Prasan R. Bhandari ·
Kala Kumar Bharani ·
Amit Khurana *Editors*

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Dedicated to

ALMIGHTY CREATOR to who, what, and why I am.

My family Mr Ramchandra G Bhandari and Ms Asha R Bhandari, Mr Dayanand A Kamath and Ms Sharada D Kamath, Ms Veebha V Prabhu and Mr Vishnu R Prabhu, Ms Savita V Shanbhag and my co-brother Mr Vinayak P Shanbhag, Ramnath V Prabhu and Siddhant V Shanbhag, and Ms Smriti Madkaiker (Springer Nature Technology and Publishing Solutions).

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Lastly, but most importantly my wife Ms Sangeeta P Bhandari and

My two lovely daughters Purva P Bhandari and Neha P Bhandari.

Dedicated to my Lord and Saviour Jesus Christ.

Preface

The use of animal models has become progressively imperative for biomedical research over the past few years, permitting an enhanced recognition of pathogenic pathways entailed in a diversity of human disorders. Within the ambit of research, animal models have played a critical role in the progress of new understandings and concepts of pathogenesis. Animal models such as mice, rat, guinea pigs, hamsters, and rabbits have been employed in an array of studies, providing important experimental information that directs researchers to an improved understanding of issues. As scientific research advances, investigators are endeavoring to recognize more unique animal models to exploit new opportunities of research.

Zebrafish (*Danio rerio*) has evolved as an increasingly widespread model in biomedical research. Research performed on these marine vertebrates has created substantial findings not only in the expanses of genetics and embryology, but also fields like cancer, cardiology, endocrinology, and neuroscience. Zebrafish are encouraging animal models due to their high genetic homology with humans and measurable phenotypes analogous to humans.

The utility of zebrafish to explore the pathological mechanisms underlying various disorders and its quantification is delved in depth in this book.

Largely, this book highlights the rising significance of zebrafish in biomedical research. As a capable substitute to mammalian animal models, zebrafish produce robust physiological reactions comparable to humans; however they do not retain the intricate phenotypes displayed by several other animal models. This book describes a broad, systematic outlook on the rise of zebrafish as a robust animal model in biomedical research. The contributing authors of this book are prominent international researchers whose work forefronts advanced research ventures in research laboratories around the world. The subjects deliberated within this book include a varied gamut of the usefulness of zebrafish within different biomedical fields. This book will aid as a valuable basis for scientists from all biomedical fields new to the discipline, along with recognized investigators in quest of cherished comprehension into the mounting usefulness of zebrafish in biomedical research.

Pune, Maharashtra, India — Prasan R. Bhandari
Warangal, Telangana, India — Kala Kumar Bharani
New Delhi, Delhi, India — Amit Khurana

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Kala Kumar Bharani is currently working as professor and head of the Department of Veterinary Pharmacology and Toxicology at the College of Veterinary Science, Warangal, Telangana. Prof. Bharani graduated from the Andhra Pradesh Agricultural University (APAU), Hyderabad, India, and completed his PhD from the Sri Venkateswara Veterinary University (SVVU), Tirupati. He is a recipient of the Telangana State Best Teachers Award, Dr. Lalitha Kameswaran Award, and Dr. S. B. Pandey Award. Currently, he is serving as the secretary-general of the Indian Pharmacological Society (IPS). Prof. Bharani has published more than 50 peer-reviewed research and review articles.

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ABCD of Zebrafish Culture

1

Yogesh Bhargava

Abstract

Zebrafish is not a new name as model organism for biomedical researchers around the world. Though institutional zebrafish culture facilities usually cater to the need of researchers for the zebrafish supply and maintenance, such facilities are very limited globally. Therefore, researchers are compelled to culture zebrafish in their lab. The literature and science behind zebrafish culture and care are scattered and limited which makes zebrafish culture a daunting task and might have discouraged new researchers from embracing zebrafish as an animal model. Therefore, this chapter has presented an overview of information on the know-how and science behind zebrafish culture. First, a note on static, flow-through and recirculating systems is described as these are used to maintain zebrafish in lab conditions. Each system has a different utility in rearing zebrafish. Apart from commercial systems, cost-effective, open-design options are also discussed to suit custom requirements of the researchers. Next, a discussion on the factors affecting the health of zebrafish and their optimum range required for the well-being of zebrafish is presented. Finally, the role and types of zebrafish diets are described. Overall, this chapter provides the readers a comprehensive guide on the maintenance of zebrafish for research purposes with ease.

Keywords

Zebrafish culture · Diet · Open-design · Recirculating · Vertebrate model

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1.1 Zebrafish: A Brief Background

During the early nineteenth century, Dr. Francis Buchanan (later known as Francis Hamilton), a Scottish surgeon, first described zebrafish in his book when he was stationed in West Bengal during his visit to India with East India Company. He presented an account of ten *Danio* species he sampled from the paddy fields in and around the West Bengal area (Hamilton 1822). However, zebrafish as a vertebrate-animal model was popularized by Dr. George Streisinger from the University of Oregon during the 1970s (Streisinger et al. 1981).

Zebrafish belongs to phylum Chordata, family Cyprinidae and genus *Danio*. Its scientific name is *Danio rerio*. Zebrafish is a freshwater fish with natural habitat along the regions of river Ganges and Brahmaputra especially in India, Nepal, Bangladesh and Myanmar (Spence and Smith 2006). Zebrafish has been reported predominantly in paddy fields, shallow ponds or river drainages with slow moving water at various sites in northern Indian states present along the bank of Ganges (McClure et al. 2006). The overall size of the adult zebrafish is so small that it can fit in a 1.5 mL Eppendorf tube. It has got its name "zebrafish" due to the presence of black and white stripes on the lateral side of its body. Zebrafish shows sexual dimorphism (having morphologically distinct male and female) which makes it easy for researchers to visually inspect and select them for mating purpose.

1.2 Zebrafish: A Vertebrate-Animal Model

To understand various life processes including any deviation from the normal course leading to the onset and progression of the disease, a model organism is required. Animal models are also required to test the therapeutics' effectiveness before it can be used for human purposes.

Now zebrafish is not a new name in the field of biomedical research. Since the availability of zebrafish transgenic and tools to study them, several labs have started to embrace this model over other mammalian models which is quite evident from the ever-increasing number of publications that employ zebrafish in their research. Zebrafish has served the need for a suitable animal model ranging from cell-based studies to the whole organism behavioural studies (Sarasamma et al. 2017). The genome of zebrafish is also fully sequenced. Its sequence revealed that the zebrafish and humans had a common ancestor nearly 450 million years ago (Woods et al. 2000). Though zebrafish is a model organism of choice, it has a few limitations. The following are the pros and cons of using zebrafish (Lawrence 2007; Spitsbergen and Kent 2003).

1.2.1 Pros

- The maintenance of a large number of zebrafish in small area is possible with little effort and cost.

- Zebrafish shows sexual dimorphism, meaning the male and females have morphologically distinct features and can be identified with a visual inspection.
- Zebrafish show high fecundity which means they can produce a large number of eggs during each mating.
- The zebrafish being a vertebrate animal shows fast and ex vivo development in which growth of the embryo occurs outside the body of mother but inside a protective layer called the chorion. This is a useful characteristic that makes developmental stages amenable for modifications by external agents like toxins, chemicals, etc. by either microinjection or via bath incubation.
- The size of various developmental stages is small, in which embryo and larval stages are optically transparent. This property is useful for optical imaging experiments.
- The genome of zebrafish shows significant sequence homology with humans especially with the disease-associated genes.
- Tools of genetic engineering are available to generate transgenic zebrafish.
- The zebrafish being small in size is compatible with the existing high-throughput screening platforms.
- Zebrafish have a well-developed sensory system. They are diurnal animals similar to humans having more cones in their eyes (unlike rodents that are nocturnal and have more rods in their eyes). Zebrafish also shows a large repertoire of behaviour similar to humans which makes them suitable for neurobehavioural studies.

1.2.2 Cons

- Our understanding of zebrafish immune system is still limited compared to other vertebrate animals like rats/mice.
- The microbiological profile of zebrafish is also limited. Moreover, the zebrafish grown in culture facilities are susceptible to microbial pathogens like mycobacterium, microsporidia, etc. The microsporidia infection is challenging to clean from recirculating systems.
- The breeding of zebrafish is highly dependent on external factors like water quality, light, etc. which are sometimes difficult to control and may affect spawning.
- We have a limited understanding of the zebrafish diet. Though several commercial pellet-based diets are available, they are far from being ideal.
- Zebrafish embryos are not differentiated into distinct male and female sexes at the time of birth; therefore, gender-based studies at embryonic stages are not possible.
- Many of the organs/structures like the breast, hippocampus, amygdale, etc. are structurally not distinct in the zebrafish and its brain. Thus limiting their use in investigations where questions related to these structures are investigated.

1.3 Steps in Zebrafish Culture

1.3.1 Acquisition and Maintenance of Zebrafish

Interestingly, zebrafish is becoming one of the popular aquarium species among pet enthusiasts due to its robust nature and the availability of various fluorescent variants called GloFish™ which express green, yellow and red fluorescent proteins in their muscles (Linney and Udvadia 2004; Shafizadeh et al. 2002). These transgenic fishes glow in the dark when irradiated with UV light. These and other wild-caught strains are frequently available in local pet shops in various countries. However, other specific strains of zebrafish are available with and can be procured from "The Zebrafish Information Network (ZFIN)" at the University of Oregon, Eugene, USA. Common strains which are popular among zebrafish researchers are AB strain, AB/Tuebingen (AB/TU) strain, Tübingen strain (TU, short fin), Tüpfel long fin (TL), etc. For zebrafish genome sequencing TU strain was used. Currently, zebrafish are cultured and maintained using the following types of systems. The applicability of these systems depends on the experimental need. The distinction between these systems mainly lies in the mechanism of water exchange (Bhargava 2018).

1.3.1.1 Static System

Figure 1.1a shows the overview of the static system. It is a tank filled with water having large number of adult zebrafish (for better reproductive health of zebrafish higher stocking density i.e. upto 19 fishes/L, should be avoided). The size of the tank may vary. Water exchange in this housing system is a single pass and discontinuous, which means that water is added once with no water exchange over a defined period (generally 1–3 days). After the intended period, the complete water is replaced (often manually) by freshwater and this cycle continues. The water quality depends on the number of zebrafish housed per litre of water. Water quality also deteriorates over time; therefore, it requires constant monitoring. Though this system is very cost-effective to set up, cleaning is time- and labour-intensive. This system can be used to maintain a small population of adult zebrafish only for a limited time due to quick change in water parameters (pH change and nitrogen content build-up). Sometimes, to enrich the tank environment, one can use artificial plants and clay items in the tank. For quarantine purposes, this system is very convenient. The static system is also best suited to rear zebrafish larvae. It is because the static system design ensures that the food is available for maximal duration in the tank for growing larvae to feed upon. In the past, several studies have used this type of system for zebrafish maintenance in the lab (Miller et al. 2013).

