

8.6 Molecular Mechanisms Involved in EMT

EMT is a process by which epithelial cells acquire mesenchymal characteristics by undergoing several changes including the loss of cell polarity. The acquisition of mesenchymal phenotype is influenced by different signalling factors, which are necessary to evade apoptosis and increase the migration potential of the cells. It also increases the secretion of degrading enzymes to penetrate the ECM, and this process promotes the invasiveness of cells [50]. EMT activates and maintains the stemness of the cells, and it plays a key role in the transition of normal stem cells to CSCs, by acquiring the invasive mesenchymal characteristics.

The EMT is a two-way process, and it can be reversed. EMT can change in either direction, and the conversion from mesenchymal phenotype to epithelial phenotype is called mesenchymal to epithelial transition (MET) (Fig. 8.4). It involves multiple signalling pathways such as Wnt/β-catenin, Hedgehog (Hh) and Notch signalling. The interaction between these signalling pathway and the immune cells, enables the normal stem cells to develop and maintain their plasticity. Various transcriptional

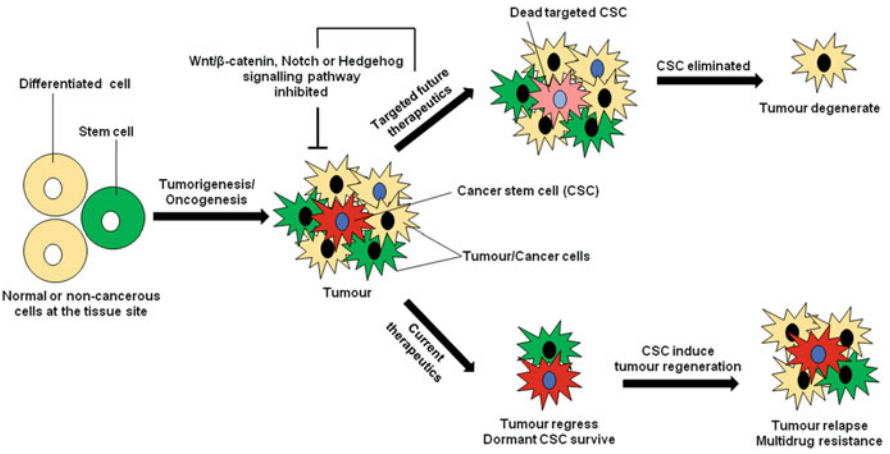

Fig. 8.4 Effect of current therapies on CSCs and future treatment perspectives. The normal stem cells undergo oncogenesis due to accumulation of mutations, and the dysregulation of oncogenes and tumour suppressor genes of the cell cycle, which lead to active proliferation of the mutated cells and development of tumour. The current therapeutics do not specifically target the CSCs, and therefore, tumour relapses with multidrug resistance. The CSCs are very plastic in nature, and they possess the characteristics of self-renewability due to their control over key signalling pathways such as Wnt/β-catenin, Notch and Hedgehog. Ongoing research specifically target CSCs, and have shown some promising results in the eradication of tumour. Cancer cells are represented with serrated margins. Red coloured cells with serrated margins are CSCs. Green and yellow coloured cells with serrated margins are different tumour cells, which are represented to show the tumour heterogeneity. Pink coloured cell with serrated margins depicts the dead targeted CSC. The blue or black coloured nucleus represents heterogeneity in the cancer genomes. *CSCs* cancer stem cells

factors such as Slug, Snail, Twist, Sox-9, etc. are involved in the transition from epithelial to mesenchymal cell characteristics [51]. The epithelial cells lose certain important characteristics, while acquiring invasiveness and metastatic properties. The epithelial phenotype markers [including E-cadherin, desmoplakin, Muc-1, cytokeratin-18, occludins, claudins and zonula occludens] are lost, while mesenchymal markers [including N-cadherin, vimentin, fibronectin, vitronectin, α-smooth muscle actin (α-SMA) and fibroblast-specific protein 1 (FSP1)] are acquired during EMT [52].

Importantly, growth factors and inflammatory cytokines, including interleukin (IL)-8 [an activator of the Janus-activated kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway] are secreted by the stromal fibroblasts, and they promote tumour progression and account for the aggressiveness of cancers [53]. Nevertheless, the aberrant activation of the signalling pathways varies with cancer types. Few proteins, which have tumour suppressor property in one type of cancer are known to initiate and cause invasiveness in a different type of cancer. But, genetic instability or mutations and epigenetic alterations can activate oncogenes or inactivate tumour suppressor genes via metabolic reprogramming, which in turn can lead to increased cell proliferation, malignant transformation, metastasis or

cancer relapse. Furthermore, CSC's heterogeneity and the process of EMT are considered to largely contribute to the complexity and organ-specific metastasis of cancers [54].

8.7 Metabolic Alterations in Tumour Survival

Due to the complex nature of various TMEs, the CSCs tend to depend on different sources for energy. It has been identified that glucose- and oxidative-based metabolisms, feed the CSCs with energy derived from vital nutrients, along with amino acids like glutamine and lysine, which act as alternative fuel sources. In a normal cell, mitochondria produce ATP using their tricarboxylic acid (TCA) cycle coupled with OXPHOS, to catabolise acetyl-CoA produced from glycolysis and fatty acid oxidation [55]. But, CSCs tend to increase the glycolytic flux in aerobic condition, thereby increasing the production of ATP multifold to feed the anabolic demands of cancer cells (Fig. 8.5). The supply of essential growth factors and nutrients from the energy provided by the oxidative and glycolytic metabolisms, favour cancer cells to survive in unfavourable hypoxia condition, and helps them to proliferate, differentiate and evade apoptosis more efficiently [56].

Metabolic alterations in the cells are considered as one of the hallmarks of cancer. Glucose is a key nutrient that is necessary for the CSCs to survive in their microenvironment. It also favours the active proliferation of CSC populations as glucose induces the transcription of specific genes associated with the pathways of glucose metabolism (c-MYC, Glut, HK-1, HK-2 and PDK-1) in CSCs [57]. Growing evidence suggest that the mitochondrial oxidative metabolism is highly favoured for energy production in CSC populations. CSCs are metabolically plastic in nature as they can switch between either glycolytic or OXPHOS pathway, depending on their anabolic needs (Fig. 8.5).

Most of the quiescent CSCs, during the initial stages of tumour development, use the oxidative metabolism to match the demands of ATP requirement by the tumour. The higher rate of oxidative metabolism results in increased oxygen consumption, along with higher mitochondrial mass and increased ROS production in the mitochondria, but with lower glycolytic rate. On the contrary, the proliferative cancer cells (i.e. non-stem cells) use the glycolytic pathway which results in higher glucose uptake, but lower oxygen consumption with the least mitochondrial mass and ROS production. This helps in efficient cell differentiation, leading to increased invasiveness [58]. Interestingly, the proliferative CSCs during metastasis get their energy from both the pathways, as more energy is required for the cancer cells to migrate and colonize a new site to form a secondary tumour. The aggressiveness of the carcinoma during metastatic dissemination or cancer relapse is doubled, due to energy availability from both the glycolytic and OXPHOS pathways.

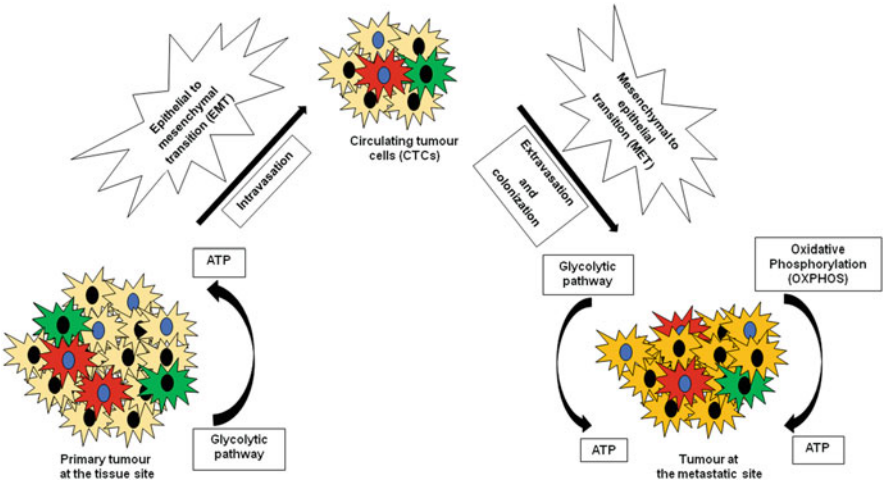

Fig. 8.5 Molecular mechanisms and metabolic alterations that drive CSCs in tumour development. The tumour cells in the primary site invade the normal tissues using various enzymes and cross the endothelial barrier, before entering into blood vessels for circulation. The tumour cells undergo EMT to gain mesenchymal properties, which aid in their rapid invasion. The circulating tumour cells (CTCs) intravasate to enter into blood vessels, then extravasate and undergo MET, which are required for dissemination and colonization of cancers at distant secondary sites in other tissues or organs. The tumour at the primary site uses the glycolytic pathway for the production of ATP, which is used as energy by cancer cells for their rapid growth. But, the tumour at the metastatic site uses both glycolytic and OXPHOS metabolisms for the production of ATP, and the tumour cells can switch the pathways depending on their need. Cancer cells are represented with serrated margins. Red coloured cells with serrated margins are CSCs. Green and yellow coloured cells with serrated margins are different tumour cells, which are represented to show the tumour heterogeneity. Orange coloured cells with serrated margins represent tumour cells with altered metabolism (i.e. using both glycolytic and OXPHOS pathways), whereas yellow coloured cells represent tumour cells using only glycolytic pathway. The blue or black coloured nucleus represents heterogeneity in the cancer genomes. *ATP* adenosine triphosphate, *EMT* epithelial to mesenchymal transition, *CTCs* circulating tumour cells, *MET* mesenchymal to epithelial transition, *OXPHOS* oxidative phosphorylation

8.8 Signalling Pathways that Drive CSCs

CSCs and MSCs employ common mechanisms, which enable them to retain their stemness and plasticity. The most crucial mechanisms that are involved in CSC self-renewal are Wnt/β-catenin, Notch and Hedgehog (Hh) signalling pathways. Wnt/β-catenin signalling is a conserved pathway present in different organisms, and it plays a key role during development. It is an essential pathway that is required for the maintenance of adult stem cell homeostasis. The intercellular Wnt signalling molecules that are usually involved in the embryonic development, are altered during EMT, which leads to continuous activation of Wnt signalling and

persistent synthesis of oncogenic proteins, thereby enhancing CSC's self-renewal potential [59].

β-Catenin is the key molecule, which regulates the activation of the Wnt signalling pathway. Importantly, aberrant activation of Wnt/β-catenin pathway plays a crucial role in cancer growth, invasion, stemness and angiogenesis [60, 61]. In the absence of Wnt ligand, the multi-protein destruction complex containing Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3β (GSK-3β) phosphorylates β-catenin, which is followed by ubiquitination and proteasomal degradation of β-catenin. However, when Wnt ligand bind to its receptor, conformational change occuring at its binding site activates downstream signalling, and β-catenin molecules are released from its destruction complex and accumulates in the cytoplasm. Later, the accumulated cytoplasmic β-catenin is imported into the nucleus, where it binds to the transcription factor Tcf/Lef, and activates Wnt target genes transcription. The target genes of Wnt/β-catenin signalling such as c-Myc, cyclin D1, MMP-7, CD44, COX-2, Axin2, etc. upon expression promote the adhesion and migration of CSCs. Furthermore, Wnt target gene expression also promotes the cellular differentiation and proliferation of CSCs, which lead to increased invasiveness of CSCs [62].

Notch signalling pathway plays a key role in cell-to-cell communication during the developmental stages. It also regulates cellular proliferation and differentiation, and apoptosis. It is essential for neural stem cell maintenance, immune regulation and normal haematopoiesis. The Notch signalling pathway is activated during cell-to-cell communication, when membrane-bound Jagged or Delta ligand bind to its specific receptor. The Notch receptor is a heterodimer, which is composed of non-covalently bound extracellular and transmembrane domains. After the binding of ligand to Notch receptor, the receptor undergoes a conformational change, which leads to its proteolytic cleavage by the metalloproteinase and γ-secretase, and the release of extracellular and intracellular fragments. The proteolytic cleavage of the heterodimeric Notch receptor releases the Notch intracellular domain (NICD) into the cytoplasm, which upon translocation into the nucleus activates transcription factors, resulting in the upregulated expression of Notch target genes such as c-Myc and HES-family members [63].

The Hedgehog (Hh) signalling pathway is intrinsic for stem cell maintenance as it controls tissue polarity and maintains patterning during embryogenesis. The genetic or epigenetic alterations in cells can generate CSCs, which hijacks the Hh signalling pathway for the maintenance of its plasticity or tumour growth. In the inactive state, the absence of Hh ligand results in the inhibition of Smoothened (Smo) receptor by the transmembrane receptor Patched (Ptch). This in turn activates a series of events in the cytoplasm, which subsequently phosphorylates and degrades Gli1/2 through proteasomal degradation. When adjacent cells secrete Hh, it binds to its receptor Ptch and activates Smo. Then, Gli1/2 molecules, which are bound to the complex in the cytoplasm are released from the Smo protein complex and translocate into the nucleus. This, results in the activation of transcription factors and the expression of Hh-associated genes [64]. The activated genes also include various genes that are directly and indirectly involved in the maintenance of the CSCs. Hh signalling

upregulates JAG2, and also indirectly upregulates bone morphogenic protein 4 (BMP4) via FOXF1. Hh pathway can crosstalk with Wnt pathway, and can upregulate crucial Wnt proteins such as Wnt2B and Wnt5A. Furthermore, Hh signalling can induce stem cell markers such as LGR5, CD44 and CD133, upon interaction with Wnt and other signalling pathways [65].

In most types of cancers, CSCs hijack all the signalling pathways, which are functionally related to the maintenance of the adult stem cells, thereby promoting the growth and invasiveness of tumour. Recent studies suggest that CSCs are mostly maintained and driven in the TME with the help of these signalling pathways. Therefore, targeted inactivation of these pathways in most carcinomas may have clinical implications, as it would inhibit the self-renewability of CSCs. This approach could be used to target both CSCs and tumour to inhibit metastasis and tumour relapse, and therefore, would enable effective future treatments and ultimately cure for cancers [66].

8.9 Conclusions

CSCs possess the plasticity and self-sustaining ability to survive longer in dormant state within the TME, by evading cell death and remaining refractory to cell signalling or communication mechanisms. Importantly, CSCs can initiate tumours and cause relapse of cancers, which subsequently could lead to more aggressive carcinomas with increasing resistance to treatments. Cancer patients show poor survival rate, when they have metastatic cancer and cancer relapse, because these cancers possess CSCs. Hence, patients with metastatic and relapsed cancers cannot be cured with currently available cancer therapies. Therefore, developing advanced novel therapeutics, which can specifically target and eliminate CSCs in tumours, is an urgently required endeavour to treat, cure and eradicate cancers in the future.

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Therapeutic Implication of Cancer Stem Cells

9

Sudeep Bose, Sartaj Khurana, and Shrey Ashley Philip

Abstract

Most of the conventional cancer treatments have limited selectivity, are temporarily effective, and have adverse side effects. The potential of cancer stem cell (CSC)-based therapies has recently attracted much attention to override the detrimental impact of conventional therapies. Here we have highlighted potential strategies including identification of cancer stem cell biomarkers, interfering with circuitry network associated with drug resistance, sensitization of CSC to chemotherapy, and radiation therapy through protein targeting. CSCs display differential metabolic activity, specific signaling pathway activity in tumor initiation, metastasis, and drug resistance. Thus identification of CSC-specific markers distinct from the total cancer cell population is essential. Given the fact that the stem cell is one of the key components of organogenesis and maintenance of homeostasis throughout life, improvement of treatment modalities based on CSC therapies holds wish for better overall survival and better quality of life of cancer sufferers, specifically for patients with metastatic disorder. Therefore, in this book chapter, we have mainly discussed aberrant regulation of gene expression and some signaling pathways in CSCs and implication of CSC surface markers for designing new therapies for better clinical outcome.

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Keywords

Cancer stem cells · Biomarkers · Therapeutics · Stemness · CSC signaling · Targets

9.1 Introduction

Cancer development is a multistep process which includes dysregulation in the complex network of genes like accumulation of series of mutations and epigenetic changes in tumor suppressors and proto-oncogenes in addition to other hallmarks like sustained proliferative signal, apoptosis resistance, metastatic ability, etc. [1]. With the fact that normal stem cells has self-renewal property, cancer stem cells (CSCs) may exhibit replicative potential for many cancer types [2, 3]. CSCs originate from tumor which sustain proliferative signal and clonal selection as in the case of normal malignancies [4, 5]. With the fact that the stem cell is one of the key components of organogenesis and maintenance of homeostasis throughout life, improvement of treatment modalities based on CSC therapies needs proper in-depth understanding of biological consequences of existing therapies for better patient outcome.

9.2 Roles of Stem Cells in Regulation of Key Biological Functions

Stem cells have profound biological significances like being pioneer of progenitor cells required for repair of the tissues of particular germ line. It is one of the key component of developmental processes like organogenesis and maintenance of normal homeostasis. Pluripotent cells which are embryonic stem cell by origin [6] give rise to a variety of cell types of the body, including regeneration of blood, skin, or intestinal tissues [7].

9.2.1 Characteristic Features of Cancer Stem Cells and Its Biomarkers

Cancer stem cells exhibit a small fraction of the entire population of tumor cells with distinct characteristic features of stemness, display of differential metabolic activity, distinct molecular switch activity regulating cell signaling, and deregulation in cell cycle function [8, 9]. Identification of such population of cells may hold tremendous potential for targeted therapies. Various cell surface proteins like ABCG2, ALDH1, CD44, CD24, and CD133 are overexpressed as stem cell markers [10, 11]. Interestingly, these cell surface markers segregate subsets of CSC population in multiple types of solid tumors. This variation in CSC phenotype in patient tumors of the same

subtype raises the question whether difference in clinical outcomes within the tumor subtype is due to variation in CSC population.

A comprehensive list of common marker proteins of CSCs and their roles in normal biological processes is represented in Table 9.1.

9.2.2 Molecular Players of CSCs Stemness

The diagnostic and prognostic significance of CSCs is documented through gene expression profiling authenticated by various molecular techniques. For instance, the molecular analyses of leukemia stem cell populations from AML patients showed a pattern of gene expression that was found to be a strong predictor of poor prognosis [12]. In colorectal cancer, EphB2-positive CSC population was identified in tumors correlated with patient relapse [13]. Subsequent clinical and laboratory studies of patients in multiple tumors based on these signatures' involvement of CSC in drug resistance and cancer metastasis are well documented now [14].

9.2.3 Therapeutic Targeting of CSCs

Cancer stem cells can be selectively targeted without disturbing the homeostasis of adjacent normal cells. These strategies include molecular players of various hallmarks of cancer such as self-renewal pathways, resistance to various types of radio- and chemotherapies, and targeting various CSC-specific cell surface proteins. For instance Notch and Hedgehog signal transduction pathway-associated pharmacological inhibitor -based targeted therapies in human and mouse leukemia inhibited the expansion of imatinib-resistant CML [15]. Thus CSC-based targeted therapies are gaining much attention due to the inefficiency of conventional cancer therapies, failure to kill CSCs, thereby resulting in multiple malignancies, and also toxic to the healthy tissues [16]. Various approaches of targeting CSC are summarized below.

9.3 Signaling Pathways

Various inhibitors have been developed for targeting signal transduction pathways including Hedgehog (Hh), Notch, Wnt/b-catenin,, Bcl-2, Bmi-1, etc. (Fig. 9.1) in CSCs and are a promising step in cancer therapeutics [17–19]. However, these inhibitors have adverse effect on the normal stem cells. Therefore, improvisation in drug formulations is required with other CSC-targeting therapies for better therapeutic outcomes.

The goal of the current therapeutic regimen should be to target circuitry network of CSCs to trigger CSC-specific apoptosis and alter the microenvironment (niches) supporting these cells. Toward this goal, modifications in ABC superfamily, antiapoptotic factors, detoxifying enzymes, DNA repair enzymes, and histone deacetylation [20, 21] play a very important role. For example, the PI3K/AKT

Table 9.1 A comprehensive list of common marker proteins of CSCs and their roles in normal biological processes is represented

Cancer types	CSC markers	Role of CSC markers	Reference
Glioma/medulloblastoma, head and neck cancers, lung, prostate, melanoma, osteosarcoma	ABCG2 (ATP-binding cassette transporter)	ABCG2 is a protein that plays a role in host detoxification of various xenobiotic substrates in various organs like the liver, intestine, placenta, and blood-brain barrier. The bone marrow is reported to express ABCG2	Guo et al. [10]
Glioma/medulloblastoma, head and neck cancers, lung, breast, pancreas, bladder, prostate	A1/ALDH1A1 (aldehyde dehydrogenase 1)	ALDHs are NAD $(P)^+$ −dependent enzymes, in the human system. ALDH1 oxidizes retinaldehyde to retinoic acid and acetaldehyde to acetic acid and is expressed in the epithelia of the brain, liver, testis, eye lens, and cornea	de Beça et al. [50]
Glioma/medulloblastoma, head and neck cancers, breast, pancreas, bladder, prostate, ovarian, osteosarcoma, leukemia	CD44	CD44 is a multistructural and multifunctional cell surface molecule and hyaluronic acid receptor, whose role is primarily governed by various posttranslational modifications CD44 is involved in cell proliferation; differentiation; migration; angiogenesis; presentation of cytokines, chemokines, and growth factors to the corresponding receptors; and docking of proteases at the cell membrane, as well as in signaling for cell survival. Such biological properties are essential not only for the physiological activities of normal cells but also for the pathologic activities of cancer cells	Wang et al. [21]
Glioblastomas, prostate, gastric, and breast	CD133/ Prominin-1	CD133 are hematopoietic stem cells, endothelial progenitor cells, glial stem cells, and kidney, mammary gland, salivary gland, testes, and placental cells. However, CD133 is also reported being expressed by glioblastomas	Yasuda et al. [51] and Brescia et al. [52]

(continued)

Table 9.1 (continued)

Cancer types	CSC markers	Role of CSC markers	Reference
		and pediatric brain tumors and gastric and breast CSCs	
Head and neck cancer	CD44+		Prince et al. [53]
Pancreatic	CD133+, CD44+, EpCAM+, CD24+		Li et al. [47] and Simeone [54]

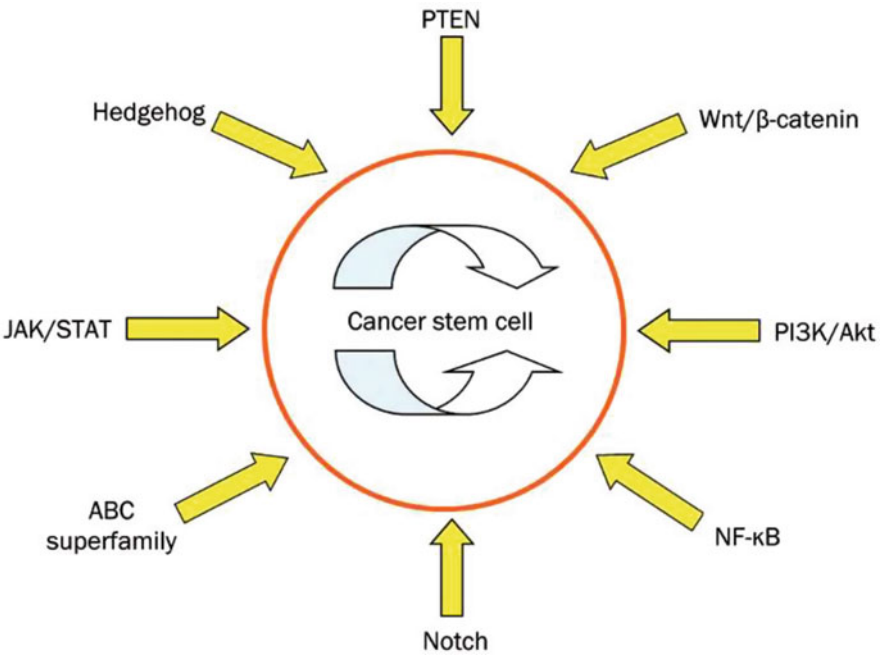

Fig. 9.1 Cancer stem cells associated different types of signal transduction pathways. The various molecular players such as tumor suppressors, apoptosis regulators, cell survival genes and drug transporter mechanism along with epigenetic pathways becomes defective during cancer stem cell formation

signaling pathway which is involved in numerous cancers, including leukemia, induces resistance to apoptosis through Bcl-2 overexpression and the phosphorylation of the pro-apoptotic protein BAD. A summary of new CSC-targeted therapeutic strategies is shown in Fig. 9.2.

Fig. 9.2 Emerging trends for targeting cancer stems cells. Identification of cancer stem cells with surface markers helps in targeting complex signalling networks such as—inhibiting drug transporters, alteration in tissue microenvironment targeting with nanoparticles, natural products and miRNAs

9.3.1 Nucleic Acid-Based Targeting CSC Markers

Certain cytotoxic drugs like Bcl-2 inhibitors, angiogenic inhibitors, short hairpin RNA molecules, antibodies, DNA methyltransferase inhibitors, etc. might be a better choice of treatment by targeting CSCs, for example, downregulation of $CD133^{+}$ CSCs using short hairpin RNA in human metastatic melanoma [22]. Similarly, breast cancer cells can be targeted with an anti-CD44 antibody-conjugated gold nanorod which displays significant cancer stem cell characteristics [23].

9.3.2 Cancer Stem Cell Targeting by Inhibitors of Detoxifying Enzymes and Drug Efflux Pumps

Cancer aggressiveness can be reduced, and sensitivity of cancer cells to chemotherapeutic drugs can be enhanced using specific drug detoxifying inhibitors like diethylaminobenzaldehyde (DEAB) or all-trans retinoic acid (ATRA), ALDH inhibitor against breast CSCs [24]. Similarly, some novel ABC transporter inhibitors like MS-209 and VX-710 [25, 26] have shown promising results in enhancing drug sensitivity in various solid cancers [26].

9.3.3 Role of Tissue Microenvironment Niche of CSCs

The advancement of in situ applications of in vitro reprogrammed stem cells and targeted tissue-specific stem cell expansion in tissue regeneration .requires understanding of how abnormal microenvironments can contribute to cancer initiation and progression. The extracellular matrix (ECM) and stromal cells enriched with a variety of proteins, growth factors, etc. of bone marrow and secondary lymphoid organs favor disease progression and resist conventional therapies [27]. Tumor angiogenesis is well known for CSC survival and drug resistance. For example, brain tumor stem cells and leukemic stem cells promoting blood vessel formation can be targeted by angiogenic inhibitor VEGF-neutralizing antibody bevacizumab that reduces CSC pools followed by tumor growth.

9.3.4 Emerging Trends of Noncoding RNA as Potential Drug for CSCs

Certain noncoding RNAs including micro-RNAs have been found to be the direct targets of CSC markers. MiRNA-mediated targeting like miRNA mimics, miRNA antagonists, and nano-delivery of synthetic oligos [28] to suppress oncogenes or activate tumor suppressor proteins was reported which plays significant regulatory roles in CSC apoptotic and antiapoptotic pathways, proliferation, survival, differentiation, migration and invasion, drug resistance, and radiation resistance [29, 30].

9.3.5 Natural Product-Based Targeting of CSCs

Natural products are the products obtained from plants or any other organism and have always been a rich source of novel compounds that can be used for cancer therapeutics. In this scenario, curcumin, a key compound obtained from the rhizome of *Curcuma longa* (turmeric), holds promising outcome as anticancer agent by inhibiting metastasis, suppressing cancer signaling pathways, sensitizing tumor cells to cancer treatment, and finally inducing apoptosis [31]. Besides curcumin, other natural products, like chrysotoxine isolated from *Dendrobium* species and

parthenolide isolated from the shoots of feverfew (*Tanacetum parthenium*), possess anticancer features that include targeting various cancer hallmarks in lung cancer.

9.3.6 Dissecting the Role of Apoptotic Players in CSC Stemness

One of the major obstacles of cancer chemotherapy is resistance to apoptosis and drug resistance. Majority of the cancer types including colon cancer, pancreatic cancer, glioblastoma, and prostate cancer are typically resistant to cancer chemotherapy due to intrinsic defects or post-chemotherapy effects [32, 33]. There are several mechanisms by which CSC triggers drug resistance including mitochondrial defects, mutations of death receptors, overexpression of antiapoptotic Bcl-2 family members and inhibitors of apoptosis proteins (IAPs), etc. [34]. Monensin and salinomycin ionophore antibiotics are currently recognized as promising anticancer agents in CSC apoptosis [35]. Obatoclax, a pan-Bcl-2 inhibitor, was recently shown to overcome resistance to the histone deacetylase inhibitors SAHA and LBH589 and to act as a radiosensitizer in patient-derived GSCs [36].

9.4 Relevance of CSC-Based Targeted Therapies in Different Malignancies

9.4.1 Leukemia

One of the most commonly diagnosed malignancies in people of all age groups [37], leukemia has different subcategories such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and multiple myeloma (MM). There is distinct patient-to-patient variation in the morphology of leukemic blast cells within these groups. Stem cell markers like CD34, CD38, HLA-DR, and CD71 have been reported in leukemic cells [38, 39]. A few of the cell surface markers, for instance, CD90 (Thy-1), are differentially expressed between normal hematopoietic stem cells and leukemia CSCs, with CD90 underexpression in leukemia [39, 40] indicating the potential of CD90 as a differentiating marker of leukemic CSC subpopulations [39, 41]. Loss of expression of CD117, also known as c-kit, is a prominent characteristic of AML CSCs ($CD34^{+}c\text{-}kit^{-}$) [42]. A monoclonal antibody, CSL360, targeting CD123 was the first-in-human trial (NCT00401739) in high-risk AML patients. In multiple reports it was evidenced that hematological neoplasms, a fusion protein like SL-401, made up of human IL-3 and diphtheria toxin truncated version of the protein directly target CD123 [17]. These studies reveal some novel approaches of targeting CD123 for leukemia treatment.

9.4.2 Breast Cancer

Continuously holding the banner for the second most deadly malignancy for females is breast cancer, where one out of eight women have a tendency to develop this malignancy in their lifetime [37]. Breast cancer is most prevalent in Afro-Americans compared to other ethnic groups where the ratio of prevalence stands at 121 per 100,000. A minor subpopulation of breast cancer cells known as breast CSCs (0.1–1%) is found in primary tumors. Upon transplantation into NOD/SCID mice, a rare subtype of breast CSCs possesses a high tendency for self-renewal and has the capacity to initiate tumorigenesis [4, 43]. CD133, CD44, ALDH, c-kit, ESA, and ABCG2 are among the most common CSC markers reported in primary breast cancer samples [44]. Targeting BCSC populations with glutathione S-transferase omega 1 apart from other cell surface marker, resulted in elevated levels of intracellular calcium and activation of STAT3 signaling, along with enriched BCSC microenvironment and reduction in metastasis.

9.4.3 Pancreatic Cancer

Presently the fourth major reason for mortality in the United States is pancreatic ductal adenocarcinoma [37] which is among the most lethal malignancies, with a 5-year survival rate of $<5\%$ [45]. Less than 1% of all pancreatic cancer cells are cancer stem cells with important attributes like self-renewal and uncontrolled potential of differentiated progeny. $CD44^+$, $CD24^+$, and epithelial-specific antigen $(ESA)^+$ are important cell surface markers expressed by pancreatic CSC populations [43, 46]. These pancreatic cancer CSC phenotypic cells also demonstrate a significant upregulation of Sonic hedgehog (SHH) and the polycomb group (PCG) gene family member Bmi-1, unlike normal pancreatic epithelial cells and non-CSC-like cancer cells. All of these have been well known for maintaining CSC characteristics [46, 47].

9.4.4 Lung Cancer

More than two lakh people are diagnosed with lung cancer every year in the United States, with a yearly morbidity rate of 160,000 individuals [37]. Tremendous growth has been witnessed in the past decade pertaining to the diagnosis and management of this condition; however due to factors such as resistance to treatment, uncontrolled tumor growth, and metastatic capacity, the prognosis still remains poor. One of the main reasons behind the aggressive phenotypes of lung cancer is the presence of a small subpopulation of lung CSCs capable of expressing certain stem cell markers, such as CD133, CD44, ALDH, Oct4, and Nanog [48, 49].

It is interesting to note that the development of immunotherapy has led to scientific advancements in using CSC-specific antigen presentation in addition to targeted small molecule inhibitors for the improvement of cancer therapies.

9.5 Conclusion

A substantial amount of evidence reveal that a puny CSC population has been known to be associated with an aggressive phenotype of tumors portraying vital characteristic features such as increased cell survival, migration, invasion, metastatic capacity, treatment resistance, and tumor recurrence eventually contributing to poor prognosis. Regardless of the number of efforts that have been made toward characterization of CSCs, their pathogenesis and molecular interactions in the tumor microenvironment are still elusive. Identification, isolation, and characterization of CSCs and CSC-specific markers in malignant tissues will enhance our knowledge and assist us in designing strategies for the development of chemotherapeutics aimed at reducing tumor aggressiveness by targeting CSCs. Recent evidence states that preclinical and clinical trials have highlighted the cruciality of CSC markers in cancer detection, screening, and CSC-based targeted therapies such as epigenetic targeting and immunotherapy which is aimed at improving patient outcomes. Current scientific advancement on CSCs has widened our horizons and provided a new dimension in developing new strategies in order to curb malignancies, thereby allowing researchers and clinicians to alleviate the burden of cancer.

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Glioblastoma Stem Cells as a Therapeutic Target

10

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Abstract

Glioblastoma (GBM) is a deadly brain tumor with poor prognosis despite the improvement in the diagnosis of GBM and innovative treatment strategies. Chemotherapy and radiotherapy could only help the GBM patients to a mean survival of 15 months. One of the key reasons for this poor outcome is a complex tumor heterogeneity and the presence of cancer stem cells (CSCs). CSCs in GBM (GSCs) are responsible for drug resistance and relapse. Cancer cells (non-GSC)

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are normally sensitive to drug treatment, whereas GSCs are resistant to treatment. This chapter describes the complexity of GSC and their microenvironment niche, GSCs as a therapeutic target, and details on clinical trials that target GSCs. This knowledge may help us in better understanding CSCs in glioblastoma and developing new therapeutic strategies for this deadly disease.

Keywords

Cancer stem cells · Glioblastoma · Brain tumor · Radioresistance

10.1 Introduction

Gliomas are a group of aggressive and most common types of primary central nervous system cancers. The incidence and mortality of gliomas have increased globally during the last two decades [1]. Histologically, glioma can be classified as high-grade glioma [glioblastoma (GBM)] and low-grade glioma (astrocytoma and oligodendroglioma) [2]. According to the cell of origin, gliomas can be classified as astrocytic tumors (astrocytoma, anaplastic astrocytoma, and GBM), oligodendrogliomas, ependymomas, and mixed gliomas [3]. Based on histopathology and level of malignancy, the World Health Organization (WHO) classified glioma under grade I to IV. Grade I glioma is less proliferative and can be treated by surgical procedures, while grade II to IV types are highly invasive and fatal. Due to the high malignancy and aggressiveness, WHO designated GBM as grade IV astrocytoma [3, 4]. Glioblastoma is one of the fatal and malignant forms of tumors from a glial origin in the human brain with a median survival period of 16–21 months [5]. Glioblastoma accounts for >60% of all brain tumors in adults with high mortality rate and a poor prognosis due to the infiltration and migration of GBM cells, high degree of intratumoral cellular heterogeneity and plasticity, and a high degree of recurrence [6, 7]. In a cancer perspective, GBM is a rare tumor with a global incidence rate from 0.59 to 3.69/100,000 population [8]. However, due to poor prognosis, GBM has a survival rate of 14–15 months and only <5% of patients reportedly surviving 5 years after diagnosis [9]. The incidence of GBM directly proportional to age and the median diagnosis age was reported as 64 [8]. Epidemiological studies revealed that the male population is highly prone to GBM than females [10]. Previous studies suggested that gliomas have higher incidence only in developed western countries [10]. However, according to a recent study, the highest gliomas incidence has been reported in eastern countries like China and India [1]. Geographic variations in the GBM incidence have been studied in the US population, and the findings revealed that South Americans are highly affected by GBM than that of other regions [11]. Among White non-Hispanic, Black non-Hispanic, Asian/Pacific Islanders non-Hispanic (API), and Hispanic adults GBM patients, a better survival rate was observed in API patients when compared to other races and ethnicity [12]. These studies indicate that GBM incidence and patient survival are dependent on race, ethnicity, and geographic region. The exact etiology of GBM remains unclear. However, studies have shown that high dose

exposure to ionizing radiation is considered as confirmed etiological factor. Though mobile phone radiation is associated with an increased risk of low-grade glioma [13], there is no conclusive evidence reported for the risk factors such as mobile phone radiation or electromagnetic field [3, 14]. Occupational risk factors such as exposure to toxic chemicals such as carbon tetrachloride have been associated with the progression of GBM [15]. Glioblastoma is a highly aggressive and invasive cancer, and therefore, even with surgery and radio/chemotherapy, there is poor prognosis in GBM patients [16]. In the last two decades, several drugs such as temozolomide (TMZ) and bevacizumab are used in GBM patients as adjuvants along with radiotherapy to improve quality of life and survival [17, 18]. TMZ is recommended for newly diagnosed GBM patients, while bevacizumab is used to treat recurrence [18]. Currently, TMZ is used as a potential chemotherapeutic agent for the treatment of GBM. TMZ metabolites are reportedly causing DNA damage; off-target effects and its continuous administration caused ineffectiveness in GBM patients due to resistance [16].

10.2 Glioblastoma Stem Cells (GSCs)

10.2.1 Historical Perspective of GSCs

A pioneering study from Bonnet and Dick (1997) confirmed the existence of heterogeneity of tumor cells, and they successfully isolated the leukemia-initiating cells, and they were considered as the first purification of cancer stem-like cells [19]. To the best of our knowledge, the human neural stem and progenitor cells (NSPCs) were first isolated by Uchida et al. (2000) using the marker CD133, and this study paved the way in the quest of brain tumor cells that shared the characteristics of NSPCs [20]. Since then, several studies described the cancer stem cells (CSCs) in a variety of brain tumors such as glioblastoma, anaplastic and pilocytic astrocytoma, ganglioglioma, medulloblastoma, and ependymoma [21–24].

10.2.2 Nomenclature

The heterogeneity of the cell populations within CNS tumors is well documented. Several terms are used to describe these cell populations; however, individually they have unique characteristics and functions. The brain CSC nomenclature is used interchangeably. However, they have unique characteristics. For instance, (1) *tumor or glioma or brain tumor stem cells* have the capacity to self-renew and give rise to differentiated progeny. Their functional characteristics include tumor generation upon secondary transplantation, and progeny contains CSCs and non-stem tumor cells; (2) *tumor or glioma or brain tumor-initiating cells* have the ability to initiate tumor after transplantation; (3) *tumor or glioma or brain tumor-propagating cells* have the ability to propagate tumor after serial transplantation.

10.2.3 Origin of GSCs

Ample evidences reported that a variety of cancers have a population of cells with stem cell characteristics that are CSCs [25]. Brain tumors consist of heterogeneous cell populations and are reported to arise from CSCs (Fig. 10.1). Clinically, GSCs have been functionally identified in brain tumors in humans [23, 26]. These stem cells have been reported to mediate treatment resistance to chemotherapy [27, 28], radiotherapy [25], and also resistant against markers involve in angiogenesis, invasion, and recurrence [28]. In origin point of view, it has been reported that GBM may arise from (1) a subpopulation of neural stem cells (NSCs), (2) transformation and proliferation of differentiated astrocytes in the subventricular zone (SVZ), and (3) the increased somatic mutations in NSCs in the SVZ [29, 30]. Moreover, an innovative experimental and clinical finding from Lee et al. (2018) confirmed that astrocyte-like NSCs in the SVZ are the cell of origin, which is the main driver mutation in human GBM [31]. This study reported that the acquirement of the telomerase reverse transcriptase (TERT) promoter mutation allows the prolonged self-renewal ability of NSCs and subsequent somatic mutation development causes GBM [31]. However, the origin of GSCs is still subjected to debate, and more studies are warranted on these lines.

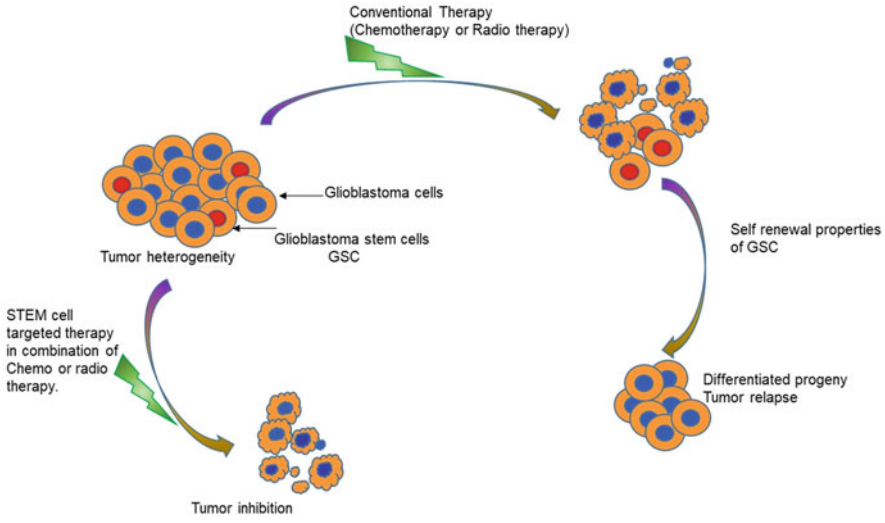

Fig. 10.1 GBM is heterogeneous which contains cancer cells and a small subset of CSCs. The CSCs can be distinguished from other cell populations. In this model, CSC-specific targeted therapies are proposed in combination with conventional chemo- and radiotherapies to kill both CSC and other cancer cells to prevent subsequent relapse

10.2.4 Cellular Hierarchy of Brain Tumors

The embryonic stem cells are the most primitive cells derived from the inner mass cells of the human embryo. They are pluripotent and capable of differentiating into any type of cells in an organism. Downstream from embryonic stem cells are multipotent progenitor cells that include NSCs, endothelial progenitor cells, hematopoietic stem cells, and mesenchymal stem cells (MSCs). These cells have restricted differentiation potential and self-renewal. The multipotent NSCs can give rise to more downstream progenitor cells with restricted self-renewal potential, differentiation, and mitosis [32]. In a hierarchical viewpoint, CSC cancer models reported to arise from CSCs by mutations in embryonic stem cells or in progenitor cells at birth or accumulate over time resulting in cells possessing the ability for uncontrolled growth and propagation [33, 34]. Studies have also shown that non-CSCs can also dedifferentiate into CSCs through epigenetic and environmental factors, thereby increasing the complexity of the tumor and thus the treatments [35].

10.2.5 Biomarkers of GSCs

Prominin-1 (PROM1/CD133), SSEA-1 (CD15), integrin-α6, and L1 cell adhesion molecule (L1CAM) are considered as markers for GSCs. PROM1 is a 5-transmembrane (5-TM) protein first recognized in the prominin family [36], and its expression was identified as a tumor initiator in a variety of cancers including GBM. Experimental studies proved the tumor-propagating potential of $PROM1^+$ cells in immunodeficient xenograft mice model [37]. Ironically, $PROM1^-$ cells also contributed to the tumor initiation [38]. This study indicates that PROM1 is not only related to tumor initiation. $PROM1^+$ GSCs are tumorigenic and exhibit self-renewal and differentiation properties [39]. Clinically, overwhelming evidences indicate that PROM1 expression and neurosphere formation were associated with short survival of GBM patients and PROM1 expression was correlated with short survival of mice transplanted with tumor cells [39, 40]. Therefore, PROM1 could serve as a prognostic marker for GBM.

Some studies have also reported the expression of stage-specific embryonic antigen-1 (SSEA-1) or CD15 in cells with tumor initiation. It has been experimentally and clinically proven that CD15 expression has been found in $PROM1^-$ tumors and nearly 40% of the freshly isolated GBM specimens did not contain CD133-positive cells [41, 42]. CD15-positive cells have the ability to generate the cellular heterogeneity of the primary tumor [43]. An experimental study from Kenney-Herbert et al. (2015) reported that CD15 was not a useful marker to distinguish a fast-proliferating, tumorigenic, or stem-like population in GBM [44]. Further, overexpression of CD44 was related to poor survival rates, and studies suggest that CD44 could act as an independent prognostic factor in patients with low-grade gliomas [45]. In GBM, high CD44 expression in GSCs promotes the tumor progression and invasion, which lead to short survival [46]. A recent study

demonstrated that high CD44-expressing GSCs in GBM are resistant to radiotherapy leading to therapeutic failure [47].

Integrins play a pivotal role in CSC biology. GSCs with high integrin-α6 expression are highly tumorigenic [48]. Integrin-α6 was reported to promote radioresistance [49]. In a recent experimental study, the functional role of integrin-α10β1 was established. siRNA-mediated knockdown of integrin-α10 in GBM cells decreased the neurosphere formation and migration and reduced the viability [50]. The neuronal cell adhesion molecule L1CAM (L1, CD171) regulates the growth, migration, and survival of neural cells and is essential for preserving the proliferation and survival of CD133-positive glioma cells with stem-like properties [43]. SRY-related HMG-box 2 (SOX2) is a glioma stem cell marker [51], and its high expression is implicated in tumor formation and chemotherapeutic resistance [52]. In a recent clinical study, Takashima et al. (2019) reported that nearly 22 sets of genes including L1CAM and sirtuin 1 can be used as the prognostic markers of GBM [53]. Though these markers help to identify the GSCs, the conclusive evidence linked to a stem cell phenotype remains elusive.

10.3 Isolation and Cultures of Primary GSCs

Fresh brain tumor specimens can be collected from patients who underwent surgical resection of histologically confirmed GBM. Specimens can be collected after obtaining necessary human ethics committee permission and with the 1964 Helsinki Declaration. Then, the collected specimen can be immediately transported in phosphate-buffered saline containing antibiotics (preferably 10–15% penicillin/streptomycin). GSCs can be isolated from the fresh tissue with trypsin. Under the sterile conditions, tumor tissue can be cut into small pieces and placed in trypsin-ethylenediaminetetraacetate solution (0.25%) and incubated for 10–15 min at 37 °C. After incubation, Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium containing 10% fetal bovine serum (FBS) can be added to stop trypsin activation and then centrifuged at 3000 rpm for 10 min to recover the cells from the enzyme. The supernatant is discarded, and complete medium is added to the cell pellet, mixed well, and filtered through a 70-μm cell strainer. Then the filtered cells are washed by centrifugation, and fresh culture medium containing 10% FBS and penicillin (100 U/mL), and streptomycin (0.1 mg/mL) is added. Further, 0.1 mL of cell suspension and 0.1 mL of trypan blue solution are added, mixed well, and counted under the inverted microscope using a cell counter. Then approximately 1×10^6 cells are seeded in culture flask containing complete medium and incubated in a standard culture condition at 37 °C and 5% CO_2. After 72 h of incubation, the flask can be observed for cell adherence and sphere generation, and the medium can be replaced until enough cell confluency is attained for passage [54, 55]. Then, the characterization should be done for the expression of tumor stem cell markers reported. Glioblastoma stem cells are cultured in Neurobasal medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to

stimulate growth, maintain the stem cell-like characteristics, and preserve the genetic profile of the GSCs [56].

10.4 Glioblastoma Cell Lines

Rat gliomas cell lines such as C6 and RG2 [D74] are commonly used to study the GBM due to their high angiogenic, migratory, and invasive properties [57]. Other human GBM cell lines such as R1, T2, A-172, T98G, U-251, U-87, A172, U-118, U-138, LN-229, and SNB-19 are characterized and also used by several workers for in vitro GBM research [58, 59]. These cell lines have unique angiogenic, migratory, invasiveness, and in vivo metastatic ability after transplantation in animals and xenograft cancer properties. For instance, LN-229 xenografts were reported to grow faster than U-251 and U-87 cell lines. Similarly, U-251 xenografts increased the tumor size. A well-defined cancer mass in the brain parenchyma was established after the transplantation of the U-87 cell line [59]. Currently, several new human-derived GBM cell lines developed by various research groups are characterized [58, 59]. However, none of the studies have recommended any GBM cell line as the ideal one, and the selection of cell lines is under the discretion of individual researchers.

10.5 Glioblastoma: Microenvironment and Niche

The niche concept was developed to describe the location of GSCs in the tumor and where the tumor microenvironment (TME) exerts its maximum influence. It is an established fact that GSCs in tumor reside in the niche that is like those hosting normal NSCs in the SVZ. The astrocytes and ependymal cells present in SVZ regulate stem cell niche, as the former establish close contacts with all cell types and with blood vessels, sensing and integrating any signals from germinal regions and vasculature within stem cell niche. The paracrine role of the niche plays a vital role in the survival of GSCs and resistance to therapy. Cells like neuroblasts, transit-amplifying cells, and quiescent NSCs also occur in the same niche [60]. These niches are surrounded by ependymal cells projected towards the ventricle and are essential for stemness maintenance.

Evidences suggest that perivascular space as a niche for GSCs survival, resistance to therapy, progression, and dissemination. The perivascular niche of GBM includes endothelial cells, astrocytes, differentiated and undifferentiated tumor cells, immune cells, pericytes, vascular basement membrane glioma-associated microglia/macrophages, myeloid cells, fibroblasts, and obviously, GSCs and normal NSCs [61, 62]. Upon specific stimuli, GSCs can transdifferentiate into pericytes or endothelial cells and directly contribute to the perivascular niche. Perivascular niches are represented by capillaries or arterioles where ECs are in direct contact with stem cells. Larger vessels like transport vessels cannot be considered as niches as they do not have direct contact with GSCs and endothelial cells [60, 63]. When niches are

found in the most invasive area of the tumor, they are called invasive niches [63]. Oxygen concentration plays a pivotal role in maintaining the stemness of GSCs defining perivascular and the hypoxic niches [64]. The existence of enriched hypoxic region GSCs has been reported as one of the hallmarks of GBM [65]. Hypoxia in GSCs involve plasticity and self-renewal and modulate the functions of non-GSCs via metabolic reprogramming and transcriptional regulation by activation of hypoxia-inducible factors 1α and 2α (HIF1α, HIF2α), Notch signaling, and epigenetic regulations [66, 67] (Fig. 10.2). Hypoxia in GSCs induces the secretion of various soluble factors such as transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), etc. which are crucial for dedifferentiation and angiogenesis in the tumor microenvironments [67]. Hypoxia condition also induces factors like C-X-C motif chemokine receptor 4, glucose transporter 1, hypoxia-inducible gene 2, serpin B9, octamer-binding transcription factor 4, and lysyl oxidase for the stemness maintenance and proliferation of GSCs [65]. The niche cells are responsible for the synthesis of a variety of signaling molecules, and at the same time, they respond to the stimuli from paracrine signaling.

10.6 Signaling Pathways Involved in GSC Regulation

Several signaling molecules secreted by the cells in tumor niche are responsible for tumor suppression and also pro-tumorigenic potential. TGF-β signals via its downstream serine/threonine kinase receptors to activate messenger proteins (SMADs) and induce the upregulation of several genes/proteins associated with the progression of GBM [68]. High TGF-β expression is involved in tumor cell proliferation, invasiveness, immunosuppression, and renewal of GSCs [69]. Interacting with fibroblast growth factor (FGF) and VEGF, TGF-β contributes to angiogenesis for rapid tumor growth [70]. Moreover, TGF-β also induces radioresistance [71]. In human GBM, the isoforms of TGF-β_1 and TGF-β_2 levels were found 33- and 11-fold, respectively, higher than in the healthy brain, and their expressions were inversely correlated with overall survival (OS) of GBM patients [70]. Bone morphogenetic protein (BMP) signaling is also a part of the TGF-β family. In GSC's point of view, BMP induces differentiation of these cells and is reported as a tumor suppressor [72, 73]. For instance, it has been reported that BMP 7 could induce the transcription factor Snail and decrease the tumor growth via activation of astrocytic differentiation in GSCs in an orthotopically xenografted immunodeficient mouse model. The same study has used a GBM cell line in vitro and confirmed the repression of the TGF-β_1 promoter upon Snail binding through its N- and C-terminal domains that interact with BMP and SMADs of TGF-β signaling [74]. These studies have proposed that BMP agonist can be developed as promising GBM-suppressive drugs. However, BMP signaling extends beyond the GSC compartment and fosters tumorigenesis in neoplastic astrocytes through the promotion of proliferation and invasion, and this experimental study suggested the differential regulation of BMP in GSCs and in other GBM compartments [75].