1.3.1.2 Flow-Through System

Figure 1.1b shows the overview of the flow-through system. In this system, water exchange is continuous with a single pass. It means that the clean water, which is stored in an overhead tank, is allowed to pass through the zebrafish tanks via an inlet port continuously. The outlet port discards the overflow water from the zebrafish tank, thus making this system continuous in which water enters and leaves

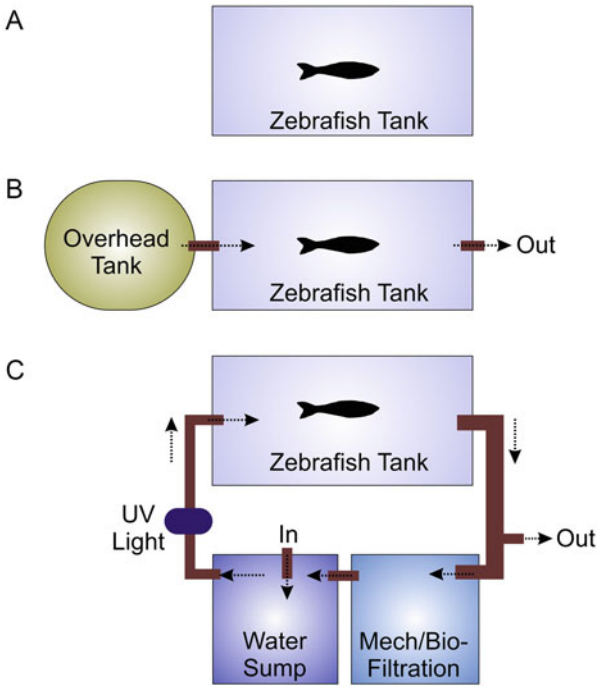

Fig. 1.1 Schematics of zebrafish maintenance systems. (**a**) Static system is a tank-based system in which water exchange is discontinuous and single pass. (**b**) Flow-through system offers continuous water exchange in which water passes zebrafish tank singly. Continuous water supply is maintained using an overhead tank which hold filtered water. Water is discarded after it leaves (out) zebrafish tank. (**c**) Recirculating system is a continuous system in which water exchange occurs multiple times. It requires daily exchange of ~10% water in the water sump. It also requires installation of mechanical and biological (mech/bio) filtration unit to filter out particulate matter and to lower nitrogen content in the recirculating water

the zebrafish tanks singly. As the water passage occurs singly, this system is devoid of any biological filter to check the nitrogen build-up. It is easy to maintain water quality in the zebrafish tank as freshwater is continuously being fed into the system; however, water wastage is relatively high in such system as the water is not being recycled after it leaves the zebrafish tank. This system is useful to maintain a pathogen-free zebrafish colony. Few studies have used this system in their lab for research (Hohn and Petrie-Hanson 2007).

1.3.1.3 Recirculating System

Recirculating system is an ideal system to maintain adult zebrafish in lab conditions because water exchange is continuous and multipass (Fig. 1.1c). In this system, water is not discarded but fed back in the system multiple times after passing through mechanical and biological filters and UV treatment chamber. Thus, the recirculating system reduces water wastage significantly as compared to the flow-through system.

To save space, the system can be made in the form of an upright rack with multiple shelves to hold several zebrafish tanks (of appropriate size) in a compact space. Zebrafish larvae can also be reared in the recirculating system with few modifications, e.g. slow water exchange and fine-mesh baffle installation in the zebrafish tanks. Due to several advantages, nearly all the commercially available zebrafish maintenance systems are recirculating systems with modular rack assembly. Table 1.1 shows comparative components of recirculating systems offered by major commercial suppliers. The only limitation of commercially available recirculating systems is their significantly high cost, which may prohibit many start-up labs or young researchers with limited funding to establish zebrafish lab with such systems. Therefore, to ease these and other limitations, "open-design" options were also built and explored in past. Under this, progress was made to replicate the function of a commercial recirculating system using custom-designs/parts. The main purpose of open-design efforts was to reduce the system cost and to equip the specific lab in custom designing the setup for their own requirement. Recirculating systems offer regulating different parameters like space requirement, water quality, salinity, sediment removal, minimizing water wastage, scale-up of zebrafish holding capacity, etc. with ease. Therefore, many researchers have already employed such systems (commercial or open-design) in their lab for research (Castranova et al. 2011; Nema and Bhargava 2017).

Major Components of the Recirculating System

Due to several advantages, nearly all the zebrafish research facilities worldwide rely on the use of rack-based recirculating system to maintain zebrafish. If cost is the prohibiting factor, one can even construct their rack-based recirculating systems to suit their custom requirement in a cost-effective manner using open-design options. A recirculating system has several components (Bhargava 2018) whose functions are listed below:

1. **Rack**: Rack is often made with metal-angle parts that provide a space and strength to hold various components.
2. **Pipes and connectors**: For water recirculation in the system, PVC pipes of schedule 40 or 80 are used. There are two types of pipes used in the recirculating systems. One is ascending pipe (1/2 in. diameter) which circulates water from the water sump to the zebrafish tanks. The other is descending (collection) pipe (1 or 2 in. diameter) which collects water from the zebrafish tanks and transports it to the water sump via filtration module. To feed the water from ascending pipe to each zebrafish tank, connectors/valves with flexible tubing may be used (narrow tubing with push-fit connectors).
3. **Filtration units**: The water collected from zebrafish tanks has both particulate and dissolved matter in it. Therefore, it requires appropriate filtration before the water can be fed back into the system sump. The particulate matter is filtered using mechanical filtration. These are sieve-based filters often made with polyesters/sponge. Activated charcoal in a nylon bag is placed in the water sump to remove odour and dissolved traces of chlorine gas in water. Biological

Table 1.1 Popular commercial suppliers of zebrafish culture system

Type of units	Properties	Popular commercial setup			
		Tecniplast aquatic solutions	Yakos65	Aquaneering Inc	Thoren aquatic systems Inc.
Zebrafish holding tanks	Capacity (in litres)	1.0, 3.5, 8.0	1.5, 3.0, 10.0	0.8, 1.4, 1.8, 2.8, 6.0, 9.5	1.0, 3.0
	Self-cleaning	Yes	Yes	Yes	Yes
	Autoclave compatible	Yes	Yes	Yes	Yes
	Materials	Polysulfone, polycarbonate	Polycarbonate	Polycarbonate	Aquatic compatible plastic
	Tank colour	Blue	Blue	Green	Transparent or light grey
Water sump/ reservoir	Capacity (in litres)	Variable	Variable	Variable	Variable
	Materials	NL[a]	NL[a]	NL[a]	NL[a]
Structural components	Rack material	Plastic coated, movable shelves, adjustable structure, rubber feet and identification marks	PVC coated, corrosion resistant	Stainless steel	Steel
	Pipes (inlet, outlet and connectors)	Grey PVC pipe, flow controllers	White PVC pipe, flow control nozzles	PVC pipe, flow regulators	Grey PVC plastic
	Lights and auto-timer	Lights (with automation)	Lights (with automation)	Lights (with automation)	NL[a]
	Pump	Variable frequency, submerged, an additional backup pump	External	External	External
	Heaters	Heating coils	Auto controlled heater	Digital control heater	Automated heaters
	Aeration	Air diffusers	Air diffusers	Air diffuser	Air diffuser in linear position

(continued)

Table 1.1 (continued)

Type of units	Properties	Popular commercial setup			
		Tecniplast aquatic solutions	Yakos65	Aquaneering Inc	Thoren aquatic systems Inc.
Filtration and disinfection	Mechanical	Fine mechanical filtration	Yes	Yes	Yes
	Chemical (activated charcoal)	Yes (pre-fill compact)	Yes	Yes (in line fitting)	Yes
	Biological (Bioballs)	Yes	Yes	Yes	Yes
	UV (disinfection)	Yes	Yes	Yes	Yes
	Other	Drum filter optional	Gravitational filtration system and pre-filter washable pads	Fluidized bed filtration, pad filters	Bag filters
Accessories	Accessory parts	Breeding and auto feeding systems, biochemical probes, cooling unit etc.	Stand-alone racks in various size, French-SMOFY technology for noise reduction, reduced electricity consumption	Chillers, dosing systems, dissolved oxygen probe, brine shrimp hatchers, breeders, etc.	Low water sensor, block filter indicator and auto fill valve, different modular rack designs
Contact info	Web links	http://www.aquaticsolutions.it/	https://www.yakos65.com/	http://www.aquaneering.com/	http://www.thoren.com/

[a]*NL* not listed

filtration is used to remove ammonia and lower the nitrate/nitrite (nitrogen content) in the water. It is done using biomedia/bio-balls or gravels (wet/dry or wet/wet method). This media will allow growth of the bacteria, e.g. *Nitrosomonas* sp., *Nitrobacter* sp., *Pseudomonas* sp., *Alcaligenes* sp., etc., which use dissolved nitrogen contents and reduce its amount in the system water. UV assembly will further reduce the number of actively growing microbial species. UV assembly is installed in the ascending pipe so that water entering the zebrafish tank is first sterilized in the UV light. The zap-dose/kill-dose depends on the wattage of the UV lamp and duration of the UV light exposure.

4. **Chambers/tanks**: A water reservoir chamber (water sump) and tanks (to hold zebrafish) are used in the recirculating system. Commercial zebrafish tanks are self-cleaning tanks that are made of polycarbonate material. The water sump is also made of polycarbonate material. It houses a pump, carbon filter, heater and/or chillers, aerators, pH sensor, etc. Tanks made with food-grade plastic are commonly used in the open-design zebrafish tanks. Size of the zebrafish tanks may vary based on the need of the individual lab (ranging from 2 to 8 L). The utility of self-cleaning feature in commercial zebrafish tanks is enormous as it significantly reduces build-up of particulate matter at the bottom of zebrafish tanks. For the first time, our work has extended the auto-cleaning feature to custom-made zebrafish tanks and found that the installation of "self-cleaning unit" in zebrafish tanks results in efficient removal of sediments from the bottom of zebrafish tanks. The design of our self-cleaning unit was also found to be independent of tank geometry (Nema and Bhargava 2016).
5. **Accessories**: It includes components like light, cooling, feeding, temperature sensing, etc. Though these components are "essential", they are not required to be an integrated part of zebrafish recirculating rack. Bright lights can be provided using standard ceiling lights in a room, e.g. LED lights, CFL lights, etc. To maintain ~29 °C during summers, cooling is required. Cooling can be provided by installing the zebrafish recirculating rack in a temperature-controlled room or a room with installed AC/water cooler. During winters, heating of the system water is required to maintain the appropriate temperature. This can be done by installing an underwater heater in the water sump. Automatic feeding machines can dispense a pre-set amount of pellet-based food and thus reduce manual labour. However, it will add to the extra cost of zebrafish maintenance.