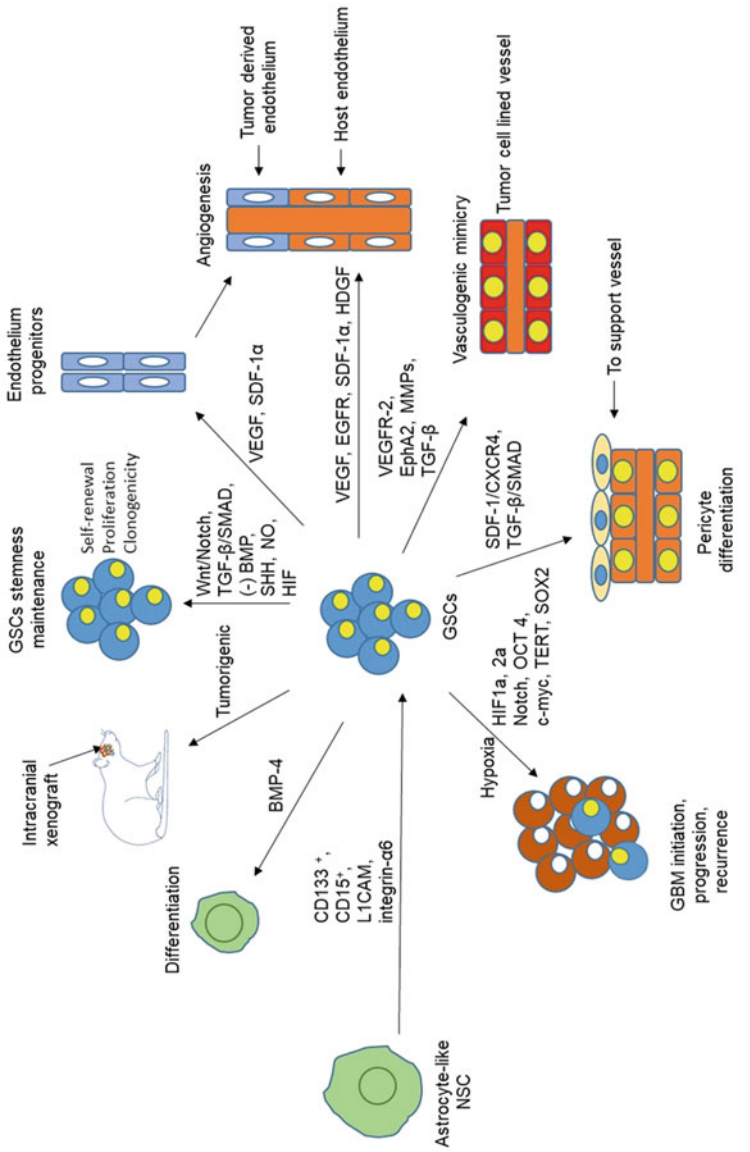

Fig. 10.2 Characteristic features of glioblastoma stem cells (GSCs). Neural stem cells (NSCs), L1 cell adhesion molecule (L1CAM), Sonic hedgehog (SHH), transforming growth factor beta (TGF-β), bone morphogenetic protein (BMP), nitric oxide (NO), hypoxia-inducible factors (HIFs), vascular endothelial growth factor (VEGF), stromal cell-derived factor-1α (SDF-1α), epidermal growth factor receptor (EGFR), hepatoma-derived growth factor (HDGF), ephrin type-A receptor 2 (EphA2), matrix metalloproteinases (MMPs), C-X-C motif chemokine receptor 4 (CXCR4), octamer-binding transcription factor 4 (OCT 4), telomerase reverse transcriptase (TERT), SRY (sex-determining region Y)-box 2 (SOX2)

Notch and Wnt/β-catenin signaling pathways are strongly implicated in GBM progression. Notch signaling via NO plays a critical role in both GSC maintenance and GSC radioresistance [76]. Gersey et al. (2019) reported the increased Notch activation and expression in human GBM cell lines [77]. Wnt/β-catenin and Notch signaling are involved in tumor progression by promoting proliferation and clonogenic ability and inhibiting neuronal differentiation in GSCs. This has been confirmed in a study in which dual inhibition of Wnt/β-catenin and Notch signaling in GSCs that express high levels of the proneural transcription factor ASCL1 leads to robust neuronal differentiation and inhibits clonogenic potential [78]. However, studies have also demonstrated that treatment with Wnt ligands and β-catenin overexpression may induce neuronal differentiation and inhibit the proliferation of primary GBM cells [79]. Several studies illustrated the Wnt activation in human GBM and suggested the novel Wnt signaling antagonists for the treatment of GBM [80].

GSCs secrete several cytokines or growth factors, such as epidermal growth factor receptor (EGFR) and VEGF, to promote angiogenesis through endothelial cell proliferation and recruitment [76]. Like other cancers, EGFR and VEGF expressions are strongly implicated in human GBM. Increased expression of EGFR has been reported in GSCs, and EGFR is essential for proliferation, survival, and invasiveness of GSCs [81]. In an in vitro study, ZR2002 (a small-sized and novel inhibitor developed with haloalkyl arm capable of reacting with the receptor itself and with DNA and bases and crossing the blood-brain barrier) induced cytotoxicity in GSCs resistant to TMZ and gefitinib, a clinical EGFR inhibitor [82]. The VEGF is a highly specific endothelial cell mitogen that has been shown to promote vascular endothelial cell proliferation, migration, and survival, resulting in tumor angiogenesis, a requirement for glioma [83]. In another study, glioma stem cell-derived exosomes promoted the angiogenic ability of endothelial cells through miR-21/VEGF signal growth [84].

10.7 Glioblastoma Stem Cells as a Therapeutic Target

Glioblastoma is highly heterogeneous involving several cell types, and GSCs secrete a variety of cell signaling molecules and respond to various paracrine signaling. Therefore, targeting with a single agent is a challenging task. However, studies have reported targeting specific GSC receptors and came out with promising results. For instance, in an epigenetic point of view, histone deacetylases (HDACs) are implicated in several cancer cell types including GBM. Therefore, HDAC inhibitors such as TSA and SAHA have been tested against the U87-MG cell line and primary tumor (GBM011) cells. HDAC activity blockade downregulates the GBM progression due to the modulation of plasticity in vitro [85]. Integrin-α7 (ITGA7), a major laminin receptor in GSCs, has been identified as a therapeutic target in GBM patients. Targeting of ITGA7 by RNAi or blocking mAbs impaired laminin signaling and delayed tumor engraftment and invasion [86]. TGF-β inhibitors like

AP12009, LY2157299, and GC1008 have been tested in clinical trials; however, they had limited therapeutic potential in GBM patients [87].

In the hypoxia perspective, GSCs are maintained in the hypoxic niche and are responsible for GBM initiation, progression, and recurrence. Notch pathway is important for hypoxia-mediated maintenance of GSCs, and therefore, inhibition of Notch signaling or depletion of HIFs can inhibit the hypoxia-mediated maintenance of GSCs. In previous studies, blocking Notch signaling through γ-secretase inhibitors caused depletion of $CD133^+$ in GSCs, decreased neurosphere formation, and inhibited xenograft tumor growth through decreased Akt and STAT3 phosphorylation [88]. Combination therapy using Notch and Akt inhibitors, MRK003 and MK-2206, respectively, tested in the GBM cell line indicates that combination therapy is useful in controlling invasion but not the proliferation of GBM cells [89]. However, a phase 1 clinical trial encountered Notch inhibition via RO4929097, gamma-secretase inhibitor in glioma patients, and reported that the inhibition of Notch signaling alone is insufficient to fully control tumor progression; however, this study is not GSC specific [90].

Epithelial-to-mesenchymal transition (EMT)-like process is considered to play an important role in the invasiveness in GBM. In a study, metformin has been reported to inhibit the self-renewal ability of GSCs and decrease the expression of stem cell markers such as Bmi1, Sox2, and Musashi1, indicating that metformin could inhibit cancer stem-like properties of GBM cells. Metformin also inhibited the Akt and TGF-β2 and its downstream SMAD signaling [91, 92]. Interestingly, a plant-derived drug resveratrol is reported to inhibit EMT-assisted self-renewal capacity of GSCs and EMT-induced cancer stem cell markers Bmi1 and Sox2. These effects were also confirmed in the xenograft experiments in vivo [93].

In the immunology perspective, GBM patients have been targeted with several immune modulators. For instance, cetuximab, trastuzumab, and panitumumab are EGFR monoclonal antibodies that have shown effectiveness in preclinical studies. However, in phase 2 clinical trial, cetuximab had no therapeutic effect and no changes in OS [94, 95]. GSCs specifically express SOX6 and are killed by cytotoxic T lymphocyte (CTL) primed against SOX6-derived peptides [96], and therefore, GSC antigen peptides recognized by CTL can be applied to immunotherapy that targets GSCs [97]. Dendritic cell-based vaccination-induced CTL recognized GSCs and prolonged the survival of tumor-bearing xenograft animals [98]. The AC133 epitope of CD133 is a vital GSC marker for GBM; therefore recombinant AC133 × CD3 bispecific antibody was developed. This bispecific antibody redirected to polyclonal T cells to CD133+ GSCs and decreased colony-forming ability of human glioma cell line (U-251), induced their lysis, and prevented the xenograft growth in vivo [99]. Similarly, several immune targets have been tried in GSC for the treatment of GBM. Clinical trials targeting GSCs in GBM condition has been listed in Table 10.1.

Table 10.1 Clinical trials targeting GSCs/CSCs in GBM

Study title	Intervention	Outcome	Study type	ClinicalTrials.gov identifier
Study of the capacity of the MRI spectroscopy to define the tumor area enriched in GSCs	Surgery (based on preoperative multimodal MRI) followed by the standard radio/chemotherapy Stupp protocol	Not available	Prospective biomedical study of interventional type	NCT01872221
Standard chemotherapy vs. chemotherapy guided by Cancer stem cell test in recurrent GBM	Chemotherapy	Not available	Phase 3	NCT03632135
Cancer stem cell high-throughput drug screening study	Combination drug cocktail therapy—Antineoplastic, anti-infective, antiemetic, antihyperlipidemic, anti-inflammatory, antihistamine, antihypertensive, antidepressant, cardiotonic alcohol antagonist, diuretic, antipsychotic, NMDA receptor antagonist, antidiabetic, immunosuppressant, anticonvulsant, anti-methemoglobinemic, sclerosing agent	Not available	Prospective single-center phase 0/1 open-label study	NCT02654964
Dendritic cell immunotherapy against cancer stem cells in Glioblastoma patients receiving standard therapy (DEN-STEM)	*Biological:* Dendritic cell immunization *Drug:* Adjuvant temozolomide	Not available	Phase 2/3	NCT03548571
Phase I/II trial of vaccine therapy with tumor stem cell derived mRNA- transfected dendritic cells in patients receiving standard therapy for GBM	*Biological:* Dendritic cell vaccine with mRNA from tumor stem cells	Not available	Phase 1/2	NCT00846456
A prospective trial of neural progenitor cell sparing radiation therapy plus Temozolomide for newly diagnosed Glioblastoma Multiforme	*Radiation:* Radiation *Drug:* Chemotherapy	Not available	Interventional (open-label clinical trial)	NCT01478854
Benzylguanine-mediated tumor sensitization with chemo-protected	*Radiation:* 3-dimensional conformal radiation therapy	Not available	Phase 1/2	NCT00669669

autologous stem cells for patients with malignant Gliomas	*Procedure:* Autologous hematopoietic stem cell transplantation *Drug:* Carmustine *Biological:* Filgrastim *Procedure:* In vitro-treated peripheral blood stem cell transplantation *Radiation:* Intensity-modulated radiation therapy *Other:* Laboratory biomarker analysis *Drug:* O6-benzylguanine *Drug:* Plerixafor *Radiation:* Proton beam radiation therapy *Drug:* Temozolomide			
STRONG trial—stem cell radiotherapy (ScRT) and Temozolomide for newly diagnosed high-grade Glioma (HGG): A prospective, phase I/II trial	*Radiation:* Stem cell radiotherapy (ScRT) and temozolomide	Affected/at risk (%)—2/4 (50.00%)	Phase 1/2	NCT02039778
Recurrent GBM stem cell tumor amplified RNA immunotherapy trial	*Biological:* BTSC mRNA-loaded DCs	Not available	Phase 1	NCT00890032
Phase I study of vaccination with dendritic cells loaded with Brain tumor stem cells for recurrent or progressive malignant Gliomas	*Biological:* Dendritic cells Drug: Imiquimod	Not available	Phase 1	NCT01171469
A phase 1 trial of tumor associated antigen pulsed dendritic cell immunotherapy for patients with brain stem Glioma and Glioblastoma	*Biological:* Autologous dendritic cells	Not available	Phase 1	NCT00576641

Ref. [100]

10.8 Conclusions

Till now, GBM is the most deadly and aggressive cancer. Several types including GSCs in the TME contribute to the progression and relapse. Available studies demonstrated that GBM microenvironment has a variety of CSC population including GSCs which display stem-like properties. GSCs are resistant to chemo- or radiotherapy, and hence, GSC-specific targeted therapies are need for the hour. Three main features of the GBM that hamper the treatment are (1) occurrence of chemo- or radioresistant GSCs in the TME, (2) heterogeneity of tumor, and (3) the microenvironment and the niche responsible for the secretion of various oncogenic soluble factors. Often, glioma treatments with various strategies affect the normal cells residing near the cancer environment. Till now, only a basic understanding of plasticity, differentiation, and colony formation of GSCs has been reported and the results derived from such studies are inconsistent. Though studies have reported the modulation of several cell signaling molecules in GSCs, several aspects have not yet been fully established. For instance, BMP is differentially expressed in GSCs as well as in other areas like solid tumors. The reason behind this kind of discrepancies needs to be addressed soon. Some plant-derived drugs have high molecular weight with poor bioavailability that hamper its ability to cross the blood-brain barrier. Therefore, care should be taken on drug size and bioavailability. Moreover, most of the therapeutic interventions are tested against in vitro and in vivo using xenograft animal models. Such studies are useful for interpretation of the results, and per se they will not recapitulate the TME of humans, and it will not give the conclusive evidence, and therefore, more studies are warranted on GBM patients.

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Cancer Stem Cells and Therapeutic Angiogenesis

11

Sambhavi Bhagavatheeswaran and Anandan Balakrishnan

Abstract

Angiogenesis is a highly regulated process of formation of new blood vessel from preexisting blood vessel during fetal development, ovulation, and wound healing. Tumor growth and maintenance are critically controlled by tumor angiogenesis by facilitating the ingress of tumor cells into the circulatory system and in turn metastatic spread of the tumor. Apart from self-renewal and proliferating capabilities, cancer stem cells (CSCs) are also involved in tumor angiogenesis. CSCs establish a vascular niche by expressing vascular-related mediators to induce neovascularity around tumors. Developing antiangiogenic agents that also targets CSCs and evaluating its effect on a three-dimensional (3D) angiogenesis spheroid model are significant cancer therapeutic measures as the interactions between niche and CSCs and the heterogeneity can be understood better by using 3D spheroid. Furthermore exploiting the antiangiogenic effect of phytochemicals is beneficial over other available conventional drugs as they have relative pharmacological safety and target multiple molecular pathways to exert its anticancer effect.

Keywords

Cancer stem cells · Vascular niche · Phytochemicals · Spheroid · Antiangiogenesis

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Abbreviations

ABCG-2	ATP-binding cassette subfamily G member 2
ALK1	Activin A receptor-like type 1
BM	Basement membrane
BMP	Bone morphogenic proteins
BMPR	Bone morphogenetic protein receptor
COX-2	Cyclooxygenase-2
CSC	Cancer stem cell
EC	Endothelial cell
ECM	Extracellular matrix
EGCG	Epigallocatechin gallate
EPC	Endothelial progenitor cell
FGF	Fibroblast growth factor
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
MVD	Microvessel density
PD-ECGF	Platelet-derived endothelial cell growth factor 1
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
SDF-1	Stromal cell-derived factor 1
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor 2

11.1 Angiogenesis

Angiogenesis is a complex process that involves the activation, proliferation, and directed migration of endothelial cells to form new capillaries from existing blood vessels [1]. This highly regulated process occurs during various physiological conditions like human development, reproduction, and wound repair [2]. Angiogenesis also has its role in pathological conditions like cancer, rheumatoid arthritis, psoriasis, and proliferative retinopathy [3]. Various factors like soluble polypeptides, cell-cell and cell-matrix interactions, and hemodynamic effects influence angiogenesis. Various inducers of angiogenesis include vascular endothelial growth factor (VEGF) family, angiopoietins, transforming growth factors (TGF), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), interleukins, and members of the fibroblast growth factor (FGF) family [4, 5]. Among these VEGF-A is the most potential proangiogenic protein which helps in induction of proliferation, sprouting, and tube formation of endothelial cells (ECs). Under hypoxic condition VEGF binds to its tyrosine kinase receptors (VEGFRs) present on endothelial cells and triggers the signal transduction pathways that kindle the cells to undergo sprouting angiogenesis [6, 7].

11.1.1 Tumor Angiogenesis

Angiogenesis plays a key role in the metastatic spread of the tumor by facilitating the ingress of tumor cells into the circulatory system and hence instrumental in the formation of pre-metastatic vascular niche [8]. As the tumor progresses, it becomes malignant and attains the hypoxic condition. Hypoxia induces the release of angiogenic growth factor molecules. This dominating proangiogenic signaling turns on the angiogenic switch. Upon binding of these growth factors to the endothelial cell receptors of the blood vessels in close proximity arises new blood vessel that invades the tumor and aids in its progression [9] (Fig. 11.1). The blood vessels formed during tumor angiogenesis are different from the normal vessel in that the walls of tumor vessels are made of both tumor cells and ECs [11]. Peripheral blood vessels are often devoid of functional pericytes [12] and thus an incomplete basement membrane which makes those vessels to be leaky and dilated [13]. Analogous to normal angiogenesis, tumor angiogenesis also relies on VEGF and other angiogenic proteins for tumor vasculature maintenance. Elevated expression of VEGF and its receptor VEGFR-2 was reported in many cancers, including metastatic human colon

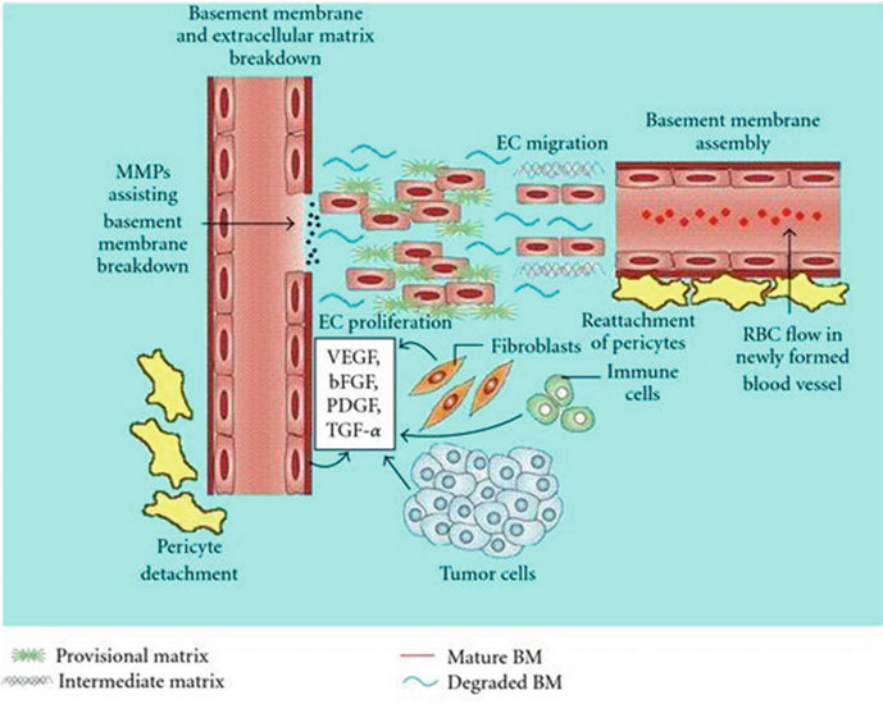

Fig. 11.1 ©Tumor-influenced angiogenesis. The stepwise process of angiogenesis begins with ECM and BM breakdown, followed by EC proliferation, EC migration, and finally re-formation of stable blood vessel. Tumor cells will secrete a variety of factors to ensure that the new blood vessels formed are fed directly to the tumor tissue [10]

carcinomas, which results in enhanced tumor vascularization [14]. Ang-2 (angiopoietin-2) expresses greatly in ECs of tumor vessel than in normal vessels. It imparts plasticity to developing vasculature in the presence of VEGF and plays an important role in initial stages of tumor angiogenesis [15, 16]. TGF-β signaling activates tumor growth and metastasis through induction of stromal reaction by neoplastic cells which results in the promotion of angiogenesis and tumor growth [17–19].

11.2 Cancer Stem Cell (CSC) and Angiogenesis

Apart from self-renewal and proliferating capabilities, CSCs are also involved in tumor angiogenesis. CSCs establish a vascular niche by expressing vascular-related mediators to induce neovascularity around tumors [20]. Tumor heterogeneity is influenced extrinsically by a major factor called tumor microenvironment or niche. A niche is a composition of stromal cells, immune cells, endothelial cells, and cancer cells per se, as well as connective tissue components, growth factors, and cytokines [21]. Niche plays an essential role in the maintenance/enrichment, preservation of the phenotypic plasticity, immune surveillance, differentiation/dedifferentiation, angiogenesis activation, and invasion/metastasis of CSC [22–24]. CSCs and angiogenesis thus exhibit positive feedback and contribute to tumor angiogenesis.

11.2.1 Molecular Interplay of CSC and Angiogenesis

At both normoxic and hypoxic conditions, CSCs secrete greater levels of VEGF than the non-CSC population. Such CSC-mediated VEGF production leads to enhanced endothelial cell migration and tube formation in vitro (Fig. 11.2). These newly generated vessels supply nutrients for the growth and development of cancer [25]. Diverse studies confirmed the potential of CSCs to encourage angiogenesis and produce angiogenic cells that interact with vascular niche and promote angiogenesis through the secretion of VEGFs, stromal-derived factor 1 (SDF-1), and

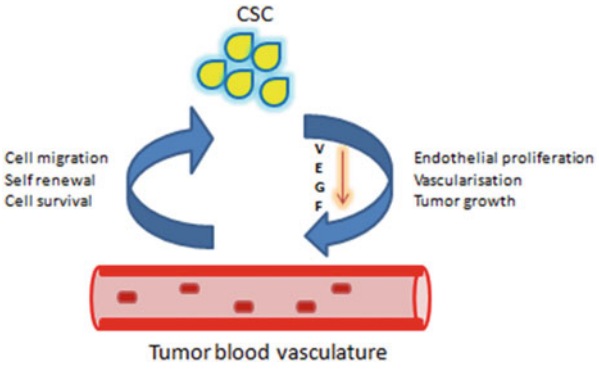

Fig. 11.2 Interplay of CSC and angiogenesis

tumor microvesicles [26–28]. As to confirm this, Grange et al., in 2011, showed that microvesicles that shed from CSCs from human renal cell carcinoma carry a set of proangiogenic mRNA and microRNA, which bestow the angiogenic phenotype to the normal human endothelial cells and thus trigger angiogenesis by promoting their growth and vessel formation.

11.2.1.1 Signaling Pathways Linking CSC and Angiogenesis

To understand how vascular endothelial cells maintain CSC proliferation, it is essential to be aware of the signaling pathways that relate CSC and angiogenesis. BMP (bone morphogenic protein) signaling is one such critical pathway. BMP which is known to help in bone formation is also involved in angiogenesis inhibition. BMP-9/ALK1 inhibits angiogenesis by suppressing VEGF expression through BMP-9 which is against the effect of the TGFβ1/ALK5 pathway that enhances VEGF expression and angiogenesis. BMP-4 balances these two pathways and maintains the vascular integrity [29]. This BMP-4 is also shown to exert anti-tumorigenic effect through BMP-4/BMPR/SMAD signaling pathway in glioblastoma cancer stem cells [30].

Notch signaling pathway is yet another pathway that relates CSCs and angiogenesis. Vascular development and survival of normal stem cell are supported by the Notch/NICD/Hes/Hey signaling pathway [31, 32]. Studies show that renewal of CSCs and angiogenesis in glioblastoma are also supported by Notch pathway. Inhibition of this Notch pathway using DAPT (γ-secretase inhibitor) reduced the number of CD133+ tumor cells and the ability of CSCs to self-renew. Vascular markers such as CD105, CD31, and von Willebrand factor also exhibited reduced expression [33].

11.2.1.2 CSCs Expressing Angiogenic Markers

A variety of CSCs that express vascular endothelium markers to support tumor blood vessel formation have been discussed in this section. Nestin+/CD133+ brain cancer (oligodendroglioma and glioblastoma) stem cells are in close interaction with endothelial cells and provide factors that are responsible for self-renewal mechanisms of CSCs. Further these stem cells are to be found in close proximity to CD34+ capillaries and correlate to microvessel density (MVD) [34]. In malignant gliomas, Bao et al. observed that CD133-enriched SCLGC (stem cell-like glioma cells) are located near blood vessels. CD133+ SCLGC enhanced tumor vascularity, necrosis, and hemorrhage when compared to CD133- SCLGC. CD133+ SCLGC also elevated VEGF expression around 10- to 20-fold, and vascular density was significantly increased [25]. Yang et al., in 2010, showed that in hepatocellular carcinoma, the MVD and angiogenic factors like VEGF and PD-ECGF are dramatically expressed in the high hepatic stem/progenitor cell (HSC/HPC) profile group (CD133, nestin, CD44, and ABCG2) than in the low HSC/HPC profile group. In the tumors of glioma condition, huge CSC population recruits enormous amount of endothelial progenitor cells (EPC) which in turn stimulates VEGF and SDF-1 and thereby brings about local angiogenesis and mobilizes EPC. These data are partly an indication to the fact that cancer stem cells induce tumor angiogenesis by introducing angiogenic factors to the cancer microenvironment (Table 11.1).

Table 11.1 CSCs expressing angiogenic markers

Tumor type	CSC marker	Angiogenic marker	Function
Malignant glioma	CD133	VEGF	Cause MVD increase
Oligodendroglioma and glioblastoma	CD133, nestin	CD34	Cause MVD increase
Hepatocellular carcinoma	CD133, ABCG2, nestin CD44	VEGF, PD-ECGF	CSC and angiogenic markers co-expressed
Glioblastoma	Nestin	VEGF, SDF-1	Induce angiogenesis
Pancreatic cancer	CD133	VEGF-C	Cause MVD increase

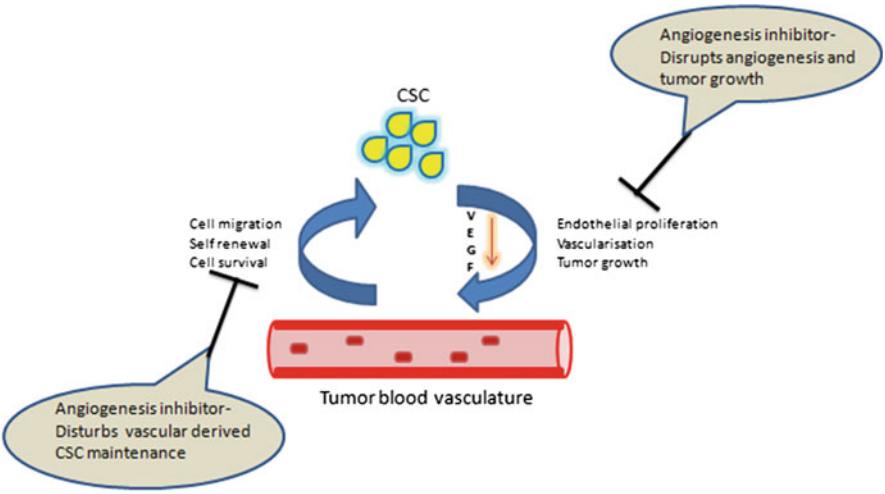

Fig. 11.3 Antiangiogenic therapy by targeting CSCs and tumor angiogenesis

11.3 Antiangiogenic Therapy by Targeting CSCs as a Significant Therapeutic Measure

Tumor vasculature that regulates the tumor microenvironment also contributes to stem and progenitor cell formation [35]. Angiogenesis inhibitors are thus expected not only to disrupt vessel formation but also target the CSC that contributes to tumor angiogenesis (Fig. 11.3). The available antiangiogenic agents like bevacizumab, thalidomide, sorafenib, sunitinib, and pazopanib along with targeting the vascular niche are also involved in the commotion of the CSC microenvironment [36] (Fig. 11.3). Antiangiogenic therapy in treating brain cancers is executed by targeting VEGF, which inhibits the tumor vasculature, partly disturbs the CSC vascular niche, and ablates self-renewing cancer stem cells [34]. Celecoxib, an anticancer drug, is cytotoxic for CSCs. It exerts anticancer effect by inhibiting the COX-2/PGE2/VEGF

and WNT/LGR5 stemness pathways. COX-2 produces prostanoids PGE2, which are released by tumor and stromal cells. PGE2 acts in an autocrine/paracrine manner binding to surface members of the prostanoid receptor family (EP1–4) to increase cancer cell stemness and angiogenesis (via production of VEGF and FGF) [37]. All the above findings infer the fact that developing antiangiogenic agents that also target CSCs is a significant cancer therapeutic measure. Such a combination of therapy serves the dual purpose of depriving the tumor of vascular supply and preventing the recurrence of tumor by debulking the tumor mass.

11.3.1 Implications of Phytochemicals as Potential CSC-Targeting Therapeutics

Phytochemicals are naturally occurring bioactive compounds derived from plants. They represent a good candidate for chemopreventive and chemotherapeutic applications. Phytochemicals are also reported to modulate the CSC phenotype by intervening the signaling pathways critical for stemness maintenance of CSCs [38]. Cyclopamine, initially found in the corn lily, targets hedgehog signaling [39–42], while EGCG and retinoic acid inhibit Wnt/−catenin signaling [43–45] and Notch signaling [46, 47], respectively. These signaling pathways are responsible for CSC self-renewal, differentiation, and invasive abilities. Furthermore, phytochemicals from turmeric (curcumin) and long pepper (piperine) are known to bring about anticancer effect upon targeting breast CSCs by inhibiting Notch and/or Wnt/−catenin signaling [48]. On the basis of the above accumulating evidence, it is evident that phytochemicals have beneficial effects against CSCs as well as cancer cells. However there seems to be no studies supporting the effect of phytochemicals on targeting CSCs expressing angiogenesis markers. Future studies should hence be directed toward identifying antiangiogenic phytochemicals targeting CSCs as use of phytochemicals is beneficial over other available conventional drugs as they are naturally present in edible plant materials and have relative pharmacological safety. Moreover they bring about anticancer effect by targeting multiple molecular pathways.

11.4 Angiogenesis Spheroid Models: Connecting Vascular Niche and CSCs

The interactions between niche and CSCs and the heterogeneity can be understood better by using three-dimensional (3D) spheroid. 3D spheroids recapitulate the spatial dimension, cellular heterogeneity, and the molecular networks of the tumor microenvironment. Sophisticated 3D models are proposed with the potential to further understand the CSCs in a more appropriate condition resembling the in vivo microenvironment. In vitro spheroid forming ability is considered as a substitute to examine the functionality of CSCs as CSCs pose the tendency to propagate as spheroid bodies [49]. As the spheroid models mimic the in vivo cancer

microenvironment, it is essential for any anticancer drug to be tested upon the 3D spheroid models than the 2D monolayer cultures. Any antiangiogenic drug thus developed needs to be checked upon the angiogenesis spheroid model to test the antiangiogenic effect by its ability to inhibit sprouting (tube formation) of blood vessels as well as spheroid development which is an indication of cancer stemness.

11.4.1 Sprouting Spheroid-Based 3D Angiogenesis Model

A 3D spheroid system involving co-culturing of endothelial cells, fibroblasts (fibroblasts in direct contact with endothelial cells allow formation of endothelial cell tubules in vitro), and the tumor cell line mimics the complex cancer-stromal interactions and tumor angiogenesis condition. This model system bridges the gap between 2D monoculture and in vivo systems and serves as potential platform to test the efficacy of various antiangiogenic drugs on a 3D in vitro model of tumor [50].

A 3D spheroid co-culture system is generated by adding HUVECs, fibroblast cells, and tumor cells to each well of a 96 U-well suspension plate. The cells form spheroids overnight at 37 °C. The spheroids are then transferred to a collagen type-I-coated 24 well plates. Angiogenesis inhibitors were added to the wells, and the spheroids were allowed to form sprouts for 2 days at 37 °C. Spheroids incubated in type-I collagen form capillary-like sprouts that mimic early stages of tumor angiogenesis. Angiogenic sprouting can be evaluated by analyzing the sprouts arising from the spheroid core using phase contrast microscope. Number of sprouts and length (mean and cumulative values) of the sprouts are the important parameters to be analyzed. Further the spheroid cells can be sorted using FACS for identification of altered angiogenic and CSC markers upon drug treatment [51] (Fig. 11.4).

11.5 Perspectives and Future Direction

Critical progress has been made in the field of cancer stem cell biology over the years. The accumulating evidences stated in this chapter reveal the importance of antiangiogenic phytochemicals targeting the CSC population. Application of in vitro angiogenesis spheroid models that mimic the capillary-sprouting mechanism and cancer stemness of tumor microenvironment in anticancer drug development has significant ramification for the future of cancer therapeutics.

Clinical manipulation of the interplay between CSCs, angiogenesis, and the tumor vasculature opens up new therapeutic windows in the area of tumor biology to provide antitumor effect. Integrating antiangiogenic therapy with anti-CSC therapy in treatment paradigm may improve the efficacy of current cancer therapies. Furthermore, it is essential to take into account that this integrated therapeutic strategy should have minimal or no effect on normal stem cells. As there are no studies reporting the role of phytochemicals in targeting CSC expressing angiogenic markers, future studies should be directed toward involving the antiangiogenic phytochemicals as a novel paradigm for potential CSC-targeting therapeutics.

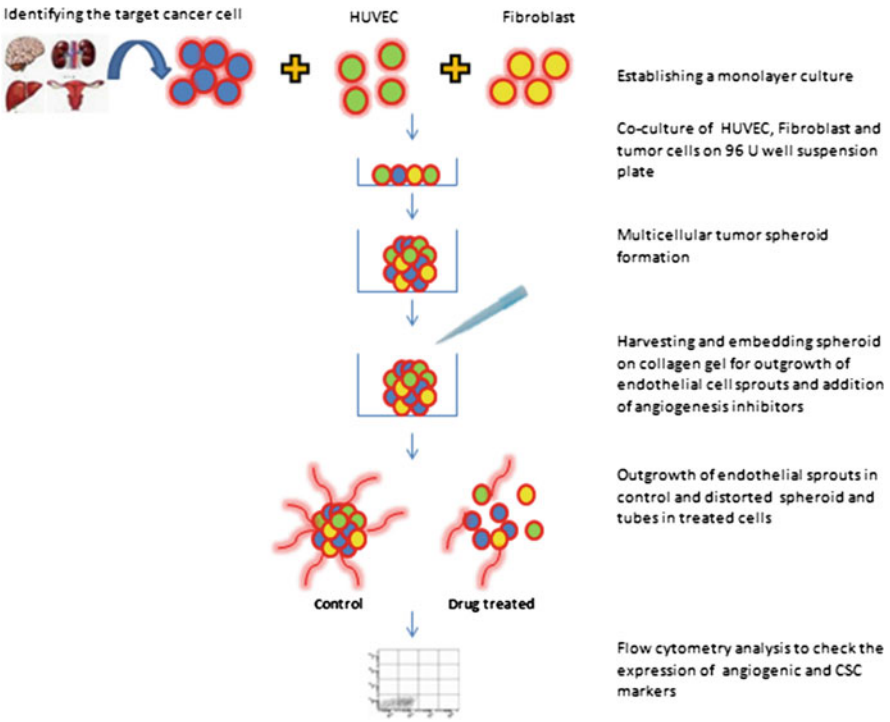

Fig. 11.4 Angiogenesis spheroid assay

CSCs often exhibit EMT (epithelial-mesenchymal transition) properties. EMT confers tumor angiogenesis by upregulated expression of the proangiogenic factor VEGF-A. In recent years studies are involved in delineating the complex EMT network using CRISPR/Cas technology [52, 53]. Therefore as an alternative approach, further research is warranted to apply emerging CRISPR/Cas9 gene editing technology to target EMT expressing CSC-related genes to alleviate the tumor burden.

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Cancer Stem Cells as a Seed for Cancer Metastasis

12

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Abstract

Cancer is one of the leading causes of death worldwide. Recent report from the World Health Organization suggested that, globally, one in six deaths is owing to cancer. In 2018, it was accountable for nearly 9.6 million deaths, and it is expected to be 14.6 million by the year 2035. The worldwide burden of cancer increase is due to aging and growth of population. In addition, cancer-associated lifestyle choices like smoking, sedentary habits and westernized diets increases the risk. Metastasis is complex and multistep process that results in the spread of cancerous cells from the primary site of the tumor to the surrounding tissues and to distant organs. Metastatic cancer is the primary cause of cancer morbidity and mortality. Several studies suggest that tumor has heterogeneous cell population and have numerically less cancer stem cell (CSC) population with self-renewal characteristics. CSCs are shown to drive tumor initiation, progression, metastasis, recurrence, and resistance. In addition, acquisition of epithelial-mesenchymal transition, expression of aberrant RNA-binding proteins, dysregulated microRNA expression, and increase in intercellular transfer of molecules via exosome cargo have been correlated with tumor progression, invasion, metastasis, poor survival, and an increased risk of cancer recurrence. Given the tumor initiating capacity, resistance, migratory potential and invasiveness, CSCs are the

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seeds of metastasis. This review article attempts to provide the details of the critical importance of CSCs on metastatic process and to offer a basis for the investigation of novel targets to curtail this deadly disease.

Keywords

MicroRNA · Epithelial-mesenchymal transition · Exosomes · RNA-binding proteins

12.1 Introduction

Cancer is one of the leading causes of death worldwide [1]. Recent report from the World Health Organization suggested that, globally, one in six deaths is owing to cancer. In 2015, it was accountable for nearly 8.8 million deaths, and it is expected to be 14.6 million by the year 2035. The worldwide burden of cancer increase is due to aging and growth of population. In addition, cancer-associated lifestyle choices like smoking, sedentary habits, and westernized diets increases the risk. Cancer is a complex disease with various cellular alterations that will result in self-sufficiency in growth signal leading to abnormal cell growth, evading apoptotic and growth suppressor signals, and increased angiogenesis, wherein, network of blood vessels develop and penetrates into the tumor to supply nutrients and oxygen for the cancerous cells. Some of the tumor cells invade surrounding tissues and distant organs through the blood circulation or lymph vessels. This spread of cancer cells from primary tumor to other sites is termed metastasis, which is shown to be responsible for more than 90% of cancer-related death. Cancer stem cells (CSCs) are cells within a tumor that exclusively have self-renewal capacity and can give rise to all cancer cell lineages within a tumor and are exclusively tumorigenic in vivo. They undergo asymmetric/symmetric cell division, can maintain and expand themselves, and also have a distinct profile of surface marker expression that has been linked to poor prognosis [2]. Intriguingly, it has been shown that CSCs drive tumor initiation, progression, metastasis, recurrence, and resistance. Identification of CSC-specific surface markers has provided opportunity to characterize CSCs and their role in tumor progression and metastasis. In addition, acquisition of epithelial-mesenchymal transition (EMT) features, expression of aberrant RNA-binding proteins, dysregulated microRNA expression, and increase in intercellular transfer of molecules via exosome cargo have been correlated with tumor progression, invasion, metastasis, poor survival, and an increased risk of cancer recurrence. Given the tumor initiating capacity, resistance, migratory potential and invasiveness, CSCs are the seeds of metastasis [3]. This review article attempts to provide the details of advances in the role of CSCs on metastatic process that will aid in better understanding of the involvement of cancer stem cells (CSCs) in the metastatic processes and to offer a basis for the investigation of novel targets to curtail this deadly disease (Fig. 12.1).

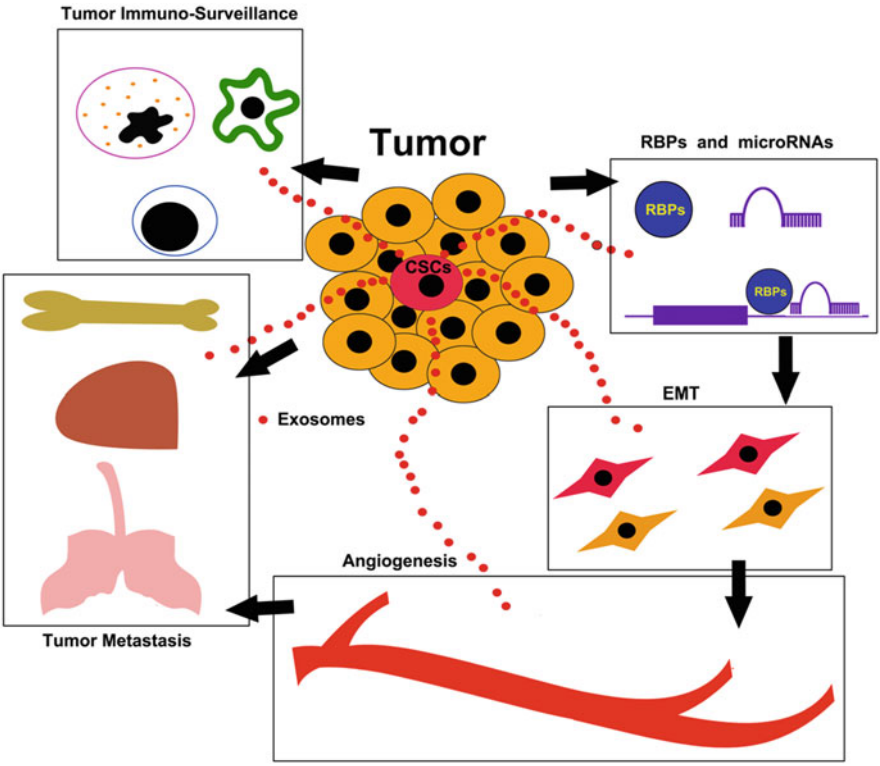

Fig. 12.1 Schematic representation for cancer metastasis

12.2 Epithelial-Mesenchymal Transition in Regulation of CSCs and Metastasis

Epithelial-mesenchymal transition (EMT) is a process where the cancer epithelial cells lose many of their epithelial characteristics and acquire various mesenchymal cell characteristics such as cell morphology, cytoskeletal organization, and cell junctions that will enable cell invasion and migration. Cancer cells have been observed in the circulation, whether as single or in clusters; these cells display signs of at least partial epithelial-mesenchymal transition [4]. Earlier reports have provided evidence that in the tumor, only CSC-enriched subpopulation exhibits aspects of EMT-related gene activation [5, 6]. In addition, induction of EMT-related gene expression in epithelial tumor cells has increased their capacity for tumor progression and metastasis [7]. Eliminating CSCs alone will not be sufficient to prevent tumor recurrence as the non-CSCs can undergo EMT and dedifferentiate into CSCs [8]. Therefore, for effective cancer therapeutic strategy,

Table 12.1 Cell surface markers of CSCs in different types of cancer

Cancer types	Cell surface markers of CSCs	References
Acute Myeloid Leukemia	$CD34^+$, $CD38^-$	Won-Tae Kim and Chun Jeih Ryu [9]
Cervical cancer	$CD133^+$ and CD49F	Ruixia Huang and Einar K. Rofstad [10]
Bladder cancer	$CD44^+$ and CD67LR	Yi Li et al. [11]
Oral squamous cell carcinoma	$CD44^+$	Weiming Lin et al. [12]
Renal cell carcinoma	$CD133^+$, CXCR4, $CD105^+$	Zhi-Xaiang Yuan et al. [13]
Hepatic/liver cancer	Laminin-332	Olivier Govaere et al. [14]
	$CD133^+$, $ALDH^+$, $CD45^-$, $CD90^+$, and $CD44^+$	Jing-Hui Sun et al. [15]
Esophageal squamous cell carcinoma	Integrin X7 (ITGA 7)	Xiao-Yan Ming et al. [16]
Colon cancer	$CD133^+$, $CD44^+$, and $CD24^+$	Sahlberg SH et al. [17]
Lung cancer	$CD133^+$, ABCG2 (high)	Shaheenah Dawood et al. [18]
Thyroid cancer	$CD133^+$ and $CD144^+$	Zhenying Guo et al. [19]
Breast cancer	$CD44^+$, $CD24^-$, $CD133^+$, and $ALDH^+$	Bin Bao et al. [20]
Ovarian cancer	$CD44^+/CD117^+$	M-Q Gao et al. [21]
Gastric cancer	$CD44^+$	Yoshiro Saikawa [22]
Pancreatic cancer	$CD44^+$, $CD24^+$, and ESA^+	Chenwei Li et al. [23]
Glioblastoma multiforme	$CD133^+$	Shideng Bao et al. [24]
Melanoma cancer	$CD20^+$	Dong Fang et al. [25]
Prostate cancer	$CD44^+/CD24^-$	Eun-Jin Yun et al. [26]
Brain cancer	$CD133^+$	Sheila K Singh et al. [27]

both CSCs and non-CSCs should be simultaneously targeted. We have listed the most important CSC markers for various cancer subtypes (Table 12.1).

The traits of EMT are the loss of epithelial cell surface marker, E-cadherin, and the gain of mesenchymal traits [28]. The initiating factors are seen to be mostly because of networks of transcriptional, translational, posttranscriptional, and post-translational modifications seen in the cells [29]. The ALDH+ cells strongly displayed stem cell-like properties plus higher invasiveness, EMT, and antiapoptotic phenotypes [30]. In the case of human breast cancer cells, it was observed that a small population of cells, which exhibited EMT, also displayed stem cell-like phenotypes. Fascinatingly studies performed in transgenic cancer models in combination with S100A4 lineage tracing have stated that EMT of the breast cancer cells is not responsible for their metastasis to the lungs, but they had a significant role in promoting chemoresistance. The lowering the levels of E-cadherin in the mouse models showed that inhibition of the epithelial traits may promote migration but does not result in metastasis [31]. CD44 is a popular cell surface glycoprotein, which is strongly associated to the stemness of the cancer and its aggressiveness. In the case

of ovarian cancer cells, the overexpression of CD44 resulted in population of cells with mesenchymal-like phenotypes (CD44S) and decreased the number of epithelial-like cells. The downregulation of ESRP1 and upregulation of TGFPs1 promoted EMT, invasiveness, and the gain of stem cell-like phenotypes and chemoresistance in CD44 cells [32].

In the colorectal cancer cells, abnormal expression of miR-26b induced EMT and stem cell-like characteristics. Lymphatic metastasis shows significantly upregulated levels of miR-26b. miR-26b directly targets many tumor suppressors along with phosphatase and tensin homolog (PTEN) and wingless-type MMTV integration site family member 5A (WWT5A) [33].

The role of IncRNAs was evaluated in two sets of cells: colorectal cancer with liver metastasis and colorectal cancer without liver metastasis. The expression levels of UICLM (upregulated in colorectal cancer liver metastasis) IncRNA was upregulated in the CRC with liver metastasis, and the knockdown of UICLM prevented cell proliferation, invasion, epithelial-mesenchymal transition, and CRC stem cell formation. Further experiments found that IncRNA UICLM regulated ZEB2 [34, 35].

MYC (c-Myc) is regarded as a very strong proto-oncogene observed to be highly expressed in many cancers. The (PARPI)-poly (adenosine diphosphate (ADP)) ribose polymerase inhibitor effect on triple-negative breast cancer can be chemically improved upon a blockade of MYC. Dinaciclib a cyclin-dependent kinase inhibitor downregulates Myc expression; this, administered along with PARPI-niraparib, downregulated EMT by reducing homologous recombination which resulted in reduced cancer stem cell-like phenotype. Also dinaciclib re-sensitized TBNC cells which displayed resistance toward niraparib. This combination of therapy also worked on ovarian, prostrate, pancreatic, lung, and colon cancer cells [36].

Claudin-6 (CLDN6) is a tight junction protein functioning as a tumor suppressor and also a stem cell marker. Triple-negative breast cancer (TNBC) cells show low levels of CLDN6. A study involving the overexpression of CLDN6 in TNBC cells (MDAMB231 cells) showed increase in epithelial marker E-cadherin and reduction in vimentin (mesenchymal marker); stem cell markers such as OCT4, SOX2, and Nanog were upregulated [37].

Another long noncoding RNA called the nuclear-enriched abundant transcripts (NEATs) plays a significant role in Non-small-cell lung carcinoma (NSCLC) stem cells. Experimental results suggest that NEAT1 was overexpressed and copper transporter 1 (CTR1) was downregulated in the NSCLC stem cells. NEAT1 knock-down reduced the cancer stem cell-like phenotype in these cells. NEAT1-expressing cells also exhibited Wnt pathways and EMT process [38].

In triple-negative breast cancer cell line SUMI159pr, low expression of miR-105 was recorded. Its targets were identified to be VEGFA, Erb33, Zab1, Fyn, and Lyn A/B, thus reducing cell proliferation and c-Myc with upregulated levels of participants.

Overexpression of MiR205 inhibited the anchorage-independent growth, migratory and invasive nature of SUM159PT cell line with activated src kinases, and low levels of MMPs. The pathways and proteins associated with EMT like CD44, TAZ,

E2AE12, twist, Snail A, and CK5 were also highly reduced with the expression of miR-205. The miR-205 also plays a critical role, and its co-expression along with anti-miR-205 reverted back all the reduced and the inhibited pathways of the triple-negative breast cancer cell line SUM159PT [39]. Ursodeoxycholic acid is an epimer of chenodeoxycholic acid found in the mammalian bile secretions, commonly abbreviated as UDCA. Reactive oxygen species (ROS) plays a critical role in cancer progression and advancement, and UDCA inhibited intracellular ROS. Pancreatic cancer cell lines treated with 0.2 mM UDCA showed elevated levels of E-cadherin and lower levels of N-cadherin and downregulation of sex-determining region Y-box 2 (SOX2). It reduced the sphere-forming abilities; thus it is evident as an effector inhibitor of cancer stem cell-like and EMT phenotypes [40].

Carnosol (CAR) is naturally found in our body that inhibits the MDM2/p53 complex. Its effects on U87MG, a glioblastoma-derived cancer stem cell line, showed that it reduced the CSC formation and promoted apoptosis of the cancer stem cells by functionally reactivating P53. Furthermore it also controlled the effects of TNF-alpha/TGF-beta and inhibited the effects of cytokines associated with EMT-regulating genes (slug, Snail, twist, ZEB1). It also promoted the activation of miR-200c, which is associated with EMT; adding on this it also increased the antiproliferative effects of temozolomide (TMZ) [41]. Lagunas et al. demonstrated that telomere DNA damage signaling regulates cancer stem cell evolution and metastasis. Telomeres are protected by the double-stranded DNA-binding protein TRF2 and maintained by telomerase or a recombination-based mechanism known as alternative lengthening of telomeres (ALT). Loss of TRF2 and Terc expression gives telomere DNA damage, severely decreases CD34+ and Lgr6+ cancer stem cells, and induces terminal differentiation of metastatic cancer cells [42]. The natural sphingolipid phytosphingosine (PHS) suppresses the stem cell-like phenotype and EMT-associated proteins and the highly malignant basal-type breast cancer cells (CD44+/CD24-) by downregulating EGFR/JAK1/STAT3 [43]. Slug and twist are important transcriptional factors that are highly associated with EMT; they are found to be regulated by two processes, namely, ubiquitination and degradation. Slug and twist are found to be in very stable conditions inside the cancer cells. It can be speculated that the stabilization of the slug and twist is because of the loss of ubiquitin by deubiquitinase (DUB). DUB3 was identified to be the deubiquitinase for both slug and twist. The upregulation of DUB3 amplified the expression levels of slug and twist in a dosage-dependent manner, also protecting the two genes from being degraded. IL-6, which plays a significant role in the metastasis of breast cancer cells, seemed to induce the expression of DUB3. Thus DUB3 is identified to play a critical role in stem cell-like phenotype, metastasis, and invasive and migratory traits in breast cancer cells [44].

Fusobacterium nucleatum has been identified to play a role in colorectal cancer. A study was conducted on stage 3 CRC patients. The *Fusobacterium nucleatum* levels were significantly high and were associated with the invasion, lymph node and metastasis and distant metastasis. Analysis showed the presence of Nanog, OCT4, and SOX2 (stem cell markers) and N-cadherin levels [45]. SOX8 was overexpressed in tongue squamous cell carcinoma (TSCC) resistant to cisplatin, which exhibited

EMT and CSC-like (Wnt) phenotypes. It is found to be upgraded in chemoresistant patients affected by tongue squamous cell carcinoma (TSCC) and also correlated with normal lymph node metastasis [46].