1.3.2 Breeding of Zebrafish in Lab Conditions

Zebrafish are dimorphic animals (male and female sexes are present in different bodies) with external fertilization. For mating in the lab conditions, male and female adult zebrafish in 2:1 ratio are placed in the dark for overnight in a mating chamber. The dark phase is essential as it is required to enhance melatonin production in zebrafish which is shown to regulate sexual behaviour in zebrafish (Lima-Cabello et al. 2014). The mating chamber is a tank with a barrier (barrier can be a base with glass balls/marvel or a sieve/net) to prevent the access of adult zebrafish to the

fertilized eggs as they tend to feed on them. The next day, spawning (a process of egg laying) is stimulated by light and fertilized eggs are collected at the bottom of the tank. Sometimes, a physical partition can also be used to separate males and females in the mating chamber for overnight to stimulate the mating process and increase the chances of spawning. In our lab, we have observed a good spawning rate even without physically separating males and females during overnight period.

It is visually easy to distinguish fertilized eggs from unfertilized ones. Fertilized eggs have a clear chorion (the protective layer around a central mass of cells), whereas unfertilized eggs are greyish and uniform (in contrast, dead eggs are white and cloudy). Zebrafish produce several eggs during each mating process; however, the clutch size (number of eggs per spawning) may vary between batches, strains and environmental conditions. A detailed comparative account on the variety of breeding and husbandry methods is discussed by others elsewhere (Tsang et al. 2017).

1.3.3 Care of Zebrafish

For the well-being of zebrafish, maintenance of various physical parameters are required. The water conductivity (300–1500 μS), pH (6.6–8.2, with ideal pH ~7.5), temperature (24–35 °C with ideal temperature 28 ± 2 °C), oxygen concentration (4–6 mg/L), photoperiod (14 h light, 10 h dark), nitrogen load (ammonia, 0 mg/L; nitrite, <10 mg/L; nitrate, <75 mg/L), UV-C light zap dose (20,000 μW/cm^2 to kill common bacteria and viruses) and ~10% water exchange daily are the optimum requirements which can also be adjusted by the individual labs to suit their requirements (Bhargava 2018; Avdesh et al. 2012; Kwong et al. 2014; Lawrence and Mason 2012). In recirculating systems, a small portion of water needs to be exchanged daily in the sump to reduce the nitrogen load and build-up of chemicals leaching out from the plastic components, e.g. bisphenol A or bisphenol S. Over time, zebrafish tanks may show the growth of algae and biofilms on its wall. The growth of algae can be retarded using blue/green shades of zebrafish tanks, but the removal of biofilms is only possible through mechanical scrubbing (Bhargava 2018).

Crowding of developing zebrafish (higher stocking density of zebrafish beyond 19 fishes/L water) has been linked to stress-induced perturbation of sex differentiation (Ribas et al. 2017). Others have found that stocking density of adult zebrafish at 5 fishes/L is optimal to express their normal behaviour without the influence of cortisol (Pavlidis et al. 2013).

Zebrafish is also susceptible to microbial infections in which mycobacteriosis and microsporidiosis are the most common infections caused by genus *Mycobacterium* and obligate parasite *Pseudoloma neurophilia*, respectively (Sanders et al. 2012; Whipps et al. 2012). The biofilm is shown to increase the chances of mycobacteriosis. If a zebrafish is infected, the infected fish should be immediately removed and discarded followed by complete cleaning of the system to prevent microbial transmission to other healthy fishes.

Importantly, whenever a fresh stock of zebrafish is procured from pet shops or transferred between labs, it is essential to first quarantine them in a separate tank(s) for at least 2–4 weeks before transferring them to the main housing system. Steps in quarantine are explained in more detail by others elsewhere (Borges et al. 2016).

1.3.4 Diet for Zebrafish Developmental Stages

Even though diet plays a vital role in the health and well-being of zebrafish of all ages, the actual nutritional requirement for them remains elusive. The zebrafish can feed on anything, but in larval stages, opening and strength of their mouth limits the size of food particles they can ingest. To this end, there are both live, natural and artificial/synthesized particulate size-based diets that are available to rear larval stages.

1.3.4.1 Live, Natural Lab-Grown Diet

Most labs grow infusoria culture to feed first feeding stage (FFS) larvae (Lawrence 2011). It is a broth culture with collection of actively growing unicellular organisms, e.g. algae, amoeba, ciliates, etc. As the maintenance of pathogen-free infusoria is a difficult task, few labs use freshwater-based defined rotifer culture/polyculture or polyculture with type L saltwater rotifers to feed FFS larvae (Aoyama et al. 2015; Best et al. 2010; Lawrence et al. 2016). The artemia culture can provide a live feed for the advanced stage zebrafish larvae (Gonzales Jr. 2012).

1.3.4.2 Commercially Available Formulated Diet

As a solution to the difficult infusoria diet, few companies are offering ready-to-use, defined size-based particulate formulated diet mainly for the FFS and advanced stage zebrafish larvae. These are compared in more detail in Table 1.2. Commercially available diets too have few limitations like different strains of zebrafish may have a varied nutritional requirement which may not be possible to supplement with a single commercial diet. Also, companies may change the product composition without notice, and the overall growth and survival may be affected if given only formulated diets (Goolish et al. 1999; Watts et al. 2012). Therefore, an option for both commercial and live feed should be used.

Feeding Regime for Zebrafish

1. From 0 to 5 dpf stage: No external feed is required. The developing embryo gets its supply from the yolk located in the yolk sac present on the ventral side of the body.
2. 6–8 dpf stage (FFS larvae): In this stage, yolk content is depleted significantly, and larvae require exogenous feeding for their survival which primarily consists of paramecium, rotifers or artemia. Some users have also tested the utility of artificial diet and found that portion of live feed can be replaced by continuous application of artificial diet without compromising the overall growth and survival of zebrafish larvae (Carvalho et al. 2006). Of note, a study found no adverse

Table 1.2 Nutritional profile of commercial feed suitable for First Feeding stage (FFS) larvae and other stages

S. No.	Companies	Product name	Lowest particle size (μm)	Nutritional analysis (%)					Remarks
				Protein	Lipid	Fibre	Ash	Moisture	
1	Sparos	Zebrafeed	<100	60	14	<1	9	8	Four types of diets are available: Zebrafeed (<100 μm); Zebrafeed (100–200 μm); Zebrafeed (200–400 μm); Zebrafeed (400–600 μm).
									https://www.sparos.pt/
2	Salt Creek Inc.	Fish Starter Diets	<200	57	14	5	11	7	Microfeast Feed at Bartlesville, OK, is acquired by Salt Creek Inc. Microfeast L-10 diet is now branded as Progression 1 (<200 μm). Higher particle sizes are also available: Progression 2 (200–300 μm); Progression 3 (300–500 μm); Progression 4 (500–800 μm).
									http://www.saltcreekinc.com/products/progfish.htm
3	Skretting	Gemma Micro	50–100	59	14	<1	14	–	Four types of diets are available: Gemma Micro 75: 50–100 μm; Gemma Micro 150: 100–200 μm; Gemma Micro 300: 200–500 μm; Gemma Micro 500: 400–700 μm.
									http://zebrafish.skrettingusa.com/collections/all
4	Zeigler Bros. Inc.	Larva Z Plus	<50	50	15	2	8	12	Five types of diets are available: 1 (<50 μm) Zoea 1 to Zoea 3; 2 (<100 μm) Zoea 3 to Mysis 3; 3 (100–150 μm) Mysis 3 to PL 3; 4 (150–250 μm) PL 3 to PL 6; 5 (250–450 μm) PL6 to PL 12[a].

									https://www.zeiglerfeed.com/shrimp/hatchery/larva-z-plus/
5	INVE Aquaculture	FRiPPAK FRESH micro encapsulated feed	5–30	52	15	3	–	10	Diet comes in three formats: #1 CAR (5–30 µm); #2 CD (30–90 µm); #3 CD (90–150 µm). This product can be procured from primo aquaculture.
									https://www.primo.net.au/shop/Prawn-Hatchery-Feeds/frippak-fresh
6	Tetra	Tetramin Tropical Flakes	Variable	47	10	3	–	6	Flakes can be converted into powder and filtered using sieve of desired size before use.
									http://www.tetra-fish.com/
7	Ocean Star International (OSI)	BSF (Brine Shrimp Flakes)	Variable	53	9	2	11	9	Flakes can be converted into powder and filtered using sieve of desired size before use.
									http://www.oceanstarinternational.com/BSFRedJungle454g.htm
8	Ocean Nutrition	Brine Shrimp Plus Flakes	Variable	55	15	<1	4	8	Flakes can be converted into powder and filtered using sieve of desired size before use.
									http://www.oceannutrition.com/dry-foods-detail/brine-shrimpplus-flakes.html

[a]Feed particles are constituted by crustacean larvae stages, e.g. Zoea (Z), Mysis (M) and Phyllosoma Larva or Post larva (PL)

effect on the growth and survival of zebrafish larvae even when there was a delay in initial feeding of exogenous diet to larvae until 8 dpf (Hernandez et al. 2018).
3. 9–11 dpf stage: The larvae can be fed twice per day with an artemia-based live feed (Farias and Certal 2016).
4. 12–30 dpf stage: The diet at this stage entirely consists of artemia fed three to four times per day (Farias and Certal 2016).
5. 31–60 dpf stage: At this stage, the diet consists of a combination of artemia and flakes/crushed pellets. The frequency can be two to four times per day (Wilson 2012).
6. Beyond 61 dpf stage to adult stage (~3 months old and beyond): At this stage, they can be fed with a normal adult diet made of commercial pellets.

1.4 Conclusion

The maintenance of zebrafish in a lab conditions is a daunting task as it requires proper care and attention. To understand the ABCD of zebrafish culture, a brief account on various steps right from zebrafish acquisition from the pet/commercial supplier to the options available to maintain them in lab conditions is presented in this chapter. For maintenance, several commercial to open-design systems are discussed. Health of the zebrafish can be influenced by water, dissolved oxygen, nitrogen content, toxins, housing density and pathogenic microbes. These environmental factors are also briefly discussed in the section related to the care of zebrafish health. Finally, the diet of zebrafish is discussed from the perspective of its developmental stage. In this section, the options of undefined infusoria-based diet to defined commercial pellet-based diet are presented and compared. Both types of diets (live and artificial) have their unique advantages and limitations due to the varied nutritional requirements of zebrafish at various developmental stages. It has also been demonstrated that the diet has a direct role in regulating the survival and reproductive health of the zebrafish which is a critical step in zebrafish rearing in lab conditions.

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Conflict of Interest Nothing to declare.

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Zebrafish: A Model Organism to Understand Tumor Angiogenesis Mechanism

2

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Abstract

Tumor angiogenesis is the most crucial step in the progression of all types of cancers. Preexisted blood vessel vascularizes into new one through sprouting or intussusceptive (splitting) mechanism. This process would facilitate the growth in the size of tumors regulated by VEGF (vascular endothelial growth factor), leading to metastasis, which ultimately increases the severity of cancer. So, it is very important to suppress tumor angiogenesis before the situation gets worse in a cancer patient. A wide variety of in vitro and in vivo models have been used to study the process of tumor angiogenesis and metastasis of cancer. It has helped us to discover new drugs and to find novel therapies for cancer, including anti-angiogenic therapy. Mainly angiogenesis is traditionally modeled in rodents and chick embryo, but of late zebrafish is emerging as the preferred model due its several advantages over the other animals. Zebrafish (*Danio rerio*) serves as the ideal model to study the various cancers, since it is possible to induce tumor growth or suppression easily, when compared to the other animal models. Also, tumor xenograft model has been studied in zebrafish extensively using many human cancer cell lines. So, in this chapter, we have reviewed some literatures that appreciate zebrafish model to study tumor angiogenesis.

Keywords

Zebrafish · Angiogenesis · VEGF · Tumor · Xenograft · Anti-angiogenic therapy

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2.1 Introduction

The term angiogenesis defines the formation of blood vessels from preexisting one, which has a vital role in supplying nutrients, oxygen transfer, and removal of metabolic waste in a cell. In a normal condition, any deviation in the vascular system causes the blood vessel tissue to collapse and die. But in cancer, when the rapid cell division occurs, a need for more supply of nutrients and oxygen arises. As a result, the blood vessels near the localized cancer sprout or split (Vimalraj et al. 2019a) to reach out to all the cells in that primary area. Nitric oxide (NO) influences the angiogenic switching from sprouting angiogenesis (SA) to intussusceptive angiogenesis (IA) at the site of tumor (Vimalraj et al. 2018, 2019b). Soon, these cancer cells would grow and develop into a tumor. When the blood vessels cover all the cells in the tumor, it further proliferates aggressively, called tumor angiogenesis. Tumor angiogenesis can further lead to intravasation, extravasation, and metastasis (Rouhi et al. 2010). The rapidly dividing cancer cells released angiogenic growth factors, including the vascular endothelial growth factor (VEGF) (Gordon 2000) and fibroblast growth factor (FGF) (Presta et al. 2005) which contributes important role in initiation of tumor neovascularization.

VEGF signaling proteins such as VEGFs (VEGF-A, VEGF-B, VEGF-C, and VEGF-D), VEGF receptors (VEGFR-1, VEGFR-2, VEGFR-3), and co-receptors neuropilins (NRP1, NRP2) are involved in the stimulation of new blood vessel development in the tumor microenvironment (Song 2007; Hooper et al. 2009; Duan et al. 2013; Zhou et al. 2013; Ebos et al. 2009; Anderson et al. 2011; Skirnisdottir et al. 2016). Proteins like netrins (Daluk et al. 2013), G protein γ2 (Nandagopal et al. 2018), and galectin-1 (Pulkkinen et al. 2008) which are required for VEGF signaling promoted tumor angiogenesis. In addition, FGFs (fibroblast growth factor) are often upregulated in tumors that escape anti-VEGF treatment. The regulation of tumor angiogenesis involves signaling pathway of angiopoietin 1 (ANG-1), angiopoietin 2 (ANG-2), and TIE-2 receptors. The endothelium and tumor cells both express ANG-1, which supports the integrity of blood vessels. ANG-2, an endothelial cell adhesion protein that promotes blood vessel movement, is produced by sprouting endothelial cells (Daluk et al. 2013). Some well-known anti-VEGF agents to treat cancer are sorafenib, sunitinib, pazopanib, and axitinib (Nandagopal et al. 2018).

Additionally, hypoxia has been shown to enhance angiogenesis via the activation of HIF1 (hypoxia-inducible factor 1) in order to maintain homeostasis. Both HIF and VEGF regulate blood vessel formation in tumors in accordance with the degree of oxygenation in the tumor microenvironment (Pulkkinen et al. 2008; Chen et al. 2013). Hypoxia-induced angiogenesis is mediated by VEGF stimulation of the prolyl hydroxylase hypoxia-inducible factor (HIF) signaling pathway. By boosting tumor cell dissemination through leaky blood vessels into the circulation, VEGF-induced vascularization may increase tumor invasion and metastasis (Moshal et al. 2010; Lin et al. 2009; Jensen et al. 2011; Zhang et al. 2009). SDF-1 (a family of low molecular weight cytokines) is increased during hypoxia and promotes vascularization via interacting to the CXCR4 receptor on endothelial cells, in addition to growth

factor signaling. S1P (sphingosine-1-phosphate) is another well-known chemokine factor that regulates blood vessel integrity in the tumor microenvironment by binding to its G-protein-coupled receptors (S1PRs) (Singh et al. 2007).

MicroRNAs (miRNAs) play a crucial role in regulating vasculogenesis and angiogenesis (Fish and Srivastava 2009; Wang and Olson 2009; Patella and Rainaldi 2012). MiRNAs in the pro-angiogenic subclass target negative angiogenesis regulators to increase angiogenesis, whereas those in the anti-angiogenic subclass target positive regulators. Several studies have demonstrated that miRNAs play a role in changing tumor environment and releasing pro-angiogenic factors (Zhou et al. 2013; Suárez et al. 2008; Santoro 2011; Ho et al. 2018). Hypoxia affects cancer cells differently than endothelial cells when regulated by MiR-210 (Pulkkinen et al. 2008). MiR-503 acts as an anti-angiogenic factor in malignancies by targeting FGF2 and VEGFA (Zhou et al. 2013). VEGF-A and N-RAS are also transcriptionally regulated by MiR-145 to inhibit tumor angiogenesis (Zou et al. 2012).

To unravel tumor angiogenesis and metastasis, zebrafish are the ideal model. Humans share many similarities with zebrafish with regard to their vasculature. A good deal of clinical research studies have reported that the advancement of capillary such as the dorsal aorta, posterior cardinal blood vessel, subintestinal capillaries, and inter-segmental vessels in very early zebrafish embryos is well characterized with analysis of anomalies impacting their formation in both regular and also transgenic fishes (Staton et al. 2004). It is a suitable model because of its small size, inexpensive ability to produce many offspring, and easy to maintain long-term and easy to carry out in vivo manipulation (Thisse and Zon 2002; Ma et al. 2011). The transparency of zebrafish embryo until 5 days post fertilization enables straight in vivo monitoring of blood vessels through fluorescent microscopy growth, coupled with the availability of reporter fluorescent transgenic lines for endothelial markers (Santoro 2011). In addition, the transparent embryos develop outside the mother fish, using only a low-power light microscope to directly observe the formation and growth of blood vessels (Lawson and Weinstein 2002). It allows us to see fluorescently tagged tumor cells in a complete embryo that cause vascular remodeling, spreading and infecting adjacent organs. The embryo model demonstrates the tumor stroma's role in long-term tumor angiogenesis and metastasis. The biocompatibility of anti-angiogenic medicines affecting malignant cell survival and migration can be assessed by feeding the agents to zebrafish embryos in a live state (Tulotta et al. 2016). The majority of pro-angiogenic and anti-angiogenic compounds screening is usually done on rodents and chick embryo model systems (Hasan et al. 2004). Several reports proposed zebrafish angiogenesis as an alternative model for drug screening (Serbedzija et al. 1999) and tumor angiogenesis (Tobia et al. 2011). By administering angiogenic factors continuously to tumor cell xenografts in animal models, we may be able to simulate the early phases of tumor angiogenesis and metastasis.

Human melanoma cells injected into the embryos of zebrafish are being studied for their influence on the development of zebrafish vasculature (Topczewska et al. 2006; Haldi et al. 2006). The main premise is that pro-angiogenic chemicals produced locally by the tumor graft would disrupt the normal growth pattern of subintestinal veins (SIV) by promoting the migration and proliferation of sprouting

capillaries toward the implant (Nicoli et al. 2007). When compared to chick embryo chorioallantoic membrane (CAM) assay, the zebrafish xenograft model exhibited a similar ability to discriminate between highly angiogenic and less angiogenic tumor cell lines (Ribatti et al. 2001). MicroRNAs that promote or inhibit angiogenesis are appealing medicines for the treatment of cardiovascular and cancer diseases (Patella and Rainaldi 2012). The zebrafish tumor xenograft model will assess if it affects the tumor cells' angiogenic response when angiogenesis-related microRNAs (miRNAs) are overexpressed (Chiavacci et al. 2014).

2.2 Tumor Induction in Zebrafish by Chemical or Carcinogen Exposure

Zebrafish can be used to develop tumor or cancer models by treating them with chemicals that mutate or cause carcinogens. Many studies have utilized chemical treatment methods to model cancer in zebrafish. Compounds like ethylnitrosourea (Beckwith et al. 2000), nitrosomorpholine, and diethylnitrosamine cause the formation of hepatocellular carcinoma and liver tumors (Topczewska et al. 2006; Pliss and Khudoley 1975; Spitsbergen et al. 2000). More cancer-causing agents, for example, dibenzo(a,l)pyrene (DBP), 7,2-dimethylbenz(a)anthracene (DMBA), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), *N*-dimethylnitrosamine (DEN), *N*-nitrosodiethylamine (NDMA), and *N*-ethyl-*N*-nitroso-urea, can artificially initiate tumors that are equivalent to hereditarily caused tumors and can take after human malignant growth highlights (Letrado et al. 2018). The majority of genes deregulated in chemically induced zebrafish live tumors are similar to those deregulated in human malignancies, according to a gene profile study of zebrafish microarrays (Lee et al. 2005). By using chemical and genetic approaches, zebrafish cancer can be modeled in many different ways. Developing temperature-controlled Cre/Lox and HSP70-inducible systems in zebrafish remains challenging in cancer research. The differentially methylated genes in hepatocellular carcinoma of zebrafish have been documented to be aberrantly methylated in humans during tumorigenesis (Mirbahai et al. 2011).

Aflatoxin, particularly aflatoxin B1/AFB1, is a genotoxic hepatocarcinogen and a type of cocarcinogen. In nature, AFB1 is processed by cytochrome P450 enzymes to reduce its toxicity. The very reactive genotoxin, on the other hand, is aflatoxin B1-8,9-epoxide (AFBO), a reactive intermediate chemical compound. Both AFB1 and AFBO attach to DNA in hepatocytes, causing DNA chain breaking, base damage, and oxidative damage (Liu and Wu 2010; Hamid et al. 2013).