12.3 Role of MicroRNAs on CSCs and Metastasis

The microRNAs are short noncoding segments of RNA with 21–25 nucleotides seen in plants, animals, and certain viruses. Their function is "RNA-mediated gene silencing" at the posttranscriptional stage by attacking the 3′-untranslated regions of the "target gene," thereby degrading the specific mRNA [47]. miRNAs play a vital role in the human cancer progression and metastasis. The expression levels of the oncogenic miRNAs can be observed to be increased as cancer progresses. The improper regulation of the expression of the miRNAs influences the processes of the progression like antiapoptotic activity, drug resistance, tissue invasion, and metastasis [48] (Table 12.2).

12.3.1 miR-200 Family

miR-200a, miR-200b, miR-141, and miR-429 are the members of the miR family. Their regulations have a strong association with the cancer stem-like features and metastasis via EMT [63]. Experiments on human mammary epithelial cells show that these cells were able to transit from non-stem like to stem like upon the loss of miR family [64]. A strong connection between the levels of miR-200s and E-cadherin in the cancer cell lines and clinical samples showed that miR-200s maintained tumor epithelial traits and prevented EMT. This was achieved by the direct interaction of miR-200s with ZEB1 and ZEB2 transcription factors. Suppression of metastasis has been observed upon the upregulation of miR-200s, but the miRNAs are also known to promote metastasis by recent studies as higher concentration of miR-200s promoted the development of metastasis in breast cancer patients and promoted invasion of the lung by murine breast cancer cells. The direct downregulation of Sec23a resulted in loss of expression of metastasis-suppressive proteins IGFBP4 and Tinagl1 in the murine breast cancer cells [63].

Tumor suppressor p53 is shown to be associated with both EMT and breast CSCs associated with EMT by transcriptionally activating the miRNAs associated with stemness including miR-200C. The loss of P53 in mammary epithelial cells downregulated miR-200C expression and initiates EMT and increases CSCs. It also directly interacts with EMT and increases CSCs. It also directly interacts with the protein line ZEB1, ZEB2, and BMI1 [65].

Table 12.2 miRNA involved in cancer stem cell metastasis

Cancer type	miRNA	Targets	Functions in CSCs-Metastasis	References
Breast cancer	miR-199a	FOXP2	Enhanced CSC properties	Lei Zhou et al. [49]
	miRNA-600	PORCN	Differentiation	Rita El Helou et al. [50]
	miR-200c	BMI1	Inhibit the clonal expansion	Zheng-ming Wang et al. [51]
	Let-7	H-RAS and HMGA2	Suppresses self-renewal and differentiation	Yohei Shimono et al. [52]
	miR-205	BMI1	Regulate EMT, migration, and invasiveness	Xiao et al. [53]
	miR-141 miR-183	BMI1	Regulate the self-renewal abilities	Yohei Shimono et al. [52]
Pancreatic cancer	miRNA-1246	CCNG2	Chemoresistance and stemness	Sabrina Bimonte et al. [54]
Acute Myeloid Leukemia (AML)	miRNA-22	TET2	Self-renewal and transformation	Ryou-u Takahashi et al. [48]
Colon	miR-193 miR-145 miR-200 miR-203	PLAU and K-RAS ZEB1	Inhibition of tumorigenicity and invasiveness Maintenance of stemness EMT activation	Ryou-u Takahashi et al. [48] Sabrina Bimonte et al. [54]
Lung cancer	miR-145	OCT4	Inhibited the proliferation	Hu et al. [55]
Prostate cancer	miR-143	FNDC3B	Differentiation of prostate cancer stem cells and promoted prostate cancer metastasis	Xinlan Fan et al. [56]
	miR-34a	CD44	Inhibit prostate cell proliferation, tumor regeneration, and metastasis	Zheng-ming WANG et al. [51]
	miR-708 and miR-199a-3p	CD44	Regulate the proliferation	Can Liu et al. [57]
Hepatocellular carcinoma	miR-22	PTEN, p21, and p53	Reduces cell growth, invasion, and metastasis	Bin Bao et al. [58]
Head and neck squamous cell carcinoma	miR-200c	*BMI1*	Regulating self-renewal, radio/chemoresistance and metastatic properties	Yu et al. [59]
Brain tumors	miR-34a	c-met	Induce the differentiation of CSCs	X J Li et al. [60]

(continued)

Table 12.2 (continued)

Cancer type	miRNA	Targets	Functions in CSCs-Metastasis	References
Ovarian cancer	miR-199a	*ABCG2*	Increased the chemosensitivity of ovarian CSCs	Yongchao Wang et al. [61]
Glioblastoma	miR-128	BMI1	Inhibit glioma stem cell proliferation	Can Liu and Dean G. Tang. [62]
Gastric cancer	miR-34	p53	Control the biological properties of gastric CSCs	Can Liu and Dean G. Tang. [62]

12.3.2 miR-203

miR-203 inhibits the colony formation, migration, and invasion of many cancer cells. Enhanced regulation of Snail and downregulation of miR-203 in CD44+ human colorectal carcinoma cell lines showed higher metastasis; further miR-203 is suppressed by Snail [66].

miR-203 reduced the sphere-forming ability of the nearby cells by indirectly prompting DKK 1 (inhibitor of Wnt signaling) [67]. The effects of miR-203 are observed in CD44+/CD88− leukemia cancer stem cells by directly interacting with BMI1/survivin [68].

12.3.3 miR-34a

miR-34a has been called as a "star" miRNA in cancer research, acts as tumor suppressor, and is downregulated in many human cancers, and also studies have shown that the aberrant miR-34a expression has been linked to chemotherapy resistance in a variety of cancers [69].

miR-34a is a mediator of the p53 transcriptional network and has been identified as a tumor suppressor that contributes to the inhibition of the invasion and metastasis in various types of epithelial cancers [69]. miR-34a expression is significantly downregulated in primary tumors from head and neck cancer patients as well as in head and neck cancer cell lines. Ectopic expression of miR-34a in head and neck cell lines significantly inhibited tumor cell proliferation, migration, and colony formation by downregulating the expression of E2F3 and survivin [70]. Expression of miR-34a in bulk can inhibit prostate cancer cells ($CD44^{+}$) through inhibition of clonogenic expansion, tumor regeneration, and metastasis, and expression of miR-34a antagomirs in $CD44^{-}$ prostate cancer cells promoted tumor development and metastasis [71], and miR-34a performs a key role in suppressing colorectal cancer metastasis by targeting and regulating Notch signaling [72].

12.3.4 miR-22

miR-22 epigenetically promoted stem-like traits and metastasis in breast cancer cells. Studies have shown miR-22 capable to directly inhibit TET expression and EMT induction in breast cancer. Ten eleven translocation (TET) enzyme has been linked to the demethylation of miRNA-200 promoter region. miR-200 is an anti-metastatic microRNA that inhibits stemness and EMT, and miR-22 is observed to be in association with TET family, thus promoting CSC-like properties and metastasis by repressing miR-200 family [48].

12.3.5 miR-17

Significant overexpression of miR-17 was seen in CD133+ cells of glioblastoma cell lines. The miR-17 is said to directly target calmodulin-binding transcriptional activator (CAMTA1), which is a transcription factor of antiproliferation cardiac hormone natriuretic peptide A. Downregulation of miR-17 in these cells reduced neurosphere formation and promotes cell differentiation. This shows that miR-17 is significantly correlated to stem (CD133+)-like traits in cells [48]. In osteosarcoma the levels of miR-17 were seen to be higher, and its inhibition resulted in reduced or suppressed cancer cell proliferation, migration effects, and invasion/metastasis. PTEN homolog was identified to be directly targeted by miR-17 [73]. PTEN levels are critical in maintaining stemness, and its suppression leads to promotion of cancer stem cells [74]. However, A549/DDP (cisplatin resistance) non-small cell lung cancer cells showed downregulated levels of miR-17, miR-20a, and miR-20b. The downregulated levels suppressed the TGF-beta signaling pathways and inhibited EMT pathways, thus affecting metastasis [75]. Cancer stem cells seem to have developed their stem-like properties via stem cell pathways like Wnt, TGF-beta, STAT, and Hippo-YAP/TAZ [76]. Colon cancer cells that overexpressed phosphatase of regenerating liver 3 (PRL-3) inducing the expression of miR-21, miR-17, and miR-19 by activating STAT3 [77]. Cancer stem cells are strongly associated with stemness-related STAT pathway [76]. Thus these miRNAs have increased the proliferation of primary colon cancer cells and the metastatic growth [77]. In ovarian cancer metastasis, the expression of miR-17 is inversely related to the levels of ITGA5 and ITGB1. Lower level of ITGA5 and ITGB1 suppressed peritoneal metastasis. The abnormal expression of miR-17 in ovarian cancer resulted in lowered expression of ILK phosphorylation and MMP-2. Thus higher levels of miR-17 suppress ovarian cancer cell peritoneal metastasis [78].

12.3.6 miR-124

SNAI2 has been found to be upregulated in glioblastoma cells, and miR-124 has SNAI2 as its functional target. SNAI2 has also been associated with stemness. Experimental evidences showed that upregulation of miR-124 and SNAI2

knockdown reduced neurosphere formation, and the expression of stem cell markers like BMI1, Nanog, and Nestin was substantially reduced, and the effects can be reverted by the re-expression of SNAI2 in in vivo [79]. miR-124 was also seen to directly target STAT3 signaling. STAT3 is identified to have positive effects on T-cell-mediated suppression in tumor microenvironment. miR-124 is observed to be lost in all grades and in all pathological types of gliomas. The upregulation of miR-124 in glioma cancer stem cells (GCSCs) resulted in the inhibition of STAT3, and it reversed the GCSC-associated immunosuppression of T cells and the induction of FOXP3 and regulatory T cells (Tregs). T cells from immunosuppressed glioblastoma patients when treated with miR-124 resulted in upregulation of interleukin (IL-2), IFN-gamma, and TNF-alpha [80]. The abnormal expression of miR-124 in MDA-MB-231 cells (known for high invasiveness) suppressed spindle formation, invasive capacity, and adhesion to fibronectin and anoikis. These results show that miR-124 plays a critical role in the multistep process of metastasis in breast cancer cells [81].

12.3.7 miR-128

Patients with advanced glioma show downregulation of miR-128. miR-128 targets BMI1 [48]. miR-128 is reported to target VEGF-C and reduce the proliferation and the invasive properties of bladder cancer cells. The knockdown of miR-128 upregulates VEGF-C and induces proliferation, migration, and invasion of bladder cancer (BC) [82]. The metastatic and the stem cell-like properties of the hepatocellular carcinoma were inhibited by the upregulation of miR-128, and they were identified to target ITGA2 and ITGA5 [83]. The chemosensitivity is increased and invasive properties of prostate cancer cells were inhibited following upregulation of miR-128. Experimental results suggest that miR-128 directly targets zinc finger E-box-binding homeobox 1 (ZEB1) in prostate cancer cells and induces the sensitivity toward cisplatin and inhibits invasion [84].

12.3.8 miR-199b-5p

Studies show that miR-199b-5p is downregulated in medulloblastoma which results in its invasive properties. This happens by targeting HES1 transcription factor in Notch signaling pathways, thus inhibiting the self-renewal properties of Glioma Stem Cells (GSCs) by targeting the CD133+ cells [48]. But its expression plays an opposite role in the case of human osteosarcoma. The elevated levels of miR-199b-5p correlate to cell proliferation, invasion, and migration of these cells. Its expression levels are seen amplified in the higher grades of osteosarcoma [85]. In breast cancer cells, miR-199b-5p suppresses HER2 expression by negatively conferring with ERK1/2 and AKT pathways. This shows loss of migration, wound healing, and colony formation. This has also improved the sensitivity of HER2 cells towards trastuzumab, thus hampering cells' migratory and clonogenicity properties [86]. miR-199b-5b

targets N-cadherin and promotes cell aggregation and suppresses migration/invasion of hepatocellular carcinoma (HCC). This inhibits metastasis of tumor xenografts. It was also shown to reduce the effects of TGF-beta-induced AKT phosphorylation which results in EMT features [87].

12.3.9 miR-451

The lower expression of miR-451 shows enhanced levels of cyclooxygenase-2 (COX2) and macrophage migration inhibitory factor (MIF); this results in acquisition of stem cell-like properties. COX2 and MIF have been shown to be associated with Wnt pathway, which is a major regulator of cancer stem cells [48].

Papillary thyroid carcinoma with lymph node (PTCLN) metastasis shows amplified expression levels of four miRNAs: miR-2861, miR-451, miR-193b, and miR-1202. When compared with PTC without lymphoid metastasis, it was found that PTCLN had high levels of miR-2861 and miR-451 especially in lateral and lymph node (LLN) [88]. Zhang et al. reported that miR-144/451 re-expression markedly suppressed the migration and invasion of breast cancer and HNSCC cells through ADAM10 and ADAMTS5 modulation. PAX4 promoted migration and invasion in human epithelial cancers by decreasing miR-144 and miR-451 (miR-144/451) expression levels.

Paired box gene 4 (PAX4) has been promoting metastasis in human epithelial cancer by downregulating miR-144 and miR-451, while miR-144/451 has been observed to inhibit cancer migration even in PAX4-expressing cells by targeting a disintegrin and metalloproteinase (ADAM) protein family members in ADAMT55 and ADAM10 [89]. MiR-451 has also been shown to suppress cell proliferation and metastasis by targeting chemokine ligand 16 (CXCL16) in osteosarcoma patients [90]; it has been shown to promote significant metastasis in various cancer types like hepatocellular carcinoma by targeting c-Myc [91]. In neuroblastoma miR-451 has been shown to target macrophage migratory inhibitory factors [92], in A549 lung cancer cells miR-451 inhibits metastasis by targeting PSMB8 and NOS2 thereby reducing the expression of MMP-2, MMP-9, VEGF. miR-451 has also been associated with stemness and CXCR4 [93]. This miR-451 plays a vital role in inhibition of stem cell-like features by inhibiting stem cells and metastasis through numerous pathways.

12.3.10 miR-320

The miR-320 directly targets Wnt/beta-catenin expression in prostate cancer stem cells. Thus the expression of CD44+ PCa cell expressing Wnt is inversely proportional to miR-320 level [48]. Fatty acid synthase (FAS) was previously reported to be correlated with various clinicopathological features of cancer. Overexpression of FAS in NSCLC has been shown to be significantly associated with bone metastasis. Thus miR-320 contributes to cell proliferation, migration, and invasion by directly

targeting FAS in NSCLC, and overexpression of miR-320 in NSCLC cell lines inhibits cell proliferation, migration, and invasion via downregulation of FAS. miR-320 may act as a tumor suppressor by inhibiting the oncogenic activity of FAS [94]. In addition, miR-320 inhibited migration by targeting FOXM1 in cervical cancer cells [95].

12.4 Functions of RBPs on CSCs and Metastasis

RNA-binding proteins (RBPs) act as epigenetic regulators of various RNA processing events, such as splicing, localization, stabilization, and translation, and can regulate various types of stem cells. Many RNA-binding proteins are overexpressed in cancers [96, 97]). Deregulation of RBPs affects every step of cancer development, such as sustained cell proliferation, inhibition of the apoptosis process, avoiding immunosurveillance, inducing angiogenesis, and activating metastasis. Some RBP proteins recognize cis-acting elements to translationally regulate proto-oncogenes, cytokines, and growth factors [98]. RNA-binding proteins that are abnormally expressed in cancers are the IMP-3, the CRD-BP/IMP-1, the p62, as well as members of the ELAV/Hu protein family, e.g., HuR. Upon binding to the AU-rich instability element (ARE) in the 3′Untranslated region (UTR) of rapidly degraded mRNAs of proto-oncogenes, cytokines, and growth factors, HuR regulates nucleocytoplasmic transport, stability, and translation. However, only very few of these RNA-binding proteins have been demonstrated to regulate tumor progression and metastasis and control the cancer stem cell self-renewal [97].

12.4.1 RBM3

Colorectal cancer mostly demonstrates the overexpression of Wnt/beta-catenin in the colon cancer stem cells. The RNA-binding protein RBM3 promotes cancer cell proliferation, angiogenesis, and resistance against apoptosis and even induces metastasis at higher levels by acting as a proto-oncogene [99]. Two cell lines HCT116 and DLD1 were taken to study the effects of RBM3 on colon cancer; upon the upregulation of RBM3, the number of sphere-forming cells increased in the HCT116 cells along with the increased expression of cancer stem cell marker DCLK1 in DLD1 cells. Further analysis have shown that RBM3 upregulated the levels of Wnt/beta-catenin but suppressed the expression of Notch [100].

12.4.2 PTBP3

The RNA polypyrimidine tract-binding protein (PTBP3) at upregulated state leads to the acquisition of cancer stem cell-like and EMT phenotype in breast cancer cells, thus enhancing metastasis. Mechanistically, the EMT regulatory transcription factor ZEB1 is upregulated due to PTBP3 binding to its mRNA at the 3′UTR [101].

12.4.3 Lin 28

Recent studies have shown that LIN 28A/B plays an important role in the formation of CSCs and is involved in tumor aggressiveness and metastasis. Higher expression rates of RNA-binding protein LIN 28 is correlated with the exhibition of malignant, cancer stem-like phenotypes in breast, colorectal, and esophageal cancer cells. Ovarian cancer cells with CSC-like trait express both LIN 28A and OCT4. LIN 28B is expressed in the colorectal stem cells with CSC markers like LGR5, KIT, and PROM1 (CD133) in colon cancer. LIN 28B might be correlated with intestinal CSCs, and LIN 28B regulated by IKKβ are able to maintain stemness by interacting with Wnt pathways as LIN 28 is expressed only by CSCs. A crosstalk between LIN 28 and let-7 is observed in CSCs; thus blocking this axis might be a solution to target the CSCs. In non-small cell lung cancer patients, higher levels of LIN 28 and lower levels of let-7 are associated with chemo- and radiotherapy resistance [102].

12.4.4 MUSASHI-1

MUSASHI-1 (MSI-1) is a stem cell marker in both normal and cancerous stem cells; in malignant colorectal cancer cells, MUSASHI1 is upregulated by the expression of Notch-3 [103]. In another experiment on gastric cancer cells, it was identified that MUSASHI1 played a significant role in the prognosis of the metastatic gastric cancer. The expression of MSI-1 can be associated with tumor node metastasis (TNM), Lauren's classification, depth of invasion, vessel invasion, lymph node metastasis, and distant metastasis. The prognosis at each stages of TNM is worse than its previous one as there is an increase in MSI-1 expression [104].

12.4.5 MUSASHI-2

Elevated levels of MSI-2 is observed in metastatic non-small cell lung cancer cell lines; the suppression of MSI-2 led to decrease in metastatic potential and promoted the expression of claudin 3 (CLDN3), claudin 5 (CLDN5), and claudin 7 (CLDN7) and downregulated the expression of TGFβR1, SMAD3, and zinc finger proteins SNAI1 and SNAI2 (Slug) [105].

12.5 Effect of CSCs on Immunosurveillance and Metastasis

A perfect display of chemoresistance and immune resistance is seen during the escape phase when detectable tumor bodies of CSCs/TICs reappear after a long dormant phase. They have been reported to produce immunosuppressive molecules and recruit cells that suppress the immune system like Treg cells more tolerant toward the immune system via loss of expression of tumor antigen, loss of processing and presentation machinery, and downregulation of MHC1 and MHC2.

All these factors along with aging related immune deficiency is utilized by CSCs to promote cancer. CSCs/TICs are able to escape the immune response because they are equipped with co-inhibiting molecules like cytotoxic T-lymphocyte antigen (CTLA4), B7-H2, B7-H3, and programmed death receptor 1 (PD-1). The lack of MHC2 leads to downregulation of low molecular weight proteins (LMP), transporter associated with antigen processing (TAP), and beta-macroglobulin, which assists immune escape. Cytokines like transforming growth factor-beta (TGF-beta), IL-10, and IL-13 were secreted in in vitro conditions. The CSCs/TICs from glioblastoma required expression of VEGF, macrophage chemoattractant protein-1 (MCP-1), macrophage inhibitory factor (MIF), and growth-related oncogene (GRO2) for their survival. Breast cancer stem cells and glioblastoma CSCs produce more TGFs than usual cancer cells; IL-4 produced by colon CSCs promotes drug resistance and stops antitumor immune responses. CSCs also produce CD200 that aids immune escape. The dissemination of CSCs of the melanoma is assisted by the expression of ATP-binding cassette subfamily member 5 (ABCB5) and shows low levels of lineage-related and Cancet-testis (CT) antigens. But the CSCs expressing CD133+ have upregulated levels of NY-ESO1 cancer testis antigen; and they produce specific T-cell response.

TAADDX3X expressed by CD133+ CSCs is susceptible to T cells in massive models, while the CD271+ CSCs do not express both lineage-related and CT antigens; hence their susceptibility toward T cells is not possible. Thus all these traits help the CSCs/TICs during tumor progression and metastasis.

Cancer stem cells of the lungs have developed ways to protect themselves from T cells and induce apoptosis in them. T cells express CTLA4/CD152 after exposure to antigen. The binding of CTLA4 to its ligand (CD80/CD86) on the antigen presenting cells (APC) induces T-cell apoptosis. Specifically lung cancer cells induce T cells to produce more CTLA4. Similarly PD4 is produced by the T cells, B cells, and some myeloid cells. Cytotoxic T cells with PD4 interacting with its ligand are marked for apoptosis. Upregulation of ALDH and B-cell lymphoma-2 (BCL-2) protein and its family seems to enhance the chemoresistant phenotype [106].

Cancer stem cells derived from histopathologically negative prostrate training lymph nodes (PDLN) in mice with prostrate intraepithelial neoplasia (mPIN) controlled by oncogene was similar to the CSCs from mPIN tumor bodies. CSCs from both PDLN and mPIN produced extracellular matrix protein tenascin-C (TNC) and CXCR4. TNC interacts with α5β1 integrin on the cell surface of the T cell and inhibits T-cells receptor dependent T cell activation proliferation and cytokine production [107].

Macrophages play a vital role during the stages of metastasis of the cancer cells. Macrophages are made tumor friendly by various cytokines and chemokines like colony-stimulating factor 1 (CSF1), vascular endothelial growth factor A (VEGF-A), semaphorin3A (SEMA3A), CC-chemokine ligand 2 (CCL2), and CXC-chemokine ligand 12. Tumor-associated macrophages (TAM) are known for their CD8+-suppressing properties by directly producing DDL1 and BT-H4 or indirectly Treg cells [108].

The vascular endothelial growth factor A (VEGF-A) is a vital oncogenic factor that also plays important role in cancer stem cell maintenance, proliferation, malignancy, immunosuppression and also EMT. Myc and SOX2 were upregulated in the breast and lung cancer stem cells through VEGF receptor 2 (VEGFR2)/STAT3 [109]. VEGF has been identified to target dendritic cells by disrupting its maturation from the progenitor cells. It also plays a role in the T cells and macrophages. The functional maturation of the DCs from its progenitor CD34+ was identified to have disturbed the VEGF produced by breast and colon cancer cells. The M2-polarized macrophages that are tumor friendly express VEGF, hence promoting angiogenesis. Initial experiments on mice with elevated levels of VEGF equivalent to the amount found in advanced cancer patients showed that the number of CD8/CD4 thymocytes was reduced. The VEGF affected the progenitor of the T cells rather than on the T cells themselves [110]. The human cancer cell A549 and breast cancer cells MDA-MB-231 recruit many tumor-associated dendritic cells (TADCs) overexpressing CCL2. CCL2 increases the stem cell-like features, migratory/invasive properties, malignancy, and also the immunosuppressive tumor-associated macrophages. The CCL2 amplifies the phosphorylation of STAT3 in the cells. 6-Shogaol can inhibit CCL2 and suppress the proliferative and the metastatic properties of the lung and the breast cancer cell lines [111]. In case of the hepatocellular carcinoma, increased expression of CXCR4 corresponds to lymph node metastasis [112], but the cytoplasmic expression of CXCR4 does not seem to play a role in the lymph node metastasis [113].

A cross talk between the CXCR4 pathway and TGF-beta pathways has been reported in the Hepatocellular carcinoma (HCC); CXCL12/CXCR4 has also been identified to promote the expression matrix metalloproteinase 10 (MMP10) that enhances the migration and metastatic properties of the HCC. A cross talk between CXCR4 and Sonic hedgehog (SHH) pathways has been reported in the human pancreatic cancer and medulloblastoma as well. SHH/CXCR4 interaction has been associated with promotion of stem cell properties and malignancy in these cells.

A cross-link between alpha-fetoprotein (AFP) and CXCR4 has been observed. AFP promotes migration by activating AKT/mTOR signaling through CXCR4 in HCC [114].

The regulatory T cells are utilized by the breast cancer cells with lung metastasis by expressing CCL22/CCL5, which is very immunosuppressive expressing alpha-chain/CD25 and forkhead boxp3 (FOXP3). Suppressing of metastasis in the breast cancer models has shown to deplete number of CD4 + CD25+ cells; overexpression of prostaglandin E2 by the breast cancer cells also recruits Treg cells there by inducing CD8+ T-cell apoptosis and enhances cancer cell bone metastasis. Melanoma utilized Treg cell to overexpress TNR to promote lung metastasis [108]. The galectin-1 produced by the breast cancer cells suppresses the immune system by regulating clonal expansion and via linker for activation of T cells resulting in the promotion of breast cancer metastasis. Galectin-1 has been reported to be highly expressed in CD133+ cells (stem cell marker) [115, 116]. Treg cells seem to target NK cells and induce apoptosis by expressing BETA-galoctoside-binary protein (BETA-GBP) in lung metastasis that Treg cells suppressed the cytotoxicity of the

NK cells via cell to cell contact and expressing TGF-beta [108]. Neutrophils have an interesting role in cancer promotion. The human fibrosarcoma and prostate cancer cells associated neutrophil promote angiogenesis by secreting MMP9. In intrahepatic cholangiocarcinoma xenograft model, CXCL15 recruits neutrophils and enhances lung metastasis. But other studies show that reducing the neutrophil contact increases the lung metastatic loci in breast cancer models. The neutrophils extracted from tumor-bearing mice can kill cancer cells in other mice models by producing Hydrogen peroxide (H_2O_2). However TGF-beta has been reported to reverse the antitumor properties to tumor-promoting traits of neutrophils [117].

12.6 Overview of CSC-Derived Exosomes on Metastasis

There are many evidences proving that exosomes from cancer cells cause organ-specific metastasis. Their specificity can be identified by the presence of certain ECM, membrane proteins, lipids, and adhesion molecules present within the exosomes. Tumor-derived exosomes (TDEs) initiate metastasis by three ways: firstly, autocrine and paracrine signaling which initiates EMT formation. Secondly, they help in the formation of pre-metastatic niche. Thirdly, they modulate the body's immune system promoting metastasis.

Exosomes from various cancer cells have Notch-1 (MMPs), miR-100, HIFα, casein kinase Iiα, and annexin A2. Hypoxia is a popular condition associated with the development of metastasis particularly; EMT-inducing molecules like TGFβ, MMPs, TNF-α, IL6, AKT, ILK1, caveolin 1, PDGFs, and β-catenin are the contents of exosomes under hypoxic conditions. Nasopharyngeal carcinoma (NPC) (CNE2) cell line co-cultured with exosomes expressed more of N-cadherin and vimentin and downregulated expression of E-cadherin [118]. Primary tumors targeting the lungs express integrins like α6β4 and α6β1. The integrin αvβ5 promotes metastasis to the lungs [119]. In another experiment where exosomes from fluorescently labeled B16-FI0 melanoma cells were injected into a mice, the exosomes combined together with the regional lymph node nearest to the point of injection. Tumor and organ-specific metastasis is a characteristic behavior of cancer stem cells [120]. CSCs secrete and uptake exosomes; hence their metastatic phenotype can also be determined by studying the contents of their exosomes. The CD105+ exosomes from the renal cancer stem cells when injected into SCID mice developed only lung metastasis [118]. Breast cancer stem cells recruit Treg cells and promote lung metastasis [108]. Tumor-derived exosomes (TDEs) use Treg cells and induce CD8+ T-cell apoptosis and suppress NK cells. The exosomes from NPC recruit Treg cells and confer with T helper cell (Th1) and Th17 differentiation; also these cells recruit CD4 + CD25- T cells and convert them to CD4+CD25+ T cells. Tumor-tropic patient-derived adipose cancer stem cells when treated with exsosomes from prostate cells induced mesenchymal to epithelial transition (MET) and lead to the development of a more aggressive prostate like secondary tumor [118]. Malignant breast cancer stem cells are also associated with CD4+CD25+ T cells [108]. Thus it is possible for the Breast cancer stem cells to utilize exosomes in a similar manner.

The paracrine activity of adult stem cells and cancer stem cells is mediated by the release of exosomes [121]. Cancer stem cells communicate with nearby cancer cells and stromal cells by uptaking the exosomes by the cells. The exosomes derived from the fibroblast mediate Notch signaling, overexpress ALDH, and promote stemness in the breast cancer cells. The intake of exosomes from melanoma cancer cells by the bone marrow progenitor cells leads to the acquisition of malignant phenotype [122]. The exosomes from more aggressive TNBC cell lines Hs578Ts (i) transmitted their aggressive phenotype to secondary breast cancer cells (Hs578T, SKBR3, MDA-MB-231, and HCC1954). The noninvasive nature of the mammary epithelial cell line HMLE was reversed upon its exposure to miR-10b from MDA-MB-231 cells. miR-10b has been reported to be highly expressed by the TNBC cells. In an in vivo study mice were intravenously injected with exosomal miR-105 from MDA-MB-231 cells, and the MDA-MB-231 cells also were intracardially injected resulted in the development of lung and brain metastasis. Under hypoxic conditions the exosomes from breast cancer cells have been shown to promote invasiveness and malignant phenotypes. The expression of RAB22A by the breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-435) is seen. The knockdown of the RAB22A by shRNA showed suppressed invasion and long colonization. The fibroblast promotes metastasis by Wnt signaling. The CD81 secreted by the fibroblast L cells through exosomes was taken up by the breast cancer cells and induces metastasis of the MDA-MB-231 cells. The knockdown of CD81 in L cells suppressed the malignancy [123, 124].

12.7 Future Prospects

Understanding the roles of CSCs on tumor progression and metastasis will provide the strategies for targeting CSCs to prevent the seed for cancer metastasis. This chapter has highlighted the need for future research on the various factors that regulate the dissemination of cancer from its primary site. The RNA-binding proteins and their role in posttranscriptional regulation during cancer progression and metastasis have provided various targets for regulating cancer stem cells. In addition, the development of combination therapies for the above highlighted multiple targets will improve patient's outcome. The latest development in the field has enabled us to understand the contents and role of CSC-derived exosomes on metastasis. There is a clear lack of information on content loading of exosomes and target or recipient cell identification. The epigenetic regulation of CSCs by microRNAs and RNA-binding proteins has highlighted the targets and identified the biomarkers for tumor progression and metastasis.

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Functionality of Intron-Specific Genes and Cancer Stem Cells in the Progression of Colorectal Cancer

13

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Abstract

This review article deals with comprehensive information about the evolutionary history of introns with their localization and functions in the gene transcripts of colorectal cancer precisely. In this way, the major breakthrough in the molecular biology discipline was the discovery of introns by Richard Robert and Phil Sharp in 1977. Firstly, noncoding regions are recognized by various assortments of regulatory ncRNA sequences such as circular RNA, telomere-associated RNA, small nuclear RNA, Piwi-interacting RNA, small interfering RNA, small nucleolar RNA, microRNA, and long noncoding RNA. Fortunately, splicing process of mRNA strand deals with the excision of introns via spliceosomal proteins into mature mRNA which is witnessed only in eukaryotic organisms and devoid of the splicing machinery components in the prokaryotic organisms. The major focal point relies on intronic genes mainly involved in the progression of colorectal cancer with preliminary information. An alternative splicing process takes place in mRNA that implicates in intron retention leading to varied gene expression in cells and tissues and their promotion in colorectal cancer. Therefore, colorectal cancer-associated diseases have paved the way to know more about the intronic genes mainly concentrated among them in the progression of the related diseases. Hence, the focus of the researchers is toward the fascinating cellular and molecu-

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lar biology aspects of the regulatory intronic sequences known to enhance as well as repress particular gene expression in tumor microenvironment of colorectal cancer by analyzing the genome and proteome levels for the betterment of human kind that is intended for various therapeutic purposes.

Keywords

Introns (noncoding sequences) · mRNA · Spliceosomal proteins · Alternate splicing process · Intron retention · Colorectal cancer

13.1 Introduction

Eukaryotic genome consists of DNA sequences which are transcribed into pre-mRNA, which is exemplified by the organization of intron-exon structure and endures posttranscriptional and posttranslational process to produce a desired protein. Consequently, intervened introns are excised among the adjoining exons, to form the pre-mRNA [1]. In particular, the coding regions specifically undergo translation, while in the noncoding regions, introns also undergo the translation process but the protein produced is acclaimed to be regulated in various splicing processes which in turn advance varied gene expression and cancer development [2]. Introns appear to influence any phase of mRNA maturation together with transcription process such as mRNA stability, nuclear transport, and polyadenylation. Splicing introns imply an expurgation of spliceosomal introns from the genome where the spliceosome consists of five snRNAs and more than 150 proteins which are coded by intron-bearing genomes itself. The transcription of lengthy introns persists up to several hours as the elongation time is found to be 60 bases for every second facilitated with the help of RNA polymerase [3]. Therefore, the host of cis-regulatory facet assists in the identification of splicing joint by the spliceosome. As a consequence, intrusion of normal splicing pattern paves the way to more than half of the genetic disorders in humans (Table 13.1).

Table 13.1 Life span of introns in five different phases

Origin of introns	Role of introns	Intronic effects
Genomic site	Genome organization, Transcription process	Sequence site and length
Transcription site	Time impediment	Length
Splicing site	Control of transcription, Alternate splicing	Splicing, sequence
Excision site	Articulation of non-coding RNAs	Splicing, sequence
Exon junction complex- anchorage transcript site	Non sense intervened decay	Splicing, sequence
	Nuclear export	
	Translation yield	

13.1.1 Elucidation of Intron Evolution

The preponderance of the existing intron-rich mammals is elevated in contrast to the preceding eukaryotic ancestors populated with introns. As a result, when the intron is excised from the gene, it turns out to be an element of post-splicing complexes that follow de-branching and destruction. Therefore, the RNA gene is entrenched within the intron; its expression is witnessed on the intron exclusion and outlasts its intronic mass (Fig. 13.1). Functional consequence of an intron might be compatible with the point of conservation of intron position. Excision of intron exon structure indicates sequence construction of orthologous genes which helps in the assessment of intron position (Fig. 13.2). Such constructions reveal the intron position is at times preserved throughout the long evolutionary times in orthologous genes [1].

The eukaryotic hierarchy reveals an array of intron concentration (Fig. 13.3). An investigation of intron mass and eukaryote phylogeny illustrates that it is not always the instance that early division eukaryotes are intron deprived and that late division eukaryotes are luxuriant intron. Analysis of eukaryotic genome aids in empathizing the intron gain or loss. During evolution, the locations of definite introns are preserved between extremely divergent and vibrant among eukaryotes [2] (Fig. 13.4).

Consequently, the life expectancy of an intron is of five stages which independently allude to the capacities that are related with each phase. Diverse functional antisense elements arise from intronic regions and are considered as intron-hosted RNA genes that are triggered during transcription process and are also required for alternative splicing (Fig. 13.5) [3].

13.1.2 Different Classes of Introns Residing in Prokaryotes and Eukaryotes

Spliceosomal introns, group I self-splicing intron, group II self-splicing introns, and tRNA introns are categorized as the four main arrays of introns to be inherent in pre-nuclear mRNA. Moreover, the intronic form is discrete due to its construction and phylogenetic dispersal [4, 5] (Table 13.2).

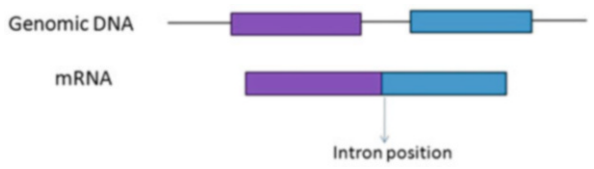

Fig. 13.1 Intronic position in site of intron insertion along the mRNA

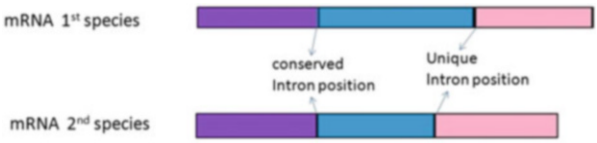

Fig. 13.2 Comparison of intron positions between orthologous genes

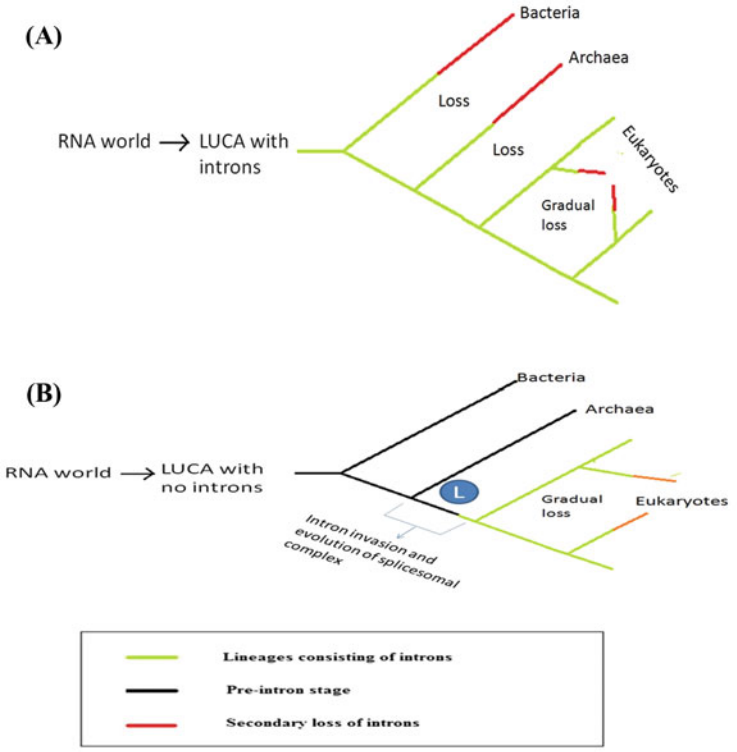

Fig. 13.3 The hierarchy of the origin of introns. (**a**) Early theory of introns. (**b**) Late theory of introns (LUCA, last universal common ancestor)

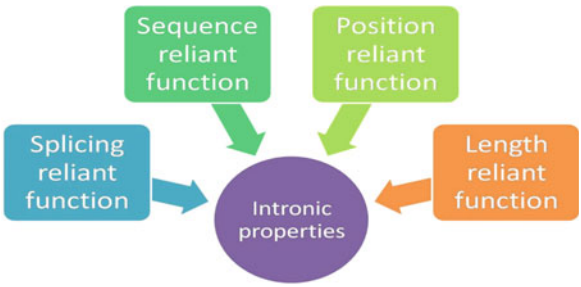

Fig. 13.4 Various types of intronic functions

13.1.3 Noncoding RNAs in Eukaryotes

In human beings, approximately 25,000 genes are recognized which effectively transcribe into mature RNA, where it is comprised of 20% coding (exons) and 80% noncoding (introns) sequences. Consequently, the genome contains greater

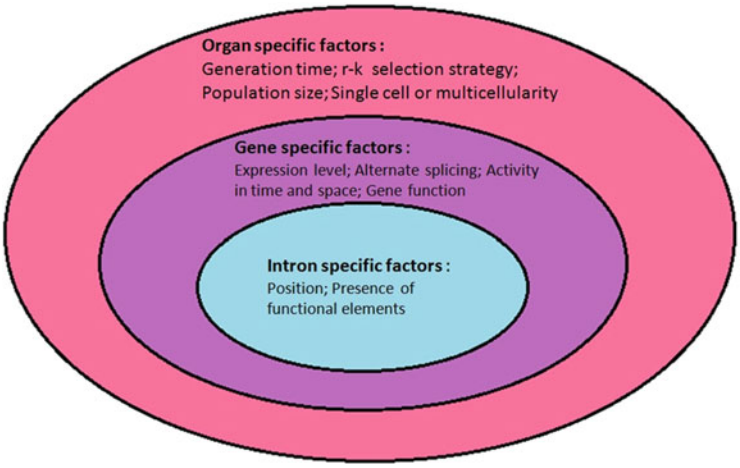

Fig. 13.5 Dynamic aspects of gain and loss of introns

Table 13.2 Differential kind of introns in prokaryotes and eukaryotes

Spliceosomal introns	Group I introns	Group II self-splicing introns	tRNA introns
• Splicesomal are mostly expounded in eukaryotes	• Group I introns are present in nuclear or mitochondrial genome in eukaryotes	• Group II introns impart in bacteria as well as in chloroplast or mitochondrial genome	• tRNA introns generally reside in nuclear genome of eukaryotes and also in archael genome
• Therefore, the contrivance of splicing process involve an expurgation of introns by intricate ribonucleproteins such as U1, U2, U3, U4, U5 and U6 of small RNAs	• They have a distictive splicing process enhanced by an exogenic guanosine as a cofactor	• An intron coded protein assist in the self-splicing process which also have reverse transcriptase enzyme which transcribes group II introns into DNA, ultimately producing transposons	• They are really diminutive introns, where the splicing process is organized by protein enzymes

part of noncoding sequences also known as "junk DNA." The major focal point is on functional noncoding RNAs which are generated in a small percentage which is an assorted regulatory biological mechanism, for instance, like gene expression, propagation, differentiation and senescence of cell, and epigenetic modification along with other cellular processes which lead to numerous diseases by the dysregulation of human genome [6].

13.1.4 Description of Different Types of Regulatory ncRNA

- *MicroRNAs (miRNAs)*
  MicroRNA is a lavish group of small noncoding RNAs which do not encode for protein synthesis. They have a significant task as a tumor suppressor or an oncogene which is misled by mutations and abnormal gene expression. Thus, the microRNA modification will prompt the development of various types of cancer [7].
- *Circular RNA (ciRNA)*
  Circular RNA is a sort of contended endogenous RNA in the lineage of long noncoding RNAs which is known to be stable in eukaryotic cell. ciRNA has diverse functionalities such as the capacity of reorganizing the genomic sequences, fortification against exonuclease at the 3′ poly(A) tail, and also an epigenetic regulator [7].
- *Long noncoding RNA (lncRNA)*
  Resistance of colorectal cancer to chemotherapy is due to the encoding long noncoding RNA which restrains the cell multiplication, differentiation, programmed cell death, and metastasis [8]. Some of the colorectal cancer-associated long noncoding RNAs influence the gene expression by epigenetic alteration that necessitates DNA methylation, histone scaffolding, chromosomal instability, and pseudogenes appropriately (Fig. 13.6).

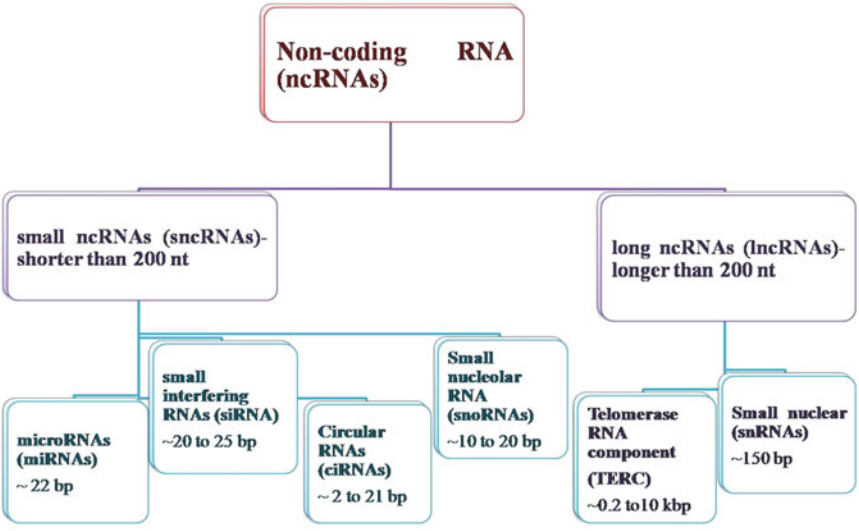

Fig. 13.6 Classification of noncoding RNAs

13.1.5 Functional Characteristic of Noncoding Sequences in Cancer

Tumor is an unrestrained proliferation of cells residing in a tumor microenvironment of a specific tissue site triggered by the dysregulation of the signaling mechanism by the "oncogene" or "tumor suppressor gene" appropriately. Consequently, it advances in their abnormal gene expression, cell growth, protein profiles, differentiation, and epigenetic modulation, and very few instances may be due to familial inherited gene in their germ line. Genome-correlated analysis in cancer divulges in the fact that nearly 75–85% of cancer-linked single-nucleotide polymorphisms transpire in the regulatory noncoding sequences such as intergenic or intronic regions [9] (Fig. 13.7).

13.2 Colorectal Cancer and Associated Factors

Colorectal cancer is the third most prevalent disease affecting both the genders worldwide which is mostly predominant in men with >50 years of age in Western countries while found in minor incidence in India. The dynamic power of tumorigenesis is due to the chromosomal mutations and epigenetic modifications, which either activate oncogenes or cease the task of tumor suppressor genes, which subsequently progress in the development of cancer from neoplasia to metastasis. Initial genetic changes start in an early adenoma and accumulate as it transforms to carcinoma and ultimately to invasive and metastatic tumor. So, the molecular pathogenesis of colorectal cancer includes the familial adenomatous polyposis (FAP) and Lynch syndrome and hereditary nonpolyposis colorectal cancer (HNPCC) (Fig. 13.8).

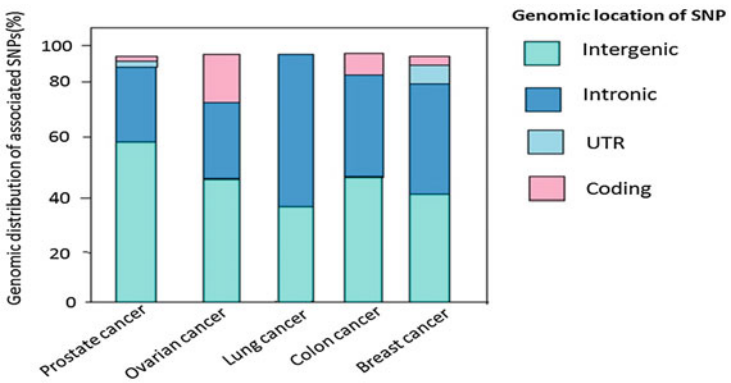

Fig. 13.7 Single-nucleotide polymorphisms (SNPs) existing in the genome dispersal (%) of a range of cancers. It is revealed to be widely encoded by the noncoding sequences (intergenic, intronic) and minority of them by the coding sequences

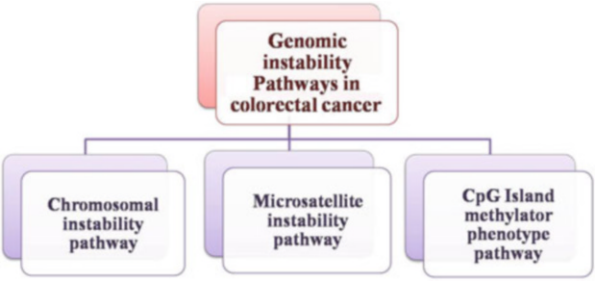

Fig. 13.8 Precariousness in genomic pathways engaged in colorectal cancer

13.3 Genetic Background of the Colorectal Cancer-Associated Diseases

13.3.1 Adenomatous Polyposis Colon Cancer

Adenomatous polyposis coli (APC) is recognized to be a tumor suppressor protein concealed by the APC gene which is endowed as the pioneer gene transformed in sporadic and inherited colon cancer. The APC gene is frequently mutated by either frameshift or nonsense mutation generating a misfolded protein which leads to various syndromes related to colorectal cancer. Normally, the cell cycle checkpoints involve G1/S (start or restriction point) and G2/M checkpoint, and spindle checkpoints are the barricade which is known to regulate cell cycle appropriately without any errors. If the mutational sequences are replicated and synthesized, it will advance in various genetic diseases. In adenomatous polyposis colon cancer, the alteration from G1 to S phase cell cycle is obstructed by the tumor suppressor gene, i.e., APC gene. Subsequently, the Gardner syndrome, familial adenomatous polyposis, Turcot syndrome, and attenuated familial adenomatous polyposis are the APC-related polyposis conditions in colorectal cancer. Wnt signaling and β-catenin pathway take part in the colorectal tumorigenesis of sporadic and familial colorectal cancer (Fig. 13.9).

13.3.2 Lynch Syndrome

Lynch syndrome is also known as “hereditary nonpolyposis colorectal cancer” which is transmitted by germline mutations through microsatellite instability and mismatch repair pathways in colorectal cancer. MLH1, MSH2, MSH6, and PMS2 are the most frequently mutated genes in case of mismatch repair pathway which is known to be the diagnostic marker in colorectal cancer [10]. Therefore, deficit of DNA in mismatch repair activity acts as an indicator of microsatellite instability. Consequently, the preponderance of mismatch repair deficiency in sporadic colorectal cancer is suitable to the epigenetic silencing of MLH1 gene expression that is induced by overmethylation of the promoter [11] (Fig. 13.10).

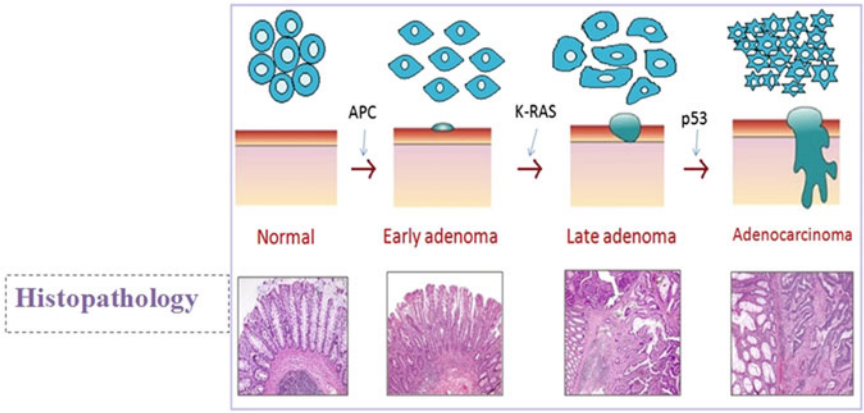

Fig. 13.9 Pathological interpretation of colon cancer tissue prototype

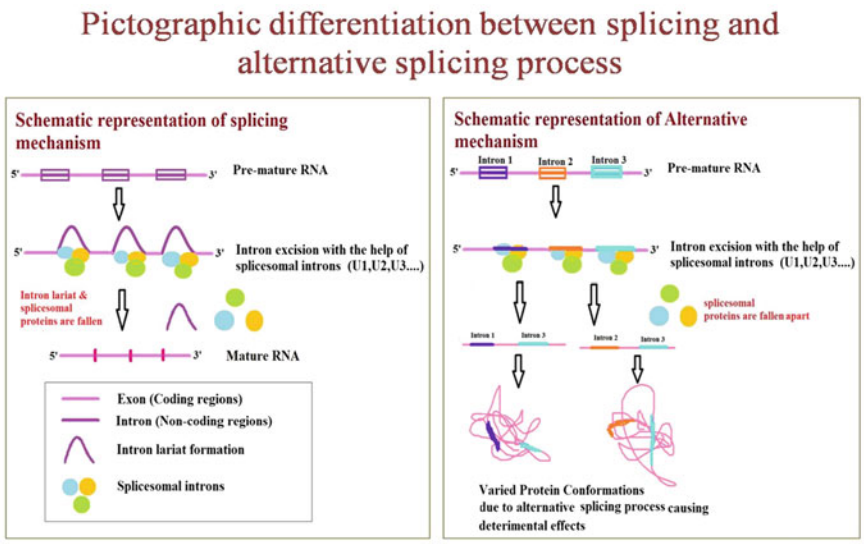

Fig. 13.10 Properties of introns

13.3.3 Intron Retention

Alternative splicing is described as an arbitrary splicing of the introns from premature mRNA which eventually affects the multiple exon genes in humans. Intron retention, alternative 5′ or 3′ control, and exonic regions are the three major divisions of alternate splicing. Among the three divisions mentioned above, intron retention plays an intense role in causing cancer of various types. Intron retention is characterized as the conservation of introns in the coding vicinity or flanked by the introns in the untranslated region which directs the way to mis-splicing of mature

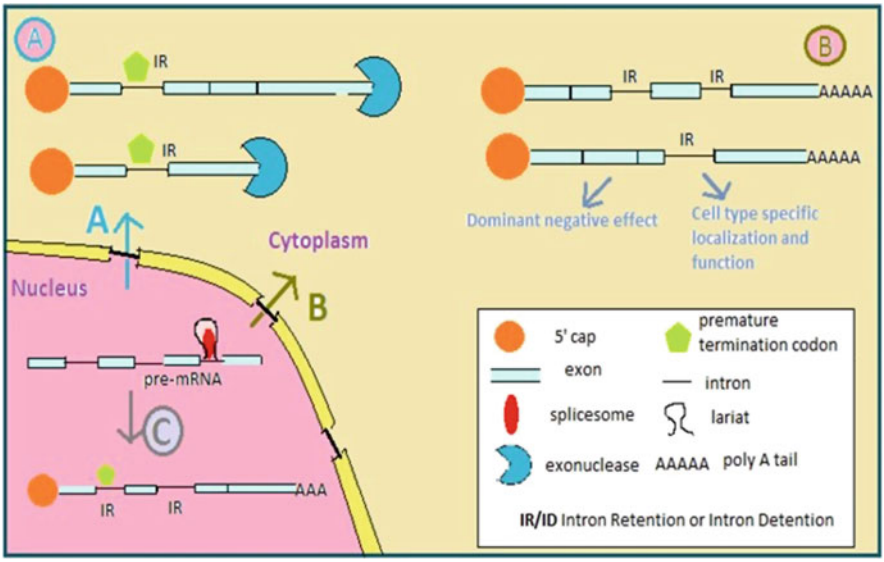

Fig. 13.11 Role of intron-sustained transcripts. (**a**) Downgrading the gene expression through eliciting the nonsense-intervened decay. (**b**) Intron-preserving transcripts might endure deprivation in the nucleus as a result of inhibiting the transport of mRNA which in turn obstructs the translation process. (**c**) Creation of new isoforms along with precise biological act

RNA during the transcription process which ultimately end results in the origination of destructive proteins during the translation process [6]. Thus, the generation of detrimental proteins may lead to an assorted gene expression which roots the basis of various genetic diseases. Therefore, the translation process is inhibited by the contrivance of nonsense-intervened decay or exosome deprivation which facilitates in the excision of introns [12]. Transcripts containing introns often contain the premature termination codons which instigate the nonsense-intervened decay (Fig. 13.11). In general, the nonsense-intervened decay disintegrates the transcripts retaining introns with a premature termination codon which is situated in greater than 50–55 nucleotides upstream of an exon to exon junction [13]. Finally, the plausibility of intron retention can be influenced by different factors such as GC content, expression of splicing factor, extent of introns, alteration of chromatin structure or nucleosome packing, and potency of splice site [14].

13.3.4 Intron Retention Performs a Crucial Role in Gene Expression

Our main focus is on intron retention where the noncoding genes are retained within the coding regions of the gene due to the alternative excision process. Theoretically, an alternative splicing process of exonic genes is known to widely influence the human population. During the transcription process, the pre-mRNA consists of 5′

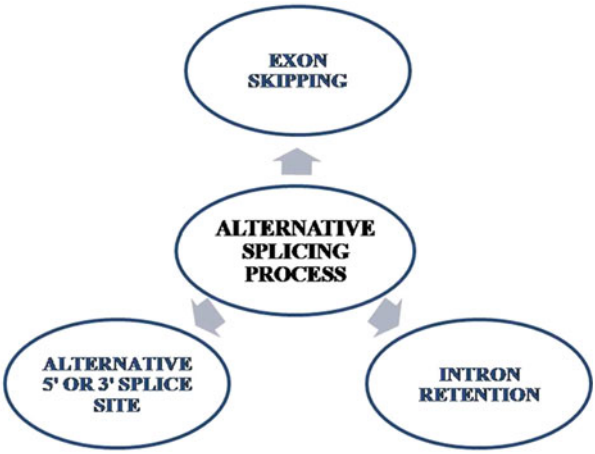

Fig. 13.12 Schematic representation of alternative splicing process

capping, 3′ poly(A) tail with interspersed exons and introns appropriately (Fig. 13.12) [15].

The captivating information about the major and minor compounds contributing to the excision process of heteronuclear RNA encompassing the dominant complex which adds to their role in nucleus, whereas, the less significant complex acts on the division of introns with lower incidence in the multicellular genomes [16]. Thus, the coding regions undergo the translation process generating a functional protein [15]. However, in alternative splicing process, the exon-exon junction complex with up frameshift proteins such as UP-1, UP-2, and UP-3 are bound to the mRNA which proceeds to the protein synthesis. In the translation process, the premature stop codons which are situated prior the termination site lead to the preservation of introns in the coding sequences which advance in the unexpected production of silencing or harmful detrimental proteins without a specific function. In general, the premature stop codons are situated in upstream of greater than 55 nucleotides in the mRNA transcript which eventually signals the nonsense-intervened decay process to activate and undergo the necessary degradation process. So, to circumvent the initiation of such forms of unknown proteins are conceded to promote the deprivation of the transcripts via nonsense-mediated decay. The nonsense-mediated decay process disintegrates the proteins which are accumulated in the processing bodies organized in the cytoplasm of a cell. Thus, the intron retention in gene expression results in varied roles in cell cycle, cell differentiation, cancer, and even genetic disorders. Consequently, our focal point will be on the intron-retaining genes extensively present in colorectal cancer to assess their diverse properties of noncoding RNAs which may be the long noncoding RNA, small interfering RNA, and various other types of noncoding RNAs. The preservation of intronic transcripts in the mature RNA is identified to cause latent destructive end product, if it undergoes translation. According to the literature analysis, intron retention is known to be associated with progression of tumor such as the instigation of oncogene besides reconciling the tumor suppressor gene. So, the profusion of

mature RNA comprising the introns residing in tumor cells is recognized to enhance multiplicity of tumor transcriptomes [17]. Abnormal excision of the noncoding regions from the mRNA leads to limited or complete preservation of the introns. Fortunately, the final intron is prone to restrain the normal as well as cancer tissues and also cause disease by the point mutation in the nucleotide sequence. The molecular level of the chromatin packing of the DNA involves the nucleosome which is compactly packed in two forms such as euchromatin and heterochromatin in which the intron retention has a crucial role in influencing the histone variation, nucleosome compaction, as well as the chromatin modifications at the gene promoter level expressing a range of tumor development with distorted gene expression in their tumor microenvironment. At this point, we will discuss about "mirtron" discerned to arise from the microRNA known to be refined in the introns during the splicing process that creates a loop which is self-regulated and devoid of the microprocessor that is exported to the cytosol of the cell [13]. Frequently, retaining the noncoding sequences within the mRNA constituent and cleaving in the cytoplasm are certain alterations in the transcriptional level advancing in the cellular multiplicity of eukaryotic cells mainly human cells [16].

13.3.5 Noncoding RNA Genes [Human]

- *H19 (nonprotein coding)*
  H19 gene is found to explicit from the maternally inherited chromosome situated in p-arm (15.5) of the chromosome 11. Therefore, the consequence of the gene is the lengthy noncoding RNA which performs as a tumor repression. So, eventually the mutation in this gene will lead to diverse genetic disorders.
- *MIR137 microRNA 137* (1p21.3), *MIR126 microRNA 126* (9q34.3), *MIR33A microRNA 33a* (22q13.2), *MIR335 microRNA 335* (7q32.2), *MIR33B microRNA 33b* (17p11.2), and *MIR21 microRNA 21* (17q23.1)
  In multicellular organisms, miRNAs are short about 20–24 nucleotides ncRNAs transcribed by RNA polymerase II which can end result as noncoding or protein coding which affects the transcriptional control. Then, the initial transcript is spliced by the Drosha ribonuclease III enzyme to generate a prototype miRNA, which in turn is further excised by cytoplasmic Dicer ribonuclease to produce mature miRNA and antisense miRNA star products. Translational reticence or destability of the mature mRNA is due to the improper base pairing with miRNA which is eventually conceded by the RNA-provoked silencing complex.
- *CDKN2B-AS1 (antisense RNA 1)*
  This gene is imprinted in the p-arm (21.3) of chromosome 9 which resides within the gene cluster. Epigenetic silencing of the neighboring genes in the cluster is due to the interaction of polycomb suppressive complex-1 and complex-2 with the functional RNA molecule. Some of the alternatively processed transcript variants have been perceived in the form of circular RNA molecule. This gene seizes the prime locus for various disease abnormalities such as endometriosis,

intracranial aneurysm, glaucoma, periodontitis, type-2 diabetes, cancer, and Alzheimer's disease.

- *KCNQ1OT1 (antisense transcript 1)*
  KCNQ1OT1 gene is situated in p-arm (15.5) of chromosome 11 which is a nonprotein coding gene specifically expressed in maternally or paternally inherited chromosomes which enclose two clusters of epigenetically controlling genes. This gene is regulated by functionally imprinted control region present in the intron of KCNQ1, and the DNA is known to be unmethylated in maternally derived chromosomes. KCNQ1OT1 transcript is the antisense to the KCNQ1 gene which is an uncleaved lengthy ncRNA found to interrelate with chromatin affected by epigenetic alteration. The transcript plays a key role in colorectal carcinogenesis.
- *CCAT 1 and CCAT 2 (colon cancer-associated transcript 1 and 2)*
  CCAT gene is situated in the q-arm of chromosome 8 (24.21) which generates a long noncoding RNA gene to facilitate the tumor progression such as cell propagation, differentiation, invasion, and metastasis. It is known to be highly regulated in colon cancer which interrelates with myc oncoprotein and controls metabolism in an allele-specific manner.

Introns play a vital role in the gene expression of proteins which are translated from the intron retention segment of the messenger RNA. The alternative splicing process comes into picture with a varied multiplicity of proteins generated that alters the gene expression in eukaryotes. Thus, the crucial alternative excision process occurs in the nucleus of the eukaryotic cell with post translational variation such as methylation, sumoylation, and phosphorylation which influence the splicing mechanism appropriately. The splicing machinery affects the tumor microenvironment such as proliferation, differentiation, invasion, and metastasis of a cancer by affecting the mutations in the regulatory site. Therefore, the majority of the human genes is affected by the alternative splicing process [18]. MicroRNAs are derived from the intronic regions of the genes which are protein encoded in *Homo sapiens*, through the analysis of the genome populated with complete intron-coded genes comprised of 22–45% approximately. A phylogenetic conservation of miRNA may provide an additional advantage to the assimilation into transcriptional systems [19]. Currently, the genome-wide analysis studies gives the entire information about the gene loci, location, region, traits, disease probability, and other functional classes such as single-nucleotide polymorphism (SNPs) [20] evaluated through genotyping of wide populace. Unpredictably, the significant genome-wide analysis study of the SNPs and haplotypes is mostly resided in the noncoding regions impeding their functions during the molecular processes [21]. The cleavage of introns from the messenger RNA that in turn modifies the open reading frame (ORF) as well as the protein production. RNA sequencing analysis which utilizes the conserved introns, and predicting their gene structure and the intron prediction algorithms eliminate the introns with <50 nucleotides and STAR aligner that overcomes the RNA sequencing analysis by recognizing very short introns in the mRNA sequences [22]. Various literature reviews have suggested that intron retention mechanism paves an extensive

evolutionary conservation of the homologous and heterologous genome diversity. They aid in regulating the genome multiplicity by assisting the species predilection through the epigenetic modification [23]. Therefore, the splicing process is in particular assessed by the kinetics phenomenon, which senses the polymerase activity as well as the length of introns that may be short or long [24]. The current progression of high-throughput transcriptome method emphasizes the persistent disposition of the human genome to transcription process that divulges the nonprotein encoding genes with their functional transcripts with the aim of genome intricacy. Hence, an efficient transcription unit might produce numerous molecules consisting of regulatory noncoding RNA; proteins rely upon the requirement of a cell according to their external factors [25]. Ultimately, the variation in the expression of specific noncoding RNAs engaged in the stemness of colorectal cancer by employing miRNAs serves as a new tool to reverse the cancer stem cell phenotype and overcome the therapy resistance significantly.

13.4 Cancer Stem Cells

Initially, the cancer stem cells are derived from progenitor or differentiated cells which are usually known to reside in the inner recesses of a tumor mass that holds the capability of self-renewal as well as a diverse family of cancer cells. There are specific surface markers that typically distinguish cancer stem cells that are isolated from various solid tumors including the colon. The two hypotheses of cancer stem cells are the mutations of the oncogene that build up within the adult cells or embryonic stem cells leading to an uncontrolled multiplication of cells, and the other one is the cellular dedifferentiation into a stem cell-like state [26]. Cancer stem cells are of two subdivisions, namely, stationary cancer stem cells and mobile cancer stem cells. The stationary cancer stem cells reside in the epithelial tissues which are active in tumor mass proliferation and cannot disseminate to other distant sites, and the mobile cancer stem cells divide indefinitely that leads to the metastasis of cancer to other parts of the body. It has been suggested that the colon cancer stem express CD44 and CD166. CD133 and epithelial-specific antigen surface marker characteristics of CSCs/CSLCs are their ability to invade and metastasize by acquiring epithelial-mesenchymal transition (EMT) phenotype, which can be determined by analyzing the expression of E-cadherin and vimentin representing Wnt effectors and notch signals. Most of the human malignancies emerge from tissues that contain an active population of stem cells. The stem cells are increasingly recognized as the focus of cancer-causing events, since both genetic and epigenetic alterations may lead to carcinogenesis processes [27]. This is primarily due to the tumor bursting through the intestinal wall and spreading through the lymph nodes and systemically through the bloodstream to distant organs. The colon's luminal surface consists of one single layer of columnar epithelial cells that are folded into the lumen to form finger-like protrusions. The spaces between those folds are known as Lieberkuhn's crypts, the intestine's functional network. There are four distinct cell lineages in the colonic epithelium: enterocytes, goblet cells, endocrine cells, and Paneth cells. The

small undifferentiated cells such as the crypt base columnar cells are known to hold the intestinal stem cells that are found to upsurge to the epithelial lineage. The stem cells have the potential of asymmetrical division which arises to give similar daughter cells as well as the transit amplifying cells that multiply and single out into goblet cells, endocrine cells, and enterocyte in the course of upward movement through the crypt. Here comes the Paneth cell which maintains the microenvironment of stem cell by the release of mucosal defense barriers that change the intestinal microflora through the growth factors and regulatory molecules.

13.4.1 Exacerbation of Cancer Stem Cells in Conjunction with Intronic Genes During the Development of Colorectal Cancer

Cancer stem cells have a distinct microenvironment encompassing the inclination of oxygen levels, chemokines receptors, cyclooxygenase, cytokines, molecules, and growth factors enhancing in the progression of colorectal cancer. Pro-cancer stem cell cytokines such as hepatocyte growth factor, prostaglandin E2, bone morphogenetic protein, and tumor niche-generating interleukins are found to be intensified in the cancer stem cell assembly. The major organ involved in the metastasis of colorectal cancer is the liver and also the growth factor such as chemokine receptor 4 (CXCR4). Stromal-derived factor 1 is chiefly articulated in the liver assisting in the transit of circulating CXCR4 colorectal cancer cells [28]. Wnt, Sonic hedgehog, bone morphogenetic protein (BMP), β-catenin, tumor growth factor-beta (TGF-β), and notch are the major signaling pathways engaged in the homeostasis of colorectal cancer stem cells precisely. Some of the cellular processes such as proliferation, differentiation, migration, and cell death majorly rely on the homeostatic self-renewal of the intestine which ultimately depends on the evolutionarily conserved signaling pathway [29]. Eventually, the microRNAs control several cancer processes like transformation, tumor cell duplication, epithelial-mesenchymal transition (EMT), invasion, and metastasis which are mainly involved in the inhibition of gene expression in pathways that regulate cell processes, for instance, cell cycle, apoptosis, and miRNA migration. Intronic gene such as mir-21 is found to be overexpressed nearly in all malignancies such as breast cancer, glioblastoma, colorectal cancer, lung cancer, pancreatic cancer, and leukemia. In due course, pluripotency and differentiation are known to be through the alteration of stem cells through microRNA [30].

13.5 Future Direction

This chapter encompasses on the intronic genes mainly involved in the progression of colorectal cancer with preliminary information. Researchers turned their focal point toward the mammalian cell and determined that the noncoding sequences (junk DNAs) are known to perform a key role in the development of cancer. Then, the

alternative splicing process takes place in the messenger RNA strand that implicates in the retention of introns leading to a varied gene expression as well as promotion in colorectal cancer. So, the intron retention is known by sensing the premature termination codons in the mRNA strand and triggers the nonsense-mediated decay process appropriately. Therefore, colorectal cancer-associated diseases such as the adenomatous polyposis colorectal cancer and Lynch syndrome have paved the way to know more about the genes and what are all the intronic genes mainly concentrated among them in the development of colorectal cancer. As a result, the concentration of the protein output and gene expression is known to be influenced by the intron retention.

Intron retention ↑	Protein output ↓
Gene expression ↑	Intron retention ↓

Thus, the noncoding regions in the genome can be predicted by RNA sequencing method and interpreting the obtained results from the normal to the diseased form focusing on colorectal cancer. They can also knock down the associated intronic genes in the tumor microenvironment of colorectal cancer which may be beneficial in the tumor proliferation and differentiation. Recent advancement has hypothesized that conjunction of intronic gene with cancer stem cells is known to be progressed in the colorectal cancer precisely. Ultimately, the current circumstances of research fields are accomplished to work with the intriguing noncoding sequences engaged to play a crucial role at certain neoplastic transformation in the normal microflora of the colorectal by analyzing the domino effect of the intronic genes in the molecular phase precisely.

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Technological Advancement in Cancer Stem Cell Research

14

Tanu Sharma and Chandi C. Mandal

Abstract

Cancer stem cells (CSCs) display a significant role in cancer research, evidenced from past decade studies. Although, with the passage of time, effective cancer therapy has been developed, still up to now, cancer possesses the second highest mortality worldwide. The only defined characteristic for every therapy failure is the presence of cells with self-renewable capacity known as cancer stem cells in the heterogeneous population of tumor. These CSCs provide a tumor resistance against various therapies like chemotherapy and radiotherapy. Thus, to prolong survival time period of cancer patients, it is prerequisite to eliminate CSC population. Thus, to develop novel effective therapeutics against primary tumors, isolation and characterization of CSCs will provide a novel insight to develop cancer therapeutics. Thus, various in vitro and in vivo approaches have been developed to isolate and target CSCs. In this chapter, we will discuss about how researchers have developed various powerful tools to characterize CSCs to develop better therapeutics to target CSCs and thus cancer and also how technology has sprung up to generate advanced preclinical models of human tumors.

Keywords:

Cancer stem cells (CSCs) · Spheroids · Organoids

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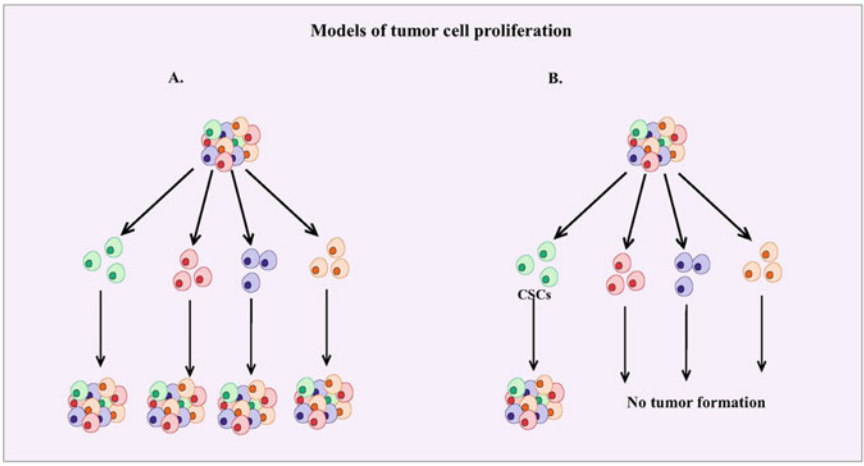

Fig. 14.1 Models for tumor cell proliferation. (**a**) Stochastic model: this model proposes that every cell has the potential to proliferate and behaves as stem cells. (**b**) Cancer stem model: according to this model, only subset of cell has the self-renewable capacity which can generate whole tumor. This distinct subpopulation of cells is known as cancer stem cells

14.1 Introduction

Despite advancement in intensive experimental approaches and progress in cancer treatment, cancer still causes the second highest death [1]. A deep insight into the mechanism of development of carcinogenesis has caused a drift toward cancer research and treatment. Previously, much attention has been paid on genetic and biochemical mechanisms that induce drug resistance. Prevailing theories have reported that tumor is not a mass of homogeneous malignant cells albeit, composed of heterogeneous population of cells. It has been well established that during carcinogenesis, treatment failure primarily occurs due to intratumoral heterogeneity. A subpopulation of cells present within a tumor is responsible for the genesis of resistance against chemotherapy and radiotherapy and the roots of tumor relapse. These minor populations of cells are known as cancer stem cells (CSCs) and can repopulate after therapies causing tumor recurrence [2].

Tumor progression has been well explained by two models, the stochastic model (Fig. 14.1a) and cancer stem cell model (Fig. 14.1b). The stochastic model is known as clonal evolution model. According to the stochastic model, all cells of the tumor possess carcinogenic potential with uncontrolled proliferation potency, and therapeutic treatment requires targeting of all tumorous cells [3, 4]. The cancer stem cell model states that tumor is originated from a stem cell with self-renewable capacity, possessing resistance to chemotherapy and radiotherapy [5]. These subpopulations of tumor are known as cancer stem cells (CSCs) due to their ability to drive whole tumor, and these cells cause tumor recurrence [5]. CSCs are involved in tumor initiation, progression, maintenance, development of metastasis, and

reappearance. Thus, specifically targeting and eliminating CSCs population from tumor could be an effective treatment strategy that can pause tumor relapse and can be sustained as a long-lasting treatment [5].

14.2 Cancer Stem Cell Models

Various tumor biology questions can be answered by studying CSCs. CSCs in a tumor population can be defined as those cells which have potential of self-renewal and are multipotent. Identification and quantification of cancer stem cells like cells can be done by either by in vitro or in vivo assays.

14.3 In Vivo Assays

14.3.1 Xenotransplantation Assay

14.3.1.1 Severe Combined Immunodeficient (SCID) Mice

Immunocompromised mice have been widely used to study CSCs. SCID mice model was first explored for the development of "leukemic stem cells (LSCs)" to study acute myeloid leukemia (AML). During acute myeloid leukemia, cells are restricted with low proliferation capacity, indicating that leukemic clones are maintained by rare population of stem cells [6]. Lapidot et al. [6], used SCID mice model and engrafted different population of cells expressing $CD34^+$ $CD38^-$, $CD34^+$ $CD38^+$, and $CD34^-$ $CD38^+$, resulting in the development of leukemia by cells expressing $CD34^+$ $CD38^-$ only. It was also observed that 1 in 2.5×10^6 cells had the potential to generate leukemic graft [1, 6]. This study provided an evidence that not all the AML cells had potency for tumor formation, but the limitation of using SCID mice was that the frequency of isolated LSCs was very low.

14.3.1.2 Nonobese Diabetic, Severe Combined Immunodeficient (NOD/SCID) Mice

Human malignant melanoma was studied using NOD/SCID mice model which is more immunocompromised than SCID mice model [7]. Xenotransplantation of human melanoma cells in NOD/SCID mice resulted in identification of only one tumorigenic cell out of million cells [7]. Researchers also observed that most cancers had less than 0.1% of tumorigenic cells when transplanted in NOD/SCID mice [7, 8]. It was questioned whether NOD/SCID assays poorly estimate the frequency of tumor generating cells [8, 9]. Thus, it was a demand to develop an efficient model which could increase the number of detection and isolation of cancer stem cells. To solve this problem NOD/SCID $IL2R\gamma^{nul}$ model was used [8].

14.3.1.3 NOD/SCID $IL2R\gamma^{null}$ Mice

NOD/SCID $IL2R\gamma^{null}$ mice is the one which lack the interleukin-2 (IL-2) receptor gamma chain and natural-killer cells and is highly immunocompromised mice model [10]. Quintana et al. showed that transplantation of melanoma cells into the

NOD/SCID IL2Rγ^{nul} mice resulted in detection of increased number of tumorigenic cells [8]. Using this model, researchers were able to generate new tumor in vivo from 25% of melanoma cells. This was quite high in comparison to tumor-initiating potential of NOD/SCID mice which was 1 in 1,090,000; in NOD/SCID IL2Rγ^{null}, it was 1 in 9 melanoma cells which had tumor-generating capacity [10].

This model then became the choice of many researchers. For example, AML cells were xenotransplanted in NOD/SCID IL2Rγ^{null} mice, and it resulted in the presence of long-term engrafting, self-renewing LSCs in very few 10^3 bone marrow hCD34$^+$hCD38$^-$ cells, but not in hCD34$^+$hCD38$^+$ or hCD34$^-$ cells [11]. Ishizawa et al. did comparative study between NOD/SCID and NOD/SCID IL2Rγ^{null} mice for human pancreatic, lung carcinoma, and head and neck cancer [12]. For all tumor under investigation, about tenfold elevation was detected for tumorigenic cells in NOD/SCID IL2Rγ^{null} mice [12].

14.3.1.4 Limitation of Xenotransplantation Assays

There are many technical errors in in vivo detection of cancer stem cells by using xenotransplantation assay and limiting dilution analysis. These errors occur due to murine microenvironment and inadequate immune response at the transplanted site and also sex of recipient mouse strain. These factors compelled scientists to develop a more accurate approach to study cancer stem cells and led to use of genetically engineered mouse models (GEMM).

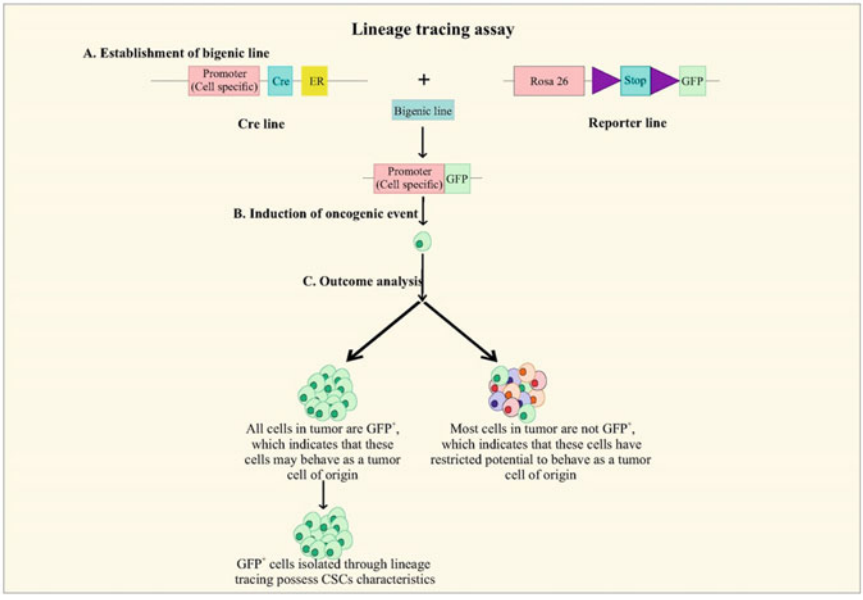

Fig. 14.2 Lineage tracing assay. Schematic representation of lineage tracing assay. The first step includes establishment of bigenic line. The second step involves induction of oncogenic event which is followed by third step, where analysis of outcome is done

14.3.1.5 Lineage Tracing Assay

In lineage tracing assay (Fig. 14.2), different cell-specific promoters are used to label different cells which enables tracking of single cells [13]. Various steps are involved in lineage tracing assay. The initial step is to generate bigenic mouse line (Fig. 14.2a). The bigenic mouse line is generated by crossing an inducible Cre (expressing Cre recombinase) with a reporter line (expressing reporter) which helps in labeling of cells [13]. The second step involves either introduction of oncogenes or deletion of tumor suppressor genes by crossing bigenic mouse generated with third conventional Tg line overexpressing either oncogenes such as Myc, Tcf, and Ras or deleted tumor suppressor genes such as p53, PTEN, and Rb (Fig. 14.2b) [13]. In spite of expressing oncogenes using Tg line, chemical carcinogens can also be used to induce oncogenic event. The most widely used carcinogen is DMBA (7,12-dimethylbenz[a]anthracene). In the final steps of tumor formation, tracing of labeled cells is done. If all the cells are reporter positive, it suggests that these cells have tumor-repopulating capacity (Fig. 14.2c). Thus, purification of these cells is done to perform serial transplantation and then CSCs are isolated. But, if majority of cells are reporter negative, then it suggests that cells do not possess CSC properties. Lineage tracing assay has gained momentum, and various studies have been performed using this assay which employs the use of genetically engineered mouse models (GEMM) [14].

For example, Chen et al. performed a lineage tracing study for glioblastoma multiforme (GBM). This study showed that dormant subset of endogenous glioma cells is responsible for tumor maintenance and recurrence of GBM after chemotherapy [15]. They used a *Nestin-ΔTK-IRES-GFP* (*Nes-ΔTK-GFP*) transgene that labels both adult NSCs (neural stem cells) and endogenous glioma tumor cells. This *Nes-ΔTK-GFP was crossed with Mut7 line which is a glioma-prone mouse line* [16]. *This Mut7 mouse line is generated by deleting three tumor suppressor genes, i.e., PTEN, p53, and Nf1* [15]. *The resultant Mut7 mice developed glioblastoma with deleted PTEN, p53, and NF1 tumor suppressor genes* [15]. *These Mut7;Nes-ΔTK-GFP* tumor cells also expressed Sox2 and had two population of cells. One subset of cells expressed $GFP^+/Sox2^+/ki\text{-}67^-$ and GFP^-/ki^-67^+. Treatment with temozolomide eliminated actively dividing GFP^-/ki^-67^+ tumor cells, and a fraction of quiescent cells responsible for tumor recurrence $GFP^+/Sox2^+/ki\text{-}67^-$ was left. GFP^+ cells could be targeted by ganciclovir; thus, ganciclovir administration significantly decreased tumor growth with prolonged survival and co-administration of temozolomide- and ganciclovir-retarded tumor growth [15]. This lineage tracing study demonstrated that dormant endogenous glioblastoma cells $GFP^+/Sox2^+/ki\text{-}67^-$ responsible for tumor recurrence possess CSC properties and are responsible for long-term tumor growth [14].

A functional evidence for the presence of stem cells in intestinal adenomas was provided by the study done by Schepers et al. [17]. In this study, they used multicolor Cre reporter R26R-Confetti mouse strain. They crossed $Lgr5^{EGFP\text{-}Ires\text{-}CreERT2}/Apc^{fl/fl}$ mice with the R26R-Confetti strain, and tamoxifen injection resulted in generation of Lgr5-GFP^{hi} and Lgr5-GFP^{low}. Gene expression and clonogenic potential analysis showed that Lgr5-GFP^{hi} had multipotent stem cell characteristics,

and retracing of these cells showed that these cells were obtained from single adenoma stem cells [17].

Another lineage tracing study done by Driessens et al. utilized a chemical two-stage carcinogenesis model to generate skin papillomas [18]. The bigenic mouse strain K14CreER/Rosa-YFP was obtained by crossing K14-driven CreER line with the Rosa26-YFP reporter line. By injection of tamoxifen, K14-expressing keratinocytes will be labeled as YFP^+ cells [18]. Administration of both DMBA and tamoxifen resulted in generation of YFP^+ cells and had majority of cells with limited proliferation capacity, while a fraction had stem cell-like characteristics. Confocal analysis of clones showed that papillomas were sustained by small population of tumor cells having characteristics like of stem cells [18].

14.3.1.6 Limitations of Lineage Tracing Assay

Lineage tracing can be performed utilizing mouse model only, and various fundamental differences exist in human and mice cells/organs. For example, mouse prostate is divided into 4 lobes that do not exist in humans, and also, mouse cells do not express PSA which is an important molecule of human prostate gland. Another difference is that mouse cells express high telomerase activity, which indicates that mouse cells may never undergo true terminal differentiation. Thus, results obtained using mouse models may not directly reflect human system [14].

14.3.2 In Vitro Assays

14.3.2.1 Side Population Assay

Side population assay has been used for the isolation and characterization of cancer stem cells (Fig. 14.3) [13]. SP assay was developed by Goodell and mulligan [19, 20]. It was observed by the researchers that a distinct population existed in murine bone marrow cells which were poorly stained for Hoechst 33342. These cells occupied a distinct position in flow cytometry dot plot, hence named as side populations [21]. The exclusion of Hoechst stain by side population is a specific property of CSCs. Interestingly, the efflux of Hoechst stain was due to ATP-binding cassette (ABC) transporter. ABC transporter uses ATP to efflux out many small endogenous molecules like peptides, cholesterol, and bile acids. These transporters help in detoxification of cells and also contribute to cancer stem cell-like properties to CSCs. ABC transporters induce chemoresistance in CSCs as chemotherapeutic drugs are also substrates for these pumps and efflux of drugs occurs by ABC transporters [21].

14.3.2.2 Retention of PKH26 and PKH6 Dye

It has been reported that CSCs proliferate slowly and remain quiescent. These CSCs when divided result in two daughter cells; one possesses stemness (remains quiescent) and other proliferates. PKH26 and PKH6 are two lipophilic dyes [21]. In this assay (Fig. 14.4), the cell membranes are labeled with these dyes. After division both daughter cells receive equal portion of theses dyes [21]. The CSCs which are

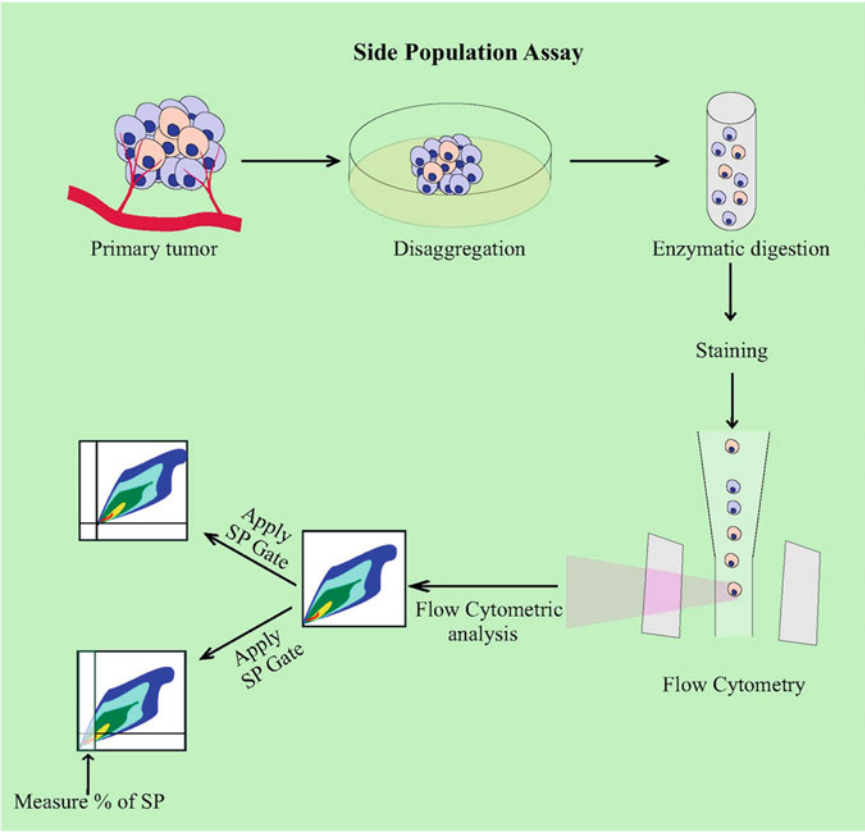

Fig. 14.3 Side population assay. The side population assay measures percent side population of cells after flow cytometric analysis. The steps include single-cell isolation of cells by disaggregation and enzymatic digestion of cells. These cells are then stained with Hoechst 33342, and then cells are subjected to flow cytometric analysis. The cells with CSC characteristics possess more ABC transporters and effluxes dye out of the cells, and these cells are obtained on side on flow cytometric plot

quiescent retain dye for longer duration as compared to non-stem cells. This method has been used to isolate CSCs from breast cancer [22].

14.3.3 ALDEFLUOR Assay

Aldehyde dehydrogenases (ALDHs) belongs to the family of enzymes that catalyzes the oxidation of endogenous and exogenous aldehyde substrates to corresponding carboxylic acids [23]. These enzymes are known for their detoxification properties as these eliminate aldehydes synthesized either by physiological metabolic products or by cytotoxic drugs like chemotherapeutic agents. This detoxification property

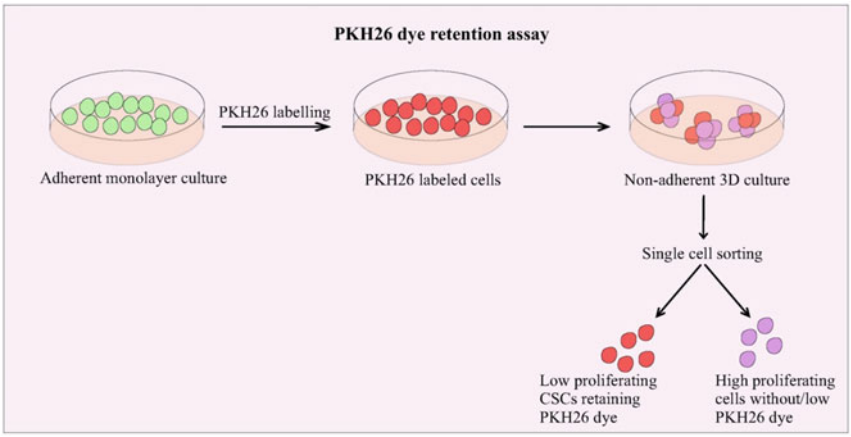

Fig. 14.4 PKH26 dye retention assay. Monolayer culture is first treated with PKH26 and then subjected to non-adherent 3D culture. After single-cell sorting, CSCs are identified as those which retained dye for long due to low proliferating potential

attributes them as a marker of cancer stem cells as they confer chemoresistance in cancer cells [24]. Study done by Hilton et al. firstly revealed that high ALDH activity is responsible for chemoresistance in leukemia stem cells against cyclophosphamide (an alkylating agent) [25]. Increased ALDH activity has been reported in lung, colon, and breast cancer stem cells [21]. *ALDEFLUOR* assay is done to identify cancer stem cells (Fig. 14.5). In this assay, CSCs with high ALDH activity become highly fluorescent and can be detected by using flow cytometer and can be isolated by using cell sorting. *ALDEFLUOR* assay works on the principle of conversion of BODIPY-aminoacetaldehyde (BAAA) substrate to a fluorescent BODIPY-aminoacetate (BAA) product [24]. Thus, this assay isolates CSCs on the basis of intrinsic functional property of CSCs.

14.3.3.1 Two-Dimensional Model

Two-dimensional cultured tumor cell lines have been extensively used to study cancer progression. Various signaling pathways have been studied using 2D cultured tumor cell lines. But, with the advancement in technology to study tumor progression, it has been reported that 2D cultured tumor cell lines provide contradictory results due to culture conditions and number of cell passages [26]. Although research using 2D cultured tumor cell lines is inexpensive, these cannot mimic three-dimensional characteristics of solid tumor models and also tumor microenvironment. Thus, researchers have developed three-dimensional tumor models that may resemble solid tumor characteristics so that more accurate therapeutics can be developed to improve survival of cancer patients (Table 14.1).

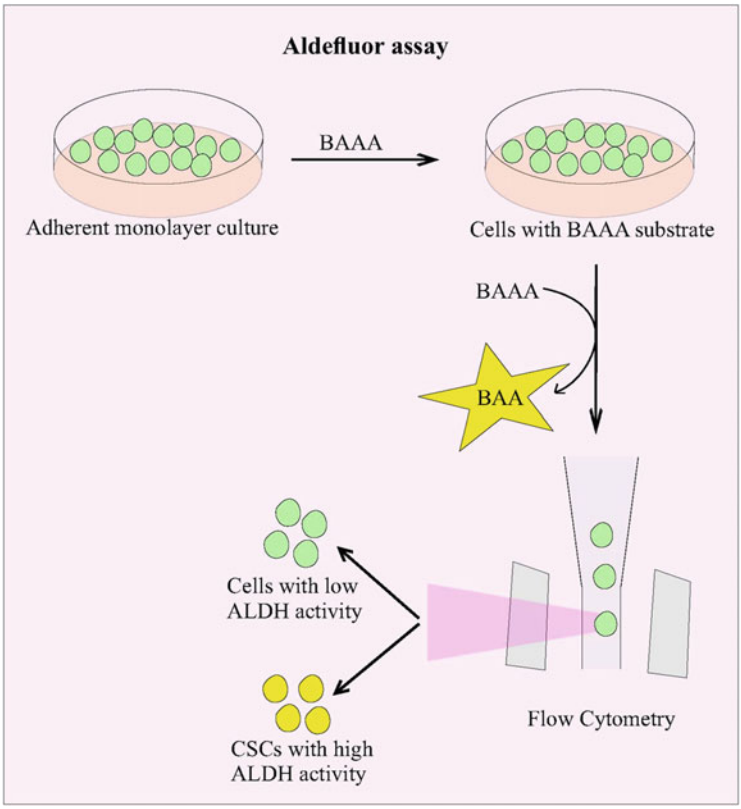

Fig. 14.5 ALDEFLUOR assay. This assay involves treatment of cultured cells with BODIPY-aminoacetaldehyde (BAAA) substrate, and CSCs are identified and isolated on the basis of high ALDH activity which converts BAAA to highly fluorescent BAA

14.3.3.2 Three-Dimensional Models

Three-dimensional tumor cultures are the recent advancement of technology to specifically generate and isolate CSCs. The main purpose of 3D models is to study the effect of tumor microenvironment on the gene expression analysis, pathogenesis, and effective drug testing to overcome chemoresistance. The two important 3D tumor models discussed in this chapter are *tumor spheroids* and *tumor organoids*.

14.3.4 Sphere Formation Assay

14.3.4.1 Tumor Spheroids

Tumor spheroids are spherical aggregates of tumor cells with self-renewable capacity and are generated by sphere formation assay (Fig. 14.6). Sphere formation assay also known as non-adherent 3D culture was firstly described as an approach to study adult stem cells [32]. The principle of this assay is that cancer stem cells in non-adherent conditions proliferate to form a sphere and non-stem cells will go for

Table 14.1 Illustration of key differences between advantages and disadvantages of two-dimensional cell lines and three-dimensional spheroids and organoids

Advantages/ disadvantages	2D cell line	Spheroids	Organoids
Advantages	• Cost-effective [2, 26] • Genetic manipulation is easy [2] • It allows high-throughput screening of drugs in short duration [2, 27]	• Provides 3D environment [28] • Allows growth of cancer stem cells [28] • Highly reproducible [29]	• In vivo-type complexity and architecture • 3D structures and resembles mini-organ to the tissue of origin [2, 30] • Patient-derived organoids enable the development of personalized medication [31]. Variable [29]
Disadvantages	• Lack of heterogeneity [2, 27] • Do not correspond to tumor microenvironment [27]	• Expansion of CSCs occurs after serial passages, thus, not efficient for investigation of drug activity [31]	• Organoids cannot mimic exact hypoxic gradient occurring in tumor microenvironment [2]

anoikis. Sphere formation assay is a powerful tool that allows to access stem cell-like characteristics residing in tumor and cancerous cells. Cancer stem cells possess the ability to generate 3D (three-dimensional) spheres in vitro when grown in serum-free non-adherent culture conditions [33]. Various 3D in vitro sphere formation assays have been developed to obtain cancer stem cells. This assay requires the growth of cells in an artificial medium resembling stem cell-like conditions which include media supplemented with epidermal growth factor (EGF), low-density condition to avoid aggregation, progesterone, heparin, insulin, and hydrocortisone [34]. Sphere formation assay is widely used as it helps to detect CSCs, and also, self-renewal and differentiation can be studied at single-cell level [32].

14.4 Critical Parameter Consideration

14.4.1 Cell Density and Clonal Formation

Cell density is the most crucial parameter as it directly affects clonality. A central focus of sphere formation assay is that each sphere is obtained from single cell and therefore must be clonal. Different research groups have proposed different cell densities for seeding. High-density seeding is not favored because interpretation of results becomes very difficult due to sphere fusion. Spheres have the potency for aggregation due to both intrinsic and experiment-induced locomotion. It must be ensured that the sphere is formed due to proliferation not due to aggregation [32]. Thus, seeding at 0.2–20 cells per microliter is recommended [35–37].

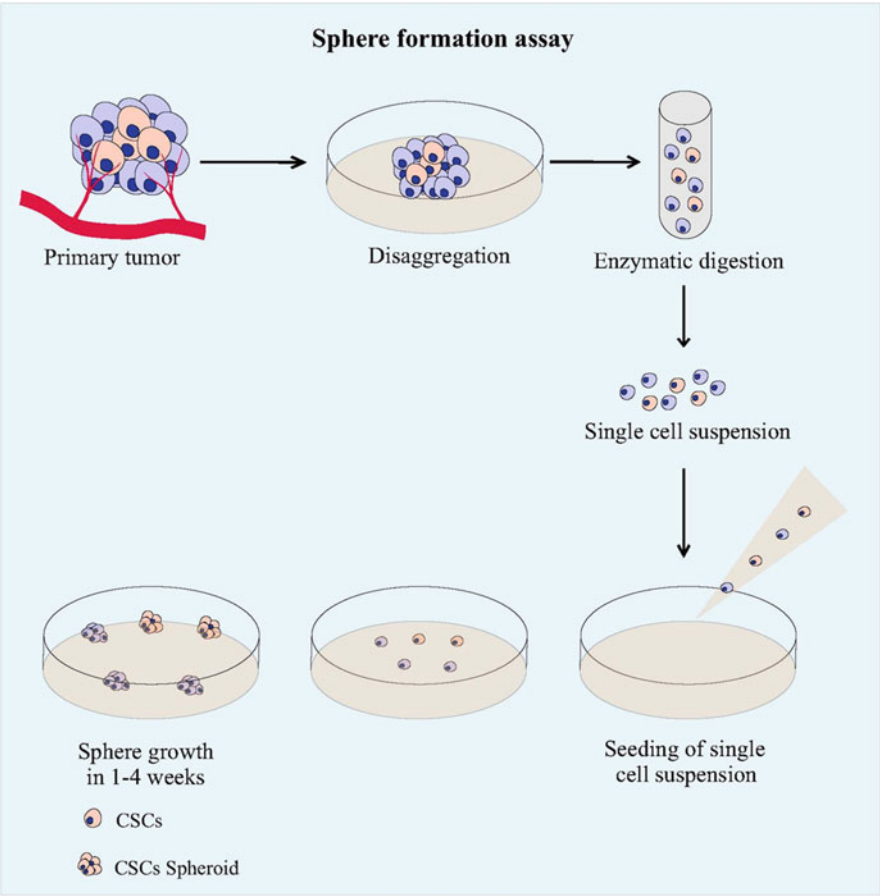

Fig. 14.6 Sphere formation assay. In this assay, firstly single-cell suspension is formed from primary tumor by disaggregation and enzymatic digestion. Then seeding of single cells is done and sphere formation occurs in 1–4 weeks

14.4.2 Mitogen Tolerance

It has been reported that spheres are cultured at very high level of EGF of about 20 ng/ml. This high concentration of EGF may alter differentiation potential of cultured cells [32].

14.4.3 Overestimation of Stem Cell Frequency

Sphere formation assay can overestimate frequency of generated stem cells because neural stem cell purification by FACS has shown that both stem cells and transit amplifying cells have potential to give rise to neurospheres [38]. Similar observation of false readout was observed by culturing mammary cells which formed mammospheres [39].

14.4.4 Tumor Organoids

Tumor organoids are 3D constructs resembling avascular tumor generated from fresh biopsy samples [26]. The process of tumor organoid formation includes mechanical or enzymatic processing of tumor samples and embedding in extracellular matrix such as collagen or Matrigels and ECM substitutes [26, 40]. The various steps used to generate tumor organoid has been shown in Fig. 14.7. Organoid technology recently has been used extensively, and various cancer organoids such as stomach cancer organoid, intestinal cancer organoid, liver cancer organoid,

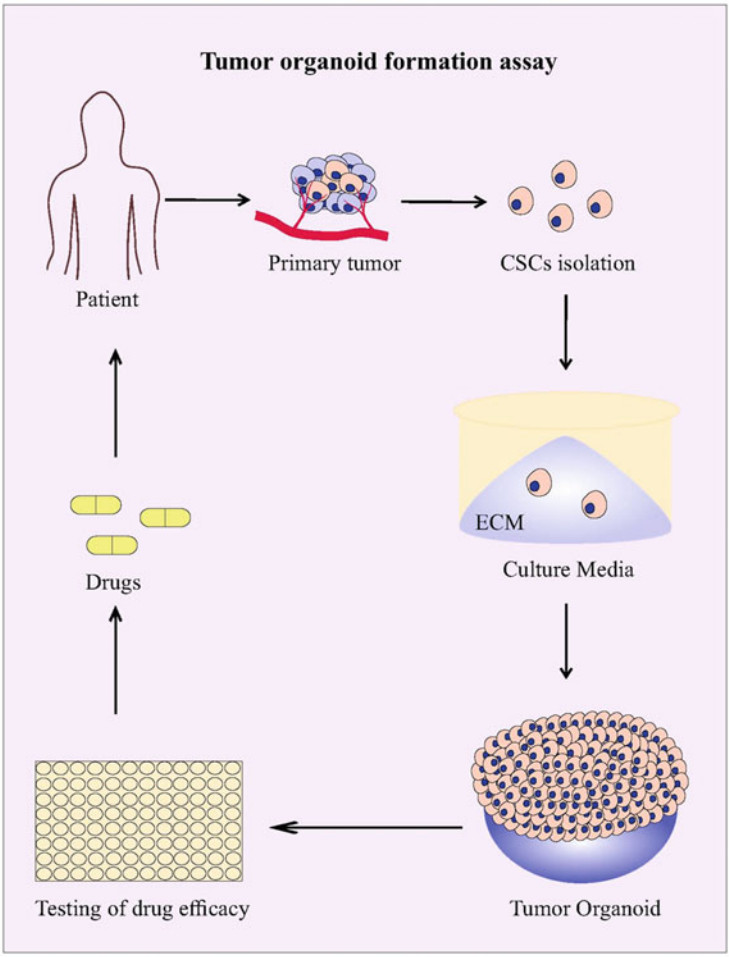

Fig. 14.7 Tumor organoid formation assay. Primary tissue from the patient is disaggregated to obtain CSCs. These cells are then cultured in three-dimensional media to generate tumor organoid, and these organoids can be used to test required drug efficacy and to develop personalized medicines

pancreatic cancer organoid, breast cancer organoid, bladder cancer organoid, and prostate cancer organoid have been synthesized [41–51].

14.5 Conclusion and Further Directions

Plethora of evidences is in agreement with current theory of cancer stem cells. It has been well established that CSCs play a vital role in tumor initiation and maintenance of tumor progression. These CSCs facilitate tumor metastasis to distant site other than the site of origin. Thus, these CSCs have the potential for tumor regeneration and recurrence; hence, these are potential therapeutic targets, and elimination of these CSCs will protect tumor recurrence. Various technological advancements have been made with the primary aim to develop effective drug treatment. Traditional 2D culture cell lines have been in long use to develop cancer treatment and have contributed significantly in cancer research. But these 2D culture cell lines fail to match accuracy to the condition of tumor development in the presence of the immune system; stromal interactions of CSCs and also lack of heterogeneity make them of least choice. To overcome these limitations, 3D tumor models are in fashion for cancer research. Tumor spheroids and tumor organoids both are widely used, but tumor organoids have revolutionized cancer research and have been proven as best models as they recapitulate whole tumor like in vivo. These tumor organoid technologies have provided a way for cancer researchers toward the development of effective drug testing and facilitation of personalized therapy.

Thus, organoid technology upholds potential for new possibilities for personalized medication which will be the best nonsurgical treatment not available today.

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Controversies in Isolation and Characterization of Cancer Stem Cells

15

Ravi Gor and Satish Ramalingam

Abstract

Cancer is an uncontrolled growth of a cell in any part of the body. It has been more than a century for identification of cure for cancer/tumor, and still, we are unable to understand and treat the cancer completely. Current therapeutic techniques such as radiation, chemotherapy, surgery, etc. are failing to eradicate the cancer cells from its root and lead to its relapse in the short or long term. This is because of the small subpopulation of the cells within the tumor that are known as cancer stem cells (CSCs). These cells play an important role in supplying differentiated cells for the growth and development of the tumor. Along with this, they also maintain their population intact for the future requirement of the cells for tumor growth and its metastasis. In spite of several studies proving the presence of CSCs in various types of tumors, there is always a question about its existence and the way we characterize the CSCs based on the histotype-specific markers. There is a dire need for the compilation of research in this area to understand whether the cells, which are being confirmed as CSCs are really CSCs or not? In this chapter, we provide the various isolation and characterization techniques along with the latest CSC identification markers for different types of cancer, in addition we highlight the arguments and the limitations regarding the isolation and characterization of CSCs in this chapter.