Alcohol is a cocarcinogen and can create damage similar to aflatoxins for liver cancer. Cirrhosis or scarring is observed in the case of alcohol-associated hepatocellular carcinoma (HCC). The activation of the JAK/STAT and p38 mitogen-activated protein kinase (MAPK) pathways is found in alcohol. By generating cytokines, chemokines, and stress, it reacts. Both cell differentiation and growth are affected by these changes (Lu et al. 2015). Acetaldehyde, an alcohol metabolite, is classified a carcinogen due to its ability to enhance oxidative stress and DNA damage (Voigt

2005). These impacts can cause liver fibrosis and cirrhosis, which can progress to HCC later in life. However, between 5% and 30% of HCC patients have no obvious identified risk factors for cancer, but insulin resistance and inflammation cause HCC (Siegel and Zhu 2009).

2.3 Zebrafish Xenograft Model of Tumor Development

So far, we have discussed induction of tumor in zebrafish through carcinogenic mutation. However, all these methods are not exactly mimicking the milieu of human tumor. The study of xenotransplantation of the human tumor in the other animal model gives us one step ahead in the cancer research. Several developed models to study cancer (murine xenograft, chicken CAM, and in vitro model) are not recapitulate or allow observation of all steps and factors involved in the metastatic cascade (Brown et al. 2017). Albeit mouse xenograft model is considered as "gold standard" of cancer research (Wertman et al. 2016), the transplantation of human cells in mouse was never be easy in that way. Among them, graft rejection plays a vital role in the xenograft models and was overcome by having immunocompromised mouse model. But this fails in providing the interaction of the immune system with tumor cells.

Recently, the study of zebrafish xenograft model (Fig. 2.1) in cancer research was a promising one and may replace or overcome the other model available. Lee et al. were the first group who studied the fate of human aggressive melanoma cells in blastula stage of zebrafish embryo with no immunosuppression drug. As a result of their experiment, they have demonstrated that both the tumor stroma and tumor itself can be xenografted, allowing the possibility of examining tumor-stroma interactions with zebrafish (Lee et al. 2005; Kocere et al. 2015). However, the malignancy of melanoma cells was suppressed in the zebrafish because of some environmental factor (Lee et al. 2005). The fluorescence resonance energy transfer (FRET)-based caspase-3 sensor detected the tracking of murine melanoma B16F10 cells proliferation in transgenic zebrafish Tg(fli1:EGFP) and in 2D and 3D culture. But none of them have shown the properties of micrometastasis except xenografted zebrafish (Fu et al. 2018). The micrometastasis proved difficult to detect, yet it plays an important role in cancer therapy. In another study, human pancreatic cancer cell lines (MiaPaCa-2 and BxPC-3) were xenografted in the perivitelline space of embryo and cardiac chamber of an adult through intraperitoneal cavity. They observed that cancer cells in the abdominal and digestive organ of zebrafish within 7 days after the injection of tumor confirm the evidence of pancreatic cell proliferation (Guo et al. 2015).

Nowadays, the real-time therapy of patient-derived xenograft (PDX) was successful personalized medication in cancer treatment. The use of PDX model in preclinical studies contributes to the development of new precision medicine applications by predicting the individual clinical response to novel compounds or a combination of those (Gaudenzi and Vitale 2019), because genetic and phenotypic expression of different tumor cells differs between individuals. Scientist widely use

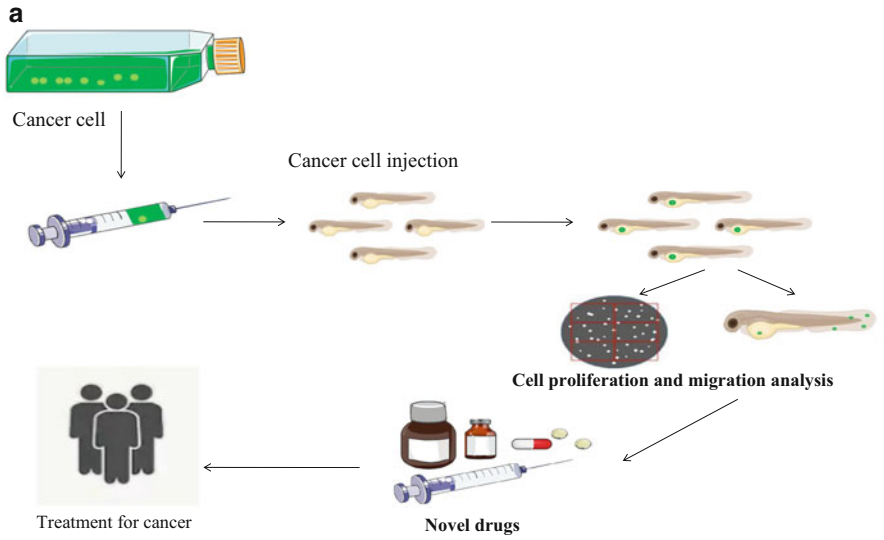

Fig. 2.1 (**a**) Schematic portrayal of age of zebrafish xenograft model. (**b**) Tumor xenograft model in zebrafish. Harmful tumor cells, CT26 (murine colon carcinoma) and B16 (melanoma) and HEK 293, non-carcinogenic (tagged with red fluorescence) were infused into the perivitelline space of stomach area of Tg(flk1:EGFP) transgenic zebrafish incipient organisms (48 hpf). Tumors (b, red) 350–450 mm in width show neo-vessels (b, green); the neoplasia were segmented and stained with H&E (b). Xenografted tumors' development rate (c) and number of neo-vessels prompted by tumor (d). (Figures obtained from Zhao et al. 2011)

mouse PDX model for the real-time therapy of cancer cell. But the transplantation of patient cells or tissue into the mouse takes nearly 2–4 months, and also it requires immunocompromised strain. Dellaire and his group successfully transplanted T-cell acute lymphoblastic leukemia (T-ALL) cells into zebrafish from the patient samples and quantified the γ-secretase inhibition ex vivo that regulates the NOTCH and PI3K/AKT pathway (Bentley et al. 2015). Another set of group used neuroendocrine tumor (NET) from patient sample engrafted into subperidermal cavity of Tg(fli1: EGFP) zebrafish embryo (Gaudenzi and Vitale 2019). However, it is not possible in some types of cancer (breast, lungs, and prostate) where the anatomical structure of zebrafish is different.

Zebrafish has distinctive advantages over using mice model. First, the engrafting tumor cells in mice require immune suppression because of host rejection. Murine xenograft studies usually use highly on immunodeficient (severe combined immunodeficient disease (SCID) or nude) mice to prevent the immune rejection. For instance, NOD/LtSz-*scid* IL2Rγ^{null} (NSG) mouse lacks common cytokine receptor γ chain, resulting in a paucity of B, T, and natural killer cells (Wertman et al. 2016). Anyhow, tumor progression was seen in NSG mice strain but the actual environment for the tumor was not achieved. However, engrafting tumor cells to the zebrafish

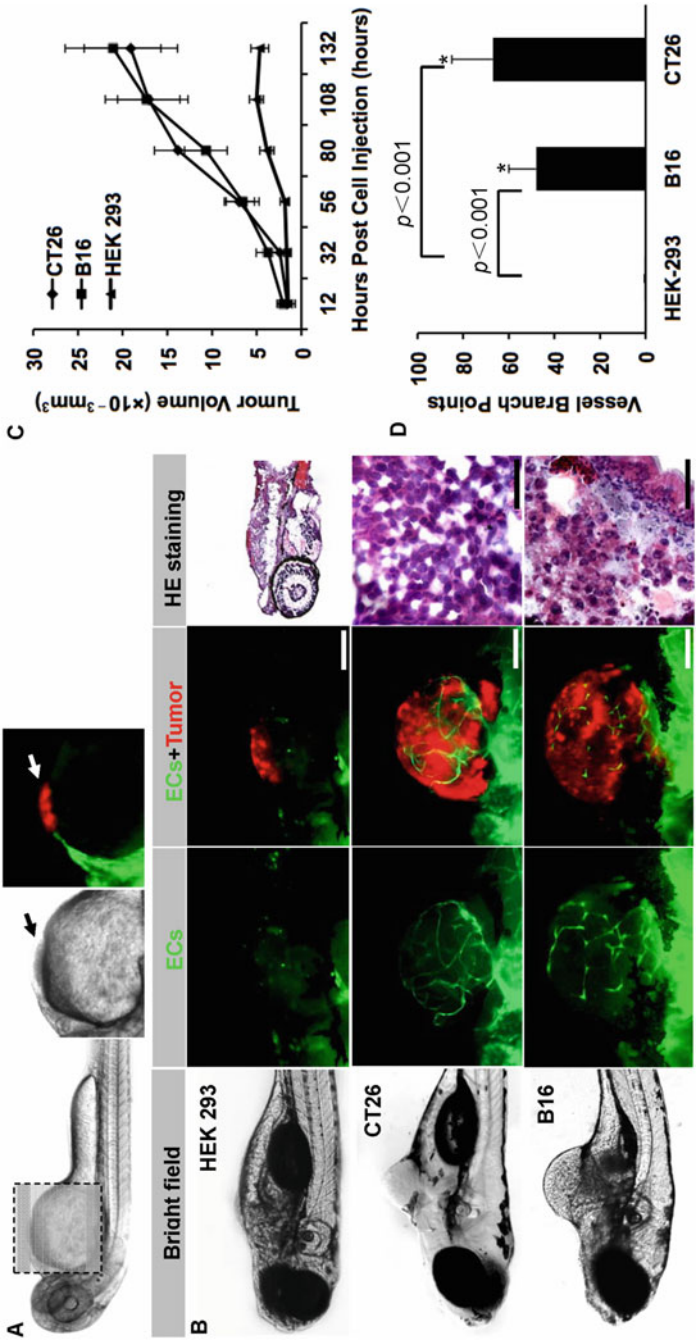

Fig. 2.1 (continued)

embryo require no immune suppression drug because of undeveloped adaptive immune system. Second, the visualization of tumor proliferation in zebrafish was much easier than the other vertebrae model because of their transparent embryo. Third, required number of tumor cells for transplant in zebrafish was very less compared to the mouse (zebrafish, 50–200 cells/fish; mouse, 0.5–1 million cells/mouse). Fourth, doubling the rate of tumor growth in zebrafish enables rapid detection of pro- and anti-angiogenic factor micrometastasis (Kocere et al. 2015). Furthermore, in cancer research, zebrafish offers advantages such as small scale, cheap cost, simplicity of study, and capacity to alter in vivo (Chiavacci et al. 2015).

2.4 Choice of Injection: Embryo vs. Juvenile vs. Adult

In zebrafish, injected tumor cells are heterotopic, meaning that the donor tissue is not in the same location as the host receiver (Wertman et al. 2016). However, the injection of tumor cell into zebrafish embryo (includes blastodisc region, yolk sac, hind brain ventricle, and duct of Cuvier (Wertman et al. 2016) (Fig. 2.2)) offers many advantages such as ease of experimentation, drug administration for their permeability to small molecules, and a transparent body wall, among others (Zhang et al. 2015a). Injection was preferred there because it is largely avascular and acellular and has nutrient-rich conditions to promote cancer cell growth, unlike the hindbrain ventricle (Wertman et al. 2016). But there are some leakage of cells which can be avoided by injecting more no. of cells (Brown et al. 2017). Usually, tumor cells are injected between 24 and 48 hpf in the zebrafish embryo. Because of a single embryonic cell, it is well suitable for antisense morpholino gene targeting approach in cancer study (Zhang et al. 2015a). Since the adaptive immune system is not developed at the time of injection, there will be no graft rejection. After 5–7 days, adaptive immunity developed and the possibility of tumor growth suppression is higher (Zhang et al. 2015a). However, embryo grafting is the best model in studying tumor-induced angiogenesis, metastasis, and determination of pro- and anti-angiogenic factor compared to juvenile and adult fish.

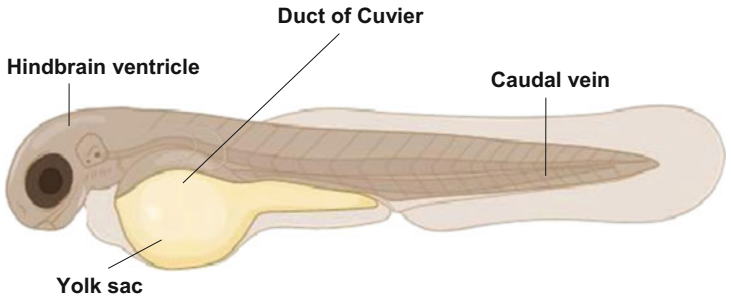

Fig. 2.2 Schematic diagram showing the location of tumor cell injection site in zebrafish larvae

Human cancer cells are transplanted into juvenile fish at 30 dpf, and the immune system is suppressed with 15 Gy of irradiation and dexamethasone (Zhang et al. 2015a; Tobia et al. 2013). At this stage, well-developed vasculature provides an unobstructed view of micro-tumor formation, cell invasion, and angiogenesis using confocal microscopy (Zhang et al. 2015a). Human cancer cells are introduced into the peritoneal cavity of zebrafish that are more than a month old. Because of the existence of adaptive immunity, zebrafish are irradiated with 20–25 Gy or treated with dexamethasone before injection (Wertman et al. 2016; Zhang et al. 2015a). But if 15 Gy is irradiated, the immune system is restored again after 20 days of the first injection and inhibits the long-term cancer research analysis (Wertman et al. 2016). The visualization of adult fish is tougher than embryo and juvenile due to presence of melanocytes and iridophores in their stripes. Noninvasive imaging of nontransparent zebrafish adults has also been tried utilizing whole mount staining, AP staining, and other methods. Ultrasound biomicroscopy has been used to follow the formation, vascularity, and reaction of liver tumors to treatment. For studies using adult zebrafish tumor xenotransplantation, a number of other imaging methods can be used, such as micro computerized axial tomography and magnetic resonance imaging. Casper mutant (cross between *nacre* mutant and *roy* mutant) that lacks all pigment in fish made them translucent in viewing (Zhang et al. 2015a). Transgenic fish with fluorescently tagged vasculature or internal organs are another option for a recipient that may be used to examine tumor-host interactions in adult zebrafish using multiple optical platforms (Zhang et al. 2015a), for example, transgenic line of friend leukemia integration 1 with expressed green fluorescence protein (*fli1:EGFP*) for the vascular visualization, macrophage expressed 1 with fluorescent protein mcherry (*mpeg1*:mcherry) for macrophage visualization, and myeloperoxidase with expressed green fluorescence protein (*mpx:EGFP*) for neutrophil visualization (Kocere et al. 2020). We have mentioned the available zebrafish xenograft model in Table 2.1 and their site of injection.

However, each site of location has some disadvantage in studying the entire process of cancer. Interestingly, Zhang et al. created a novel model for cancer study with immunological tolerance in zebrafish. Initially, the irradiated cancer cells (not killed, only proliferation is controlled) are injected in the yolk sac of the embryo. Here cells are not proliferated and destroyed after the development of adaptive immune system. Now then, nonirradiated cancer cells are injected in the dorsal aorta of same 3-month-old adult fish with no need of immunosuppression drug. The cancer cells grew and metastasized in the absence of host immunological inhibition, making them an ideal model for researching immune-related cancers (Zhang et al. 2015b).

2.5 Tumor-Induced Angiogenesis in Zebrafish Model

Primary tumor invasion and removal, intravasation into the blood or lymph vessels, extravasation onto specified secondary tissues, engraftment into a new microenvironment, and eventually tumor proliferations are all part of the metastatic process.

Table 2.1 The tabular column given below represents the zebrafish tumor/xenograft model

Cancer source	Strain type	Age	Injection site	Reference
Human umbilical vein endothelial cells (HUVECs), DU 15 prostate cancer cells	Tg(Kdrl: eGFP)	48 hpf	Perivitelline space	Chiavacci et al. (2014)
Red fluorescent mouse melanoma B16 breast cancer cells	*Tg(fli1a: EGFP)*, *Tg (mpeg1: mcherr y)*, *Tg (mpx:GFP)*	3 dpf	The neural tube of embryo	Kocere et al. (2020)
CM-DiI-labeled T-cell acute lymphoblastic leukemia (T-ALL) cell line	Casper mutant zebrafish	48 hpf	Yolk sac	Bentley et al. (2015)
FRET-labeled murine melanoma B16F10	*Tg(fli1: EGFP)*, wild type	72 hpf	Beneath the yolk sac	Fu et al. (2018)
Neuroendocrine tumor (NET) cell line	*Tg(fli1a: EGFP)*	48 hpf	Subperidermal cavity	Gaudenzi and Vitale (2019)
CM-DiI-labeled Mia PaCa 2 cells, BxPC-3 pancreatic cancer cell line	*Tg(flk1: EGFP)*	Embryo: 48 hpf, Adult: 6 mpf	Perivitelline space of embryo; cardiac chamber of adult fish through intraperitoneal	Guo et al. (2015)
CM-DiI/DiO-labeled Ep Ras cells	*Tg(fli1: EGFP)*	48 hpf	Yolk sac	Marques et al. (2009)
CM-DiI-labeled gastroenteric cancer HGS and SGC7901	*Tg(fli1: EGFP)*	48 hpf	Yolk sac	Wu et al. (2020)
RFP-labeled U87 glioma cell line	*Tg(fli1: EGFP)*	48 hpf	Embryo	Yang et al. (2013b)
Human cancer cell lines (293T, 3T3, and MDA-MB-231), MCF10A-derived breast epithelial cell lines M1, M2, and M4	*Tg(fli1: EGFP)*	48 hpf	Duct of Cuvier	Drabsch et al. (2013)
Human breast carcinoma (MDA435), fibrosarcoma (HT1080), melanoma (B16)	Immune suppressed AB, *Tg(fli1: EGFP)*	1 mpf	Peritoneal cavity	Stoletov et al. (2007)
Human glioblastoma (U87MG)	Wild type	4 dpf	Yolk sac, brain	Lal et al. (2012))

The interactions of cancer cells with their external environment, the microenvironment, and the metastatic niche are intriguing targets for preventing or slowing tumor progression (Brown et al. 2017). However, in most cancer type, the detection of metastasis was after the appearance of secondary tumor in early prognosis. Zebrafish tumor model serves as the best model to study the tumor-induced angiogenesis, early micrometastasis because of its rapid development, and unobstructed view of vasculature. The vascular development of zebrafish begins at around 12 hpf, with the formation of vascular cord where vascular endothelial cell (EC) migrated from lateral plate mesoderm. In 20 hpf, the complete lumen of the dorsal aorta (DA) and the ventral cardinal vein (PCV) is formed by ephrin-b2a and ephrin-b4a. Blood flow starts with circulation, but the specification of the DA and PCV lumen and arterial versus venous blood flow is genetically determined and is not dependent on circulation or blood flow. As each somite grows vertically, an intersegmental vessel (ISV) grows dorsally and laterally and involves notochord and neural tube. This is the first blood vessel of the zebrafish. A second vein, the subintestinal vein (SIV), originates from the PCV, which supply the gut, liver, and pancreas with blood (Fig. 2.3). The entire process regulated by sonic hedgehog and vegfa stimulation involves several pathways such as notch signaling, Eph-ephrins, Vegf - Neuropilins cytoplasmic scaffold molecules, i.e., synectin, and transcription factors such as Ets family members, Scl/Tal1 and Coup-TFII (Brown et al. 2017; Baldessari and Mione 2008; Guerra et al. 2020). The profoundly specific tumor-prompted angiogenic measure is trailed by a progression of steps: (1) enactment and advancement of the angiogenic endothelial cell aggregate; (2) changes in the extracellular network and cellar film corruption; (3) expansion and relocation of endothelial cells; (4) improvement of primer tubules; and (5) rebuilding of recently molded miniature vessels (Yang et al. 2013a).

To read the tumor angiogenesis, we look into the ISV and SIV growth in the zebrafish body to get whether newly formed vessels help the tumor cells to metastasize. For instance, tumor cells sprouted and lumenize into vessel within 48 hpi (hour post injection) after the complete development of SIV and cardinal vein in PDX-neuroendocrine tumor model (Gaudenzi and Vitale 2019). And also in the colorectal cancer, the no. of SIV and ISV is increased compared to non-tumor model (Petrovi et al. 2020). However, the formation of new vessels in any cancer model requires tumor proliferation, intravasation, extravasation, and dissemination. For example, MDA-MB-231 breast cancer cells injected in the duct of Cuvier are disseminated to the DA, caudal vein, and smaller optic vein, and also in ISV (Drabsch et al. 2013). When mouse melanoma cells are injected into the neural tube of transgenic embryo fish, the ISV becomes thinner after 7 dpi, and there is a strong proliferation of blood vessels near the tumor cell mass (Kocere et al. 2020). The injected tumor cells need not be form tumors as with Lee et al. group where less aggressive human melanoma cells failed to form tumor when injected in the blastula stage of embryonic zebrafish, but cells were found in the near vicinity of DA (indication of dissemination) and gone after 3-month developed fish (Lee et al. 2005). So, the malignancies of each tumor type differ based on the phenotype and microenvironment of that cancer. To provide evidence for this conclusion, Drabsch