Keywords

Cancer stem cells · Histotype-specific marker · Metastasis

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15.1 Cancer Stem Cell: Brief History and Current Status

It all started with one question, "How many cells are required for the induction of tumor?". Back in the year 1937, J. Furth and his group reported the transmission of mice leukemia with the transfer of a single transformed cell. The leukemia cell suspension was obtained by isolating the leukemic tumor and filtering it to remove larger particulates and this results in the single cell suspension. The cell suspension is diluted and a single cell is injected in the mice and checked for its tumor forming ability. Out of 97 mice, five developed carcinoma. Finally, they concluded that leukemia can be transmitted with a single transformed cell in an adult individual [1]. Later after half a decade, a publication by John E. Dick's and colleagues in 1994 and 1997 demonstrated that only a few rare cells (undifferentiated) of mouse acute myelogenous leukemia (AML) are capable of initiating this leukemia in other mice on transplantation. They concluded that these rare groups of cells ($CD34^+$ $CD38^-$) can produce a different lineage of cells and also maintain the undifferentiated form of themselves for a longer duration of time [2, 3].

Currently, we call them cancer stem cells, which are mostly like the hematopoietic stem cells present in the human body, which give rise to the blood cells, immune cells, etc. Cancer stem cells are called by many different names like "cancer-initiating cells," "cancer stem-like cells," and "tumor-initiating cells," but they all mean the same and possess key characteristics of stem cells. These are the population of cells found in the tumor which possess the features such as multipotentiality, self-renewal, clonogenicity, and treatment resistant which are the key features of a stem cell. Although these cells are found to be capable of recreating the original tumor independently, we are unable to uncover the origin or mechanism by which the tumor has got Cancer Stem cells. There are only possible theories suggesting that the CSCs came into existence due to mutation(s) in the tissue stem cell, or the transformed cancer cell has gained stem cell property by mutation(s) and became a Cancer Stem Cell. With these characteristics of CSCs, we are unable to eradicate the CSCs by conventional cancer therapy (Fig. 15.1) which will only wipe out the non-tumorigenic cells and CSCs will remain even after the treatment. Current methods of treatment includes chemotherapy, surgery, radiation, immunotherapy, etc. even if one CSC is left after the treatment, then it can regrow the tumor in the same place resulting in tumor recurrence. If the CSCs are killed along with the non-tumorigenic cells, it can result in the total eradication of the tumor. As a result of cancer stem cell targeting therapy the cancer can be eradicated from its root and there won't be any relapse of cancer.

The real challenge is how to select the putative cancer stem cells from the heterogeneous population of the cells in the tumor. Since they only comprise less than 1% of the total tumor cells, it's like finding a single nucleotide polymorphisms (SNP) in the whole genomic DNA sequence. We need to have some identification markers to target them. Around 40 cancer stem cell surface markers are being published and still counting for various types of cancer [4]. Including these cell surface markers, there are other stemness genes like BMI, β-catenin, OCT3/OCT4, SMO, SOX2, NANOG, NOTCH, etc. They play an important role in maintaining

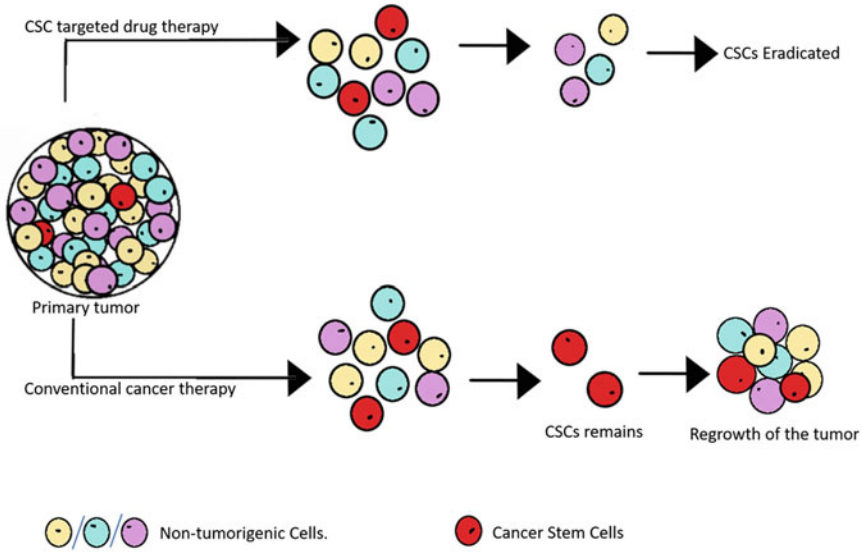

Fig. 15.1 Cancer stem cell hypothesis

the character of the cancer stem cell; evaluation of the expression of these genes by real-time PCR analysis can be used to characterize the isolated CSCs and to understand the molecular mechanism required to maintain the stemness. In the case of breast cancer, SOX2 levels are used as a prognostic marker for early detection of cancer recurrence [5]. In renal cell carcinoma, OCT4 and NANOG can be used as markers for prediction of poor prognosis of the disease [6]. This information regarding different cancer types can further be used for targeting them and fully eradicating them.

15.2 Isolation and Characterization Techniques

There are two major ways by which cancer stem cells can be identified, one with the help of cell surface marker dependent and another is independent of cell surface marker identification. The cell surface marker-dependent techniques involve fluorescence activated cell sorting (FACS) as a critical step in sorting the cells based on their surface markers. On the other hand, FACS is also used for detecting the intracellular marker such as ALDH1 to isolate cancer stem cells in different tumor types. Other identification techniques include phenotypic assay, cytotoxic drug effluxing assay, side population assay, sphere formation assay, somatic stem cell property, pulse-chase approach, etc. Almost all the isolation techniques have their pros and cons in isolating and characterizing the alleged cancer stem cell population, therefore combination of these techniques needs to be utilized for efficient isolation of CSCs.

15.2.1 Isolation Based on Cell Surface Markers

As the cells in the body have an identification mark showing self-cells, immune cells, etc. with the help of a cluster of differentiation protein collectively called CD, similarly, the CSCs can also be identified based on the CD proteins or other surface markers like EpCAM/ESA, etc. This can be achieved by advanced high-throughput machine called FACS; it can sort cells based on their surface marker using fluorescent labelled antibodies. For CSC characterization, different markers specific for mesenchymal and hematopoietic stem cells can be used, such as CD133, CD44, CD90, etc. Different combinations of these markers tell us about the presence of a very small population of the putative CSCs. A population of cells can be defined as a cancer stem cell if it can develop tumor after implantation to the immunodeficient host [7]. Also, it must continuously do that for multiple subculturing. To validate the ability of the isolated cells to imitate the tumor in vivo, the isolated cells are xenotransplanted to immunodeficient host (mice usually). If it gives rise to the tumor and further transplantation reproduces the result, then it can be concluded that the isolated population of cells are cancer stem cells.

The isolation protocol can be divided into three major steps, isolating the tumor sample from the patient, making a single cell suspension, and lastly cell labelling and flow cytometry analysis. The solid tumor is isolated from the patient with proper concern and must begin processing as soon as possible to maximize viability. To prepare a single cell suspension sample can be processed by a mechanical or enzymatical (overnight) method to make a suspension of cells from solid tissue. The goal is to break all the cell-cell connections or junctions so that we get a suspension with individual cells floating. To achieve better results, combination of chemical and mechanical dissociation is performed to provide maximum yield and viability of the cells schematic of the method is depicted in the figure (Fig. 15.2).

Further, the suspension is filtered through the cell strainer, and homogeneous cell suspension will be used in the process. Multiple cell wash is done and labelled with the fluorescent labelled antibody specific to the surface marker under study. Cells are sorted and transplanted to the immunodeficient mice to check its ability to form a tumor. FACS can also be used for isolation of the CSCs from the cell lines from the cell banks/working laboratory cell lines [45, 46]. $CD44^+$ gastric cancer stem cell that has been isolated by FACS shows stemness properties like differentiation and self-renewal. In gastric cancer, $CD44^+$ cells show more resistance for chemotherapy and also radiation-induced cell death. Also, knockdown of CD44 showed reduced tumor production in SCID mice and shows reduced spheroid colony formation [28]. When working with a large number of cells, magnetic bead-assisted sorting will be quicker than flow cytometry. Commonly used methods with magnetic beads are Dynabeads, magnetic-activated cell sorting (MACS), etc. According to the sample acquired and the number of marker needs to be accessed to separate the CSCs, FACS or magnetic bead-assisted sorting is carried out [45].

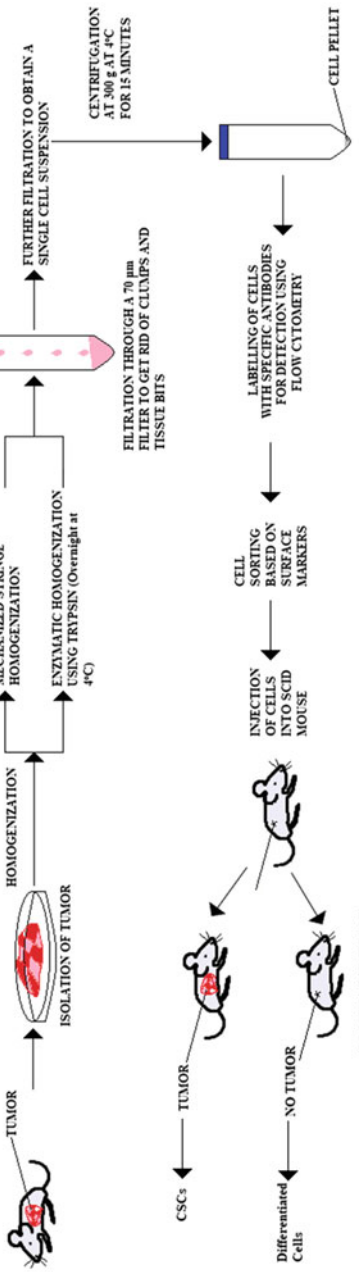

Fig. 15.2 General overview of cancer stem cell isolation by FACS

15.2.2 Isolation Independent of Cell Surface Marker

15.2.2.1 Side Population (SP) Assay

Stem cells have a high capacity to outflow antimitotic drugs. These cells come under a subset of stem cells and called "side population." Side population assay checks for the ability of the CSCs to remove the drug out from the system rapidly as their characteristic for chemoresistance. Normally the differentiated cells will take up the chemical in and process it or be targeted by the same. CSCs can achieve drug resistance with the high expression of ABC transporter protein family members. It is an ATP-dependent transporter or also called a drug effluxing pump and is used to translocate molecules across membranes [47–49]. It has been analyzed in glioblastoma [50], colon carcinoma [51], breast cancer [52], and other types of cancer [53, 54] that ABC proteins provide high chemoresistance to the normal stem cells as well as CSCs with comparison to the differentiated cells [55–57]. Hoechst 33342 dye is used in this assay; it is a nucleic acid stain and emits blue fluorescence when bound to dsDNA. In the heterogeneous population, differentiated tumor cells will keep the dye, whereas the CSCs stream out the dye with high efflux capacity. In the case of neuroblastoma cells, SP was capable of sustaining expansion in vitro and also shows the asymmetrical division, generating SP and non-SP (differentiated) cells. High level of ABCG2 and ABCA3 expression was found to help in better survival by expelling cytotoxic drugs [58]. The advantage of SP assay is there is no requirement of the cell-specific marker to isolate the CSCs. Since the population of CSCs are less in number which makes it difficult to even isolate them with the help of FACS (<2%) [59], the longer incubation with the dye increases apoptosis in a glioma cell line [60].

15.2.2.2 Sphere Formation Assay

Sphere formation assay can enrich CSCs from the solid tumor without utilizing the cell sorting and surface marker. The solid tumors are grown in the non-adherent condition. In this assay, mitogenic growth factors are provided, and the media is devoid of the serum to provide a non-adherent environment. Mitogenic growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), etc. are supplied depending on the specific cell line. As a result of providing a non-adherent environment, the primitive cells will form a sphere by clustering together, and the differentiated cells die because of no communication to the neighboring cells. Cell sphere was first found in the culture of adult mouse striatum (part of the basal ganglia of the brain) forming sphere in the absence of adhesion factors or supplementary substrate. With all the provided mitogenic growth factor, only the primitive cells survived and the differentiated cells died [61]. With this finding even only cancer stem cells can be grown in the non-adherent environment and be isolated from the heterogeneous population of cells. After a decade it has been shown that a CD133+ cell from human brain tumor grew as a neurosphere in a non-adherent environment [62]. In case of the C6 glioma cell line, only the SP cells survived in the serum-free, growth factor supplemented media and the non-adherent environment by forming a tumorsphere. It has also reported that the C6SP cells can

generate the SP and non-SP cells in the culture, and they are responsible for the malignancy [63].

15.2.2.3 Pulse-Chase Approach

One characteristic of a CSC is slow proliferation, i.e., as compared to the other cells in the surrounding, they will be in the quiescent stage and only divide when needed to produce the differentiated cells. Slowly dividing cells will retain DNA analog for a long time because of no cell division taking place, and this will be termed as a label-retaining cell or stem cell. The more the label retained in the cell, chances of it to be CSCs are more. Firstly, the cells are labelled with BrdU (^{3}H-thymidine or 5-′-bromodeoxyuridine), and then it will be examined regularly to check for the cells with label retention. As the dividing cell's label will be diluted due to the DNA replication during cell division, the selected cells with high label retention are tested with other assays to confirm the CSCs [64–66]. In the case of prostate cancer (PCa) cells with the help of BrdU, pulse-chase assays reveal that $CD44^+$ cells colocalize with intermediate label-retaining cells. Further, it was concluded that these cells are more proliferative, tumorigenic, metastatic, and clonogenic than the $CD44^-$ PCa cells [67].

15.2.2.4 Aldehyde Dehydrogenase (ALDH) Activity

ALDH1 is used as an internal marker for the identification of CSCs in many cancers like breast, colon, etc. ALDH is an enzyme which catalyzes the pyridine nucleotide-dependent oxidation of aldehydes to acids. For example, in case of retinoid signalling ALDH1 catalyzes the conversion of retinol to retinoic acid (RA) in the cytoplasm and finally the RA activates genes which will help in the regulation of stem cell and cancer stem cells. ALDH is substrate nonspecific; by this property it protects the organism from potentially harmful xenobiotics and makes stem cells resistant to the aldehyde-specific xenobiotics [68]. Commercially available fluorescent ALDEFLUOR assay kit can be used to identify the ALDH activity with the help of the ALDH substrate and an ALDH inhibitor (diethylaminobenzaldehyde) as a negative control. By ALDEFLUOR assay, isolation of CSC from breast cell line is done [69] also from acute myeloid leukemia, and multiple myeloma CSCs are isolated with this method [70, 71]. Further, isolated cells, i.e., ALDELFUOR positive and negative cells, are collected, and tumor xenograft studies, expression of stemness genes, etc. can be studied to validate the isolated cancer stem cells. It is found that in renal cell carcinoma (RCC), the number of $ALDH1^+$ cells is doubled in the metastatic ACHN cell line than compared to the primary KRY/Y cell line. Also, the $ALDH^+$ cells show higher sphere-forming ability than that of $ALDH^-$ cells [72].

15.2.2.5 Tumorigenicity Assay Also Known as Reestablishing Heterogeneity

Along with the slowly dividing, self-renewal property, a cancer stem cell must be able to generate a heterogeneous population again at the new site of growth; this property is called tumorigenicity. All the cells which are isolated as prospective cancer stem cells are further analyzed to check its ability to imitate the same cell

heterogeneity when injected into immunocompromised mice. This was first demonstratd by J. Furth et al., to know how many cells are required for it to generate leukemia in immunodeficient mice. As per their research a solid tumor is isolated, and after processing tumor to a single cell suspension and limiting dilution, the specific amount of the cells was counted and injected subcutaneously into NOD-SCID mice [1]. This research has made one thing clear that there is a small population of cells responsible for the tumor regeneration, and the easier way to know them well is by tumor xenograft model. It's the best alternative to the marker-dependent CSC isolation. Limiting dilution assay is a labor-intensive process and time demanding, but the results are highly acceptable. As the smaller number of cells are being introduced to check tumorgenicity, the identity of the same can be known very well.

15.3 Controversies in Cancer Stem Cells

Discovery of the CSCs is a great finding that has provided a reason behind resistance to cancer therapy or cancer survival even after an enormous effort or ways to treat cancer, and tumor recurrence. This in turn also enabled us to identify new approach for cancer therapy, that is, to target the CSCs to totally eradicate the roots of the cancer. However the CSC hypothesis remains controversial; it is because of the divergence in defining cancer stem cells, reliance only on cell surface markers, and lack of standard functional assays [73]. CSCs are known by many different terminologies like cancer stem cells, tumor-initiating cells, or cancer stemlike cells. The CSCs and tumor-initiating cells cannot be considered as a same population of cells. This is because the tumor-initiating cells means that the isolated cell can generate tumor after implantation, whereas CSCs can repopulate the tumor as well as the original heterogeneity. Assays must be done to check the tumorigenicity as well as to demonstrate the cellular heterogeneity.

15.3.1 Relying on the Cell Surface Markers

As depicted in Table 15.1, there are multiple CSC markers for a single type of cancer based on different research group findings. CD133 marker is expressed in multiple tumor-like glioblastoma [42], ovarian cancer [20], prostate cancer [8], etc. One research group found that CD133 is not found to be a CSC marker for non-small cell lung cancer [74]. Whereas the other group found that CD133 shows the ability of a tumor-initiating cell by resistance to cisplatin in case of NSCLC [75]. Apart from this, the combination of markers shows tumorigenic properties rather than individual markers alone [75]. All these finding suggest that we do not have a universal marker for individual cancer type, and we must find the distinctive small population of cell lines on the top hierarchy. As different research groups utilizes various combination of cell surface markers to isolate CSCs. Since these subpopulations of CSCs in the heterogeneous tumor are very less, as reported in the case of pancreatic cancer, only

Table 15.1 List of cancer stem cell markers of various cancer types

Sr. no	Cancer type	Markers	References
01	Prostate cancer	$CD44^+/\alpha_2\beta_1^{hi}/CD133^+$	[8]
02	Colon cancer	$EpCAM^{hig}/ CD44^+/ CD166^+$	[9, 10]
		$CD26^+$	[11]
		$DCLK1^+$	[12, 13]
03	Pancreatic cancer	$CD44^+/CD24^+/ESA^+$	[14]
04	Breast cancer	$CD44^+/CD24^-/$ $lowLineage^-$	[15]
		$CD44^+/CD24^{-/low}/$ $ALDH^{high}$	[16]
		$Thy^+/CD24^+$	[17]
05	Lung cancer	$CD133^+$	[18]
06	Non-small cell lung cancer	$CD24^+/CD38^-$	[19]
07	Ovarian cancer	$CD133^+$	[20]
		$ALDH^+$	[21]
		$CD44^+/CD177^+$	[22]
08	Liver cancer	$CD133^+$	[23–25]
		$EpCAM^+/AFP$	[26]
		$CD13^+$	[27]
09	Gastric cancer	$CD44^+$	[28]
		$CD133^+/CD44^+/ CD24^+$	[29]
10	Melanoma cancer	$CD271^+$	[30]
		$CXCR6^+$	[31]
11	Acute myeloid leukemia (AML)	$CD34^+/CD38^-$	[2, 3]
12	Chronic myeloid leukemia (CML)	$CD34^+/CD38^-$	[32]
13	Acute lymphocytic leukemia (ALL)	$BCR/ABL^-/ALL^-$	[33]
14	Chronic lymphocytic leukemia (CLL)	$CD19^+/CD5^+$	[34]
15	Head and neck squamous cell carcinoma (HNSCC)	$ALDH^+/CD133^+/CD44^+$	[35]
16	Cervical cancer	ABCG2-positive	[36]
		$OCT3/4/BCRP/CD133^+$	[37]
		CD49f	[38]
		ALDH1	[39]
17	Renal cell carcinoma	$ALDH1/CD44^+/CD24^{-/}$	[40]
		$CD133^+/CXCR4^-$	[41]
18	Glioblastoma	$CD133^+/SSEA1^+$	[42]
19	Esophageal carcinoma	$\alpha6^{bri}/CD71^{dim}$	[43]
20	Bladder cancer	$EMA^-/CD44v6^+$	[44]

0.2–0.8% of cells show increased tumorigenic potential compared with non-tumorigenic cancer cells [14]. Also in the case of glioblastoma (GBM), $CD133^+$ is a putative CSC marker, but recently it has been challenged by other

groups. In the culture of $CD133^+$ CSCs, the $CD133^-$ population of cells are unable to form a tumor. But individually isolated $CD133^-$ shows tumorigenic potential which was not reported earlier [76]. The $CD133^+$ CSC culture only maintains a small set of primary glioblastomas. This means that $CD133^+$ cells are an early differentiated cell from the parental CSC, and we need to trace the first line of cells which give rise to the different progenies making a heterogeneous population of tumor. From this, we can assume that there might be another tumor-initiating cell that exists in the heterogeneous population of tumor cells which recapitulates the original tumor and maintains the heterogeneity. As we have advanced in the CSC isolation techniques from serial dilution to FACS, MACS, and transplantation to NOD/SCID mice, maybe soon we might be able to isolate and characterize CSCs with a universal marker for each cancer type.

15.3.2 Model for Tumor Heterogeneous Population

As we call tumor a heterogeneous population of cells, this plays a key role in the development and growth of the tumor. Currently, there are two models representing the heterogeneous population in the tumor, CSC model and the stochastic model. As depicted in Fig. 15.1, the CSC model suggests that the growth and progression of many cancers are driven by a small uncommon subpopulation of cells called CSCs. They mimic normal tissue development by working as stem cells in the normal tissues. Whereas the stochastic model predicts that the reaction of a cancer cell is random and influenced by the environment in which it is, i.e., intrinsic and/or extrinsic factors [77]. Interleukin 6 (IL6) can induce transformation of non-stem cancer cells (NSCCs) to form CSCs. CSCs are shown to have more amount of IL6 as compared to the NSCCs and therefore it is hypothesized that the non stem cancer cells having increased IL6 expression can instruct the NSCC to dedifferentiate to CSCs. This has been reported in breast and prostate cancer cell lines and also from the human breast tumors [78]. In the case of colon cancer, $CD133^+$ cells are potential cancer stem cell population from SW620 human colon cancer cells [79]. A recent study has demonstrated that with in situ immunofluorescence the division types of CSC from the non-stem cancer cell (NSCC) are observed. Results show that even non-stem cancer cells can differentiate into CSCs due to extrinsic factors like radiation in their study [80]. The CSCs which we are identifying may not be the universal CSCs for a particular cancer type, because of the plasticity of cell and their niche. Although both the models are based on theoretical and experimental studies and support the cancer therapy targeting CSCs along with the heterogeneous populations, it would be better if a combination of these two models is created which will provide clarity regarding the cell responsible for repopulating and maintaining the heterogenicity of the parent tumor.

15.3.3 The Problem in the NOD/SCID Mice System and FACS-Mediated Isolation of CSCs

The presence of CSCs in the heterogeneous population of cells in the tumor is very rare. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice are used as an in vivo model system to validate the tumorigenic ability of CSCs. Studies have indicated that only a rare 0.1–0.0001% of the heterogeneous population of cells in the human cancer cells can initiate a tumor, in diverse cancer types. Not all the NOD/SCID mice are equally immunocompromised, which can lead to variation in the result from one research group to another research group. There are no defined criteria for the compromised immunity of NOD/SCID mice, how much of their immunity is compromised is not mentioned while injecting the cells for tumor formation. In the case of acute myeloid leukemia (AML), it has been shown by Quintana et al. using higher immunocompromised NOD/SCID mice as a xenotransplantation system there is increase in the percentage of the tumorgenic cells in the tumor population. Their study focuses on injecting the isolated cells from tumor into two different mice one with higher immunocompromised and against the regular NOD/SCID mice and comparing the percentage of tumor-initiating cells in both the experiment. Results from that study suggested that the percentage of tumor-initiating cells increased in number by 25–27% by limiting dilution and single-cell transplantation in NOD/SCID mice [81–83]. In AML, it has been shown that $CD34^{+}/CD38^{-}$ cells have an ability to repopulate the tumor. Fluorescent-activated cell sorting uses the fluorescently conjugated antibodies for the cell surface marker, and based on the expression of the antigen, different fractions were collected and transplanted into NOD/SCID mice. In this study, $CD34^{+}/CD38^{-}$ cells were isolated from AML, the antibodies itself affected the survival of transplanted cells which is Fc-mediated, and this was overcome by treating the mice with immunosuppressive antibodies. Further when the inhibitory effect is prevented, most of the cells were found to be leukemia-initiating cells. This is another example to show the increase in the leukemia-initiating cells [84]. This finding was carried out on the same AML on which the CSC hypothesis is established, resulting in having multiple tumor-initiating cell phenotypes. So, it can be concluded that if the test system is not evenly immunocompromised from one laboratory to another, the data generated can be error-prone. Also, the antibodies we use to isolate the single cells from the population of cells must also be studied for its effects on the sorted cells and their ability to produce a tumor. It questions all the studies based on the CSCs in a different solid tumor, whether the cells isolated are really CSC or not, and further validation of the isolated cells needs to be done.

15.4 Conclusion and Future Perspectives

Multiple evidences and researches prove that only a small distinctive population of cells are capable of generating tumors and original heterogenicity in many different cancer types. Many methods are being developed to isolate and characterize the

CSCs from the mixed population of the cells. There are different types of cancer, and current research has found multiple combinations of the CSCs in a single type of cancer which is identified, verified, characterized, and published. But, can we rely on these data? Since multiple CSCs are identified for a single cancer type, how do we decide which CSC needs to be targeted for eradication of cancer from its root? It's been more than half a decade but still, there is no exact definition for the CSCs, and different researchers call it with different names like CSCs, tumor-initiating cells, etc. but there are no standard meaning or definition. CSC means the cell which can recapitulate the parental tumor and keeps original heterogeneity, whereas tumor-initiating cells are the cells which can form a tumor after transplanted into NOD/SCID mice. Recent publications raise questions regarding the existence of CSCs based on the experiment performed by John E. Dickand colleagues based on which the CSC theory is established. Research shows the standard assays to identify tumor-initiating cells fail to detect the exact population of cells which are responsible for tumor regeneration. A proper experimental system must be established to perform a solid functional analysis of CSCs isolated from the parental tumor. The in vivo xenograft assays must be refined for proper characterization of CSCs. Not only the isolation and identification of CSCs are important but also the understanding of the gene expression of the CSCs versus normal stem cancer cells is imperative. Unanticipated intrinsic or extrinsic factors also play an important role in the fate of the cells to be normal or cancer stem cells. Also, the stemness genes are the same that help the CSCs and normal stem cells to maintain their stem cell property. A better understanding of it helps in targeting the cells overexpressing those genes and not only relying on the cell surface markers. Further, we need to design a better model for tumor heterogenicity to make a clear understanding regarding the cell responsible for the heterogeneous population. We conclude that, although we have come a long way with the understanding of CSCs but with many assumptions has lead to the controversies like broad definition, limited assays and their standard of quality to determine the CSCs, relying mainly on the surface markers, etc. A standard definition and list of rigorous assays need to be made mandatory for the isolation and characterization of the CSCs and this needs to be followed by the research group aound the world to isolate, analyse and understand the CSCs. Analysing the cells by using surface markers, Side population analysis, intracellular markers, spheroid assays etc. alone will not provide a strong supporting data for a population of cells to be CSCs, because of the limitations in each of these methods, however combining these methods together could provide a strong supportive evidence. Therefore a guidlines listing a combinative approach to be made for the identification of CSCs is obsolutely essential. This will further reduce the possible controversies arising due to the limitations of different methods that are currently used by the research groups worlwide. The current strategies to understand CSCs is not bound to any rules and to get more clarity about these small subpopulation of cells a standard international guidelines must be made and followed. It's high time now to clear these black spots in the CSC theory and direct research in the aim of curing this dreaded disease and eliminating its existence from mankind for a better future.

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Targeting Therapies for Cancer Stem Cells

16

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Abstract

Cancer stem cells (CSCs) represent a small subpopulation of the bulk of a tumor. The CSCs possess the characteristics of self-renewal, clonal repopulation and resistance to conventional therapies, and thereby contribute to cancer metastasis and relapse. Moreover, CSCs establish homeostasis under stress via autophagy, endoplasmic reticulum (ER)-stress-mediated unfolded protein response (UPR) pathways, and mitophagy. Recent evidence indicate that besides many protein molecules, the noncoding RNAs (ncRNAs) also play a significant role in CSC growth and maintenance, as well as in cancer metastasis and therapeutic resistance. Therefore, targeting the CSCs has evolved as an important strategy for cancer therapy. Recent advancements in cancer immunotherapy has shown excellent application of its potential in targeting CSCs. Various immunotherapy approaches like immune checkpoint inhibitors, dendritic cell (DC)-based vaccines, adoptive T-cell therapy, oncolytic viruses, and combination therapies are currently used to target the CSCs. Also, recent multi-omic technologies can divulge exclusive CSC-associated cell surface markers, which can be used in detection or therapeutics of CSCs for various cancers. Additionally, detection of CSC-specific neoantigens can help in the design of new immunotherapeutics for cancers. Available literature suggests that many types of cancers have CSCs located in anatomically distinct niches within the tumor microenvironment (TME), which help in CSC's survival and maintenance. Unique pro-survival and anti-survival intercellular and intracellular cross talk also exists among the CSCs, its niche and/or TME. Modulating unique CSC-niche/TME interaction(s) can reduce the maintenance potential of CSCs, and thereby prevent tumor development and progression or cancer metastasis. Many important cell signaling pathways play a key role in the maintenance and regulation of CSCs. Several new potential therapeutic molecules that could specifically target the CSCs or their signaling pathways to overcome cancer metastasis, treatment-resistance or relapse, are being developed. Furthermore, the emerging clinical studies strongly support the use of drugs as a monotherapy or in combination with other available standard therapies. This chapter highlights the roles of various critical CSC markers and pathways in or around the CSCs, and the several CSC-targeting approaches or therapies that are used or being developed to treat cancer for a cure.

Keywords

Noncoding RNAs · Unfolded protein response (UPR) · Endoplasmic reticulum (ER) stress · Autophagy · Cancer stem cells (CSCs) · Immunotherapy · Dendritic cell (DC)-based vaccine · Adoptive immunotherapy · Oncolytic virotherapy · Combination chemotherapy · CSC cell surface markers · CSC-associated tumor microenvironment · CSC signaling pathway · Hedgehog · Notch · Wnt/β-catenin

16.1 Introduction

Cancer is a major public health problem and remains the second most common cause of death globally, despite the intense efforts in cancer research and rapid improvement of treatment strategies in the past decade. Resistance to cancer therapeutics and relapse are the most pertinent problems in cancer drug development. It is estimated that a total of 9.5 million people die every year worldwide [1], and in the USA alone, an estimated 606,880 people died of cancer in 2019 (https://seer.cancer.gov/statfacts/html/all.html). Tumor phenotypic heterogeneity along with the complex tumor microenvironment (TME) presents an exciting challenge in targeting therapy resistance. Tumor heterogeneity here refers to the existence of subpopulations of tumor cells, with distinct genotypes and phenotypes that may harbor different biological behaviors, within a primary tumor or after it metastasize and forms secondary tumors, i.e., between tumors of the same histopathological subtype (intra- and inter-tumor variability, respectively) [2]. It has been noted that tumor heterogeneity is, in part, controlled by a small population of cells called cancer stem cells (CSCs). The CSC model was first identified in leukemia, which proved the existence of a small population of cells capable of initiating leukemia, also generally called as tumor initiating cells (TICs) [3].

Generally, there are three types of normal stem cells that exist in mammals including human namely, embryonic stem cells, adult germinal and somatic stem cells. These normal stem cells can renew themselves, differentiate into multiple cell types or lineages, and maintain a balance between self-renewal and differentiation [4]. Like normal stem cells, CSCs also have the capacity to self-renew and differentiate into different types of cells. But, CSCs differ from normal stem cells by having the potential to induce tumorigenesis. So far, studies have proposed that CSCs form a subset of the tumor cells within TME and are ultimately responsible for tumor initiation, progression, and recurrence [5]. In addition, CSCs exhibit the properties of resistance to chemo/radiotherapy, cellular apoptotic pathways and immune evasion. CSCs can also potentially confer these properties to the cells that reside in their niche or TME or tumor. The knowledge acquired from CSC signaling pathways in recent years has enabled a better understanding of CSC biology and improved the strategies on therapeutic drug development. In the Chapter 2 of this book, “Types of Cancer Stem Cells” are discussed in more detail.

The discovery of CSCs brought a new perspective in the search for therapeutic targets to tackle cancer. CSCs are a rare subpopulation of the tumor cells, which possess self-renewal properties, and contribute to tumor heterogeneity and therapeutic resistance. Mounting experimental evidence suggests the existence of CSCs in several human cancers, suggesting a commonality among different tumor types, and therefore a potential therapeutic target for various cancer treatments [6, 7]. Heightened DNA-damage response, accumulation of drug efflux transporters, dysregulated apoptosis, cellular quiescence and self-renewal are the unique attributes of CSCs [8]. Therefore, a patient-specific combination therapy approach using CSC-specific targeting along with other available therapeutics to attack the bulk tumor, could improve the therapeutic outcomes in cancer patients [9].

CSCs achieve apoptotic resistance by activating the unfolded protein response (UPR) in response to extrinsic and intrinsic stress. A strong association exists between the upregulated UPR, autophagy and tumor phenotype in many cancers. Therefore, targeting the CSCs and tumor cells-specific-UPR and autophagy pathways can help to induce cellular apoptosis, and kill these cells to eliminate tumor cells [10]. Success in immunotherapy-based tumor targeting has provided renewed hopes in cancer therapy. CSCs exhibit resistance towards chemo/radiotherapy. Immunotherapy with two-pronged approaches of activating the body's immune system and suppressing the tumor cells-imparted immune (evasion) inhibitory effect can lead to egression of tumors and successful elimination of tumors [11]. Targeting CSCs is gaining huge attention among anticancer therapies because of its specificity and potentially very low adverse effects on normal cells [12, 13]. Different approaches to target CSCs such as dendritic cell (DC)-based vaccine, adoptive immunotherapy, oncolytic virotherapy and combination chemotherapy are discussed in this chapter.

CSCs possess normal stem cell-like characteristics and depend on the activation of stem cell-related signaling pathways. In this chapter, we will also focus on the most recent therapeutic strategies in development for targeting CSC's signaling pathways such as Hedgehog (Hh), Notch and Wnt/β-catenin (Fig. 16.1), and other pathways. In normal stem cells, these pathways are highly regulated, but show abnormal activation in CSCs, causing uncontrolled proliferation, dysregulated apoptosis and differentiation. The evolutionary uniqueness, underlying genetic signatures and associated networks create unique phenotypic signatures of CSCs. Harnessing, the strengths of genome-wide screening, proteomics and flow cytometry techniques can help to identify CSC-specific and unique cell surface marker profiles to distinguish CSCs from other tumor and normal cells [14]. A unique patient-specific cancer or CSC marker signature will contribute to a better understanding of the underlying regulatory mechanisms and more effective targeted therapies. Recent studies have suggested the existence of both protumor and antitumor signaling interactions between the CSCs and TME. The TME consists of several cell types and is a place for reciprocal interactions, leading to a favorable niche for the maintenance of the CSCs [15]. Targeting the CSC-associated niche and TME can curb the tumor proliferation and metastasis, as discussed in this chapter. Moreover, therapies targeting CSC's markers and signaling pathways or immunotherapies, and epigenetic modifiers that are used, or currently being explored in clinical settings are also dicussed in this chapter.

There are many options to treat cancers depending upon the stage or severity and may involve the use of single or combination treatments such as surgery, chemotherapy, radiation therapy, and targeted therapies. However, currently mono- or combination therapies targeting CSCs remain the most promising mode of cancer treatment. Therefore, this book chapter focuses on CSC-targeting strategies for cancer therapy.

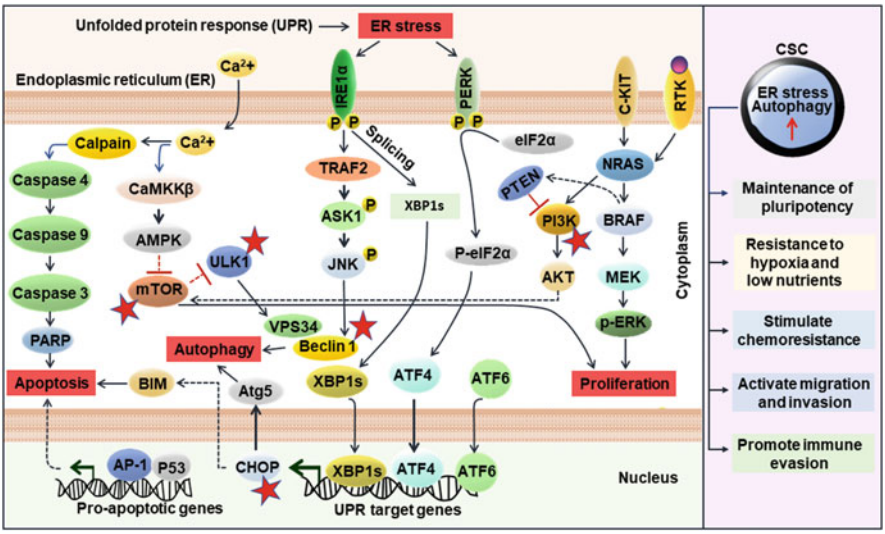

Fig. 16.1 Schematic representation of the molecular pathway linking endoplasmic reticulum (ER) stress response, autophagy, and apoptosis in cancer stem cells (CSCs). Accumulation of unfolded proteins triggers the unfolded protein response (UPR) and creates ER stress. UPR activation is initiated by the stimulation of stress sensors on the ER membrane. The important components include protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1 alpha (IRE1α), and activating transcription factor 6 (ATF6). IRE1 RNAse domain mediates mRNA splicing and activation of the functional transcription factor X-box binding protein 1 spliced form (XBP1). PERK inhibits global protein translation by phosphorylating eukaryotic initiation factor 2 alpha (eIF2α) and activating the translation of autophagy (ATG) related genes via activating transcription factor 4 (ATF4). ATF6 translocates from the ER to the Golgi, followed by protease-mediated cleavage and the release of the active form ATF6 basic leucine zipper domain (ATF6bZIP). After translocation into the nucleus, XBP1s, ATF4, and ATF6bZIP activate the translation of specific genes involved in ER stress regulation. Several other pathways also interact with the ER stress pathway and induce autophagy. A high-level or continuous UPR signaling can activate apoptosis. CSCs activate the autophagy pathway to gain survival advantages. Autophagy is also important for maintenance of CSC pluripotency, imparts protection against cellular stress, activates chemo- and immune resistance, regulates cell migration and invasion, and thereby influences metastasis

16.2 Targeting CSC-Associated Endoplasmic Reticulum Stress and Autophagy Pathways

The endoplasmic reticulum (ER) is a membrane-bound organelle and a site for protein folding, posttranslational modification of proteins, lipid synthesis, glycogen metabolism and calcium homeostasis. The ER homeostasis may get perturbed in some pathological state or stressful conditions, which can lead to an abundant accumulation of misfolded and/or unfolded proteins or vice versa. Then, adaptive signaling mechanisms, senses and detects these stress-inducing conditions or events,

and evokes the ER's "unfolded protein response" (UPR) stress pathways, leading to the restoration of ER homeostasis [16]. Different ER membrane sensors, including inositol-requiring enzyme 1 alpha (IRE1α), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6), are some of the key molecules that play a role in sensing the stress and activating the UPR mechanisms, and they help to restore and maintain ER homeostasis (Fig. 16.1) [17]. IRE1α, PERK and other receptor tyrosine kinases that are located in the ER membrane, intiate the autophosphorylation cascade and downstream signaling pathways as shown in Fig. 16.1. Autophagy, an evolutionarily conserved process of lysosomal degradation and recycling of defective macromolecules, and pernicious organelles, is tightly regulated by the UPR pathway. Autophagy is a complex multistep process involving cargo recognition, packaging, vesicle nucleation, sequestration and complete formation of the autophagosome, and finally fusion with the lysosomes [18, 19]. IRE1α phosphorylates mitogen-activated protein kinase 8 (MAPK8), which then interacts with c-Jun N-terminal kinase (JNK) and triggers the downstream effectors followed by autophagy. During ER stress response, JNK indirectly activates the Beclin 1, a coiled-coil myosin-like BCL2-interacting protein-mediated autophagy pathway. ER stress can also stimulate calcium release from the ER, followed by calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) phosphorylation and activation of AMP-activated protein kinase (AMPK). The AMPK removes the mammalian target of rapamycin (mTOR)-induced inactivation of the unc-51 like autophagy activating kinase 1 (ULK1) complex, and thereby activates autophagy. ER stress-associated calcium release can also activate death-associated protein kinase (DAPK)-Beclin 1 pathway for autophagy induction. ER stress also induces another arm of UPR response via PERK-mediated autophagy [20]. PERK-eukaryotic initiation factor 2 alpha (eIF2α)-ATF4 pathway activates CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP)-mediated autophagy. An overview of the ER stress signaling and its association with apoptosis and autophagy is shown in Fig. 16.1.

Tumor cells and CSCs are subjected to extrinsic and intrinsic stress, and they activate the UPR pathway as a survival strategy. A strong association exists between the upregulated UPR and cancer progression in several tumor types. CSCs activate autophagy to achieve a significant pro-tumorigenic advantages in many cancer types. Activated ER stress and autophagy in CSCs provide several pro-tumorigenic advantages including self-renewal, resistance to stress from adverse environmental conditions such as hypoxia or low nutrients, resistance to drugs during therapy, modulation of anticancer immunity, and activation of migration and invasion properties (Fig. 16.1). Hence, targeting the CSC's UPR and autophagy pathways, can sensitize CSCs to apoptosis and thereby help to strategize cancer treatment therapies [10, 21, 22].

$ALDH^+$ CSCs from mammary ductal carcinomas are dependent on Beclin 1 autophagic flux for survival and tumorigenesis [23, 24]. Autophagic flux imparts survival advantage, plasticity and stemness in $CD44^+CD24^-$ breast CSCs [25]. shRNA screens have also helped to confirm the role of autophagy in the maintenance of breast CSCs and revealed autophagy-related protein 4 homolog A

(ATG4A), a cysteine peptidase and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathways as important mediators [26–28]. Autophagy inhibition, through targeted deletion of the FIP200 component of the $CD29^{hi}CD61^{+}$ mouse mammary CSCs in MMTV-PyMT and MMTV-Wnt1 transgenic mice, showed strong autophagic dependence [29, 30]. ER stress and other stresses [hypoxia and transforming growth factor-beta (TGF-β)] are prevalent in the TME and induce both EMT and autophagy gene expression in CSCs. EMT induction also promotes a CSC phenotype in breast cancer [31–33]. Other CSCs also exhibit autophagic dependence, including those from human hepatocellular carcinoma [34, 35], pancreatic cancer [36, 37], bladder cancer [38], colorectal cancer [36, 39], chronic myeloid leukemia [40], and glioblastoma [41]. Hypoxia-induced autophagy activates immune resistance from T cell cytotoxicity [42]. Thus, UPR and autophagy pathways have become an exciting target for therapeutic exploration to eliminate CSCs.

Chloroquine (CQ) and hydroxychloroquine (HCQ) are the main autophagy inhibitors that have been tried and tested in the clinic [10, 43, 44]. Small-molecule inhibitors targeting the catalytic components of the autophagosome biogenesis pathway (ULK1, VPS34, and ATG4B), Phosphoinositide 3-kinase (PI3K)/AKT/mTOR and the mitophagy pathway are also being evaluated for their antitumor properties. The important druggable targets include mTOR, ULK1, VPS34, Beclin 1, ATG7, and ATG4. Lys05 is a dimeric chloroquine that has been reported to be significantly more potent than HCQ and is under preclinical trials [45, 46]. A quinacrine derivative (DQ661) showed a robust anticancer effect in several cancer types [47]. Several clinical trials are underway exploring the synergistic cytotoxicity of anticancer drugs with autophagy inhibitor CQ and HCQ on different cancer types [10, 43]. Further investigation to better understand the tissue and cancer-specific role of the UPR, autophagy flux, and apoptosis is needed to facilitate the design of better pharmacological modulators for targeting CSCs.

16.3 Cancer Stem Cell (CSC)-Specific Immunotherapy

The immune system combats the initiation and progression of cancer, and it's failure leads to tumor progression. CSCs exhibit resistance toward small-molecule inhibitors and thereby limit the therapeutic response. Recent advances in immune oncology have raised hopes for targeting CSCs using immunotherapy-based approaches. Currently, immunotherapy is aimed not only at potentiating and harnessing the strength of the immune system but also to suppress the immune inhibitory effect imparted by the tumor cells. Recent evidence has clearly shown that inhibiting tumor cells' immunosuppressive effect can lead to the successful elimination of the tumor. CSCs, by virtue of their inherent properties like self-renewal, low abundance, and chemo- and radiotherapy resistance, present a significant therapeutic challenge. Preclinical and clinical efforts are underway to harness the immunotherapy-based strategies to successfully eliminate CSCs and induce tumor

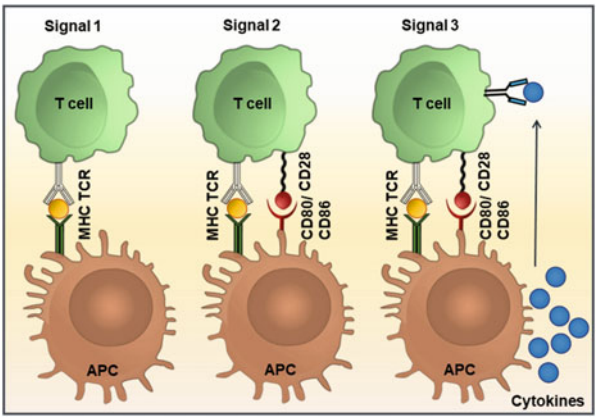

Fig. 16.2 Immune synapse between antigen-presenting cells (APCs) and T cells. Signal 1 involves the presentation of an antigenic peptide by MHC II molecule and its identification by the antigen-specific T cell receptor (TCR). Signal 2 corresponds to the synapse stabilization by co-stimulatory molecules, leading to an activation signal. Interaction with cytotoxic T lymphocyte-associated protein 4 (CTLA4), on the other hand, produces inhibitory signals (not shown). Signal 3 helps to generate a T cell effector phenotype and is dependent on cytokine secretion by APCs

regression [11]. Different immunotherapy approaches to target CSCs are elaborated under specific subtitles.

16.3.1 Dendritic Cell (DC)-Based Vaccines for CSCs

Alterations in the genomic DNA or mutations can cause changes in protein sequence and function contributing to tumorigenesis. The patient's human leukocyte antigen (HLA) molecules can present the novel tumor-specific peptides or neoantigens to the immune cells and thereby elicit an immune response. Innovative immunogenomic techniques, including whole genome or exome sequencing technologies, HLA haplotyping, HLA binding computational predictions, T-cell receptor (TCR) sequencing, and immunophenotyping, have collectively paved the path to elucidate the tumor-specific mutant antigens or neoantigens. The complete understanding of the neoantigens' repertoire can help design cancer immunotherapies for specific tumor types [48, 49]. Dendritic cells (DCs) are the most efficient antigen-presenting cells (APCs). DCs can conventionally present (exogenous antigens as MHC II-associated peptides to $CD4^+$ T cells; endogenous antigens as MHC I-associated peptide to $CD8^+$ T cells) as well as cross-present (exogenous antigens as MHC I-associated peptides) and thereby accomplish more efficient T cell activation. T cell activation is not only dependent on a cognate antigen (signal 1) but also on co-stimulatory signals present on the APC cell surface (signal 2; e.g., CD86, CD40, CD80) and cytokine signaling (signal 3), Fig. 16.2. An immunosuppressive

microenvironment is promoted by most tumor cells to limit immune efficacy. DCs provide a robust platform for integrating all relevant signals required for T cell priming with appropriate cancer antigens, leading to a strong antitumoral immune response [50, 51]. Autologous DC-based vaccines to activate in situ T cell mediated antitumor response and concomitant reversal of the immune suppression might lead to successful combinatorial immunotherapies in the future.

Several pioneering discoveries that showed DCs as highly specialized APCs, DC-mediated T cell priming, and the role of cancer antigen in cancer immunotherapy led to the concept of the DC vaccine. The first-generation DC vaccines relied mostly on patient-isolated DCs or ex vivo differentiated monocyte-derived immature or semi-immature DCs (mo-DCs). These DCs were then primed with tumor cell lysate and synthetic antigenic peptides and achieved limited success against certain tumor types (melanoma, non-Hodgkin's lymphoma). The second-generation DC vaccines relied on fully mature mo-DCs primed with recombinant antigenic peptides and irradiated tumor cell lysates. Results from several clinical trials suggest greater clinical efficacy. The next-generation DC vaccines focus on a specific subset of patient-derived DCs (e.g., BDCA1/CD1c^{+} myeloid DCs and BDCA3/CD141^{+} myeloid DCs) for efficient memory T cell generation and immune response. Table 16.1 shows the recent advances in targeting CSCs by immunotherapy regimens that are a part of clinical trials. Patients' peripheral blood mononuclear cells (PBMCs) can be differentiated into DCs and sensitized with cancer cell lines or CSC lysate or patient tumor lysate and can be used as vaccines to potentiate the antitumoral immune response (Fig. 16.3) [49]. The efficacy of DC-based vaccines is evident in multiple cancers. Ning et al. [52] demonstrated the cell-killing efficacy of ALDH^{hi} CSC-pulsed DCs in an immunocompetent murine model using D5 melanoma and SCC7 squamous cell cancer [52]. DCs charged with pancreatic CSC lysate (enriched from Panc-1 sphere cultured cells) induced enhanced immune killing and robust antitumor cytokine production [53]. ALDH^{hi} CSC-pulsed DCs inhibited pulmonary metastasis of primary tumor melanoma and squamous cell carcinoma and induced B cell priming toward CSCs [54]. A DC-based vaccine against CSCs in mouse malignant melanoma (B16F10 lysate) showed a decrease of metastatic tumor burden in mice model [55]. The efficacy of DC primed with CSC lysate on tumor burden is also evident in other studies [56, 57]. CSCs from pancreatic and lung cancer cell lines pulsed with DCs can lead to successful T cell stimulation [58]. Another approach includes the fusion of DCs with tumor cells, which lead to the DC/hepatocellular carcinoma stem cell vaccine that provides the potential to generate polyclonal immune responses and can provide a unique method to target CSCs [59]. Examples of clinical trials based on DC-based vaccines and cancer immunotherapies aimed at targeting CSCs are shown in Table 16.1.

Targeting the CSCs by DC immunotherapy can be challenging as CSCs express low MHC I molecules and other innate immune receptors and are resistant to immune effector cells. Concomitant overexpression of pluripotency markers renders the CSCs immune resistant. Moreover, cytokines and chemokines released by the stromal cell population and some immune cells suppress antitumor immunity.

Table 16.1 Clinical trials of immunotherapies that targeted CSCs

Study title	Conditions	Interventions	NCT number	Study period	Locations
Dendritic cell (DC)-based cancer immunotherapy against CSCs					
DC immunotherapy against cancer stem cells in glioblastoma patients receiving standard therapy	Glioblastoma	Biological: DC immunization Drug: adjuvant temozolomide	NCT03548571	Apr 2018 to May 2023	Oslo University Hospital
Phase 1 study of a DC vaccine for patients with either newly diagnosed or recurrent glioblastoma	Glioblastoma Glioblastoma multiforme Glioma Astrocytoma Brain tumor	Biological: DC vaccination, in addition to standard temozolomide chemotherapy and involved field radiation therapy Biological: DC vaccination, with optional bevacizumab treatment for patients previously treated with bevacizumab	NCT02010606	Jan 2014 to Apr 2021	Cedars-Sinai Medical Center
Safety study of DC-based therapy targeting tumor stem cells in glioblastoma	Glioblastoma Brain tumor	Biological: DC vaccine with mRNA from tumor stem cells	NCT00846456	Jan 2009 to Feb 2013	Oslo University Hospital
Vaccine therapy in treating lung cancer patients with cancer stem cells	Lung	Biological: cancer stem cell vaccine	NCT02084823	Mar 2014 to Dec 2014	Biological treatment center in Fuda Cancer Hospital

A phase 1/2 study of active immunotherapy with cancer stem cell vaccine for colorectal cancer	Colorectal	Biological: cancer stem cell vaccine	NCT02176746	Jun 2014 to May 2015	Biological treatment center in Fuda Cancer Hospital
Vaccination with DC loaded with brain tumor stem cells for progressive malignant brain tumor	Brain tumor Glioblastoma Medulloblastoma Ependymoma Anaplastic astrocytoma	Biological: dendritic cells Drug: imiquimod	NCT01171469	Sep 2010 to Jun 2012	Masonic Cancer Center
Vaccine therapy in treating patients undergoing surgery for recurrent glioblastoma multiforme	Recurrent Central Nervous System Neoplasm	Biological: brain tumor stem cells mRNA-loaded DCs	NCT00890032	Sep 2009 to Feb 2016	Duke University Medical Center
Study of DC vaccination against glioblastoma	Glioma Glioblastoma multiforme Neoplasms	Procedure: surgery Drug: chemotherapy Radiation: radiotherapy Biological: DC vaccination	NCT01567202	Mar 2012 to Feb 2020	Huashan Hospital, Fudan University
Adoptive T-cell transfer (ACT)-based cancer immunotherapy against CSCs					
A clinical research of CAR T cells targeting EpCAM-positive cancer	Colon cancer, Esophageal carcinoma, Pancreatic cancer, Prostate cancer, Gastric cancer, Hepatic carcinoma	Biological: CAR T-cell immunotherapy	NCT03013712	Jan 2017 to Dec 2020	IEC of Chengdu Medical College

Examples of dendritic cell (DC) and adoptive T-cell therapy (ACT)-based cancer treatment clinical trials conducted in patients with different types of cancers are represented

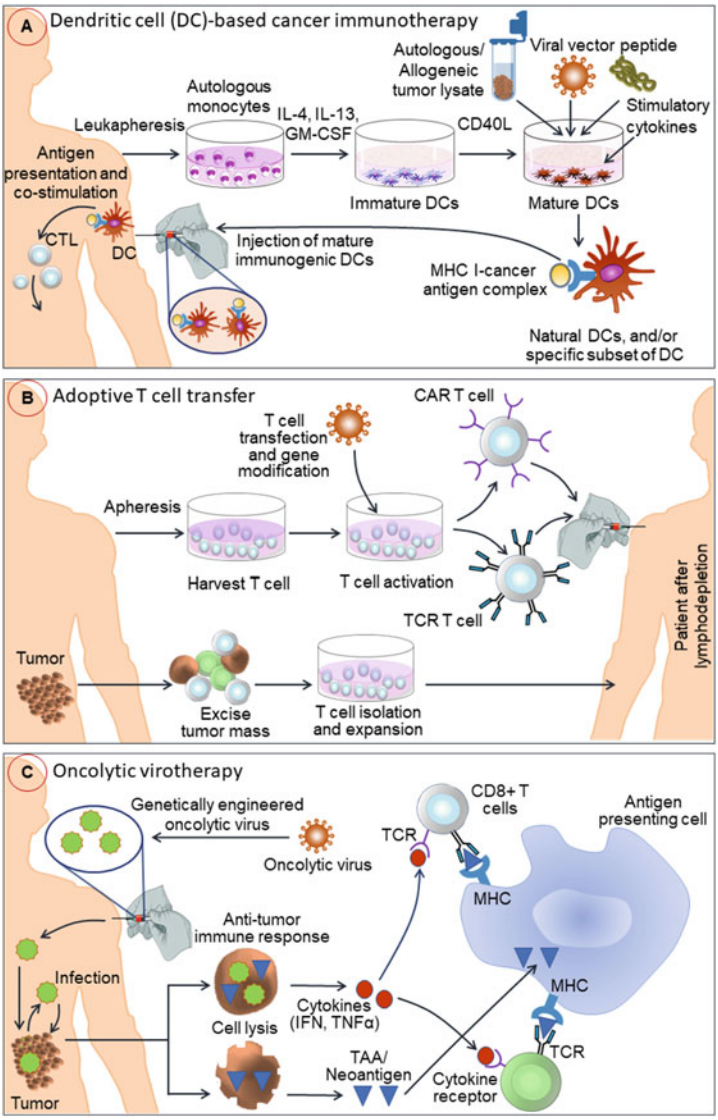

Fig. 16.3 Cancer stem cell (CSC)-specific immunotherapy. (**a**) Dendritic cell (DC)-based cancer immunotherapy: Autologous $CD14^{+}$ monocytes are isolated through the process of leukapheresis from the peripheral blood of a cancer patient. The monocytes are then differentiated in the presence of GM-CSF, IL-4, and IL-13 to produce immature DCs, which are then primed with tumor-associated antigen (TAA)/CSC antigen and finally subjected to maturation cocktail to obtain mature DCs. The maturation process is followed by the injection of mature immunogenic DCs back to the patient. The immunogenic DCs facilitate antigen presentation and co-stimulation. *GM-CSF* granulocyte-macrophage colony-stimulating factor, *IL-4* interleukin 4, and *IL-13* interleukin 13 (**b**) Adoptive T cell therapy (ACT): T cells are isolated from the patient's peripheral blood via leukapheresis and then subjected to genetic modification using a retroviral or lentiviral vector to express a specific artificial chimeric antigen receptor (CAR). CARs comprise of an extracellular antigen recognition domain, which is then fused with an intercellular T cell signaling domain and also merged with co-stimulatory domains. CAR T cells are then administered to the patient. For

Potential new strategies like adoptive T cell therapy may be useful in tackling immune resistant CSCs.

16.3.2 Adoptive Immunotherapy for CSCs

Passive immunization or popularly referred to as adoptive T cell therapy (ACT) represents the extraction of T cells from tumor-infiltrating lymphocytes (TILs) or antigen-specific T cells, followed by ex vivo genetic modification and culture and the subsequent autologous transfer of the T cells to the cancer patient. ACT is achieved based on two strategies. One approach is the genetic engineering of autologous T cells to overexpress T cell receptors (TCRs) that can identify tumor-associated antigens (TAA) by viral transduction. Genetic engineering of autologous T cells to express the chimeric antigen receptors (CARs) is the other approach. CARs combine multiple signaling domains, including the single-chain variable fragments (scFv), antigen binding domain to an intracellular T cell signaling domain, and one or two co-stimulatory domains (e.g., CD3 ζ-CD28-41BB, CD3 ζ-CD28-OX40). CAR T cell therapy is an innovative and promising immunotherapy approach as it leads to T cell activation and is non-MHC restricted.

CSCs play a vital role in tumorigenesis and subsequent metastasis; hence the adoptive transfer of CAR T cells may lead to the elimination of CSCs and provide long-term disease-free survival or cure. The use of ACT targeting CSCs is limited, but scientists are exploring the possibility. Tettamanti et al. [60] transduced the adoptive transfer of cytokine-induced killer cells to target leukemia progenitors and leukemia stem cells by targeting IL3RA or CD123. A strong response against $CD123^+$ cell lines and AML blast cells and a concomitant minimal killing of normal monocyte and CD123-low expressing cells were observed [60]. Deng et al. [61] used CAR T cells specific for CSC-specific marker EpCAM and showed significant response on PC3M tumor cells both in vitro and in vivo [61]. CAR T cells engineered to coexpress CAR with a membrane-bound chimeric interleukin (IL)-15 (mbIL15) promoted a stem cell memory T cell subset in $CD19^+$ leukemia [62]. Though colorectal cancer exhibits a low CSC number, Miyamoto et al. [63] using an HLA ligandome analysis identified ASB4 as a colorectal CSC-specific marker. The ASB4 peptide as an epitope primed a potent $CD8^+$ cytotoxic T cell (CTL) response and demonstrated significant elimination of the CSCs [63].

Adoptive transfer of natural killer (NK) cells has also shown significant success and is a potent alternative adoptive transfer strategy. Interestingly, NK cells show a

Fig. 16.3 (continued) T cell receptor (TCR) therapy, conventional TCR is inserted into a patient's T cells, followed by T cell expansion, and then injected to the patient. TCRs are composed of an α chain and a β chain and can recognize antigens only when presented on an MHC molecule. Alternatively, tumor-specific T-cells can be isolated from the tumor of a patient, expanded and injected into the patient. (**c**) Oncolytic virotherapy (OVT): OVT involves infection of a tumor with a genetically modified oncolytic virus to induce tumor cell killing and activation of local inflammation, followed by immune infiltration. *CTL* cytotoxic T lymphocyte, *TAA* tumor-associated antigen, *MHC* major histocompatibility complex, *TNF-α* tumor necrosis factor-alpha, and *IFN* interferon

preferential targeting of the CSC phenotype and thereby highlight the possibility of the use of NK-based immunotherapy [64]. Multiple exciting studies have shown the potency of NK cell-based adoptive therapy in CD54/ICAM-1 in breast cancer [65] and IL-2- and IL-15-mediated activation of NK targeting of chemoresistant bladder cancer stem-like cells [66]. Though several laboratories worldwide are exploring the benefits of adoptive immunotherapy, very few studies have reached the clinical trial level, suggesting an unexplored area that is worth to investigate (Table 16.1).

16.3.3 Oncolytic Virotherapy (OVT) for CSCs

Viruses can infect healthy cells and cause diseases. Certain viruses are known to target cancer cells specifically and are called oncolytic viruses. Oncolytic viruses can kill cancer cells by multiple approaches, first by infecting, followed by intracellular replication, and finally destroy by inducing lysis of the cancer cells [67]. The second method involves the production of cancer antigens when the cancer cells die, subsequent uptake of the cancer antigens by immune cells, and T-cell activation leading to tumor cell killing [68]. Viruses can also hijack the cancer cell's apoptotic machinery and induce cell killing. Various groups have demonstrated the therapeutic efficacy of OVT on multiple cancers and CSCs [12]. Cancer cells show susceptibility toward specific viral infections because of defective interferon pathways (e.g., myxoma virus, raccoon poxvirus, vesicular stomatitis virus) [69]. Cancer cells also exhibit expression of specific viral receptors leading to enhanced intracellular uptake of certain viruses (e.g., adenovirus, poliovirus, measles virus, etc.) [70]. Advances in the genetic engineering of viruses, success in the clinical trials of oncolytic viruses, and a better understanding of immunotherapy led to the progress of virotherapy from the laboratory to the clinic. Genetically modified oncolytic adenovirus armed with apoptosis-inducing gene tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and using human telomerase reverse transcriptase (hTERT) promoter for driving the viral E1A gene leads to significant cytotoxicity of radioresistant esophageal cancer stem-like cells [71]. Cheema et al. [72] engineered and armed the oncolytic herpes simplex virus with the immunomodulatory cytokine interleukin 12 (G47-mIL12). G47-mIL12 showed significant therapeutic benefits in a CSC model of glioblastoma [72]. The genetically modified oncolytic adenovirus (using hTERT promoter for driving the viral gene E1A) on gastric cancer stem cell lines (MKN45 and MKN7) showed enhanced cytotoxicity toward gastric CSCs [73]. Oncolytic adenovirus targeting CD133 exhibited an antitumor effect on colorectal cancer and corresponding CSCs. The application of oncolytic viruses has also been used on breast cancer [75, 76], ovarian cancer [77], and colon CSCs [78]. Many different oncolytic viruses are being tried and tested in clinical trials. T-Vec is the first to receive US Food and Drug Administration (FDA) approval for the treatment of metastatic melanoma, suggesting the need for further investigations in the field of OVT.

16.4 Targeting CSCs via Combination Chemotherapy

Immune checkpoint inhibitors and monoclonal antibodies have proven effective in clinical trials, but only a small fraction of patients benefit from them. Zhang et al. [79] investigated the combinatorial effect of CSC-DC vaccine and with a dual blockade of programmed death ligand 1 (PDL1) and cytotoxic T-lymphocyte-associated protein (CTLA-4) on CSC targeting using B16-F10 murine melanoma tumor model. The effect of combination therapy in less immunogenic tumors raises hope. Saha et al. [80] used a immunovirotherapy (G47Δ-mIL12) in combination with immune checkpoint blockade (CTLA-4, PD-1, PD-L1) and obtained a synergistic therapeutic response to eradicate glioblastoma. Combination chemotherapy approaches can be more efficient in combating the multiple immune escape mechanisms of the CSCs.

16.5 Targeting CSC-Associated Cell Surface Markers

Rudolf Virchow and Julius Cohnheim proposed similarities between fetal tissues and cancers more than 150 years ago [13]. Experiments on acute myelogenous leukemia by John E. Dick's laboratory first demonstrated a rare leukemia-initiating cells in mice [81]. Since this pioneering finding, many other laboratories have identified CSCs in other cancer types as well [13]. In an interesting study, Gerlinger et al. [82] analyzed nine different areas of a renal tumor including three metastatic tumors from the same patient. The data from this study revealed intra-tumoral heterogeneity and nonlinear evolution of cancer, resembling that of branched normal stem cell lineage specification. This study not only highlighted the heterogeneity but also suggested that a small population of the cells possessed the primary evolutionary stem cell-like properties [82]. CSCs constitute a subpopulation of cancer cells that are difficult to target using chemotherapy and radiation therapy. Increasing evidence suggests that CSCs lead to tumor recurrence and metastasis after cancer therapy [6]. Understanding the origin, source, regulatory mechanisms, and the process of malignant progression of CSCs has received significant attention from scientists [12]. Distinguishing CSCs from other tumor cells depends on the use of antibodies that detect cell surface markers that are sometimes nonexclusively expressed on CSCs. The underlying networks of the transcription factors that create the CSC-specific phenotyping identity may lead to a unique cell surface marker pattern relevant for targeting CSCs. Rapid application of genome-wide screening, proteomics, and cell sorting techniques have helped elucidated the CSC-specific cell surface markers in tumors from different organs. The fundamental evolution of CSCs and a list of frequently used CSC markers are presented in Fig. 16.4 [8, 9, 83–86]. In general, the CSC surface markers show commonality to that expressed on human embryonic stem cells (hESCs), but are rarely expressed in normal tissue. The Human Protein Atlas (http://www.proteinatlas.org) is an excellent source of histological data for different cell surface markers. The discovery of the CSC-specific cell surface markers not only paved the way to study the basic mechanisms governing

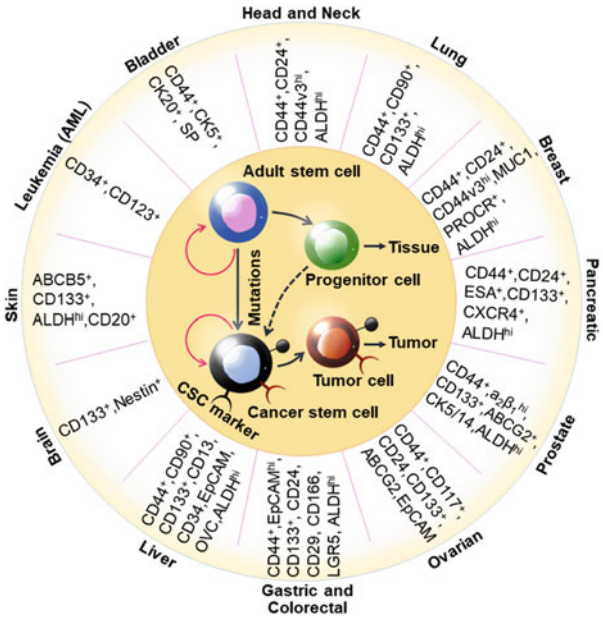

Fig. 16.4 Cancer stem cell markers. The central area shows the origin of CSCs. The outer area above the central area shows CSC-specific cell markers in tumors from different organs/tissues

cancer cell stemness but also to specifically target these resistant populations. Harnessing the strength of specificity provided by cell surface markers, several targeting approaches have been explored including antibody-based targeting, targeted chemotherapy, targeted radiation therapy, and immunotherapy.

An interesting example of CSC surface molecule based-targeting is CD133, a membrane-bound glycoprotein and one of the most frequently investigated CSC markers (Fig. 16.4) [87]. CD133 is the choice for targeting CSCs in many tumors. Cytolethal distending toxin (Cdt)A$^{C149A,\ C178A}$BC-CD133 MAb, a more specific anti-CD133 antibody conjugated with Cdt toxin, showed specific inhibition of oral cancer cell proliferation [88]. dCD133KDEL is a deimmunized pseudomonas exotoxin fused to anti-CD133 scFv fragment that has shown promising results in ovarian cancer model in vivo [89], melanoma cells [90], and breast cancer cells [91]. To increase the specificity of targeting bispecific humanized EpCAM/CD133, targeted toxin (dEpCAMCD133KDEL) was synthesized using DNA shuffling and ligation technique. dEpCAMCD133KDEL showed high selectivity and tumor regression in an in vivo model (using UMSCC-11B cells) of head and neck squamous cell carcinoma [92]. Another approach with a bispecific antibody is the use of anti-CD3/anti-CD133, which led to a decrease of CD133$^+$ pancreatic (SW1990) and hepatic (Hep3B) tumor cell growth in nude mice [93]. CD133-targeted nanoparticles or CD133NPs [poly(D,L lactide-co-glycolide) polymer loaded with paclitaxel] reduced MDA-MB-231 xenograft growth and decrease of the CD133$^+$ population [94]. A bispecific anti-CD16/anti-CD133 antibody has been developed to engage NK cells and CD133-positive CSCs (BiKEs). BiKEs lead to enhanced NK cell engagement and activity against CD133$^+$ colorectal cancer cells [95]. To improve

CSC targeting trispecific CD133EpCAMCD16 antibody tagged with a toxin (TriKE) shows three ligand-specific bindings and increased cell killing of the colorectal cancer cell line (Caco-2) [96]. Immunotherapy-based approaches (ICT-121 DC vaccine) are used for targeting $CD133^+$ glioblastoma cells. ICT-121 DC vaccine was successfully tested in a phase 1 trial, and results suggest that ICT-121 is both safe and well tolerated with specific immune response observed in a subset of patients (https://clinicaltrials.gov/ct2/show/NCT02049489). Several preliminary results suggest the possibility of the use of $CD133^+$ targeted approach in cancer therapeutics, new investigations, and stringent validation will aid the potential of better targeting the CSCs in the future.

Another exciting example is CD44, a transmembrane glycoprotein that functions as a hyaluronic acid receptor with many isoforms and is prominent on many CSCs (Fig. 16.4). CD44 is ubiquitously expressed in many healthy cells, but its differential splice variants (CD44v) generated by alternate splicing can be unique in different tumors and help target the CSCs [97]. CD44 variant CD44v6 is expressed in non-small cell lung cancer, pancreatic cancer, and gastric carcinoma [98–100], while CD44v9 is expressed in gastric cancer [101] and colorectal cancer [102], and CD44v8–10 is expressed in gastric CSCs [103]. Considering the expression pattern of CD44 on CSCs and its function in regulating cancer cell proliferation, metabolic shifting, and invasion, many targeting approaches have been formulated, including the anti-CD44 monoclonal antibody approach. In vivo administration of monoclonal antibody specific to the CD44 molecule, which shows efficient eradication of acute myeloid leukemia (AML) in SCID mice [104], is one approach to target $CD44^+$ tumor cells. Similar reports were also published for BCR-ABL expressing leukemia [105]. Verel et al. [106] designed chimeric antibodies (BIWA-1, BIWA-2, BIWA-4, and BIWA-8) and labeled with rhenium 186 (^{186}Re) isotope leading to a higher degree of specification and efficacy. Further experiments with BIWA Abs conjugated with isotope Tc-99 and cytotoxic drug mertansine showed promising results with head and neck squamous cell carcinoma in phase 1 clinical trials [107, 108].

Several nanotechnology-based platforms have also been explored to achieve CD44 targeting for cancer therapy. An anti-CD44 monoclonal antibody loaded with chitosan nanoparticles coated with polylactic acid was effective against human ovarian cancer cells in vivo [109]. A new approach to tag anti-CD44 antibody with modified superparamagnetic iron oxide nanoparticles (SPIONPs) and using alternating magnetic field treatment led to magnetic hyperthermia and significant tumor reduction in human oral squamous cell carcinoma xenograft model in nude mice [110]. Chen et al. [111] developed anti-CD44 and anti-CD133 antibody conjugated all-trans retinoic acid-loaded poly-lecithin-PEG nanoparticles (CD44/CD133–ATRA-PLPN) to target gastric CSCs and showed encouraging results. These nanoparticles provide a platform for targeting multiple CSCs populations [111]. Appropriately functionalized carbon nanotubes (CNTs), including single-walled CNTs (SWCNT) and multiwalled CNTs (MWCNT), are being used as nano-carriers for anticancer drugs and noninvasive imaging [112]. MWCNTs functionalized with hyaluronic acid (a ligand for CD44 receptors) and α-tocopherol

succinate and loaded with anticancer drug doxorubicin (α-TOS-HA-MWCNT-DOX conjugate) were used to target the CD44 receptors overexpressed in MDA-MB-231 triple-negative breast cancer cells [113]. In an another recent study, Gautam et al. [114] used a similar CD44 receptor targeting approach and used hyaluronic acid-modified PEGylated DOX-STS loaded phyto-liposome, which showed an increased antitumor effect in the MDA-MB-231 xenograft tumor model.

Interestingly, nanoparticle systems are also designed to promote an antigen-induced immune response in CD44 receptor overexpressed cells. Hyaluronic acid was also modified into the outer shell of the 3s-PLGA-PEG nanoparticles to improve immune cell uptake. 3s-PLGA-PO-PEG/HA nanoparticles (PHO NPs) were targeted to $CD44^+$ cells, and in vivo experiments resulted in avid T cell response accompanied by modest stimulation of memory T cells [115]. Several other studies have elaborated different therapeutic strategies including neutralizing antibodies, peptide mimetics, aptamers, HA-directed nanoparticles, and CAR T cell-based targeting of $CD44^+$ cancer cells and are in various stages of development.

Other CSC-specific cell surface markers have also been used for CSC targeting. Therefore, total profiling of the CSC surface markers can help differentiate CSCs from normal tissue stem cells and better understand their physiological and functional properties in terms of tumor progression and provide therapeutic benefit to the patient.

16.6 Targeting CSC-Associated Tumor Microenvironment and Metastasis

The tumor microenvironment (TME) contains several cell types, as well as their derived soluble factors (e.g., cytokines and chemokines). TME is constituted by various kinds of cells, including mesenchymal stem cells (MSCs), cancer-associated fibroblasts (CAFs), endothelial cells, immune cells, fibroblasts, tumor cells, adipocytes, etc., which utilize reciprocal interactions and influence CSC properties and functions (Fig. 16.5). Tumor cells and CSCs modulate the microenvironment via various signaling cues in the form of cell-cell contact and secreted factors [e.g., IL-6, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF)], to alter the properties of the stromal cells into a favorable support system for the maintenance of the CSCs. Like the normal stem cells, CSCs also reside in specific niches. CSC niche is a particular microenvironment, in which the CSCs can maintain their principal properties like self-renewal, ability to form a clonal tumor, long-term repopulation potential, plasticity, and potential to evade cell death and metastasize [116]. The CSC niche is an integral component of the TME and collectively considered as the adjacent stroma along with the tumor cells [117]. Understanding the spatiotemporal dynamics of TME pattern formation, maintenance of CSC functional heterogeneity, reciprocal cross talk, and immune suppression by the component cells can help target the TME and CSC niche.

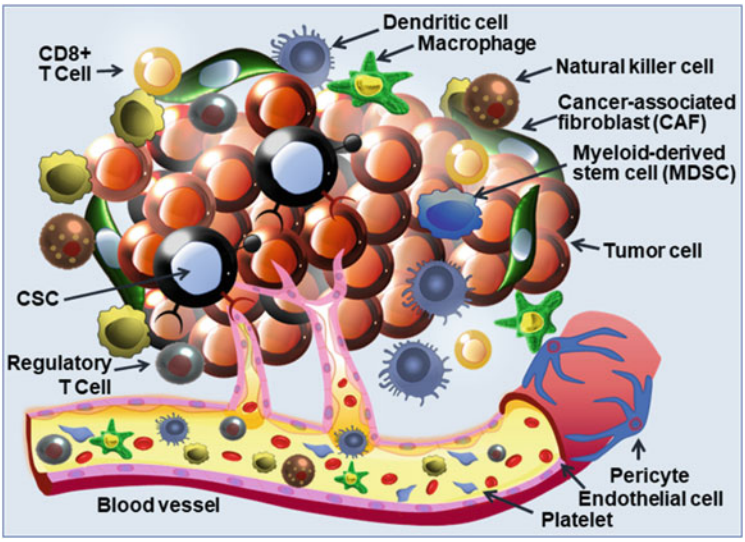

Fig. 16.5 Cancer stem cells and the tumor microenvironment (TME). TME-associated important cell types and their spatial localizations are shown

Solid tumors are complex tissues, as shown in Fig. 16.5. As the primary tumor grows, inadequate vascularization within tumor leads to increased hypoxia and ROS generation, which in turn activates the CSC's stress signaling pathways. CSCs activate the production of angiogenic factors to stimulate angiogenesis [116]. The tumor cells and CSCs also secrete cytokines, chemokines, and other soluble factors to attract the MSCs, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAM), tumor-associated neutrophil (TAN), and regulatory T cells (Treg), i.e., the pro-tumorigenic components. CSCs establish reciprocal interaction with the MSCs. While the CSCs secrete IL-6 to stimulate MSCs, the MSCs secrete C-X-C motif chemokine ligand 12 (CXCL12), IL-6, and IL-8 to activate CSC stemness. The tumor cells and CSCs also secrete macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) that attract and stimulate the expansion of pro-tumorigenic cell populations. These pro-tumorigenic cells contribute to a pro-tumorigenic and pro-metastatic effect and thereby promote tumor growth. On the contrary immune components like the cytotoxic T cells, helper T cells, natural killer cell (NK), and dendritic cells (DC) lead to an antitumor effect [118]. The pro-tumorigenic cells block the immunosurveillance of the antitumor adoptive and innate immune responses.

Cancer metastasis is a multistep process, including epithelial to mesenchymal transition (EMT), neoangiogenesis, metastatic niche creation, cell migration primarily through the blood vessel, and establishment of secondary tumors. For detailed information on cancer metastasis, please refer to our "Chapter 8: CSCs and Tumour Aggressiveness" in this book. In the TME, the CAFs and TAMs

coordinate and activate the pro-angiogenic switch to activate angiogenesis and invasiveness. The TME in the presence of cytokines secreted by the tumor stroma induces a specific population of CSCs to undergo EMT and thereby renders them invasive. CSCs and tumor cells can also hijack normal MSC niches and colonize, modify, and manipulate distant tissue niches for future colonization and create perivascular metastatic niches [119]. Mutational analysis suggests that CSCs harbor alterations in critical developmental pathways that are essential regulators of cell cycle and differentiation, including the Notch, Wnt/β-catenin, Hedgehog, and Hippo pathways [118]. The TME is characterized by the reciprocal interactions among these developmental pathways and also with other tumorigenic signaling pathways, including the nuclear factor-κB (NF-κB), MAPK, PI3K/AKT/mTOR, and EGFR cascades leading to tumor progression and metastasis. NOTCH mutations are not only associated with tumorigenesis but also reported to play a key role in EMT induction, neoangiogenesis, anoikis resistance, malignant cell expansion, and aiding in metastatic cell homing [120–122]. Activation of the Wnt/β-catenin pathway is also clinically associated with tumorigenesis, migration, and invasion [123, 124]. Similar involvement of the Hedgehog and the Hippo pathways are also implicated in metastasis [125–129]. Therefore, several studies have targeted the Notch, Wnt/β-catenin, Hh and Hippo signaling pathways as the primary targets for anti-CSC therapy, and the relevant, targeted signaling components are shown in Fig. 16.6 [7]. Many Notch pathway inhibitors are currently tested in clinical trials. The γ-secretase inhibitors (RO4929097 and BMS-906024) are in different clinical trial phases. The anti-Notch pathway antibodies are also in various stages of trials. They include anti-NOTCH1 antibodies (brontictuzumab), anti-NOTCH2/3 antibodies (tarextumab), anti-DLL4 antibodies (demcizumab, enoticumab, MEDI0639), and anti-DLL3 antibody-drug conjugates (rovalpituzumab tesirine). There are several pharmacological inhibitors aimed at the canonical as well as the noncanonical Wnt signaling pathway that are under investigation in clinical trials. Wnt pathway inhibitors include PRI-724 (β-catenin-CBP complex antagonist), DKN-01 (anti-DKK1 antibody), ipafricept (anti-FZD8 antibody), vantictumab (anti-FZD1/2/5/7/8 antibody), cirmtuzumab (anti-ROR1 antibody), and CWP232291 (also referred as CWP291; peptidomimetic small-molecule β-catenin antagonist). The Hedgehog signaling pathway plays a vital role in embryonic development, but mutations and dysregulation in the Hedgehog pathway are also associated with many tumor types. Multiple Hedgehog pathway-specific investigating agents are currently undergoing clinical trials. The principal investigated agents are glasdegib (small-molecule inhibitor of a Sonic HH), sonidegib (smoothened antagonist), vismodegib (smoothened inhibitor), taladegib (smoothened antagonist), and saridegib (smoothened antagonist). Pevonedistat (NEDD8 inhibitor) is an investigational agent that targets the Hippo signaling pathway [118]. Specific agents targeting CSC signaling pathways have received FDA approval, including the Hedgehog pathway inhibitor glasdegib for the treatment of AML patients, and have raised hopes for efficient cancer cure.

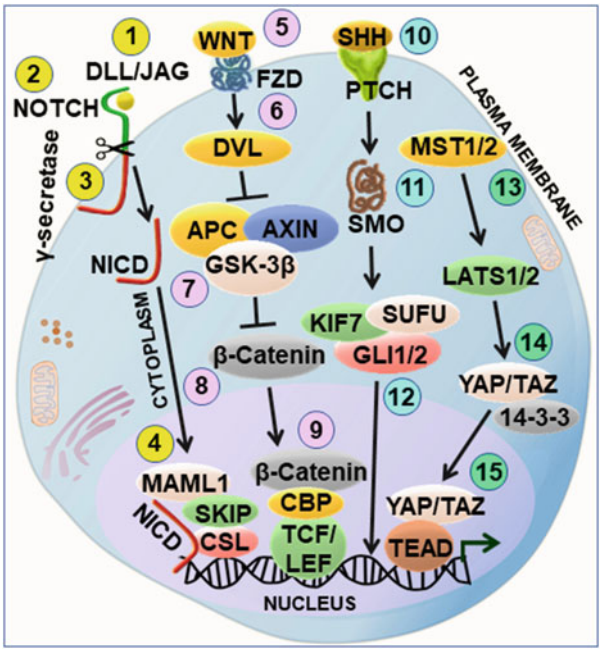

Fig. 16.6 Therapies targeting key signaling pathways associated with CSC-directed metastasis. Schematic diagram shows the specific signaling pathway components that are targeted by agents, which are currently under clinical investigation. The numbers relate to the specific target and to the type of inhibitors tested. (1) mAbs to NOTCH ligands; decoy NOTCH receptors; anti-DLL3 antibody-drug conjugates (ADCs), (2) mAbs to NOTCH receptors, (3) γ-secretase inhibitors, (4) NOTCH-specific transcription complex inhibitors, (5) WNT secretion inhibitors/porcupine inhibitors, (6) Decoy FZD-fusion proteins; anti-FZD mAbs; anti-FZD ADCs; anti-WNT mAbs; small-molecule inhibitors, (7) Tankyrase inhibitors, (8) β-catenin inhibitors, (9) the binding protein of the cAMP response element-binding protein (CREB) [CBP]-β-catenin antagonists; Traf2 and Nck-interacting protein kinase (TNIK) inhibitors, (10) HH blockers, (11) SMO antagonists, (12) Small-molecule GLI inhibitors, (13) Macrophage stimulating 1 (MST1) upstream signal inhibitors, (14) Yes-associated protein (YAP) inhibitors, and (15) Transcriptional coactivator with PDZ-binding motif (TAZ) inhibitors. The color of each number indicates the pathway being targeted: yellow, Notch pathway; pink, Wnt pathway; light blue, HH pathway; and green, Hippo pathway

16.7 Targeting CSC Signaling Pathways

16.7.1 Hedgehog Signaling Pathway in CSCs

Hedgehog (HH) pathway activation has been linked to more than a dozen types of cancers [130]. The main parts of the Hedgehog signaling contains three secreted ligands [Sonic (S) HH, Indian (I) HH, and Desert (D) HH], a negative regulatory receptor [patched (PTCH)], a positive regulatory receptor [Smoothened (SMO)], and

glioma-associated oncogene 1, 2, and 3 (GLI1, GLI2 and GLI3). The initial step of the Hedgehog pathway involves binding of the Hh ligand to PTCH leading to the lifting of SMO and translocation of GLI transcription factors into the nucleus to regulate target genes. Growing evidence suggests that targeting the Hedgehog signaling pathway in CSCs might offer a sensible as well as effective clinical choice to restrict tumor growth, overcome cancer resistance, and prevent cancer recurrence. So far, there are two orally available drugs, namely, vismodegib and sonidegib, which have been identified as inhibitors of Hedgehog signaling pathway (Fig. 16.7) [131] and investigated in clinical settings (Table 16.2). These two drugs showed exceptional activity in metastatic basal cell carcinoma (mBCC) and have been approved by the FDA [132].

A phase 2 study (ERIVANCE study) evaluated the clinical efficacy of vismodegib in 104 patients with mBCC as second-line therapy [133]. From this

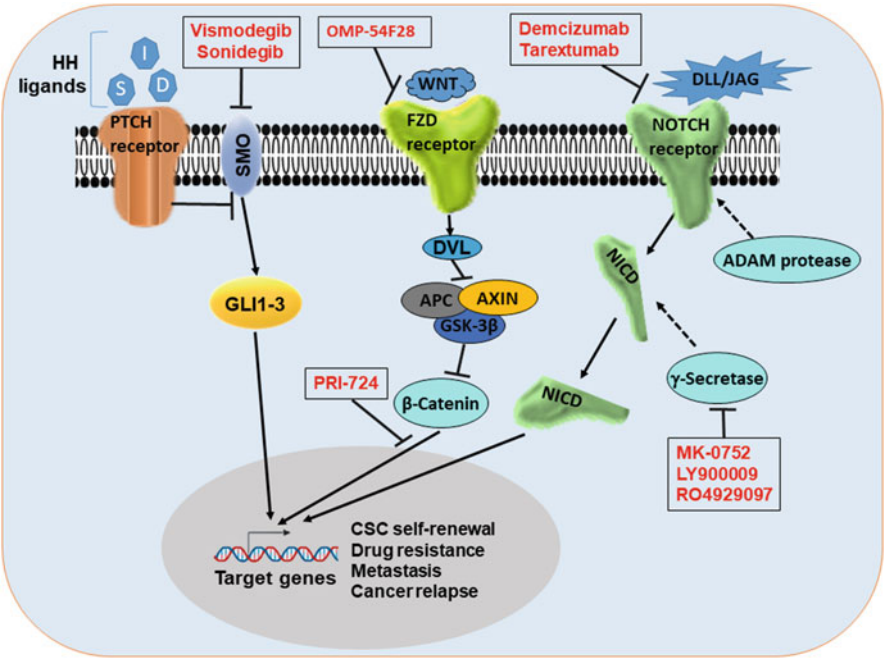

Fig. 16.7 Therapeutic strategies targeting the CSC signaling. Overview of CSC signaling and CSC signaling-targeted therapeutics: vismodegib and sonidegib are inhibitors of Hedgehog signaling pathway; Notch pathway is targeted by either GSI inhibition (MK-0752, LY900009, and RO4929097) or its receptor/ligand by use of antibodies (demcizumab and tarextumab); Wnt/β-catenin signaling-targeted therapeutics include OMP-54F28, which targets FZD receptor, and PRI-724 that targets β-catenin. Abbreviations used are as follows: HH, Hedgehog; HH ligands: Indian (I), Sonic (S), and Desert (D); SMO, Smoothened; GLI1-3, Glioma-associated oncogene 1–3; FZD, frizzled; DVL, Dishevelled; APC, Adenomatous polyposis coli; GSK-3β, Glycogen synthase kinase 3 beta; and NICD, NOTCH intracellular domain

Table 16.2 Clinical trials for targeted therapy against the signaling pathways of CSCs

Signaling	Drug	Trial/NCT identifier/ indication	Status	Outcome
Hedgehog pathway	Vismodegib	Phase 2/NCT00636610/ metastatic colorectal cancer	Completed	No vismodegib-associated benefit was observed in combination with either FOLFOX or FOLFIRI
		Phase 2/NCT00739661/ ovarian cancer	Completed	No difference in PFS for vismodegib versus placebo
		Phase 2/NCT01088815/ metastatic pancreatic cancer	Completed	Adding vismodegib to chemotherapy did not improve efficacy
		Phase 2/NCT00887159/ extensive stage small cell lung carcinoma, recurrent small cell lung carcinoma	Completed	There was no significant improvement in PFS or OS with the addition of vismodegib
		Phase 1/NCT01209143/solid cancers	Completed	Systemic exposure of rosiglitazone (a CYP2C8 substrate) or OC (ethinyl estradiol/ norethindrone) was not altered with concomitant vismodegib
		Phase 1/NCT00968981/solid cancers	Completed	Vismodegib failed to achieve unbound plasma concentrations as previously reported in advanced basal cell carcinoma and medulloblastoma
		Phase 2/NCT00833417/basal cell carcinoma	Completed	Study demonstrated durability of response, efficacy across patient subgroups, and manageable long-term safety of vismodegib in patients with advanced BCC
		Phase 2/NCT00607724/ unspecified adult solid tumor, protocol specific	Completed	Vismodegib showed antitumor activity in locally advanced or metastatic basal cell carcinoma
		Phase 2/NCT01160250/ advanced basal cell carcinoma	Completed	The results of this study suggested that patients aged $\geq$65 years were

(continued)

Table 16.2 (continued)

Signaling	Drug	Trial/NCT identifier/ indication	Status	Outcome
				likely to benefit from vismodegib
Notch pathway	MK-0752	Phase 1, phase 2/NCT00645333/ metastatic breast cancer	Completed	Clinically meaningful doses were possible, with manageable toxicity and preliminary evidence of efficacy
		Phase 1/NCT00572182/brain and central nervous system tumors	Completed	Well tolerated in children with recurrent CNS malignancies
		Phase 1/NCT01098344/ pancreatic cancer	Completed	Gemcitabine and MK-0752 can be combined as combo therapy. Thirteen patients achieved stable disease and one patient achieved a confirmed partial response
	LY900009	Phase 1/NCT01158404/ advanced cancers	Completed	No complete or partial responses were seen, but five out of 35 patients had stable disease after treatment with LY900009
	RO4929097	Phase 2/NCT01232829/ metastatic pancreatic adenocarcinoma	Completed	Of the 18 patients enrolled, 12 patients were evaluable for response based on protocol criteria; three patients (25% of evaluable; 17% of total enrolled) had stable disease at best response. There were no complete or partial responses
		Phase 2/NCT01141569/ clear cell renal cell carcinoma, recurrent renal cell carcinoma, and stage IV renal cell cancer	Completed	No objective radiographic responses were observed, and only six patients had stable disease as their best response
	Demcizumab	Phase 2/NCT02289898/ solid tumors	Completed	Well tolerated in patients with solid tumors
		Phase 2/NCT02289898/ pancreatic cancers	Completed	No efficacy benefit when demcizumab was added to gemcitabine plus abraxane

(continued)

Table 16.2 (continued)

Signaling	Drug	Trial/NCT identifier/ indication	Status	Outcome
		Phase 2/ NCT02259582/Non-squamous non-small cell lung cancer		Failed to meet studies' primary endpoint
	Tarextumab	Phase 2/NCT01647828/ metastatic pancreatic cancer	Completed	Addition of tarextumab did not improve overall survival
Wnt pathway	OMP-54F28	Phase 1/NCT01608867/solid tumors	Completed	Prolonged stable disease was noted in desmoid tumor and germ cell cancer patients
	PRI-724	Phase 1/NCT01764477/ advanced pancreatic adenocarcinoma	Completed	PRI-724 combined with gemcitabine was safe and demonstrated modest clinical activity

Clinical trials conducted using specific CSC signaling pathway inhibitors are depicted. Source of the table is https://www.clinicaltrials.gov

study, 43% of patients with locally advanced disease experienced substantial shrinkage of tumors or healed visible lesions, whereas 30% experienced mBCC tumor shrinkage. The objective response rates (ORR) for locally advanced and metastatic BCC were 60% and 46%, respectively. Subsequent to the ERIVANCE study, another open-label, phase 2 study (EAS) explored the impact of vismodegib in BCC patients [134]. The results from the EAS study were similar to those of ERIVANCE trial. Further, sequential phase 2 studies (PBTC-025B and PBTC-032) analyzed the clinical benefit of vismodegib in pediatric and adult medulloblastoma (MB) [135]. Results from these studies showed that patients in sonic Hedgehog (SHH)-MB group with active Hh signaling had significantly longer progression-free survival (PFS) than patients in the non-SHH-MB group. Despite the fact that vismodegib has excellent antitumor activity in MB, other studies with metastatic colorectal [136], small cell lung [137], and pancreatic [138] cancers had no considerable improvement in terms of clinical efficacy endpoints.

Other data indicated that cancer cells might develop resistance to the Hh inhibitors. For example, in MB and also BCC, treatment with vismodegib (GDC-0449) and sonidegib (LDE-225), two drugs that have shown better outcome in other clinical trials, triggered drug resistance in the residing cells of these cancers [139]. Hence, the efficacy of Hh inhibitors in cancers is still uncertain, and so far, they have shown activity in only a subset of tumors with active Hh signaling. A comprehensive understanding of the Hh pathway regulation could facilitate the

development of new therapeutics to deal with Hh pathway-activated cancers with better outcomes.

16.7.2 Notch Signaling Pathway in CSCs

Notch signaling represents a type of direct cell-cell communication that is necessary for the regulation of proliferation, apoptosis, and fate decisions of stem cells during embryonic development [140]. Several studies suggest that Notch signaling plays important roles in cell proliferation, survival, self-renewal, differentiation, angiogenesis, and migration of CSCs [76]. Notch signaling works through a cell-cell communication, in which a membrane-bound Notch ligand, Delta-like (DLL) or Jagged (JAG) binds with a transmembrane Notch receptor on a juxtaposed cell. This interaction initiates two proteolytic events, which are carried out by A Disintegrin And Metalloproteinase (ADAM) and γ-secretase enzymes, and results in liberation of the NOTCH intracellular domain (NICD). Later, the cleaved intracellular domain enters the nucleus to engage with other DNA-binding proteins and to regulate gene expression [76]. So far, clinical studies targeting Notch pathway have been following two approaches; one is the use of γ-secretase inhibitor (GSI) and the other is targeting the Notch receptor or ligand through the use of antibodies (Table 16.2).

Pathway inhibition by γ-secretase inhibitors has been shown to be effective in preclinical models of cancer and appears to have a safe profile based on phase 1 clinical studies [141, 142]. Inhibitors of GSI (MK-0752, LY900009, and RO4929097) (Fig. 16.7) have been investigated in clinical trials. In one study, patients with advanced solid tumors were treated with MK-0752, and clinical benefits were assessed [142]. The results from this study showed that the MK-0752 was not effective in extracranial tumors; however, one patient with anaplastic astrocytoma achieved complete remission (CR), and ten patients with high-grade glioma had stable disease for more than 4 months. However, there were two studies where MK-0752 showed minimal or no clinical benefit [141, 143]. In another study, patients with breast cancer were treated with MK-0752 plus docetaxel as combotherapy. In this study, 30 patients were enrolled and received MK-0752 along with docetaxel. Of the 30 participants, 26 entered the study with measurable disease as defined by RECIST criteria, and 2 of them were not evaluable for other reasons. Of the 24 participants, 11 showed partial response, 9 had stable disease, and 4 had progressive disease [144]. But the results from this study need to be translated with caution considering that all participants received combotherapy with the standard of care therapy, and the trial was also designed with single-arm treatment. Another drug, LY900009, has been studied in phase 1 trial and assessed for maximum tolerated dose, toxicity, pharmacokinetics (PK), pharmacodynamics (PD), and antitumor activity [145]. This study had shown a manageable safety profile and acceptable PK and PD with limited antitumor effects of LY900009 in patients with advanced cancer. The RO4929097 drug, which also inhibits GSI, has been studied in more than 35 phase 1 and 2 clinical studies, but so far, it has not

reached to the level of phase 3 studies yet. LY3039478 is another drug that targets γ-secretase and has been successful in phase 2 study with encouraging signs of preliminary clinical activity in advanced and metastatic cancers [146].

For therapies targeting Notch ligand and receptors, immunotherapies such as demcizumab and tarextumab have been developed and are currently undergoing clinical investigations. Demcizumab binds to the membrane-binding portion of DLL4 and prevents its interaction with NOTCH1/4 receptors, thereby inhibiting Notch-mediated signaling and gene transcription. This drug was tested in phase 1 clinical trial with solid tumors and tolerated well in patients [147]. However, a recent study (YOSEMITE study) in pancreatic cancer failed to demonstrate efficacy benefit when demcizumab was added to gemcitabine plus abraxane (NCT02289898, https://www.clinicaltrials.gov). Another phase 2 study (DENALI study) investigating the efficacy of combotherapy of demcizumab with standard therapy in lung cancer was terminated (NCT02259582, https://www.clinicaltrials.gov). Data from the DENALI study showed that not only did the trial fail to meet its primary endpoint of overall response rate (ORR) but also that outcomes were better for patients in the placebo group than for those in the demcizumab treatment group (NCT02259582, https://www.clinicaltrials.gov).

Another monoclonal antibody directed against the NOTCH receptor, tarextumab, has been tested in several clinical trials and has been approved as an orphan drug to treat pancreatic and lung cancers. This drug is a humanized monoclonal antibody targeting the NOTCH2/3 receptors. Preclinical studies using pancreatic xenograft models have found that treatment with the combination of tarextumab, gemcitabine, and nab-paclitaxel caused tumor regression and decreased CSC frequency compared to treatment with cytotoxic therapy alone [148]. However, the addition of tarextumab to standard therapy in advanced pancreatic cancer did not improve outcomes over the standard therapy [149].

16.7.3 Wnt/β-Catenin Signaling Pathway in CSCs

WNT ligands are secreted glycoproteins that bind to the N-terminal extracellular cysteine-rich domain of the frizzled (FZD) receptors in the presence of co-receptors, low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6). Upon activation of Wnt signaling, Dishevelled (DVL) prevents degradation of β-catenin and results in translocation of β-catenin into the nucleus, which eventually leads to facilitation of transcription of WNT target genes [130]. Abnormal activation of Wnt signaling is implicated in the CSC maintenance of colorectal, breast, hematologic, skin, and lung cancers [150–152]. Moreover, hyperactivation of Wnt/β-catenin pathway contributes to cancer cell proliferation, stemness and invasion [153, 154].

OMP-54F28 is a drug that inhibits FZD receptor binding to Wnt ligands (Fig. 16.7). It has been tested in clinical setting in patients with desmoid tumors and demonstrated clinical activity [155]. In addition, currently, it is being tested in phase 1 studies for safety profile (NCT02050178, NCT02092363, and NCT02069145). Another drug, PRI-724, which blocks β-catenin interaction with

its transcriptional co-activators, has been studied with gemcitabine in phase 1 study [156], and the results showed some clinical benefits. The preclinical data on targeting Wnt signaling have shown some promising outcome (Table 16.2); however, these drugs are still in the early phase, and these drugs' future will depend on identifying the right population who can respond really well and also choosing better combinations with available standard therapies as combotherapies.

16.7.4 Other Pathways in CSCs

Various other signaling pathways including the TGF-β, JAK/STAT and PI3K/AKT/mTOR signaling have all been shown experimentally to mediate various stem cell properties, such as self-renewal, cell fate decisions, survival, proliferation, and differentiation. There are numerous inhibitors targeting these signaling pathways that show promising treatment abilities in cancers, and some of them have been shown to utilize anti-CSC effect, but most of them are still in the early phase of development.

TGF-β signaling has been shown to be very active in cancer-initiating stem cells within the tumor tissue. Galunisertib, the first small molecule of TGF-β receptor inhibitor, has been shown to inhibit cancer-initiating stem cells and arrest the TGF-β-dependent tumor cell growth and migration [157, 158]. Another recent study has shown that galunisertib with gemcitabine resulted in improvement of survival in patients with unresectable pancreatic cancer [159]. Combotherapy having galunisertib in combination with chemo- and radiotherapy with temozolomide is being tested in glioblastoma patients (NCT01220271), and the study results have not been published yet. In another study, galunisertib plus lomustine combotherapy failed to demonstrate improved OS relative to placebo plus lomustine in patients with recurrent glioblastoma [160]. For detailed information on glioblastoma, please refer to our "Chapter 10: Glioblastoma Stem Cells as a Therapeutic Target" in this book.

JAK/STAT signaling is also involved in maintaining embryonic stem cell self-renewal properties, hematopoiesis, and neurogenesis [161]. Evidence that JAK/STAT signaling pathway is activated aberrantly in CSCs has been found in tumors of the breast and prostate [162, 163]. There has been some success in clinical trials using JAK inhibitors (e.g., pacritinib) to treat myeloid and lymphoid malignancies [164].

PI3K/AKT/mTOR signaling pathway is critical for CSC maintenance [165]. mTOR inhibitors (temsirolimus and everolimus) and PI3K inhibitors (copanlisib and idelalisib) have been under investigation in the clinical settings as anticancer drugs targeting PI3K/AKT/mTOR pathway.

16.8 Conclusions

Conventional therapies have several limitations, and therefore, there is an unmet need to develop novel effective therapies for the treatment of various cancers. CSCs possess the properties of self-renewal, plasticity, and the ability to cause cancer metastasis and relapse. Various in vitro and in vivo studies have demonstrated that conventional cancer therapeutics induce apoptosis of tumor cells but are unable to eliminate the CSCs in the tumor, thus posing a serious challenge to currently available therapies. Moreover, available evidence suggests that conventional therapies can activate CSC-like phenotype in many bulk tumor cells. In contrast to conventional therapies, CSC-based therapeutics targeting CSCs population, exhibit high pharmacological efficiency, and thus have a greater potential to eliminate CSCs and cancers. Though, several approaches targeting CSCs are feasible, targeting CSC-specific pathways and CSC-niche interactions, likely have the greatest potential to effectively control cancer progression and metastasis in cancer treatments. Multiple aberrant CSC-associated signaling pathway molecules are potential targets for inhibition and different inhibitors are currently under investigations in clinical trials.

Targeting the UPR and autophagy pathways can lead to elimination of CSCs. Treatment options using immunomodulatory approaches like antibody therapy, DC-based vaccines, adoptive immunotherapy, and oncolytic virotherapy can also efficiently target the CSCs, and should be explored more widely for various cancers. Targeting the ncRNAs that modulate the CSC's ability to induce EMT and drug resistance can serve as another potential alternative therapeutic approach to treat cancers. Combination therapy approaches targeting the CSCs and bulk tumor cells or CSCs and their niche, using different approaches can efficiently eliminate the primary and secondary metastatic tumors. Nanoparticle (NP)-based delivery systems including liposomes, exosomes, lipid NPs, protein NPs, viral NP, apoferritin-based NPs, inorganic NPs, and natural phytochemical-based NPs can also potentially target the CSCs specifically.

Advances in integrated omics, including genomics, transcriptomics, proteomics, metabolomics and other omics platforms integrated with statistical tools, machine learning algorithms or artifical intelligence (AI) platforms, can help to dissect out the specific and unique features of the CSCs as compared to normal and bulk tumor cells. The markers unique to CSCs can also be used to target CSCs. Moreover, detailed information harnessed from patient-specific CSC alterations for many cancer types, will be useful to better understand the variabilities across individual's CSCs epigenetic status, self-renewal, signaling, marker profile and CSC-niche interactions, for use in precision medicine treatments.

Current therapies targeting CSC signaling pathways demonstrate its efficacy in clinical trials against many tumor types, but exhibit poor performance during treatment of cancer patients. Therefore, targeting CSC's signaling pathways should be explored in depth to further develop and enhance tumor targeting treatments, and evolve it into an efficient method to combat cancers. The flexibility of employing CSC signaling pathway targeting drugs along with available standard therapies has

made the treatment strategies highly promising. As extensive information on the molecular biology of CSCs is garnered for various tumor types, more targeted therapies may be designed and tested in combination with each other or with standard therapies. This could ultimately lead to the development of novel therapies and potent therapeutics to effectively target CSCs, and thus, treat and cure cancer.

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Conflict of Interest The authors declare no conflict of interest.