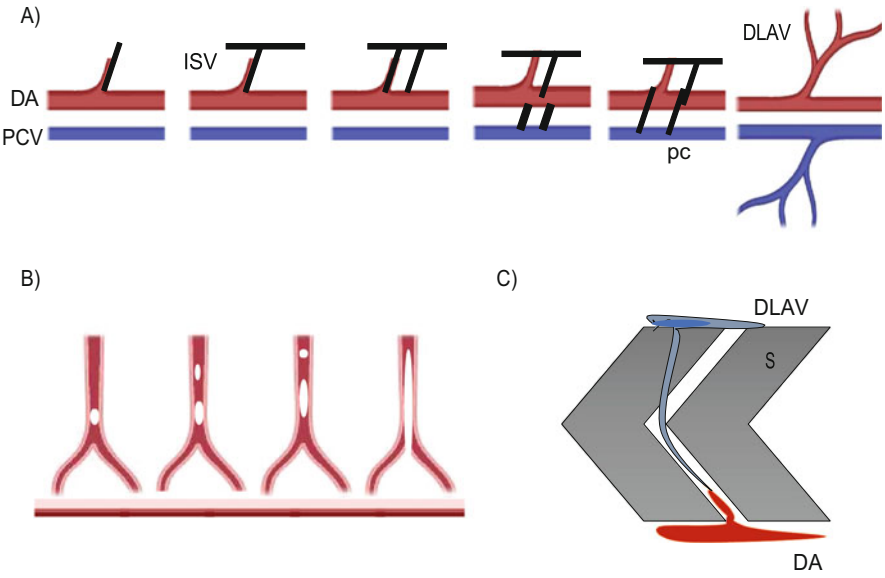

Fig. 2.3 Zebrafish blood vessel development. (**a**) Intersegmental vessel formation: An embryo of zebrafish is depicted in the scheme. The sprouts of the dorsal aorta (DA) ascend between the trunks and form two parallel dorsal longitudinal anastomotic vessels (DLAVs) between them. The posterior cardinal vein (blue) is the only vein from which secondary sprouts emerge. Most secondary sprouts form intersegmental veins (blue) and lead to parachordal vessels (pv), whereas others do not. Anterior intersegmental arteries (red) become primary segments and become affixed only to the aorta. Intersegmental veins form additional connections with the parachordal vessels at the level of the horizontal myoseptum. (**b**) Vessel lumen formation. Anatomically, the diagram shows the following processes: intracellular vesicle formation, intracellular vesicle fusion, vacuolar intertwining, blood vessel formation, and lumen formation. (**c**) Intersegmental vessel model of zebrafish (ISV). Each ISV consists of three types of endothelium. The ventral connection to the aorta is represented by an inverted "T" (red). The DLAV is connected to the T-shaped cell (blue) dorsally. A connecting cell (green) appears to run ventrally between the notochord and the neural tube between the somites, without traversing dorsally across the somite boundary. *ISV* intersegmental vessel, *DLAV* dorsal longitudinal anastomotic vessel, *pc* parachordal channel, *S* somite, *DA* dorsal aorta, *PCV* posterior cardinal vein. (Figure obtained from Baldessari and Mione 2008)

et al. group examined the tumor malignancy of MCF10A-derived epithelial cell lines M1, M2, M3, and M4 (level of malignancy increases from left to right order). Their result shows that the very less M1 and M2 cells are found in the caudal hematopoietic tissue (CHT) than the M3 and M4 cells. However, M2 cells also metastasize when it was engrafted more no. of cells to the zebrafish embryo. They also found that specific cells will reach specific site as in the case of MDA-MB-231 breast cancer cells found in collagen matrix of caudal fin (Drabsch et al. 2013).

The zebrafish tumor model is highly advantageous because solid tumors require adequate blood supply to grow, survive, and spread. A number of molecules have been found to be important in vascular and tumor biology, such as neuropilins (Nrp1, Nrp2), semaphorins (Sema3), and vascular endothelial growth factor (VEGF)

(Baldessari and Mione 2008). Tumor can grow up to 1–2 mm^3 and for further growth and metastasis, tumor cell prone to hypoxic condition which elevates the VEGF expression. The signaling cascade of VEGF interaction with the VEGF receptors (kdrl in zebrafish) promotes the formation of vessels by sprouting as well as intussusceptive. In hypoxic environment, adenosine also stimulates the production of angiopoietin-1, VEGF, and IL6 via adenosine receptor on vascular cells that may contribute to angiogenesis (Rani et al. 2016). And also other growth factors such as transforming growth factor (TGF-β1), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) stimulate the VEGF expression and promote the angiogenesis. The TGF-β in tumors can act as both suppressor and promoter, and there are several signaling molecules that are influenced by the TGF-β (Drabsch et al. 2013). Yang et al. found that the tumor invasion is increased by the TGF-β1 through JNK pathway in glioma model. And also, they found that tumor-associated macrophages (TAM) are infiltrated with blood vessel and migrated toward the brain, tail, and yolk sac (Yang et al. 2013a). So, the absence of TAM in breast cancer model provides no tumor invasion after 24 hpi of MDA-MB-231 cells injected in the duct of Cuvier in zebrafish embryo (Drabsch et al. 2013).

2.6 Potential Screening of Anti-angiogenic Drug in Zebrafish Tumor Model

Transparent zebrafish embryos or adult mutants of zebrafish offer an alternative to cell culture models and other animal models for testing and studying the effect of new chemical bioactive agents and nanomaterials on cell viability, cancer risk, and cancer-inducing activity (Mimeault and Batra 2013) (Fig. 2.4). The studies have shown that the use of anti-angiogenic drug mostly of VEGF blocker (sunitinib) with chemotherapy treatment increases the patient's survival rate. Anti-angiogenic medicines are used to prevent new blood vessels from forming, cell proliferation, and tumor growth. List of anti-angiogenic drugs used in zebrafish model and their observed result are mentioned in Table 2.2.

Researchers will be able to utilize 96-well plates (two to five embryos/well) or micro plates for high-throughput screening of different water-soluble bioactive compounds that immediately permeate into embryos due to the small size of ex utero zebrafish embryos (Mimeault and Batra 2013) or it can be administered with DMSO also. Zebrafish model is used for studying the dose-dependent drug treatment on tumor-induced zebrafish model. After the treatment of drug, the trunk of ISV and SIV was quantified using ImageJ software.

The zebrafish yolk membrane (ZFYM) assay was suggested more recently, and it is based on injecting recombinant angiogenic growth factor into the perivitelline area of zebrafish embryos in the vicinity of developing SIV. Because of well-established angiogenic stimuli in ZFYM assay, the development of ectopic blood vessel growth is calculated. This enables a low and high molecular weight antagonist to be tested for a particular angiogenic factor and/or its receptor (Tobia et al. 2013). Rani and her group studied the role of adenosine receptor in hypoxic environment of non-tumor

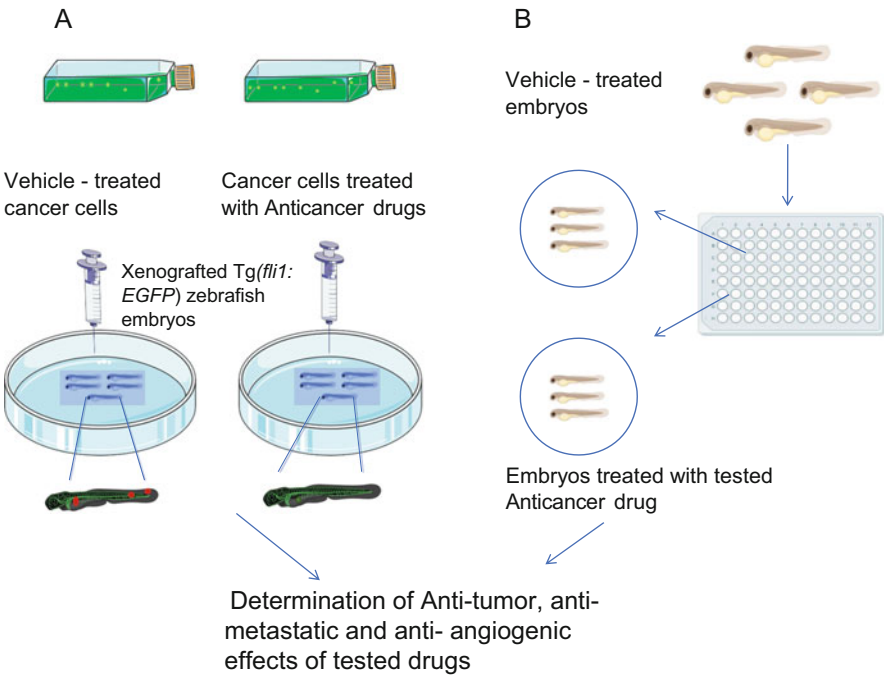

Fig. 2.4 Schematic representation of testing anticancer/anti-angiogenesis drug in zebrafish model. (**a**) Zebrafish embryos xenografted with cancer cells. (**b**) Use of mutant or transgenic zebrafish

ZFYM assay using adenosine agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) and antagonist methylxanthine pentoxifylline (PTX). They observed that NECA triggers the ISV formation and PTX completely block the ISV formation through the downregulation of vegfaa (Fig. 2.5). This was the same result when apigenin and forskolin decrease or increase the production of vegfaa, respectively (Rani et al. 2016). Our group utilized the caudal regeneration assay to study the role of NO in intussusceptive angiogenesis in transgenic zebrafish model Tg(Tie2:EGFP) (Vimalraj et al. 2019b) (Figs. 2.6 and 2.7).

However, the mechanism of angiogenesis in this model was not the same as tumor model because it is wound healing angiogenesis. There are very less models in relating to the intussusceptive angiogenesis compared to sprouting because it happens at the low rate of VEGF and others and actual mechanism of intussusceptive was not understood well.

Apart from the chemical substance, the gene knockdown of specific gene is also an effective approach in tumor inhibition. For example, using morpholino antisense oligonucleotides or the retinoic acid receptor-a antagonist Ro41-5253 to downregulate miRNA-10a expression in PaTu-8988T pancreatic cancer cells followed by injection into the yolk sac of 2 dpf zebrafish embryos was effective at decreasing invasion and dissemination compared to control morpholino oligos and untreated PaTu-8988T cell (Vlecken and Bagowski 2009). Similarly, silencing two

Table 2.2 List of anti-angiogenic drugs that are tested in zebrafish model

Cancer type	Anti-angiogenic drugs	Observed result	Reference
Murine melanoma B16F10 cell line	LY294002	Inhibits PI3K/AKT pathway—responsible for micrometastasis	Fu et al. (2018)
Human cervical cancer CaSki cell line	*N*-benzyloxycarbonyl-Ala-Asn-doxorubicin	Migration, invasion of cells blocked through legumanin; less toxic compared to doxorubicin alone	Chen et al. (2020)
HUVEC	1-methoxycarbony-β-carboline (from *Picrasma quassioides*)	Both ISV and SIV blocked completely; inhibition of cell proliferation, migration; suppress the activity of ANG, EGF, TIE-2, IGF-1, bFGF, GRO (CXCL1), MMP1	Qing-Hua et al. (2018)
Gastric cancer SGC7901 cell line	Ramucirumab, apatinib, regorafenib, cabozantinib	Decrease in cell proliferation, pro-angiogenic factors; dose-dependent ISV inhibition but ramucirumab has no effect on ISV inhibition; SIV inhibition is stronger in regorafenib and in cabozantinib; all four shows pericardial edema, tail bending, and shortened body axis	Wu et al. (2020)
U87 glioma cell line	SP600125 (JNK inhibitor)	Involves in TGF-β signaling responsible in the VEGF-mediated angiogenesis	Yang et al. (2013a)
Human breast cancer, MDA-MB 231	SB431542; LY294002 (TGF-β kinase inhibitor)	Decreases in cell migration, epithelial to mesenchymal transition, invasion, and metastasis to bone	Drabsch et al. (2013)
HUVEC, DU145 prostate cancer	Silencing miR125a, miR320, miR492, miR487b	Apoptosis increases, cell proliferation decreases	Chiavacci et al. (2014)
Red fluorescent mouse melanoma B16 breast cancer cells	Cy5-labeled poly(ethylene glycol)-block-poly (2-(diisopropyl amino) ethyl methacrylate) PEG-PDPA	Angiogenesis is decreased at the neural tube due to absence of TAM	Kocere et al. (2020)
CM-DiI-labeled Mia PaCa 2 cells, BxPC-3 pancreatic cancer cell line	U0126	Inhibits the tumor progression through KRAS signaling pathway	Guo et al. (2015)