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Targeting Cancer Stem Cells by Nanoenabled Drug Delivery

17

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Abstract

Resistance to chemotherapy and radiotherapy is commonly seen in cancer cells due to various reasons like mutation in drug target or their overexpression, drug inactivation, or drug removal from the cell, thereby rendering a problem in cancer management. The cancer stem cells (CSCs), which are responsible for cancer metastasis, are far reached from conventional therapies as these approaches are unable to eradicate the drug-resistant CSCs, and a novel approach for targeting these CSCs is warranted. Nanotechnology has occupied a huge space in drug delivery due to their unique photophysical properties and large surface area to volume ratio compared to their bulk counterparts. Targeted drug delivery can be achieved using nanoenabled drug delivery as the different nanostructures can be functionalized to tag different molecules which can identify specifically the CSCs. Moreover these nanostructures can also be used as cargo for carrying the chemotherapeutic drugs and delivering them to the target site. This chapter discusses the different types of nanocarriers used for targeted drug delivery as well as the progress in research for targeting the CSCs and destroying them.

Keywords

Cancer stem cells · Nanoenabled drug delivery · Bionanotechnology · Nanotheranostics

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17.1 Introduction

New therapeutic and diagnostic strategies for treatment of cancer have made enough progress in its preclinical and clinical research on cancer [1], but the metastasis in cancer is life-threatening as it spreads the cancer cells to other tissues from the origin [2]. The tissues found in cancer consist of heterogeneous cells with different states of differentiation and contain "tumor-initiating" cells formed by normal stem cell mutations [3, 4]. These "tumor-initiating" cells were termed as "cancer stem cells" (CSCs) which exhibit similar properties like other stem cells, self-renewal, can differentiate into any cell, and can proliferate to enhance malignant cells [4]. The strategy for cancer therapy includes the balance between self-renewal and differentiation of these CSCs to prevent formation of cancer.

In recent times, many new drugs are being invented with outstanding pharmacokinetic and therapeutic properties, but delivering those new drugs to target effectively becomes a challenge. Once targeted to the specific molecules, it can show its potential activity. Many nanotechnology-based drug delivery systems have been introduced and successfully commercialized like oncology drugs based on solid nanoparticles, liposomal formulation, conjugates of proteins and polymers, or drug-polymer conjugated nanoenabled drug delivery systems. However, the bioavailability of these drugs is dependent on several factors like size of the drug, dosages, difference in solubility of water-soluble and fat-soluble drugs, and their clearance from the blood stream. The drug designing also involves the target cells because in case of cancer it is desirable that the drug should affect the malignant cells only, not the benign ones, thereby warranting certain drug carriers which can encapsulate the drug and release them in only tumor microenvironment. Modern medical bionanotechnology has enabled us to design such nanocarriers which can target cancer cells. Targeting CSCs is much more relevant in cancer research because there are many drawbacks associated with conventional treatments using radiation and chemotherapy. But in cancer some CSCs can escape this treatment and migrate into new place through metastasis and start developing fresh tumors, relapsing the disease [5–7]. The different types of nanocarriers and their role in targeting CSCs will be discussed in this chapter.

17.2 The Different Types of Nanocarriers

The nanocarriers are helpful for the solubilization of lipophilic drugs, give protection to drugs which are fragile from degradation by enzymes or pH, and can target the drugs to be released at specific sites [8]. The different types of nanocarriers are discussed below.

17.2.1 Nanobots

Nanobots are nanorobots or nanomotors which are self-driven with submicron dimension, biodegradable nanodevices composed of bionano materials that can transport the cargo to deliver them in target sites. Zinc-based nanobots, named as PEDOT/Zn micromotor, were used to deliver payload in the stomach of a mouse model, which gradually dissolves the nanobot in the stomach acid and delivers the payload [9]. Single-molecule-based submersible nanomachines in solutions were activated using UV light, and single-molecule fluorescence correlation spectroscopy (FCS) was monitored. Designing such nanobots, which were non-unidirectional rotating motor, provided 10% enhanced diffusion, and we could monitor the behavior of these motorized molecules in solution [10]. DNA origami-based nanobots were designed to deliver payloads by designing outer functionalization using a DNA aptamer which can bind nucleolin, an endothelial cell tumor protein, and in the inner cavity had thrombin, the blood coagulation protease. They demonstrated that when these nanobots were injected intravenously, they delivered thrombin to the blood vessels associated with tumor and could induce thrombosis intravascularly. This resulted in necrosis of tumor and inhibited tumor growth [11]. Nanoactuators have also been designed which get activated using light by binding temperature-responsive polymers over gold (Au) nanoparticles which are charged. This stores the elastic energy which can be released rapidly under light for repeated isotropic nanoactuation. When the nanoactuator was heated above critical temperature ($T_c = 32\ ^\circ C$) using light from incident laser, the coating expels water and gets collapsed into nanoscale within a microsecond which is million times fast compared to the base polymer. This phenomenon triggers a small number of nanoparticles to get tightly packed into clusters. When the nanomachine is cooled below T_c, the strong van der Waals force between the cluster particles is surmounted as the expansion of polymer takes place giving rise to nanoscale forces of several nN. The intensity of the large force is dependent on van der Waals attractions between the Au cores existing very large in collapsed polymer state which sets a tightly compressed spring of polymer that can be triggered further into inflated state [12]. Nanoswimmers were designed which can be applied to swim in bloodstream to deliver the drugs. Multilink nanowire-based chains of diameter 200 nm were used to make a composite which exhibited planar undulations induced, using a planar-oscillating magnetic field. The chains were constructed by an elastic polypyrrole tail like eukaryotes and rigid nickel links which were magnetic in nature connected by hinges made up of flexible polymer bilayer. This multilink design showed high swimming efficacy and thereby could be used as a vehicle for drug delivery in body fluids [13]. These nanotechnological developments can enable nanobots useful for drug delivery.

17.2.2 Nanoneedles, Nanoclusters, and Nanobubbles

To facilitate the entry of drugs into the cell cytoplasm directly, nanoneedles are used because the biological membranes do not facilitate the drug entry into the cells. Nanoneedles are mainly used in atomic force microscopy but are applied for drug delivery to cells where they make small temporary perforation in the biological membrane and deliver the drug without perturbing the biological functions [14].

Metal nanoclusters are usually of the size of 10 nm prepared by self-assembly of polymeric or small organic molecule-based nanoparticles, cross-linked together with plasmonic metals like gold and silver or magnetic nanoparticles. These nanoclusters exhibit molecule-like properties and fluorescence; they are used for tracking the drug carried using these clusters to the target site and imaging. Peptide-protected gold nanoclusters (Pep-AuNCs) were used for self-regulated loading and release of drug vancomycin (van). The antimicrobial activity of van loaded in Pep-AuNCs was comparable to van alone, and the van released by Pep-AuNCs was proportional to the number of bacteria present [15].

Nanobubbles, on the other hand, are nano-sized spherical structures filled with gas which are usually stabilized using polymeric/lipid shells. The nanobubbles are used in combination with ultrasound, thermal, or magnetic sensitivities for efficient application in drug delivery and imaging, because of their higher stability and long time of residence in systemic circulation. For the purpose of diagnosis and therapy done together, theranostics has come into field, and researchers have developed plasmonic nanobubbles (PNBs) for tunable theranostic applications. The PNBs were designed by gold nanoparticle exposed to laser after delivering it intracellularly which generated transient photothermal vapor nanobubbles. The action of PNBs was tuned inside the individual cells from noninvasive, at lower laser fluence, to cell membrane disruption at higher fluence. The imaging was also captured with 50-fold amplification of optical scattering amplitude, and PNBs were established to support diagnosis, therapy, and image guidance at the cellular level in a single process [16].

17.2.3 Nanoghosts, Nanoclews, Injectable Nanoparticle Generators (iNG), and Nano-Terminators

Nanoghosts are based on a technology to form nanovesicles isolated from natural functionalized membranes of mammalian cell surface of complete biological cells like mesenchymal stem cells (MSCs) which do not contain cytoplasm or any organelles. These are smart delivery vehicles for drug or gene delivery. As they are derived from natural source, they do not pose difficulties related to drug loading, adverse immune response related to evading tumor etc. Moreover, it provides improved nanoparticle stability and gives a superior drug release profile. Nanoghosts were successfully isolated from cell membranes of MSCs (MSC-NGs), and in vitro and in vivo tumor targeting properties were also retained and were cleared from blood-filtering organs. These MSC-NGs were biocompatible, and drug-loaded MSC-NGs showed 80% inhibition of prostate cancer cells after systemic administration [17]. Negatively charged plasmid cDNA (pDNA) was loaded on a nanoghost

derived from MSCs which retained their unique surface-associated tumor-targeting properties. These engineered nanoghosts which were loaded with gene that is toxic to cancer cells could inhibit the growth of orthotopic lung cancer which has metastasized. These nanoghosts also proved to be effective in subcutaneous prostate cancer models and were shown to improve the survival of animals [18]. Nanoghosts derived from monocyte cell membrane were used along with doxorubicin-loaded PLGA core to make core-shell nanoghosts. The size of the nanoghosts was nearly 200 nm and was stable in serum for 120 h. These core-shell nanoghosts showed higher cellular uptake and cytotoxicity in MCF-7 cell lines compared to non-coated nanoparticles [19].

A nanoclew or nanococoon is made up of a single-stranded DNA which self-assembles to form a cocoon or yarn or a clew-like structure. The DNA amplification takes place by rolling circle model, and these nanococoons are highly biocompatible nanodrug delivery system. Sun et al. [20] first described a cocoon-like DNA-based nanocomposite as a drug delivery carrier which was associated with "caged worm" of deoxyribonuclease (DNase) that can undergo self-degradation thereby releasing the drug inside the cells. The DNA structure was a nanoclew made by weaving of DNA amplified by using rolling-circle model, and the self-assembly was facilitated by incorporating a palindromic sequence. The loaded drug was doxorubicin (DOX), and the targeted tumor delivery was achieved by folic acid (FA) conjugation with a nanoclew complementary DNA which gets hybridized to the DNA nanoclew. For self-degradation after reaching the tumor site with acidic environment, an encapsulated DNase I in single-protein-based nanocapsule (NCa) having a thin positively charged polymeric layer shell made up of cross-linkers which were acid degradable was used. This NCa which was positively charged was embedded into the nanoclew through electrostatic interactions forming a DOX-loaded DNA scaffold which was self-degradable. Under physiological pH, the DNase I was not released by the cage, but as soon as the nanoclew entered the cancer cell, the acidic microenvironment degraded the nanoclew as the pH-sensitive polymer releases the DNase I, thereby releasing the encapsulated drug DOX exhibiting higher anticancer efficacy [20].

Injectable nanoparticle generator (iNG) was first described by Xu et al. [21] and was made up of a polymer loaded with DOX which had multiple strands enwrapped over a nanoporous silicon material which is biodegradable. When these drug-loaded nanocarriers were intravenously injected, they got accumulated in tumor cells due to natural tropism. Then the silicon material slowly degraded and released the drug polymeric strands. Spontaneously, these strands formed nanoparticles which were taken up by the cancer cells, and the acidic microenvironment inside the cancer cells made the polymeric stands to trigger drug release. The iNG-based drug delivery system could cross the multiple biological barriers, and the dimensions and geometry of the silicon core could be tuned for targeting precise anatomical locations like the lung and liver. Moreover instead of DOX any other anticancer drug could also be loaded in the engineered iNG [21].

Nano-terminators were developed by Lu et al. [22] that were nanodroplets made from liquid metal loaded with drug which were absorbed by the tumor cells when

injected. In acidic tumor environment, it released the drug because the nanodroplet made from liquid phase eutectic gallium-indium core and a thiolated polymeric shell equipped with hyaluronic acid got dissolved. This nanodroplet was a core-shell nanosphere loaded with DOX and the hyaluronic acid acted as a tumor-targeting ligand. This nanoformulation when used in chemotherapy was shown to inhibit tumor in xenograft tumor-bearing mice in a much superior way than conventional chemotherapy [22].

17.2.4 Exosomes, Liposomes, and Niosomes

Exosomes, typically of the size from 30 to 120 nm, are used for transferring information from one cell to another and can be used as a natural vehicle for targeted drug delivery. The mode of transport using exosomes is depicted in Fig. 17.1.

The exosomes can be isolated from the patient's own cells which are healthy, as they can interact with its own cellular membranes when used for drug delivery without any hindrance. The exosomes have a unique property called "cell-specific tropism" which means that they can target specific cells by expressing specific receptors on the membrane, toward the cells from which they are isolated. This property can be utilized to convey drugs, microRNAs, or proteins loaded in these exosomes. Since the origin of these exosomes is biological which contains natural lipid bilayers, the immunogenicity and issues regarding clearance of drug can be reduced. Moreover, these exosomes can also cross the blood-brain barrier overcoming the challenging situation for drug delivery in the brain and for designing personalized medicine. Encapsulation of natural products and RNA has been accomplished in exosomes for the treatment of many solid tumor cancers like pancreatic,

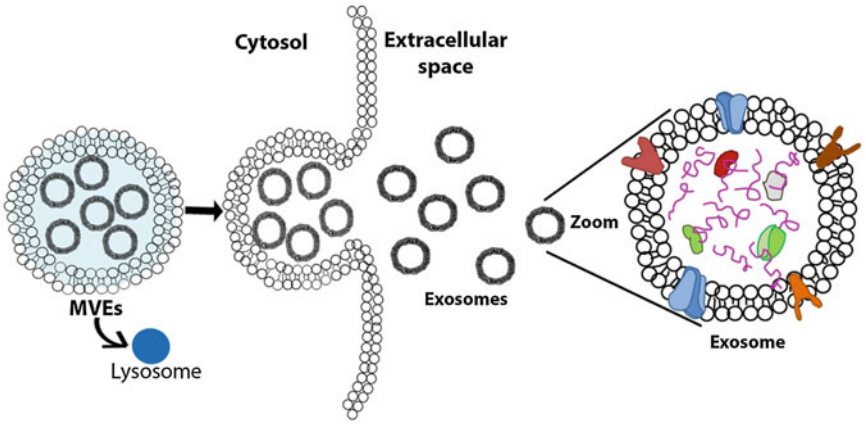

Fig. 17.1 The formation of exosomes. The multivesicular endosomes (MVEs) encompass the exosomes and these MVEs can fuse with plasma membrane to release the exosomes to the intercellular space or fuse with lysosome for their degradation. Once the exosomes are released, they can be isolated and used as a vehicle to carry DNA, RNA, protein, drugs, etc.

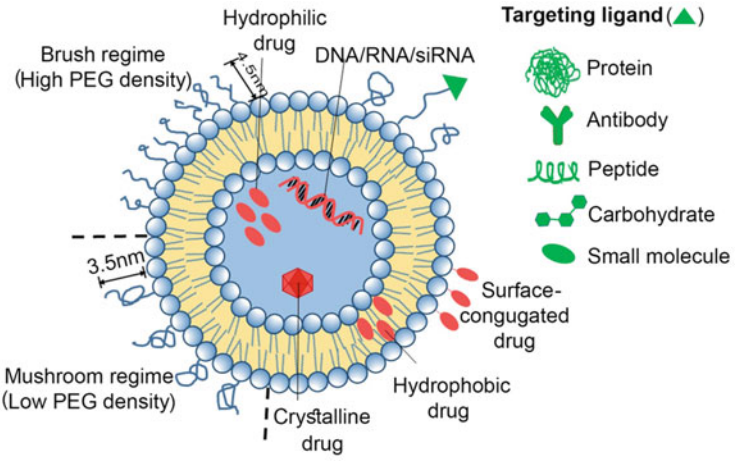

Fig. 17.2 The schematic diagram showing a liposome and the possible drug loading capacity of different types of drugs. The different surface functionalization is also illustrated for targeting the different types of cells

breast, prostrate, lung, and glioblastoma [23]. Three means in the exosomal targeted therapy can be achieved: (1) by targeting the peptides to the exosomal surface, (2) by encapsulating specific genes within the exosomes and transferring them to tumors, and (3) by targeting the exosomes that contain tumor-associated antigen. These are elaborately discussed in review by Wang et al. [24].

Liposomes are formulated spherical vesicles comprising of an aqueous core and surrounded by a lipid bilayer, used for improving the bioavailability, drug absorption, and reducing toxicity. The unique feature of liposomes is their ability to compartmentalize as well as solubilize both hydrophobic and hydrophilic materials, thereby opening a vast encapsulation capability. The different possibilities of using liposomes as drug carrier are given in Fig. 17.2.

A co-delivery system was developed based on fusogenic liposome that encapsulated chemotherapeutic agents with ATP-responsive elements and a liposome that contains ATP. When these two liposomes fuse together, there is triggering of ATP-mediated drug release. The design of the fusogenic liposome is a protein-DNA complex core consisting of an ATP-responsive DNA scaffold with DOX which could release DOX by a change in conformation of aptamer/ATP duplex in the presence of ATP. To achieve cancer cell targeted delivery, the fusogenic liposomal membrane was coated with a peptide which can open when acid-triggered to fuse the two liposomes under cancer acidic microenvironment. Thus, a pH-sensitive anticancer drug delivery system was achieved [25].

Niosomes are nonionic surfactant vesicles available in different sizes that range from 20 to 50 μm. They can be constructed by self-assembly of monomers of hydrated nonionic surfactant and are capable of encapsulating a variety of drugs [26], and their typical structure is shown in Fig. 17.3.

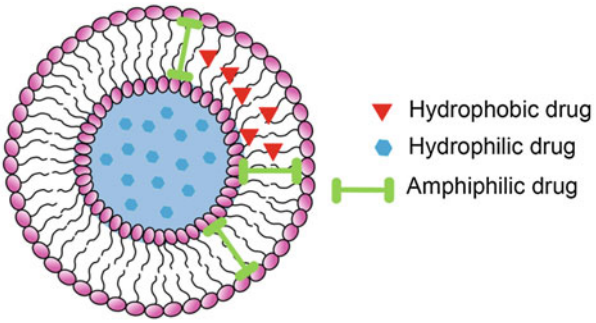

Fig. 17.3 The structure of noisome made from nonionic surfactant and cholesterol used for drug delivery. All the types of drug, hydrophobic, hydrophilic, and amphiphilic, can be loaded in the niosomes

Due to the stability problems found in liposomes, niosomes are introduced as an alternative drug delivering vehicle. Niosomes also possess the capacity to encapsulate both hydrophilic and lipophilic drug substances. The efficiency of entrapment increases with the increase in lipophilicity and concentration of the surfactant which is used to make them. Compared to liposomes, niosomes have different chemical compositions of its bilayer making them more advantageous. The components of liposomes are based on phospholipids, whereas surfactants are used to make niosomes which have improved chemical, physical, and biological stability. Moreover, by modulating the noisome bilayer composition, enhanced drug entrapment can be achieved. The industrial manufacturing of niosomes is also less expensive as they do not need special handling methods as well as storage conditions due to their high stability. The mostly used nonionic surfactants for niosome preparation used for drug delivery are alkyl ethers, sorbitan fatty acid esters, alkyl glyceryl ethers, and polyoxyethylene fatty acid esters. The correct selection of the surfactant plays an important role in designing nonionic vesicular systems. The stability, size, pharmacokinetics, entrapment efficacy, pharmacodynamics, and targeting properties of the vesicular systems are affected by the molecular structure of the surfactant used [27]. The different types of niosomes used for cancer drug delivery are well discussed in the review by Bondar et al. [28].

17.2.5 Dendrimers

Dendrimers are polymers having a well-defined structure with a core at its center made up of an atom or molecule. Branches emerge from its core comprising of repeated units of the constituent polymer with the branch junctions, known as generations [29]. There can be first-generation, second-generation, third-generation, or fourth-generation dendrimer emerging from a single core. The branching makes multiple functionalization and many molecules can be attached thereby on a single core. Dendrimer framework can be controlled and can be utilized as a good drug carrier, and their functionalizations are used for conjugation with drugs or DNA/RNA. Dendrimers can enhance the solubility and bioavailability of the drugs that are hydrophobic. The entrapment of drugs can happen in the intramolecular

cavity of dendrimers or can be conjugated to the functional groups attached at their surface [30].

17.2.6 Graphene and Carbon Nanotubes

Graphene is a two-dimensional nanostructure of carbon with one-atom thickness made from densely packed sp2-hybridized carbon atom network arranged in a hexagonal crystal lattice structure exhibiting unique nanoscopic properties [31–35]. It has profound usage in materials science as well as biomedical science [32, 35]. The graphene nanoparticles can exhibit various structural features, biological responses, and physicochemical properties based on their manufacturing methods [36]. The different types of graphene nanoparticles are graphene nanoribbons (stacks of ribbon-shaped graphene synthesized by the unzipping of the multiwalled carbon nanotubes), graphene nano-onions (spherical shaped layers of graphene which are concentric having both sp2 and sp3 hybridizations), and graphene nanoplatelets (irregular or disc-shaped multiple layered graphene nanoparticles which are synthesized from graphite, also named as graphene oxide (GO)) [36]. The promising applications of graphene in imaging, therapeutics, and drug delivery are attributed to their unique physical and chemical properties [35, 37–39], and so it is considered as a multifunctional nanoparticle. The surface of graphene nanoparticles can be functionalized covalently or noncovalently with anticancer drugs as well as functional groups that can target the cancer cells or tissues for improving the treatment efficacy. The physicochemical properties of graphene nanoparticles can be utilized to assist stimulus-responsive therapy as well as drug delivery. Scientists have targeted CSCs using graphene nanoparticles without causing any harm to normal cells [40].

Carbon nanotubes (CNTs) are made from carbon graphite nanomaterials arranged in an ordered array and hollow structure. CNTs have high surface area, ultralight weight, high aspect ratio, and high tensile strength with tube diameter ranging from 1 to 100 nm. The end of the tubes is usually capped with half-fullerene molecules on both ends and exists as one or several coaxial layers of graphite having diameters in nanometer range. Every carbon atom in CNT is joined to their three neighbors with sp2 hybridization just like graphite that gives the molecules huge strength. CNTs are classified into two types depending on their structure: single-walled carbon nanotubes (SWNT) and multiwalled carbon nanotubes (MWNT). In the field of drug delivery, CNTs have a number of advantages to deliver the drugs at specific locations in our body suggesting that CNTs may overcome the difficulties of nanoparticles. Since the CNTs have a huge inner volume, it allows more drug molecules which can be encapsulated. Moreover, these volumes are easily accessible because the fullerene caps at the ends can be removed easily and they can have different functionalizations for inner and outer surfaces [41]. CNTs can be chemically modified to attach a variety of molecules on its surface such as proteins, DNA, drugs, peptides, ligands for targeting cells, etc. which enable them to be appropriate candidate for targeted drug delivery. Although one of the drawbacks of CNTs is that

they are evidenced to show oxidative stress both in vitro and in vivo causing inflammation and damage to cells in the liver and lungs [42]. To overcome these, nitrogen can be doped in CNTs in the form of various functionalities like pyrrolic nitrogen, pyridinic nitrogen, oxidized nitrogen, and graphitic nitrogen. Further alterations of these functionalities by means of chemical reactions can be done to get desired nitrogen species [43]. When the nitrogen atoms are incorporated into the graphitic lattice of the CNTs, an additional strain to the structure of CNT results in forming "stacked cups" [44]. These stacked cups are held together with weak van der Waals forces, and when these weak interactions are disrupted, individual or short-stacked nanocups are obtained. These short-stacked nanocups are corked with gold nanoparticles, thereby yielding sealed nanocontainers for cargo delivery [45]. In this way a much biocompatible, sealed drug delivery system can be obtained and can be used to deliver drugs at targeted cells. In cancer immunotherapy also CNTs are used as an artificial substrate. Expansion of T cells isolated from mice was done using CNT-polymer nanocomposite, as an artificial antigen-presenting cell. The antigens were attached onto bundled CNTs and complexed with polymer nanoparticles which contained magnetite and interleukin-2 (IL-2), a T-cell growth factor. The results obtained were very promising, and the T cells obtained could delay tumor growth observed in murine melanoma model. Thus, CNT-polymer platform could generate a huge number of cytotoxic T cells which can be used for cancer immunotherapy [46].

17.2.7 Nanodiamonds

Nanodiamonds are carbon-based nanoparticles with 2–8 nm diameter having truncated octahedral structure which gives them multiple facets. These nanodiamonds are not recognized and carried out by the transport proteins which usually pump the drugs outside the cells, and thus the drugs attached to these nanodiamonds remain inside the cells. The synthesis of nanodiamonds can be done using chemical vapor deposition (CVD), detonation, or high-temperature-high-pressure process [47]. Nanodiamonds have good chemical stability, structural rigidity, octahedral symmetry, large surface area, and low production costs [48, 49]. The two types of nanodiamonds used in medical applications are detonation nanodiamonds (DNDs) and fluorescent nanodiamonds (FNDs). In cancer chemotherapy, nanodiamonds are coupled with chemotherapeutic drugs that enable sustained release of the loaded drug for a period of 1 month. Epirubicin, a chemotherapeutic drug, was attached to nanodiamonds of nearly 5 nm diameter to make a nanodiamond-epirubicin drug delivery complex (EPND) which could specifically kill the CSCs apart from killing normal cancer cells [50]. The other applications of nanodiamonds in cancer therapy are discussed by Gupta et al. [51] and Ho et al. [52].

17.2.8 Whole Cells

Drug delivery mediated by whole cells involves specific cells as vehicles for the drugs to deliver them into targeted sites. Therapeutic drugs or imaging molecules are loaded inside these cells and further released into the diseased sites. The cells usually used for cell-based therapy include leukocytes, red blood cells, stem cells, etc. and these cells act like a Trojan horse. The payload is carried inside the cells and gets transferred to the diseased tissue from the circulating blood. During this process these cells retain their original properties because of which they mimic the migration behavior of certain cells for carrying the drug to targeted site when administered in vivo [53]. Mesenchymal stem cells (MSCs) are recently being used as drug carriers as reviewed by Cheng et al. [54] apart from the use of genetically modified MSCs for the delivery of different pro-apoptotic, antiangiogenic, as well as therapeutic proteins to various types of tumors. Jiang et al. [55] have induced overexpression of CXCR4 in human adipose-derived stem cells and used these cells as a potential vehicle for targeting hypoxia in tumors. Paclitaxel (PTX), a potent chemotherapeutic drug, was successfully delivered using nano-engineered MSCs which acted as a tumor-specific drug delivery vehicle with improved anticancer efficacy compared to conventional chemotherapeutic drugs [56]. A hybrid spheroid/nanomedicine system was constructed from MSC spheroid entrapping a drug-loaded nanocomposite. The spheroid formulation increased the tumor tropism of MSCs and allowed the loading of various types of drugs. The system altogether acted as drug delivery platform tested in glioblastoma model integrating the properties of cell- and nanoparticle-mediated drug delivery along with tumor-homing features of MSCs, resulting in advanced combinational therapy for cancer [57].

17.2.9 Photodynamic Therapy

Photodynamic therapy (PDT) is a mechanism of killing cells using a photosensitizer, oxygen, and appropriate wavelength of light. The photosensitizer is specifically delivered into the cancer cells using a vehicle. The photosensitizer when activated moves to their excited state and generates reactive oxygen species (ROS) by two different ways. In primary photochemical reaction (type I), the electrons are transferred to oxygen or other molecule which forms a radical. This radical further reacts with molecular oxygen forming superoxide anion. In secondary photochemical reaction (type II), the main pathway involves energy transfer to molecular oxygen which further forms the ROS. Both type I and type II mechanisms can take place simultaneously, and the proportion of the two reactions is dependent on the photosensitizer type used, the substrate concentration, and the amount of oxygen present. If the accumulation of the photosensitizer can be made selective at the target site and there is delivery of focused light, it can reduce the damage to the normal cells and can eventually enhance PDT efficacy [58]. The destruction of cells by the reactions of PDT is mediated by either necrosis or apoptosis [59]. The commonly used

photosensitizers which can be administered intravenously for PDT are very rapidly cleared from our circulation, thereby rendering them safe for usage. However, the hydrophobic nature of these photosensitizers renders them to aggregate in aqueous solution and thereby reduces the efficacy of PDT. This drawback of the photosensitizer hinders their delivery inside our body and also causes a decrease in singlet oxygen formation due to self-quenching at the excited state [60]. Thus, to overcome this difficulty, to maintain the photosensitizer in their monomeric state, to give them protection from aqueous environment, and to increase the safety as well as efficacy of PDT treatment, different pharmaceutical carriers and drug delivery systems have emerged. These delivery systems for photosensitizer include liposomes, micelles, oil-based emulsions, polymeric nanoparticles, etc. [58].

For the eradication of cancer, PDT is used in combination with conventional therapy to yield superior outcomes, and nanoenabled therapy for cancer gives higher specificity for cancer cells, lowers side effects, and destructs the cancer cells with high efficiency both in vivo and in vitro. PDT is suitable for treating the types of cancers which cannot be cured by surgery, and moreover the nanoenabled drug delivery system can reach the CSC niche, thereby killing cancer cells and destroying the CSCs which are drug resistant. These nanomediated therapies give 100-fold high therapeutic efficiency compared to free drugs against the drug-resistant cancer cells. The different types of photosensitizers used in PDT are hematoporphyrin, photodithazine, methylene blue, curcumin, chlorins, hypericin, and phthalocyanines [61–63]. To enhance the therapeutic efficiency of the photosensitizers, improvements are being made through conjugation with other molecules. Previous applications of PDT were restricted to surface applications only because of the inapproachability of light in the deeper areas. It has been shown that PDT can also be used in deep-seated cancers including brain tumors and liver cancers using a wireless device capable of activating the photosensitizer inside the tumor [64]. Bakalova et al. have shown that the photosensitizer trifluoperazine when loaded in anti-CD90 antibody conjugated with water-soluble CdSe core-shell nanocrystals was delivered directly to CD90 + leukemia CSCs and could kill the CSCs when exposed to UV light via apoptosis [65]. The different aspects of PDT in targeted cancer therapy have been discussed by Crous et al. [66], but only future perspective of targeting CSCs has been discussed. Till now, not many potential PDTs have been developed to target CSCs.

The different treatment strategies involving nanoenabled drug delivery were discussed so far. Regarding the treatment of cancer, research is still ongoing concerning the target cells, vehicles for drug delivery, and their outcomes in combating the disease. Targeting CSCs becomes the most effective way of controlling the tumor outbreak and metastasis. The different targets that can be utilized for CSC destruction are given in Fig. 17.4.

The CSCs actually act as seed for the initiation of tumor, transition from epithelial to mesenchymal cells, and resistance to chemotherapy thereby resulting in metastasis [67]. A combination drug therapy can help in improving the clinical outcomes, by combination of inhibitors of CSC with conventional cytotoxic agents which can kill both CSC and bulk tumor cells simultaneously [68]. The combination therapy not

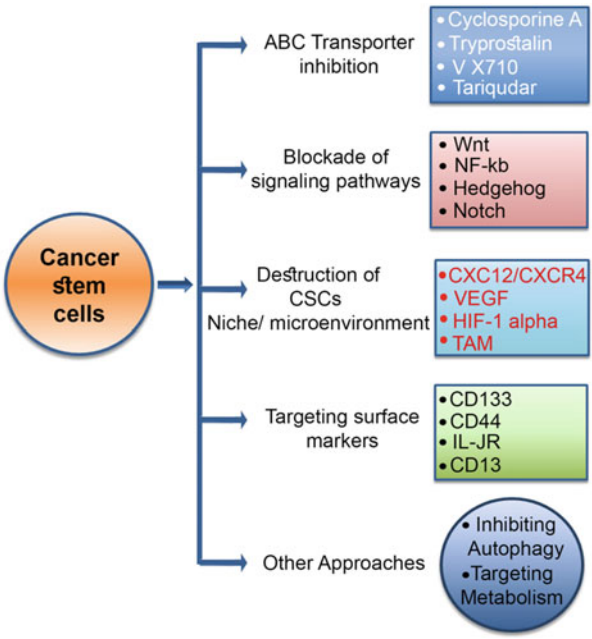

Fig. 17.4 The destruction of CSCs can be achieved by interrupting various pathways as depicted in this figure like inhibition of ATP binding cassette (ABC) transporter, blocking different signaling pathways, destruction of CSC niche, targeting the CSC markers and autophagy inhibition or targeting metabolism

only delays or suppresses the adaptation of cancer, its mutation, and progression, but it eventually decreases the individual dose and hence the side effects [69–71]. The targeting of cancer cells or CSCs can take place in two different ways:

1. Passive delivery systems: This system is based on the enhanced permeability and retention effect (EPR) in case of solid tumors. In this phenomenon, due to the increased permeability of the vasculature around the tumor tissue, low molecular weight molecules (up to 40 kDa) can enter into the tumor space, and the suppressed lymphatic filtration also allows these molecules to accumulate [72–75].
2. Active targeting system: Active targeting systems can be achieved by associating the cancer cell-specific affinity ligands to the nanostructure-based drug delivery systems [76, 77].

17.3 Nanoenabled Treatment of Cancer Stem Cells

Chemoresistance is the major cause of failure in treatment of cancer and also a common trait in the tumor-initiating CSCs. CSCs escape the chemotherapy and have enhanced tumor initiation capacity. Targeting cancer stem cells for effective therapy of cancer is being studied in the last few decades [78–80]. Targeting the CSCs using different cell markers gives a strategy for the targeted drug delivery. For example, there are several markers for ovarian cancer stem cells like epithelial cell adhesion

molecule (EpCAM), CD117 (c-kit), CD44, CD133, and aldehyde dehydrogenase isoform 1 (ALDH1) [81]. The most common cell markers of CSCs are CD44 and CD133 which are generally used for targeting the CSCs using nanovehicles. The progress in research using nanocarriers for targeting CSCs is discussed below.

17.3.1 Nanodiamonds as Drug Carriers

A nanodrug delivery platform based on nanodiamonds, to deliver epirubicin, a chemotherapeutic drug, has been used to impair the growth of tumor that is developed from chemoresistant CSCs. The nanodiamonds were attached reversibly to epirubicin through physical adsorption (nanodiamond/epirubicin ratio = 5:1) to make epirubicin drug complex (EPND). The drug complex, EPND, was characterized (size and surface charge) and found to be capable of passive targeting with enhanced permeability and retention property. The cellular uptake and cell killing capacity of EPND were monitored showing higher chemotherapeutic killing in both CSCs and normal cancer cells [50]. Previous studies have suggested that there can be covalent and noncovalent methods for functionalization of the nanodiamonds which make them more biocompatible and superior than the other carbon-based nanomaterials like SWNT, MWNT, and carbon blacks [82]. Several applications of nanodiamonds in drug delivery system for cancer have been reviewed by previous researchers [83], but very few reports exist on targeting the CSCs.

17.3.2 Polymeric Nanoparticles

Polymeric nanoparticles have also been applied for the drug delivery [84] for targeting cancer stem cells. Yang and his team [85] prepared functional micelles that were self-assembled from the mixture of polyethylene glycol (PEG) and acid-functionalized polycarbonate to make a diblock copolymer (PEG-b-PAC) and a PEG and urea-functionalized polycarbonate copolymer diblock (PEG-b-PUC) through hydrogen bonding. These synthesized micelles had high stability because of the hydrogen bond presence (urea-urea and urea-acid) and had the ability to accumulate preferably in the tumor tissues due to EPR effect [86]. They also exhibited high loading capacity for the chemotherapeutic drugs like DOX [85–87]. Phenformin is another chemotherapeutic drug, with two guanidine groups that can form hydrogen bond with urea group and can have ionic interaction with the acid group present in the micellar core. A self-assembly of PEG-b-PUC and PEG-b-PAC mixture was made and loaded with phenformin, and the drug-loaded micelles were characterized for its size, stability in serum containing solution, and drug release properties in vitro. Lung cancer cell line H460 was analyzed for its cytotoxicity using only phenformin and micelle-loaded with phenmorphin which showed promising results for the micellar form. Further the CSC population in the tumor tissue after treatment with only phenformin and micelle-loaded with phenmorphin was monitored. The

tumors posttreatment with only phenformin and micelle-loaded with phenmorphin were excised and dissociated to make a single-cell suspension and were analyzed for CD133-positive cells using flow cytometry. The results showed that free phenformin did not reduce the CSC subpopulation, whereas, the phenformin-loaded micelles could significantly reduce the CSC's population in the tumor cells compared to the control. The reason behind this may be due to the EPR effect of drug-loaded micelles in the leaky tumor tissues that led to preferential accumulation of these micelles in the tumor tissues [88]. In another study, salinomycin-loaded poly(lactic-co-glycolic acid)-polyethylene glycol nanoparticles were used for conjugation of CD133 antibodies (CD133-SAL-NP) for the purpose of elimination of CD133+ ovarian CSCs. The size of the polymer-loaded drug-antibody conjugate was 149 nm and had the property of sustained drug release with high efficient binding capacity to CD133 + ovarian cancer cells. An increased cytotoxicity was observed in CD133+ ovarian cancer cells compared to nontargeted SAL-NPs and only salinomycin. There was a reduction in the CD133+ ovarian CSCs in the ovarian cells compared to only salinomycin and SAL-NP treatment showing that the polymer-loaded drug-antibody conjugate was effective in targeting the CSCs. The nude mice were taken which bore ovarian cancer xenografts and were treated with CD133-SAL-NPs, showing enhanced therapeutic effects demonstrating that CD133-SAL-NP can be a promising target for killing ovarian CSCs [89]. Actively targeting CSCs was achieved using a multilayered core-shell polymeric nanoparticle using hyaluronic acid (HA) in place of PVA as a drug loading vehicle [90]. HA can specifically bind to CD44 antigen which is commonly overexpressed at the surface of several types of CSCs [91, 92], and the HA-decorated nanoparticles can co-deliver many drugs specifically into the CSCs. The four drugs used for co-delivery were doxorubicin hydrochloride (DOX, hydrophilic), curcumin (CUR, hydrophobic), indocyanine green (ICG, hydrophilic), and irinotecan or camptothecin (CPT, hydrophobic), using nanoparticles prepared from four polymers: pluronic F127 (PF127 with and without chitosan modification), poly(D,L-lactide-co-glycolide) (PLGA), HA, and chitosan. These polymers are approved by the US Food and Drug Administration (FDA). The combination of PLGA and PF127 yielded more uniform size and high stable nanoparticles compared to the one obtained using PF127 or PLGA alone. Chitosan was also found to bind specifically to CD44 which was overexpressed in the CSCs [93]. Drug repositioning is another strategy used for targeting the CSCs because it helps to overcome some limitations of conventional drug therapies like poor drug solubility, toxicity at off-targets, etc. A transcription factor, named STAT-3, can regulate the genes which are involved in the renewal of stem cells and has become a novel target for cancer therapy. Breast cancer stem cells' (BSCs) studies were highly correlated with STATs [94], and STAT-3 has been documented for its role in invasion, survival, and promotion of cell proliferation in tumors, immunosuppression, angiogenesis, obesity, inflammation, as well as premetastatic niche formation [95]. Moreover, STAT-3 also plays an important role as potent immune checkpoint responsible for immune response for multiple tumors that are present in tumor microenvironment for promoting tumor progression [96–99]. Thus, any therapeutic approach that can block STAT-3 can be effective in the treatment of cancer. Drug repurposing strategy was

done for delivering suitable STAT-3 inhibitor, niclosamide, incorporated in a polymeric nanoparticle (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)) and conjugated with CD44-targeting peptide yielding CD44-tagged niclosamide-loaded nanovehicles (CD44-NIC-Veh). This drug-loaded nanoparticle was used for efficient targeting of BSCs and was found to be promising in breast cancer stem cell killing capacity by altering the gene expression and protein translation. There was downregulation of CSC marker genes like MYC, BCL2, IL10, MCL1, IL11, MMP9, MUC1, EGFR, COX2, IFNG, and VEGF in the mouse xenograft tumors that were treated with the CD44-NIC-Veh in comparison to the nano-Veh-treated controls. The researchers have also found that the CSC populations were significantly decreased, as evidenced by the reduction in CD44+/CD24$^-$ expressing cell population. This showed that the "stemness" characteristics were reduced in the CSCs and the CD44-NIC-Veh could deactivate STAT-3 [100]. HA-functionalized ethylenediamine conjugated bovine serum albumin (eBSA) encapsulating all-trans retinoic acid (ATRA) (HA-eNPs) was used as a drug delivery vehicle for the targeted drug delivery to CD44-enriched B16F10 cells. In vivo imaging experiments showed that HA-eNPs could accumulate in the lungs of the tumor-bearing mouse. The ATRA-laden HA-eNPs could exert better killing ability to B16F10 cells as seen from the cytotoxicity assay compared to free drug or normal nanoparticles exposed at the same dose. Moreover, the tumor growth was inhibited significantly by HA-eNPs/ATRA as seen in the lung metastasis tumor mice. Thus, HA-eNP-loaded ATRA can be a superior drug for controlling the CSCs [101]. Active targeting of breast and colon CSCs was achieved by targeting the stem cell surface marker CD44. PLGA-co-PEG loaded with PTX micelles was used for targeted drug delivery to BCSCs and colon cancer cells showing promising results [102]. N-Isopropylacrylamide, vinylpyrrolidone, and acrylic acid polymer mixture was used in the molar ratio of 60:20:20 to encapsulate curcumin and was found to reduce the brain tumor size and also reduced the number of CD133+ stemlike cells [103, 104]. The Hedgehog (Hh) signaling pathway was interrupted by delivering GLI inhibitor through PLGA-PEG nanoparticles and showed inhibition in metastasis in hepatocellular carcinoma models [105, 106]. Anthothecol encapsulated in PLGA could alter the fate of pancreatic CSCs by inhibiting CSC proliferation and inducing apoptosis [107]. Polyethyleneimine/polyethylene glycol conjugated with mesoporous silica nanoparticles was used to load the TGF-β inhibitor, LY364947, for inhibiting the TGF-β signaling pathway of BCSCs and also to deliver siRNA to the CSCs. These nanopolymer-based delivery of siRNA caused the accumulation of the siRNA in the tumor and reduced the CSCs [108, 109]. Targeting the different CSC killing pathways using nanoenabled drug delivery is discussed in detail by previous researchers [7, 110–113].

17.3.3 Liposomal Nanocarriers

Liver cancer stem cells (LSCs) are responsible for the initiation of liver cancer, invasion, recurrence, metastasis, and further chemoresistance. Like other cancers,

targeting LCSCs using nanoenabled drug delivery can show some insight in liver cancer treatment and prevent their recurrence [114]. Nanoliposomes were prepared using a lipid mixture of hydrogenated soybean phospholipids (HSPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(PEG)-2000] (PEG-DSPE) in the ratio of 85:10:5. The nanoliposomes were used to make salinomycin-loaded nanoliposomes (SLN), doxorubicin-loaded nanoliposomes (DLN), as well as a combination of salinomycin and doxorubicin (SDLN) for targeting both normal liver cancer cells and LSCs. The mole ratio of DOX/salinomycin sodium at 1:1 had the optimum synergistic combination index value, and the same ratio was taken in SDLN. The percentage of LSCs in vivo was significantly decreased after treatment with SDLM and SLN + DLN post 12 h treatment [115]. Liposomal nanoformulations for targeting the prostate cancer cells and prostate CSCs have been engineered using cabazitaxel (CBX)- and silibinin (SBL)-loaded liposomes made from cationic phospholipid *N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium chloride (DOTAP) and cholesterol. For specifically targeting prostate CSCs, HA was coated atop the cationic liposome that had affinity for the CD44 cell surface receptors overexpressed in CSCs. The in vitro results showed that these surface-functionalized liposome-encapsulated drugs could exert specific cytotoxicity against CD44+ cells. Thus, the results showed the potential of CBX-SIL co-loaded liposomes for eradicating prostate cancer stem cells [116].

17.3.4 Exosomes as Drug Cargo

Exosomes are natural nanovehicles derived from cells and are widely distributed in body fluids for the cell-cell communication. They are involved in multiple diseases, including cancer, and they contain receptors above their lipid bilayer membrane. They carry lipids, proteins, miRNAs, mRNAs, and small DNA fragments within them to protect the degradation of these molecules [117–120]. The exosomes have specific surface markers like TSG101, Alix, Flotillin-1, CD63, and CD9 and are present in different cell culture-conditioned media as well as body fluids like saliva, synovial fluid, urine, semen, blood, and breast milk [121–124]. Different treatment strategies have been proposed for controlling the proliferation and differentiation of CSCs. Since the cell surface marker, CD44, is highly expressed in hepatic CSCs, it has been targeted for liposomal drug delivery to control hepatic CSCs [125], and exosomal delivery of anti-CD44 antibody can be a future aspect for targeting the CSCs. Similarly other CSC markers like CD24, CD133, epithelial cell adhesion molecule (EpCAM), and CD200 can also be attached to the surface of the exosomes for targeting the CSCs. Multiple clinical trials are going on for targeting the cancer stem cells using exosomes as nanocarrier [126].

17.3.5 Nanoporous Materials

Mesoporous silica is the most commonly used nanoporous materials and is synthesized using tetraethyl orthosilicate which reacts with micellar rod templates. The porous form of silica thus synthesized is a collection of rods or spheres of nano-size filled with numerous pores arranged regularly. They are usually of two types, MCM-41 and SBA-15. Mesoporous silica based-nanoparticles, encapsulating γ-secretase inhibitors (GSIs), were used to control the notch signaling driven stem cells and enhance the tumor reduction in medulloblastoma [127]. Notch signaling inhibitors were loaded in mesoporous silica to deliver the drug in BCSCs which are susceptible to more glucose consumption. The results showed reduction in CSC population as well as size of the tumor both in vivo and in vitro [128]. The typical porous nature of mesoporous silica is shown in Fig. 17.5.

Thus, the different nanoenabled drug delivery systems used so far for targeting the CSCs have been discussed, and the outcome of such treatment strategies was also reviewed in the above sections.

17.4 Conclusion

The major problem in addressing the chemoresistance and multidrug resistance in cancer therapy is the inability to combat the CSCs through any drug. These CSCs migrate to a different site and initiate different tumors, thereby spreading the cancer. This warrants the need of targeting specifically the CSCs and delivers the chemotherapeutic drug to these populations and killing them. The CSCs have been known to exhibit different cell surface biomarkers as well as pathways of internal signaling which are involved in their self-renewal and the drug resistance. CD44 and CD133 are commonly identified in many cancer types. If these biomarkers can be targeted using some novel drug delivery system, it can improve the CSC killing thereby improving the drug resistance, eradication of CSCs, and possible cure for cancer.

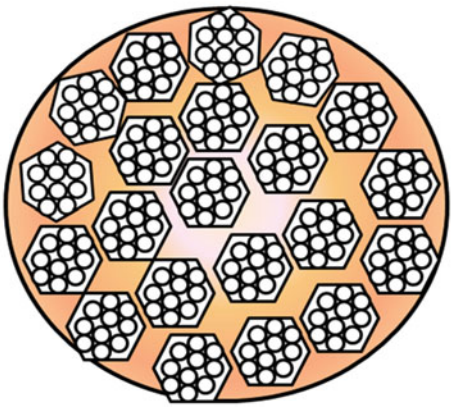

Fig. 17.5 The porous structure of mesoporous silica which enables the drug to be loaded in the small pores to reach the target site

Nanostructure-based therapeutic strategy has been recently evolved for effective cancer treatment due to their specific properties like (1) sustained drug release, (2) designing and development of personalized medicine, and (3) improved bioavailability of drugs and use in multifunctional therapy. In this chapter we have discussed about the different nanoenabled drug delivery systems and their possible therapeutic research done so far to target the CSCs. The nanodrug delivery systems could deliver a single or multidrug to the CSCs with particular biomarker targeting molecules attached to their surface for the CSC targeting. The targeting of CSCs was done using small chemical ligands, peptides, lipids, polysaccharides, and surface markers which have selective affinity for the CSCs and attaching them with the nanocarrier along with the chemotherapeutic drug. Both active and passive targeting were used to eradicate the CSCs. Thus, the different targeting strategies can enable to wipe out the CSCs and open a new avenue in the near future for the cancer treatment.

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Cancer Stem Cells in Patient Survival and Therapies in Cancer

18

Ying Yang, Chao Tian, and Wen-Jian Meng

Abstract

Cancer stem cells (CSCs) are a subpopulation of cancer cells and responsible for stemness properties of cancer cell. It is regarded as one of the major causes of cancer formation, recurrence, and metastasis. Recent studies demonstrated that CSCs are closely related to the prognosis and treatment of many tumors including lung cancer, colorectal cancer, breast cancer, gastric cancer, and melanoma by targeting cell surface markers, signaling pathways, and microRNAs (miRNAs) to affect stemness features of CSCs. In addition, the application of nanotechnology in CSCs also makes it a novel and potential target in therapy of tumor. However, given the limitations of CSCs as mentioned in this paper, its clinical applications as a target of cancer face many challenges. Further research is needed to explore its clinical application as a target for tumor therapy.

Keywords

Cancer stem cells · Cell surface markers · Signaling pathway · MicroRNA · Drug resistance · Survival

Abbreviations

5FUR	5-Fluorouracil-resistant
ABC	ATP-binding cassette

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ABCB	ATP-binding cassette subfamily B
ABCB5	ATP-binding cassette subfamily B member 5
ALDH	Aldehyde dehydrogenase
ALDH1A1	ALDH1 family member A1
ATRA	All-trans retinoic acid
BCRP	Breast cancer resistance protein
BCSCs	Breast CSCs
CAPE	Caffeic acid phenethyl ester
CDDP	Cisplatin
CDF	Difluorinated-curcumin
CRC	Colorectal cancer
CSCs	Cancer stem cells
CSLCs	Cancer stemlike cells
CTCs	Circulating tumor cells
CTL	Cytotoxic T lymphocytes
CTNNBIP1	Catenin beta interacting protein 1
DCs	Dendritic cells
DFS	Disease-free survival
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EpCAM	Epithelial cellular adhesion molecule
GCSCs	Gastric CSCs
GSI	Gamma secretase inhibitors
HA-SLNs	Solid lipid nanoparticles with hyaluronan
HH	Hedgehog
HHAT	HH acyl transferase
HIF	Hypoxia-inducible factor
HMGA2	High mobility group AT-hook 2
LAC	Lung adenocarcinoma
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
LSCC	Lung squamous cell carcinoma
LSD1	Lysine-specific demethylase 1
MAPK	Mitogen-activated protein kinase
MDR1	Multiple drug resistance
miRNA, miR	MicroRNA
MRP	Multidrug resistance-associated proteins
mTOR	Mammalian target of rapamycin
N2IC	Notch2 intracellular domain
NSCLC	Non-small cell lung cancer
OPN	Osteopontin
OS	Overall survival
PAF	Proliferating cell nuclear antigen-associated factor
PCNA	Proliferating cell nuclear antigen
P-gp	P-Glycoprotein
PRKCI	Protein kinase C iota

PTX	Paclitaxel
Rh123low	Low rhodamine 123
ROS	Reactive oxygen species
SCLC	Small cell lung cancer
SDCSCs	Spheroid-derived CSCs
SIRT1/2	Sirtuin 1 and 2
Smo	Smoothened
SP	Side population
STAT3	Signal transducer and activator of transcription 3
TAM	Tumor-associated macrophage
TNBC	Triple-negative breast cancer
VCR	Vincristine
VEGF	Vascular endothelial growth factor
VM	Vasculogenic mimicry

18.1 Introduction

Cancer stem cells (CSCs) are a minor population of tumor cells with the properties of self-renewal and differentiation, as well as tumorigenic potential. [1] The accumulated evidence suggests that CSCs play an imperative role in metastases, posttreatment recurrence, and resistance to chemoradiotherapy in cancer [2], which closely associated with the worse survival of cancer patients. Meanwhile, these properties also make CSCs become a potential therapeutic target for cancer treatment.

Previous studies have shown that conventional chemotherapy and radiotherapy could not completely eliminate CSCs in cancer patients, resulting in treatment failure. The reason is that CSCs possess the properties of slow cell cycle, detoxification or regulation of cytotoxic outflow, resistance to oxidative stress, and rapid response to DNA damage [3, 4].

Recently, several methods available to target CSCs including specific surface markers of CSCs, specific signaling pathways, tumor microenvironment, or specific microRNA (miRNA, miR) have been reported by accumulating researches. For specific surface markers of CSCs, CD44, CD133, CD24, and ALDH1 are routinely used to identify and validate CSCs [5]. Another approach could be targeting CSCs by their specific signaling pathways. Key cell signaling pathways include Notch, Wnt/β-catenin, hedgehog (HH), human epidermal growth factor receptor (EGFR), and so on [6]. These signaling pathways are of crucial importance in CSCs. Other treatment strategies include targeting the tumor microenvironment or specific microRNA (miRNA, miR). MiRNAs participate in many vital biological processes including cell proliferation and migration, tumor cell aggression, and metastasis. At present, emerging evidences suggest that miRNAs play a critical role in CSCs [7, 8]. Of course, the CSC-targeting strategy certainly goes beyond these methods above.

Therefore, in this article, we have summarized recent advancement on the therapeutic and prognostic role of CSCs in different types of cancer including lung cancer, colorectal cancer (CRC), breast cancer, gastric cancer, and melanoma.

18.2 CSCs in Lung Cancer

Primary lung cancer remains one of the most common malignant tumors, and deaths from lung cancer exceed those from any other malignancy worldwide [9]. A growing number of studies have shown that CSCs exhibited important roles in driving initiation, metastasis, recurrence, and resistance to conventional therapy of lung cancer. Hence targeting CSCs of lung cancer may provide a promising approach to improve the survival and therapies of lung cancer patients in the future.

18.2.1 Targeting Cell Surface Markers of CSCs

Many cell surface and transmembrane proteins are expressed in CSCs of lung cancer including CD24, CD133, ALDH, and so on. These surface markers could not only identify CSC population in lung cancer but also act as an approach to target CSCs.

Elevation of CD133+ and CD24+ cells has been found to be closely related to poor prognosis of cancer treatment. Stem cell surface marker CD24 has been considered as a novel prognostic marker and stem cell marker in non-small cell lung cancer (NSCLC). Overexpression of CD24 was reported to associate with tumor patients with a higher risk of disease progression and tumor-related death. In addition, this study also showed that the overexpression of CD24 could be used as an independent predictor for poor progression-free survival and tumor-specific survival in patients with NSCLC [10]. Therefore, CD24 antigen could provide numerous crucial information to research the biology of NSCLC and may be used as a beneficial tool to promote the development of novel diagnostic and therapeutic ways to eradicate CD24 function in tumor cells. As a specific marker of human hematopoietic stem cells, CD133 has been considered as a marker of CSCs in numerous cancers. In NSCLC, CD133+ cells possess a higher tumorigenicity ability compared with CD133− cells and express genes which confer to cancer cells the property of stemness, adhesion, motility, and drug efflux. And importantly, CD133+ cells of lung tumor are spared by cisplatin treatment [11].

The aldehyde dehydrogenase (ALDH) is another important surface marker of lung cancer with stem cell properties. Compared with the ALDH-CSC population, ALDH+ CSCs show longer telomeres. MST312, a novel telomerase inhibitor, plays an antiproliferative role in lung CSCs and possesses the characteristic of inducing tumor population apoptosis. The previous study demonstrated that MST312 possesses potential antitumor properties in NSCLC in vivo (mouse model): intraperitoneal injection of MST312 (40 mg/kg/day) can reduce the tumor size by more than 70%. In addition, at the end of in vivo treatment, immunohistochemical analysis of ALDH and fluorescence-activated cell sorting analysis of ALDH after removal of

the tumor showed that the population of CSCs was also significantly reduced. Thus, as a antitelomeric therapy mainly by targeting lung CSCs, MST312 may prove to be effective in treating lung cancer [12]. ALDH1-positive CSCs showed stronger proliferative ability, cloning efficiency, and tumorigenicity. And ALDH1, a marker of CSCs, may be employed as a target for the therapy of lung cancer in the future [13]. Huang et al. [14] showed that compared with ALDH1 family member A1 (ALDH1A1)-negative lung cancer cells, lung cancer cells with ALDH1A1-positive possess the property of resistance to gefitinib. Clinical sample studies showed that a significant increase in the proportion of ALDH1A1-positive cells was observed in lung cancer cells that resist to EGFR-tyrosine kinase inhibitor and chemotherapy agents. In addition, a higher proportion of ALDH1A1-positive cells was shown in PC9/gef cells (lung cancer cells showing resistance to gefitinib), compared with lung cancer cells which were sensitive to gefitinib. Another study showed that the expression level of ALDH1A1 was significantly correlated with the poor prognosis of patients with stage I and N0 disease. ALDH tends to select NSCLC stemlike cells with stronger tumorigenicity and self-renewal ability, and those NSCLC carrying tumor cells expressing ALDH1A1 tend to have a worse outcome [15].

18.2.2 Targeting Signaling Pathways of CSCs

Any deregulation of CSC-related signaling pathway will activate CSCs, which eventually results in formation, recurrence, and metastasis of numerous cancer including lung cancer [16]. Dysregulation of various signaling pathways in CSCs is expected to be a novel potential therapeutic target for human cancer.

18.2.2.1 Hedgehog Signaling Pathway

In lung cancer, hedgehog (HH) signaling pathway was found to increase drug resistance in patients and eventually leads to the failure of chemotherapy. Mutations of HH signaling pathway play a critical role in promoting tumorigenesis and activation of CSCs, thereby resulting in lung cancer. A study involving genetically engineered mice showed that the activation of Smoothened (SMO), the component of HH signal molecules, could not only contribute to the formation of cloning in human small cell lung cancer (SCLC) in vitro but also promote the occurrence and development of mouse SCLC in vivo. Furthermore, the key cell-intrinsic role of HH signaling in the progression and maintenance of SCLC has been explored, as Park KS et al. demonstrated that the use of SMO antagonists could suppress the development of SCLC in mice and humans, especially after chemotherapy. And the inhibitor of HH pathway may be a novel potential therapeutic target for human SCLC patients to slow down the further deterioration of the disease and delay the relapse of cancer [17]. GDC-0449, a HH inhibitor, effectively reduces cell growth of SCLC and enhances the inhibitory effect of cisplatin on the growth of lung cancer cells [18]. At present, there is a lack of effective targeted therapy in lung squamous cell carcinoma (LSCC). A study shows that GANT61, a HH-GLI inhibitor, could effectively induce apoptosis of LSCC cells, suggesting that inhibition of HH-GLI

may be employed as a new and effective strategy for the treatment of some patients with LSCC [19]. Protein kinase C iota (PRKCI)-mediated SOX2 is required for HH acyl transferase (HHAT) to initiate and activate the HH pathway. Justilien et al. [20] reported that PRKCI-SOX2-HH signaling pathway is crucial to maintenance of CSC in LSCC. These findings offer a strong rationale for HH inhibitors for treatment of LSCC.

18.2.2.2 Notch Signaling Pathway

Notch signaling pathway is key to maintain a cancer stem or progenitor cell compartment, which is necessary for tumorigenesis in lung cancer.

Notch signaling is one of the most activated pathways in cancer cells and crucial for the correlation between self-renewal of CSCs and angiogenesis. In addition, the growth of lung cells is regulated by it via controlling the fate of normal stem cells. By testing the effect of Notch1 blocking on the growth and viability of lung CSCs, Cai et al. [21] observed that CD44+/CD24– cells isolated from A549 cells possessed stem cell-like properties with high expression of Notch1 and blocking Notch1 by inhibitor DAPT (GSI-IX) or siRNA, both inhibiting the growth capacity of lung CSCs. In a study by Liu et al. [22] in 2014, the difference of Notch signal expression between CD133+ and CD133- cells was compared in the same human lung adenocarcinoma cell line A549 with CD133 as the marker of stem cells. And in these two cells above, the effects of DAPT combined with cisplatin (CDDP) were detected and compared. The results showed that notch signaling pathway members (Notch1, Notch2, and Hes1) were low expressed in CD133+ cells, and significant drug resistance to CDDP was found in CD133+ cells. Moreover, after combined application of GSI, the inhibitory effect of CDPP was significantly enhanced in both cells above, particularly in CD133+ cells. These findings suggest that the inhibitor of Notch pathway is expected to be a potential therapeutic target for lung cancer. Furthermore, the previous studies [23, 24] showed that the combination of the inhibitor of Src-YAP1, EGFR, and signal transducer and activator of transcription 3 (STAT3) could provide an inhibitory effect beyond its application alone or double in vivo, indicating the importance of combined treatment. Recently, they further investigated whether the expression of CSCs and EMT markers and the activity of ALDH were affected by the inhibition of EGFR. The results showed that combined inhibition of EGFR, STAT3, and Src significantly reduced CSC subsets in the cell model of EGFR mutation. Thus, a single inhibitor of EGFR may increase the number of CSCs; on the contrary, its combination with targeted Src and STAT3 might be beneficial to the treatment of lung cancer [25]. Taken together, these findings suggested that for the treatment of lung cancer, a single inhibitor of signal pathway is insufficient and it would further drive activation of parallel signal pathways, thereby leading to the failure of treatment. In contrast, combined therapy might be beneficial to the treatment of patients, especially for those NSCLC patients with positive EGFR mutations.

18.2.2.3 Wnt/β-Catenin Signaling Pathway

In a 2012 study, trifluoperazine showed an ability to inhibit the generation of CSC tumor sphere and decrease the expression level of CD44/CD133 (CSC markers). It inhibited Wnt/β-catenin signaling in lung cancer sphere with resistance to gefitinib. Furthermore, in animal models of lung cancer metastatic and orthotopic CSC, trifluoperazine has been found to inhibit the development of tumor and increase the sensitivity of gefitinib. Combined application of trifluoperazine, gefitinib, or cisplatin may effectively increase the sensitivity of lung cancer to it [26].

18.2.3 Targeting the miRNAs

Accumulating evidence suggests that as a key regulatory molecule of lung CSC-related metastasis, drug resistance, and tumor self-renewal, miRNA can effectively regulate numerous signal pathways, which participate in the regulation of proliferation, differentiation, apoptosis, cell cycles, and immune response of lung CSCs.

MiRNA plays a critical role in regulating lung CSCs, and these CSCs are closely related to the obstacles in cancer treatment including recurrence, metastasis, and drug resistance of cancer. For instance, as a tumor suppressor gene, miRNA-34a inhibits abnormal cell growth of malignancies including lung cancer [27]. The low expression level of miR-34a is key to promoting the invasiveness of lung CSCs. Moreover, with the recovery of miR-34a activity and the generation of exogenous delivery, this invasive property will also be reduced [28]. Thus, the recovery of miR-34a activity might be employed as an effective strategy for tumor treatment via downregulating the expression of Notch target genes or family members. Qi et al. [29] demonstrated that miR-214 suppressed the expression of catenin beta interacting protein 1 (CTNNBIP1), which also elucidates the mechanism of activation of Wnt/β-catenin signal in lung adenocarcinoma (LAC) tumor stem cells. Moreover, the expression level of CTNNBIP1 is also proportional to the differentiation of cancer cells and could be used to predict the survival of LAC patients. Thus, identifying miR-214-CTNNBIP1 pathways with the ability to regulate the self-renewal and stemness of CSLCs is expected to become a new strategy for the treatment of LAC patients. Recently, Dai et al. [30] identified the important role of miR-150-5p in the recurrence and metastasis of NSCLC. The result showed that the significantly low expression of miR-150-5p was observed in CSCs compared with non-CSCs. Furthermore, there was a significant positive correlation between the low expression of miR-150-5p and the disease deterioration and poor prognosis of NSCLC patients. The suppression of miR-150-5p would lead to the increase of CSC population, stemness, and metastasis of NSCLC cells. On the contrary, the high expression of miR-150-5p would markedly suppress the relapse, metastasis, and tumorigenesis of CSCs via targeting high mobility group AT-hook 2 (HMGA2) and β-catenin signaling in NSCLC. These results showed that as an inhibitor of CSC, the upregulation of miR-150-5p could be expected to a novel potential approach for the inhibition of CSC-induced metastasis and relapse in NSCLC. Taken together, these

findings suggested that miRNAs could significantly affect the biological behavior of lung CSCs by regulating the signaling pathways of LCSCs.

However, targeting CSCs in lung cancer would be a challenge due to heterogeneity of the cells and various genomic pathways involved. Therefore, many studies are focusing on using combination of cellular markers as it increases the specificity of targeted population.

18.3 CSCs in CRC

CRC is one of the most common and fatal malignant tumors in the world. It puts great pressure on medical and health care in all countries, and its incidence is gradually increasing [31]. The metastasis and recurrence in patients with CRC are known to be the main cause of failure of CRC treatment and ultimately leads to the worse prognosis. If we can early detect metastasis of CRC and take appropriate intervention before the disease progression, we can greatly improve the prognosis of CRC patients. Accumulating evidence suggests that CSCs participate in tumor formation, recurrence, metastasis, and resistance to chemoradiotherapy, which might play a crucial role in CRC. In fact, failure to completely eradicate CSCs is a major reason for the failure therapy of cancer [32]. It has been reported that the eradication of CSCs would be useful in increasing CRC patients' survival rates [33]. Thus, colorectal CSC markers can act as effective prediction factor and therapeutic targets.

18.3.1 Targeting Cell Surface Markers of CSCs

CSCs express some specific cell surface macromolecules that can be used for its identification and separation, and these macromolecules or markers can also provide a feasible target for scavenging CSCs. Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), an identification mark of CSCs, is crucial to the development of tissue and the maintenance of adult stem cells in gastrointestinal system. LGR5 overexpression has been reported to be closely related to lymphatic invasion, lymph node metastasis, vascular invasion, tumor depth, and tumor stage in patients with CRC. High level of LGR5 expression was linked to poor disease-free survival (DFS) for CRC patients [34, 35]. Further study [36] demonstrated that the inhibition of LGR5 cell such as selective ablation would suppress the development of primary tumor, but not lead to the regression of tumor. Furthermore, CSCs play a key role in the generation and maintenance of liver metastasis derived from CRCs, which indicate that CSCs may be expected to become a novel potential target for the treatment of metastatic cancers. In addition, Shimokawa and colleagues [37] showed that LGR5-positive (LGR5+) CRC cells could be acted as CSCs in growing cancer tissues. And significant regression of the tumor was observed after eradicating LGR5 + CSCs through ablation in LGR5-iCaspase9 knock-in organoids. Interestingly, the reemerging LGR5 + CSCs are shown to contribute to tumor regrowth after

LGR5 + CSCs' ablation. A previous study [38] showed that LGR5 is effective in the treatment of gastrointestinal tumors, especially colon cancer using different antibody-drug conjugates. Several studies have indicated that LGR5 has been shown to relate to increased drug resistance in gastrointestinal tumors. And in primary colon tumors, overexpression of LGR5 was shown to associate with chemoresistance and lower DFS and overall survival (OS) [39–43]. Hence, LGR5 is expected to be a new prognostic indicator and a potential target for the therapy of CRC. Nevertheless, the mechanism of LGR5 which participates in the tumorigenicity of CRC is not completely understood; therefore, larger, higher-quality studies are needed to illustrate the role of LGR5 in CRC.

CD133, CD24, and CD44 are cell surface markers which have been shown to be linked to stem cells, as well as aggressive cancer types and poor prognosis in CRC. Jing et al. [44] reported that CD44 might be used as an effective indicator to predict liver metastasis and prognosis of colon cancer patients. Du et al. [45] found strong inhibitory effect of knockout CD44 on clone formation and tumorigenicity of xenografts, indicating that CD44 is a robust marker and key to the initiation of tumor. Rao et al. [46] have illustrated that tumor correlated with macrophage (TAM) interacts with CD44-positive cancer cells and secrete osteopontin (OPN) which in turn promoted the clonogenicity and tumorigenicity of CRC. Moreover, tissue microarray data showed that the expression of OPN and CD44v6 (an OPN functional receptor) was inversely related to the survival of patients with CRC. These findings revealed that interaction between OPN and CD44 is crucial to the development of CRC and might be employed as a promising therapeutic target in CRC. However, large-scale, higher-quality studies (such as prospective trials) are needed to verify the results of these studies. Sahlberg et al. [47] reported that overexpression of CD133/CD44 was proportional to the increase of resistance to radiation in colon cancer cells. Liu et al. [48] reported that combined treatment of paclitaxel and siRNA-targeted CD133 cells could effectively reduce the expression of multiple drug resistance-1 (MDR1) in human colon CSCs (CD133+ enriched cell population), which could markedly reduce the resistance to paclitaxel. Jao et al. [49] reported that the overexpression of CD133 was markedly proportional to local relapse and prognosis of patients and could be used as an effective prognostic marker for tumor regression grading in rectal cancer patients after neoadjuvant concurrent chemoradiotherapy. Kanwar et al. [50] observed that difluorinated-curcumin (CDF) combined with 5-fluorouracil and oxaliplatin could effectively inhibit proliferation and induce apoptosis of CSCs via decreasing CD44 and CD166 drug-resistant colon cancer cells, suggesting that the combination of CDF with 5-fluorouracil and oxaliplatin is expected to be a reliable therapeutic approach to inhibit the drug resistance of colon cancer cells via eradicating CSCs. Excepting for the above cell surface markers, ST6Gal-I may also be a promising marker of CSCs. Swindall et al. [51] suggest that ST6Gal-I promotes tumorigenesis and plays a crucial role in maintaining behavior of stemlike cell; thus it might be employed as therapeutic target. Lugli et al. [52] reported that loss of membranous CD44s, CD166, and epithelial cellular adhesion molecule (EpCAM) was linked to tumor progression, invasiveness, and infiltrating tumor growth pattern. Therefore, CD44s, CD166, and

EpCAM possess the potential to predict the survival of CRC patients. Furthermore, Xiang et al. [53] developed a novel therapeutic approach based on the combination of EpCAM aptamer (a new drug delivery system) with doxorubicin which is able to target surface molecules of CSCs. This approach can effectively inhibit the growth of tumor and a prolonged longer tumorigenic latency, thereby improving the prognosis of CRC patients. Wang et al. [54] established a novel therapeutic strategy based on chitosan vesicle entrapment of oxaliplatin, which could eradicate tumor cells and CSCs (more effective than free oxaliplatin) and might be a novel strategy for treatment of CSCs. Recently, ALDH1 is one of putative CSC marker in CRC. Kahlert et al. [55] found that ALDH1 nuclear expression related to shorter survival of patients with CRC. Furthermore, Deng et al. [56] reported that high postoperative ALDH1 expression predicts the recurrence, distant metastasis, and poor prognosis for CRC patients who received neoadjuvant therapy. Also, Goosssens-Beumer et al. [57] suggested that co-expression level of ALDH1, survivin, and EpCAM was a reliable prognostic indicator to predict risk of recurrence and survival of colon cancer patients. However, further validation about these conclusions in clinical trials is warranted.

18.3.2 Targeting Signaling Pathways of CSCs

It has been shown that various signaling pathways including Wnt and Notch can control growth, differentiation, migration, and response to drug treatment of CRC by regulating CSCs. Therefore, targeting CSCs through signal pathway is a potential therapeutic strategy for CRC, but only taking such treatment does not make an effective approach at least today. The combination of conventional therapies such as radiotherapy and chemotherapy and the inhibitor of CSC-specific pathway possesses the potential to improve cancer cure compared with monotherapies [58].

Overexpression of BMI1, a signaling pathway of CSCs, induces tumor progression and metastasis and contributes to the self-renewal of CSCs. Depletion of BMI1 cancer cells can lead to suppression of CSC self-renewal. [59] The STAT3 pathway is crucial to regulate CSC self-renewal, and suppression of this pathway will lead to a decrease in the number of CSCs. Lin et al. [60, 61] reported that CD133/ALDH(+) CSC cell population expressed higher level of pSTAT3, and the effects of STAT3 inhibition in colon CSLCs were examined, which indicate that inhibition of STAT3 in CSLCs might provide a potential therapeutic strategy for CRC. And then they found that GO-Y030 acted as inhibitor of STAT3 phosphorylation and therefore inhibited colon CSCs. Napabucasin, an inhibitor of tumor stemness by targeting STAT3, possesses the ability to inhibit the recurrence and metastasis of numerous cancers [62]. A phase III clinical trial recently aimed to test napabucasin in advanced CRC showed that STAT3 may be a promising target for the therapy of CRC with elevated phosphorylated STAT3 (pSTAT3) expression [63]. Overactivation of Wnt signaling is the main reason for the pathogenesis of CRC. In colon cancer, the suppression of HOXA5 by the Wnt pathway maintains stemness of CSCs, and its

reexpression induces loss of the CSC phenotype, which will prevent tumor progression and metastasis [64].

A previous study revealed that HH-GLI1 was crucial to promote the development, metastasis, and self-renewal of stem cells in advanced colon cancers. Therefore, targeting HH-GLI1 may be used as a therapeutic strategy to reduce tumor size and metastases of colon cancer and eliminate colon CSCs [65]. These results suggest that HH-GLI1 signaling pathway played a key role in the formation of colon CSCs and may be a potential therapeutic strategy for colon cancer, especially for those with refractory and metastatic characteristics.

18.3.3 Targeting the miRNAs

A growing body of evidence suggests that miRNAs are closely related to the invasiveness and metastasis of CRC. And the aggressiveness and stemness of CRC remain a major cause for relapse and metastasis of CRC. Hongdan et al. [66] reported that miR-3210-5p could increase the characteristics of aggressiveness and stem cell-like in colon cancer via decreasing the expression of Axin2 (a regulator of Wnt signaling).Therefore, targeted inhibition of miR-3210-5p may be a promising therapeutic strategy to improve the prognosis of patients with colon cancer. Zhai et al. [67] showed that high expression of miR-140-5p was significantly correlated with the low expression of Smad2 in CRC cell lines, which would lead to the decrease of cell proliferation and invasiveness and the increase of cell cycle arrest. Furthermore, overexpression of miR-140-5p in CSCs abolished tumor formation and metastasis in vivo. The functional and clinical significance of miR-140-5p shows that it can regulate the metastasis and progression of CRC, as well as it has the ability to be a new therapeutic target for CRC in the future. Huang et al. [68] revealed that tRF/miR-1280, a 17-bp fragment derived from tRNA and pre-miRNA, affected Notch signaling pathways supporting the role of CSLCs in CRC progression. They have reported that tRF/miR-1280 could inhibit the metastasis and development of CRC via suppressing Notch signaling pathways. Furthermore, they demonstrated that miRNA with functional activity could be obtained from tRNA, which undoubtedly provides another promising biomarker for the treatment of CRC. A study showed that miR-34a directly inhibited Numb in early-stage colon CSCs and deletion of miR-34a will enhance CSC properties in colon cancer [7].

Colon CSCs have been identified as one of the main reasons for the resistance of colon cancer to chemotherapy. MiRNA is crucial to the progression of colon CSCs and might contribute to reducing drug resistance and increasing sensitivity to chemotherapy [8]. It has been shown that the expression of miR-451 leads to the decrease of colonic bulb self-renewal and tumorigenicity and the increase of its sensitivity to irinotecan by reducing the expression of ABCBA1 (an ATP-binding cassette drug transporter). The above results revealed that miR-451 may be used as a novel marker to predict the relapse and chemoresistance of CRC, especially the response of colon cancer to irinotecan [69]. A study by Xu et al. [70] in stem cell-like side population (SP) cells in CRC showed that the high expression of miR-328 could

improve the chemoresistance and suppressed the invasiveness of SP cells. These findings indicate that miR-328 plays a crucial role in maintaining cancer stemlike SP phenotype and might be a promising target for the treatment of CRC.

18.3.4 Other Strategies Targeting CSCs

Conventional therapies, such as radiotherapy and chemotherapy, play a predominant role in the management of patients with advanced-stage CRC. However, the inherent or acquired chemo- and radiation resistance of CRCs results in failure of treatment, which is partly on account of the enrichment of CSCs with resistance to conventional therapy.

A recent study shows that the combination of MST-312 (a telomerase inhibitor) with flavonoid morin can decrease the stemness of CSCs. In addition, co-treatment of the two drugs above can also enhance the therapeutic effect of 5-FU [71]. Therefore, the combination of flavonoid morin and MST-312 can be used as a novel therapeutic target to improve the prognosis of tumors. Recently, epigallocatechin-3-gallate (EGCG), an active catechin present in green tea, has been found to possess the ability to inhibit the development of CSC in a variety of tumor. Toden et al. [72] reported that EGCG could increase the efficacy of 5-FU and inhibit tumor proliferation in 5-fluorouracil-resistant (5FUR) CRC cell lines. EGCG treatment in these 5FUR cells leads to the inhibition of spheroid-derived CSCs' (SDCSCs) generation and increased sensitivity of 5-FU to SDCSCs. The above findings provide a support for EGCG to enhance the sensitivity of 5-FU by targeting CSCs of CRC and highlight the potential of EGCG as an adjuvant therapy for conventional chemotherapy in patients with CRC.

18.4 CSCs in Breast Cancer

A growing number of studies demonstrated that the formation of breast cancer was thought to be driven by CSCs. Today, acquired and inherent resistance to radiotherapy and chemotherapy represents a main obstacle in therapy of breast cancer. Breast CSCs (BCSCs) not only drive tumor formation, recurrence, and metastasis of cancer but also mediate therapeutic resistance [73]. Thus, developing strategies with effective targeting BCSCs might be beneficial to control tumor relapse, increase DFS, and increase sensitivity to conventional therapy such as chemoradiotherapy.

18.4.1 Targeting Cell Surface Markers of CSCs

Doxorubicin can achieve the therapeutic effect of scavenging BCSCs by decreasing the expression of CD44 in vitro. Moreover, decreased expression of CD44 will increase the sensitivity of CD44+CD24− breast cancer cells to doxorubicin [74]. Interestingly, another study [75] showed that knockout of the CD44 gene

makes BCSCs become non-BCSCs with less tumorigenicity, which changes the cell cycle and some CSC-related gene expression, leading to the loss of stemness and enhancing response to conventional therapy.

The malignant potential of triple-negative breast cancer (TNBC) is also relied upon a subpopulation of BCSCs. CD133 and EpCAM, two BCSC markers, are significantly related to invasiveness of breast tumors, indicating that combined therapy targeting two surface molecules at the same time might be an effective strategy for the therapy of TNBC [76]. GD2 (a glycosphingolipid) is a new CSC-specific cell surface marker. As a key enzyme of GD2 synthesis, GD3 synthase might be a promising therapeutic target for CSCs and may improve the prognosis of breast cancer patients. Furthermore, complete knockout of GD3 could eliminate the formation of tumor in vivo [77]. Hence, developing a therapy approach targeting CSCs may be beneficial to the treatment of patients with breast cancer.

18.4.2 Targeting Signaling Pathways of CSCs

A recent study has revealed that the noncanonical hedgehog inhibitor GANT61 could reduce not only the development of cells but also the number of CSC in triple-negative breast cancer cells and GANT61 could enhance the inhibitory effect of paclitaxel on the growth of these cells [78]. Likewise, GANT61 also possesses the ability to increase the proportion of CSC in ER-positive breast cancer cells excepting for its inhibitory effect on breast cancer cells [79]. These results suggest that GANT61 could be used as a target for the therapy of breast cancer patients through its inhibitory effect on cancer cells and CSCs. Genistein inhibited the breast CSCs and MCF-7 cells' growth and proliferation and promoted apoptosis via the downregulation of hedgehog-Gli1 signaling pathway [80]. These researches offer reasonable and reliable evidence to further explore the clinical application of genistein in the therapy of breast cancer via targeting BCSCs.

The Notch pathway is crucial to stem cell renewal and might be a promising target for BCSC-directed treatment. A preclinical and clinical study [81] shows that treatment with gamma secretase inhibitors (GSI) reduced BCSCs in MC1 and BCM-2147 tumor grafts via suppression of the Notch pathway. In this study, GSI shows the capability of enhancing the efficacy of docetaxel in breast cancer. These results indicate that the inhibitor of the Notch pathway could lead to the decrease of BCSCs. A 2017 study revealed that vitamin D compounds could be acted as a promising preventive drug of inhibiting TNBC via regulating BCSC differentiation and reducing its population through inhibition of Notch pathway [82].

Wnt/β-catenin pathway is critical for regulation of BCSC-mediated metastasis. Survivin was found to contribute to self-renewal of CSCs via driving the activation of PI3K/Akt-dependent Wnt/β-catenin pathway, which mediates the breast cancer metastasis [83]. Some signaling pathways are not only key to maintaining the biology of CSCs but also main reason for breast cancer patients to resist treatment [6]. For instance, a study [84] in 2018 shows that the STAT3 pathway can promote BCSC maintenance and breast cancer chemoresistance. Furthermore, the inhibitor of

JAK/STAT3 blocks BCSC self-renewal, and blocking fatty acid β-oxidation, induced by STAT3 in BCSCs, can resensitize them to chemotherapy. These results indicate that STAT3 pathway may be a promising target for BCSC-directed therapy. Sabutoclax has been reported to reduce sphere formation of drug-resistant cells and eliminated the CSC subpopulation by downregulating the IL-6/STAT3 signaling pathway. When sabutoclax was combined with chemotherapeutic agents, it presented a strong synergistic antiproliferative effect [85]. The study shows the feasibility of combination of sabutoclax and chemotherapy; however, larger and higher-quality studies are needed to verify these findings and determine the potential value of the combination of sabutoclax with conventional therapy in chemotherapy-resistant breast cancer.

18.4.3 Other Strategies Targeting CSCs

18.4.3.1 Antiangiogenic Therapies

The formation of blood vessels in the tumor provides a continuous supply of nutrition and oxygen for the frenzied growth of the tumor. BCSCs is found to favor the generation of novel blood vessels by undergoing dedifferentiation into endothelial cells, which is termed as vasculogenic mimicry (VM) [86]. The presence of the close relationship between VM and CD133(+) expression may be helpful for TNBC relapse and progression [87]. Later, USP44(+) CSCs subpopulation possess the ability to predict the generation and invasiveness of VM and could be used as a reliable prognostic biomarker of worse clinical outcomes in breast cancer patients [88].

The therapy of antiangiogenic drug can effectively suppress the growth of tumor neovascularization, thus inhibiting the development of tumor. Reversely, tumors will inevitably be resistant to antiangiogenic drugs due to the generation of HIF (hypoxia-inducible factor) with the ability to promote formation, metastasis, and aggression of tumor blood vessels and CSC self-renewal. Conley et al. [89] found that the combined administration of HIF-1α-targeted agents like CRLX101 and antiangiogenic drug such as bevacizumab could get a better effect by targeting the CSC populations. In addition, OPN also is critical for angiogenesis and tumor progression of breast cancer. A recent study [90] has revealed the prime role of OPN in controlling breast cancer progression and angiogenesis through ILK and NF-κB-mediated HIF1α-dependent vascular endothelial growth factor (VEGF) expression in response to hypoxia. Compared to the control group, the expression of OPN cells induced the progression and angiogenesis of breast cancer by upregulating the expressions of proangiogenic factors, suggesting that OPN and its controlled signal network may be a promising target for breast cancer therapy.

18.4.3.2 Radiotherapy

It was reported that CSCs show resistance to radiotherapy via enhancing the ability of DNA repairing and reducing the concentration of intracellular reactive oxygen species (ROS), due to the overexpression of ROS scavengers in CSCs [91]. These

BCSCs with low ROS level are proportional to the priority spare and smaller DNA loss after irradiation, resulting in resistance to radiotherapy in breast cancer [92].

Mammalian target of rapamycin (mTOR) activation is critical for sustaining the self-renewing ability of CSCs. In triple-negative MDA-MB-453 and MDA-MB-468 breast tumor cells, rapamycin suppression of mTOR phosphorylation helped to sensitize the resistant breast cancer to low-dose radiation therapy [93]. In addition, proliferating cell nuclear antigen (PCNA)-associated factor (PAF) is a pivotal factor with the ability to regulate the stemness of cancer cell. PAF was overexpressed in breast cancer cells, and its depletion impairs the maintenance of stemness of CSCs, suggesting that PAF may become an effective therapeutic target to restore radiotherapy sensitivity of breast cancer [94].

BCSCs are also accountable for drug resistance by expressing drug efflux proteins like multidrug resistance-associated proteins (MRP), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP) [95]. ATP-binding cassette (ABC) transporters, a class of drug transporters, possess the ability to promote resistance to drug by relying on ATP to drain the drug out [96]. These ABC efflux pumps provide shelter for CSCs to protect them from various drug treatments [97]. As an ABC transporter encoded by MDR1 gene, P-gp participated in the protecting these tumor cells from anticancer chemotherapeutic drugs. The combination of P-gp inhibitors and chemotherapeutic drugs can maintain the concentration of chemotherapeutic drugs in tumors [96], suggesting that BCSCs expressing P-gp may be significantly associated with the chemoresistance and recurrence of tumor, which could make CSCs a novel therapeutic target and beneficial to current antineoplastic therapy. The high expression of BCRP (a protein of ABC transporter superfamily) will promote the resistance of tumors to some drugs including topotecan, methotrexate, mitoxantrone, doxorubicin, and daunorubicin [98].

Recently, lysine-specific demethylase 1 (LSD1) is considered as a major contributor to EMT, tumor stemness and drug resistance of breast cancer. Circulating tumor cells (CTCs) from patients with metastatic breast cancer were found to be rich in LSD1. Furthermore, targeting LSD1 by pharmacological inhibitor inhibited the stem cell-like and mesenchymal characteristics of the above CTCs. This report suggests that LSD1 might be acted as an effective therapeutic target for the therapy of advanced and drug-resistant breast cancer [99]. Lanzardo et al. [32] reported that downregulation of xCT damaged the formation of tumor sphere and changed the balance of CSCs intracellular redox in vitro. In addition, anti-xCT vaccination was shown to increase CSC chemosensitivity to doxorubicin in vivo; therefore therapeutically targeting xCT may contribute to the therapy of patients with breast cancer. Accumulating evidence suggests that metformin can also act as a promising agent for breast cancer and may be used as an effective (neo-)adjuvant therapy to eradicate CSCs and inhibit tumor aggressiveness [100, 101].

18.5 CSCs in Gastric Cancer

Gastric cancer is one of the five most frequently diagnosed cancers and the third leading cause of tumor-related death worldwide [31]. Gastric CSCs (GCSCs) have been proved to play a critical role in gastric cancer chemoresistance, recurrence, and metastasis. As a result, GCSCs might be expected to be a potential therapeutic target for improving the prognosis of patients with gastric cancer.

18.5.1 Targeting Cell Surface Markers of CSCs

CD44, a surface marker of CSCs, can be used as a key factor to promote the development of GCSCs. In gastric cancer, the presence of CD44+ CTCs has been considered to be related to lymph node metastasis, distant metastasis, and recurrence, which indicate that CD44+ gastric cancer CTCs may be used as a prognosis indicator of gastric cancer [102]. A variant of CD44 (CD44v8–10) has been considered as a major expression of CD44 variant in gastric cancer cells, which may promote tumorigenesis by enhancing the defense against oxidative stress [103]. A preclinical study shows that overexpression of CD44 and ALDH has recently been demonstrated in gastric cancer and all-trans retinoic acid could downregulate the expression of CD44 and ALDH, which eventually leads to the inhibition of the growth of gastric cancer [104]. Another study further illustrated the great potential of CD44 in predicting the prognosis of gastric cancer. Wang et al. [105] showed that the expression of CD44 was proportional to tumor transformation, TNM grading, metastasis, and recurrence of gastric cancer. At the same time, the high expression of CD133 also tended to predict the poor prognosis of patients. However, further research is necessary to verify the potential value of above markers in predicting the prognosis of gastric cancer. As a marker of CSCs, LGR5 is also highly expressed in gastrointestinal tumors. Gong et al. [106] developed two LGR5-targeting antibody-drug conjugates with the ability to induce the apoptosis of gastrointestinal cancer cells with high expression of LGR5, but it had no effect on gastrointestinal cancer cells that do not express LGR5. These findings suggest that it might represent a novel potential therapy targeting CSCs to eliminate the LGR5-positive gastrointestinal tumors and prevent cancer recurrence.

18.5.2 Targeting Signaling Pathways of CSCs

GCSCs make use of various signaling pathways including Notch, Wnt, HH, and so on to regulate the growth, migration, and response to drug therapy of gastric cancer. There was a significant correlation between target genes with different miRNA expression levels of gastric CSCs and several critical biological pathways including the regulation of cell cycle, the property of stemness, and differentiation [107]. Hence, targeting signaling pathways might be a therapeutic strategy to eradicate CSC population of gastric cancer.

A research exploring the relationship between Notch2 signaling pathway and the progression of gastric cancer showed that the expression of N2IC, an activated Notch2 receptor, not only promoted the proliferation of human SC-M1 cells and its development of transplanted tumor but also enhanced the colony generation, migration, aggression, and wound healing ability of SC-M1 cells. Interestingly, these effects will also disappear with the knockout of Notch2. Therefore, the knockout of Notch2 may be an effective strategy to suppress the development of AGS and AZ521 gastric cancer cells [108].

HH signaling pathway is critical to maintain CSC phenotypes and malignant transformation phenotypes in CD44(+) gastric cancer cells. Moreover, the inhibition of HH can block chemotherapy resistance in CD44(+) cells. However, this study also revealed that combination of HH inhibition and chemotherapy may only be effective for a small number of gastric tumor patients with overexpression of CD44 [109].

18.5.3 Targeting the miRNAs

Recent data suggest that miRNAs are associated with gastric cancer and contribute to carcinogenesis due to abnormality in their expression, which in turn affects cell proliferation, apoptosis, motility, and invasion [110]. It has been found that the expression profile of miRNAs in tumor initiation cells (CSCs) was significant differed from that in noncancerous cells [111].

MiR-501-5p, which plays a critical role in promoting the stem cell-like property of gastric cancer, shares a negative correlation with OS of patients with gastric cancer. MiR-501-5p has been shown to be significantly associated with gastric cancer patients possessing the more aggressive traits, suggesting that miR-501-5p represents a promising target for the therapy of human gastric cancer [112]. A study showed that there was a significant negatively correlation between the expression of miRNA-20a and miRNA-92a and the survival of patients with gastric cancer. This study also illustrated miRNA-92a may be an independent prognosis factors in gastric cancer [113]. Interestingly, miRNA can regulate stemness properties of CSCs of gastric cancer by regulating signaling pathway, thereby affecting the prognosis and therapies of patients with gastric cancer. A study [114] showed that the miR-23b suppressed gastric tumorigenesis such as development, invasion, migration, and metastasis through Notch2 pathway. MiR-132 has also been shown to possess the potential to promote the cisplatin resistance in gastric cancer patients by regulating SIRT1/CREB/ABCG2 signaling pathway [115]. The miRNAs are significantly associated with numerous cellular processes such as differentiation, proliferation, motility, and apoptosis in malignancies including gastric cancer, which suggest that miRNAs may be an effective approach to target CSCs and will ultimately possess the ability to treat gastric cancer patients and affect the prognosis of gastric cancer patients.

18.6 CSCs in Melanoma

In addition to playing a role in hematopoietic cancer and solid tumors (such as brain, breast, colon, pancreas, lung), CSCs have recently been found to be correlated with tumorigenesis, metastasis, and drug resistance of melanoma [116–120].

18.6.1 Targeting Cell Surface Markers of CSCs

Numerous stem cell markers have been found in drug-resistant melanoma cells and clinical specimens like CD133, CD20, ABCB5, ALDH1, and so on. Rappa et al. [121] have shown that in childhood malignant melanoma, CD133 + CSCs may be associated not only with lymph node and/or visceral metastasis but also with lower proliferative Ki-67 index which is one of the reasons for drug resistance. In addition, the overexpression of stem cell-associated markers, nestin, and CD133 in circulatory melanoma cells is related to the worse clinical outcome in melanoma patients. However, further validation in a large study with sufficient follow-up, similar sample sources, and including patients in stages II and III is warranted. [122] Lai et al. [123] have revealed that CD133+ and ABCB5+ subpopulations were co-localized in melanomas in perivascular niches and as stem cell-like cells, CD133+ cells could promote the development of tumor via promoting VM and the morphogenesis of a specialized perivascular niche in melanoma. Furthermore, CD133 knockdown melanoma cells are related to the poorer tumor growth in vivo. Luo et al. [120] reported that the expression level of ALDH was associated with the drug resistance of human melanoma stem cells, thus regulating the proliferation and survival of cancer cells. And the inhibition of ALDH by silencing ALDH1A can not only result in cell cycle arrest, apoptosis, and inhibited cell variation in vitro but also inhibit tumorigenicity in vivo. These results suggested that ALDH was not only a marker of CSC but also a promising target for the therapy of melanoma. However, further research on the molecular mechanisms of regulating CSCs of these isozymes and genes is warranted. Vincristine (VCR) is widely used in melanoma treatment; however, it has been found ineffective to treat the specific CSCs of melanoma. Song et al. [124] found that VCR-containing immunoliposomes combined with CD20 antibody (VCR-Lip-CD20) were 1.85 times more effective than VCR-Lip and VCR in melanoma. Significantly, the results also showed that VCR-Lip-CD20 could selectively kill CD20+ melanoma cells in populations of WM266-4 cells both in vitro and in vivo. These findings indicate that VCR-Lip-CD20 may be expected to be an efficient target to kill CD20+ melanoma cells.

18.6.2 Targeting Signaling Pathways of CSCs

Accumulating studies have shown that there are many signal pathways and potential therapeutic targets in numerous malignant tumors like melanoma. It was reported

that inhibiting HH pathway by interfering SMO or GLI1 drastically attenuates the self-renewal and tumorigenicity of melanoma stem cells with overexpression of ALDH; thus SMO and GLI1 may possess the potential to become a new and effective approach for the targeted therapy of human melanoma [125]. It has been also reported that the overexpression of Notch4 contributes to the aggression and metastasis of melanoma stem cells, which indicates a poor prognosis [126]. Kumar et al. [127] have shown that the expression of CD133+ CSCs which was regulated by Notch1 pathway could activate its regulated signaling network, promoting the development, metastasis, and angiogenesis of melanoma. Furthermore, eradication of Notch1 by blocking or ablation could also suppress the expression level of CD133, which in turn inhibits the cell migration and angiogenesis of melanoma. However, these findings only verified in mice experiments, and further studies are necessary to verify the value of these findings in human melanoma. BRAF and NRAS mutations are reported to occur in about 70% of melanoma, and a study [128] shows the combination of γ-secretase inhibitors (block the activation of Notch signal) and BRAF inhibitors (block the activation of BRAF-MEK-ERK signal) is more effective in the therapy of melanoma with BRAF/NRAS mutation. A study in 2016 showed that andrographolide could attenuate melanoma growth and lung metastasis by abrogation of Notch1-mediated CD133-dependent p38 mitogen-activated protein kinase (MAPK) activation pathway in CD133+ melanoma cells. Mechanistically, Notch1 upregulates MAPK activation through CD133 resulting in the development, lung metastasis, and angiogenesis of melanoma. In contrast, inhibition of Notch1 and MAPK pathways inhibits cell migration and angiogenesis of melanoma [129]. Excepting for these signaling pathways, low rhodamine 123 (Rh123low) cells are enriched for stem cell-like activities, and Rh123low cells possess the characteristic of relative stillness and drug resistance in melanoma CSCs. A study reported that PI3K/Akt pathway was the key to maintain Rh123low in melanoma stem cell compartment [130].

18.6.3 Targeting the miRNAs

Several reports have suggested that miRNAs played a crucial role in development of tumor, angiogenesis, and metastasis in numerous tumors including melanoma. A preliminary report showed that several miRNAs (miR-21, miR-10b, miR-200c, miR-520c, and miR-373) were significantly upregulated in melanoma sphere, suggesting that these miRNAs might regulate the potential of metastasis and stemness in melanoma [131]. Noman et al. [132] have shown that miR-210, as a hypoxia-regulated miRNA in lung cancer and melanoma, could reduce the lysis of tumor cells by antigen-specific cytotoxic T lymphocytes (CTL). And low expression of miR-210 could also enhance the CTL-mediated lysis of tumor cells in lung cancer and melanoma. Thus, miR-210 could be a promising prognostic marker and therapeutic target. Moreover, a study [133] suggested that miR-33b directly binds to HMGA2 3′ untranslated region to suppress its expression and suppresses EMT and migratory potential of melanoma cells. Forloni et al. [128] found that overexpression

of miR-146a enhances the proliferation property of human melanoma cells in culture and the formation of tumor in mice by targeting Notch signaling and BRAF-MEK-ERK signaling, while knockout of miR-146a showed opposite results. Oncogenic DNp73 is a dominant-negative variant of tumor suppressor gene p73, which confers to tumor cells the characteristic of increased stemlike properties by attenuating expression of miR-885-5p [134]. It was reported that augmentation of miR-9 markedly inhibited the cell proliferation and migratory capacity of melanoma cells. In contrast, knockout of miR-9 or anti-miR-9 miRNA inhibitor could enhance not only the expression of Snail1 but also the cell proliferation and migration capacity of melanoma. Mechanistically, miR-9 binds to the 3′ noncoding region of NF-κB and attenuates its expression, thus preferentially inhibiting Snail1 and finally inhibiting the proliferation and metastasis of melanoma cells [135]. Taken together, these findings suggested that miRNAs may regulate the proliferation and metastasis of melanoma cells by modulating CSC properties and may be a promising prognostic marker and therapeutic target.

18.6.4 Targeting the Microenvironment of CSCs

Tumor progression also depends on their microenvironment. The tumor microenvironment like fibrous cells, immune cells, inflammatory cells, and blood vasculature (angiogenesis of tumor) is necessary for CSC survival since it could not only produce and maintain CSCs but also protect them from attacks by the immune system and lead to enhanced migration and recolonization as secondary tumors [136, 137].

TAMs play multifaceted roles in the growth of tumor, especially related to the aggression and angiogenesis of tumor. CSCs may regulate the surrounding niche by regulating the expression of OPN in TAM. Kale et al. [138] have shown that macrophage related to melanoma modulated tumor microenvironment by secreting OPN, triggering the angiogenesis and development of melanoma. Therefore, inhibition of OPN and its regulated signaling network may be a potential approach to eliminate melanoma through the manipulation of TAMs.

Besides, hypoxic microenvironment is linked to the worse clinical outcome of tumor and controls the number of CSCs by stabilizing HIF [139]. It has been reported that HIF1α and HIF2α drove the aggression of melanoma and related to the metastases of melanoma patients [140]. MFG-E8, a powerful angiogenic factor, increased tumor angiogenesis and the development of melanoma under hypoxic conditions via enhancing the expression of VEGF and ET-1 in MSC and M2 polarization of macrophages [141]. However, this finding which is based on a tumor model of bone marrow chimeric mice does not represent that MFG-E8 has a similar effect in vivo, and further researches are necessary to verify findings above. Another study [142] showed that hypoxia can downregulate the expression of miR-340-5p, while the low expression of miR-340-5p is negatively related to the expression of ATP-binding cassette subfamily B member 5 (ABCB5, a marker of

melanoma stem cells, is a key transmembrane transporter closely related to tumor chemoresistance).

18.6.5 Other Strategies Targeting CSCs

In addition to the above targeted CSC therapy, there are also some other methods targeting CSCs to treat melanoma. Melanoma stem cells can also display drug resistance via enhancing drug efflux, which is mediated through ATP-binding cassette subfamily B (ABCB). El-Khattouti et al. [143] revealed that caffeic acid phenethyl ester (CAPE), a bioactive molecule, could enhance the expression of E2F1 and apoptosis in CD133(−) melanoma, but not in CD133(+). Interestingly, the knockdown of ABCB5 was shown to enhance the sensibility of CD133(+) cells to CAPE. Therefore, combination of ABCB5 inhibitor and CAPE can eliminate chemoresistance in melanoma-specific CSCs. A study [144] showed that the novel sirtuin 1 and 2 (SIRT1/2) inhibitor tenovin-6 could effectively eliminate CSCs and induce apoptosis in uveal melanoma. The approach of targeting tumor stem cells can also be used in tumor immunological therapy. Lu et al. [145] developed a vaccine by using CSC lysate-pulsed dendritic cells (DCs), which could target the CSC populations of numerous cancers including melanoma.

As a CSC marker, the high expression of CD44 is significantly related to the development and metastasis of various human cancers like melanoma. CD44 can bind specially to hyaluronic acid (HA), a pericellular matrix component. Shen et al. [146] reported that solid lipid nanoparticles coated with hyaluronic (HA-SLNS) possess the ability to target paclitaxel (PTX) transport to B16F10 melanoma cells with high expression of CD44 and significantly improve their intracellular transferring efficiency. Furthermore, it will cause numerous CD44(+) cells to induce apoptosis in vitro, and the growth and lung metastasis of melanoma were also significantly inhibited. In addition, this strategy could be markedly beneficial to the survival of patients with no adverse events. Moreover, there was a significant positive correlation between the expression of differentiation inhibitor/DNA binding (Id) proteins 1 and 3 (both depend on BMP4/7) and the development and poor survival of patients with malignant melanoma. Moreover, the interaction between HA-CD44 and BMPR contributes to the expression of Id1/3 protein relying an BMP4/7 and poor survival in patients with melanoma [147]. These results suggest that targeting CD44 is a potential and effective approach for melanoma therapy by elimination of CSCs.

18.7 Application of Nanomedicine in Targeting CSCs

Although targeting CSCs is one of the most promising therapeutic approaches, the traditional methods of targeting CSCs have several flaws including poor water solubility, poor pharmacokinetics, and poor stability of CSC-specific agents [148–150]. At present, for the existing technology, it is a challenging task to target drugs

into a small amount of CSCs in tumor tissue. In recent years, nano-transport technology offers a novel approach to effectively exert the efficacy of drugs via controlling the release of drugs, prolonging the effective time of drugs, and improving the biological distribution of drugs. And this technique can effectively solve the obstacle of eradicating CSCs and improve the therapeutic effect of targeted CSC drugs. It has been proved that CSC-targeted inhibitors alone are not particularly effective in inhibiting tumor growth. The double-targeted nano-drugs containing CSC-targeted inhibitors and conventional antineoplastic drugs can not only effectively eliminate CSCs and tumor cells but also possess lower toxicity than that of free drugs [151–153]. For example, through nanotechnology, Sun et al. [154] wrapped all-trans retinoic acid (ATRA, a powerful differentiation agent of CSCs) and chemotherapy agent doxorubicin together to make into a nano-drug, which can significantly enhance the concentration of drugs in tumor tissues and CSCs, greatly enhance the inhibitory effect on tumor growth, and cooperatively decrease the population of CSC. As a result, ATRA combined with doxorubicin can be used as a promising therapeutic approach to inhibit the development and relapse of cancer via targeting CSCs and non-CSCs.

18.8 Limitation, Barriers, and Controversy in the CSCs

There are some limitations in the treatment of targeted CSCs.

Firstly, it is crucial to select the appropriate CSC markers for these researches about CSCs, as misrecognition of CSC subpopulations will lead to the wrong conclusion. Nonetheless, how to identify and isolate CSCs has always been an obstacle in this field. Some CSC populations do not express the cellular markers found so far, while some non-CSC tumor cells also express these markers. At present, the isolation and identification of CSCs is mainly based on cell surface markers like CD133. Nevertheless, the credibility of this method remains to be answered. [155, 156] Novel techniques like live-cell RNA detection and single-cell DNA and RNA sequencing methods may help to identify CSCs, but these methods are still unlikely to identify a unique CSC marker [157].

Secondly, despite CSCs having been found in numerous dysfunctional signaling pathways, these pathways also express normal stem cells that are of great significance to normal physiological activity. Therefore, drugs targeting these signaling pathways can affect not only CSCs but also normal stem cells, leading to serious side effects. Considering this limitation, we must improve the specificity of targeting CSC drugs, through continuous optimization or in combination with other technologies (such as nanotechnology) in order to gain effective treatment and avoid serious side effects [158]. In addition, the cross talk between different pathways should also be considered. Therefore, the strategy of targeted CSC-related signaling pathway in the treatment of tumor still needs to be further explored before it is used clinically.

18.9 Conclusion and Future Perspectives

CSCs have been shown to be able to evade current cancer treatments including novel immunotherapies, thereby leading to tumor recurrence, metastasis, and resistance to radiotherapy and chemotherapy. Therefore, targeted CSC treatment for more thorough treatment of tumors to achieve a better prognosis is necessary. In summary, the application of CSCs is promising, opening a new era for cancer treatment and evaluation of prognosis. However, there remain several unresolved problems in CSCs. Further research is needed to find reliable and accurate markers to distinguish CSCs from normal stem cells for developing the treatment approaches with higher specificity and fewer side effects. In addition, before the clinical application of targeted CSCs in tumor therapy, it is necessary to clarify its effective dose and side effects and other important factors. After all, the current findings from in vitro or animal experiments may not achieve satisfactory results or even cause major accidents when applied to humans. Signaling pathway and tumor microenvironment as an approach of targeting CSCs have shown encouraging results. Nevertheless, it is worth noting that the signal pathway regulating CSCs does not operate separately and the combination of drugs may be a more effective targeting strategy in the future. Although nano-carriers were able to enhance the delivery and drug activity of CSC inhibitors, only a small number of nano-drugs could be approved for clinical treatment at present. With the further research of CSCs and nano-drugs, nano-drugs loaded with different therapeutic drugs may become the most effective drugs for tumors.

In the future, the combination of traditional antineoplastic drugs and targeted CSC drugs combined with nanotechnology may be expected to be an effective approach for the therapy of cancer.

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The book was inadvertently published with an incorrect last name of the author 'Upasna Upadhyay' in chapter 7 as Upasna Reddy whereas it should be 'Upasna Upadhyay'. The chapter has now been corrected and approved by the author.

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