(continued)

Table 2.2 (continued)

Cancer type	Anti-angiogenic drugs	Observed result	Reference
MCF-7 breast cancer, A549 lung carcinoma cell lines	PTX	No ISV formation	Rani et al. (2016)

LIM serine/threonine protein kinases (LIMKs), LIMK1 and LIMK2, involved in cell cycle control, by short interfering RNAs in pancreatic cancer cells has resulted in full suppression of invasion and micrometastasis generation (Marques et al. 2009). In addition, cGAMP injected as vaccine in colorectal cancer cell line MC38 inhibits tumor metastasis and invasion by blocking the STING/STAT3 pathway and elicit the response of Th1 cytokines such as IFN-γ, IL2, and TNF-α (Jiang et al. 2019). With gold nanobubbles for mechanical tumor ablation and carbon nanotubes for local delivery of immunosuppressive drugs like thalidomide, zebrafish can be used to test the efficacy of novel nanotechnology-based tumor therapies (Tobia et al. 2013).

2.7 Challenges in Tumor Models to Study Anti-angiogenic Therapy

Anti-angiogenic therapy is challenging because there could be variations in the response to treatment with zebrafish and humans. Currently, it is impossible to adequately measure the anti-angiogenic impact in a tumor since angiogenesis suppression inhibits tumor development and vice versa (Wang et al. 2012). When human tumor cells are transplanted in zebrafish, the growing xenograft tumor stromal cells, ECM, blood components, immune cells, and circulating cells are nonhuman. Even the newly formed vasculature is also nonhuman (Sikder et al. 2003). Unfortunately, the responses of anti-angiogenic therapy are typically temporary, usually followed by resumption of development. These transient reactions could highlight creature model discoveries that enemy of angiogenic treatment can hurt vessels, bringing about a window of transient vascular normalization followed by intrusion or recovery of primarily unusual and harmed vessels, conceivably incited by the delayed enemy of angiogenic treatment hypoxia seen.

In the current standard treatment paradigm, biological markers that predict evasion of anti-angiogenic therapy are steadily increasing during the closing of this normalization window and before radiographic disease progression, allowing for a timely level of improvement in therapies rather than currently being dependent on radiographic progression. Despite the fact that many of the biomarkers that confirm anti-angiogenic therapy resistance also predict response evasion when moving in the opposite direction, animal models have reported some unique biomarkers (Bergers and Hanahan 2008). Several evasion mechanisms during anti-

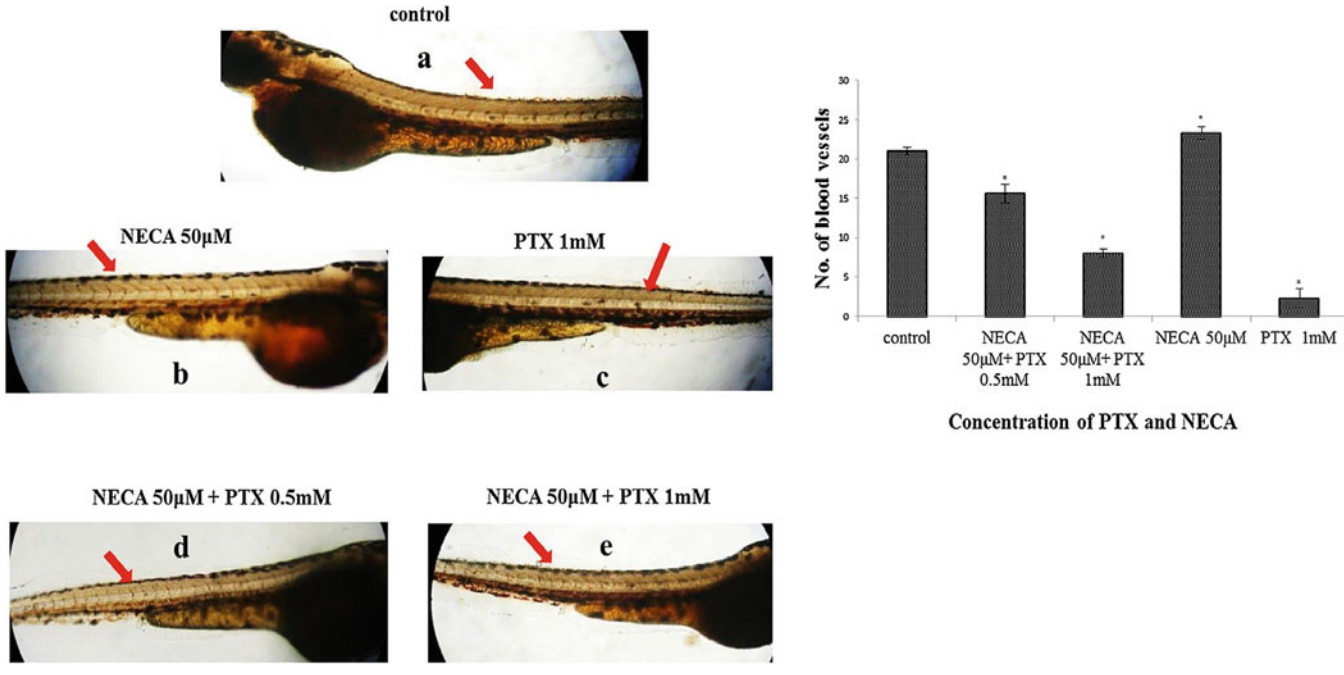

Fig. 2.5 Hypoxia-induced zebrafish model of NECA and PTX drug evaluation. RBC staining. (**a**) Embryos from control groups developed normal blood vessels. (**b**) ISV formation increased when 50 mM NECA-treated embryos were compared with control embryos. (**c**) The embryos treated with 1 mM PTX showed fewer ISVs than control embryos. (**d**, **e**) Embryos treated with both NECA and PTX had fewer ISVs. Angiogenesis is hampered by 1 mM PTX. (Figures obtained from Rani et al. 2016 with permission)

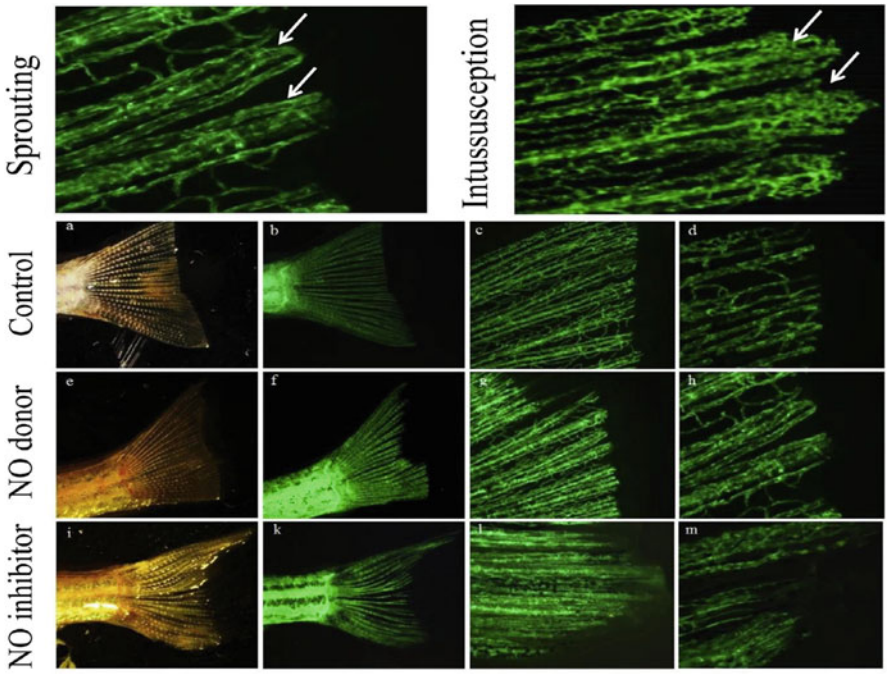

Fig. 2.6 The effects of NO stimulation or inhibition on Tie2-GFP transgenic zebrafish (adult) tail fin regeneration and angiogenesis occur after 48 h of treatment. Using a sterile razor blade, we amputated half of the fins of each adult Tie2-GFP zebrafish. We took bright-field (**a**, **e**, **i**) and fluorescent images (**b**–**d**, **f**–**h**, **k**–**m**) of both the cut and uncut fins at various magnifications. After 20 min, the Tie2-GFP zebrafish were put into 0.001% 1× PBS, 5 mM NO donor (SPNO), or 5 mM NO inhibitor (L-NAME) at pH 0.05. The growth of Tie2-GFP zebrafish treated with NO donors or an inhibitor of NO is faster than that of those treated with a control dose of NO. Further, the regrowth of blood vessels differed depending on whether the NO stimulation or inhibition was used. While NO inhibitors led to pillars in fin regeneration (**l**, **m**), NO donors increased SA (**g**, **h**). Images in the upper panels represent SA and IA. The following images are magnified 10× (**a**, **b**, **e**, **f**, **i**, **k**), 52× (**c**, **g**, **l**), and 160× (**d**, **h**, **m**). (Figures obtained from Vimalraj et al. 2018)

angiogenic treatment and molecular profilings of recurring cancers after anti-angiogenic therapy are avoided (Jahangiri and Aghi 2012).

Owing to the genetic variability among populations of neoplastic cells and the interactions between all the components present within the tumor, angiogenesis-related features may be principally tumor-specific and possibly tumor type-specific, and may be dependent on the organ site. Intra-tumoral lymph angiogenesis is reportedly absent from ectopically and orthotopically implanted tumors (Okuda et al. 2016). This may affect significant experimental end points such as metastasis formation, tumor progression, and survival data (Norrby and Norrby 2006).

Anti-angiogenic treatments can be reversed by tumor overproduction of pro-angiogenic ligands. A potential remedy in targeting phosphoinositide resynthesis is gained during intracellular processing of proangiogenic signals generated by

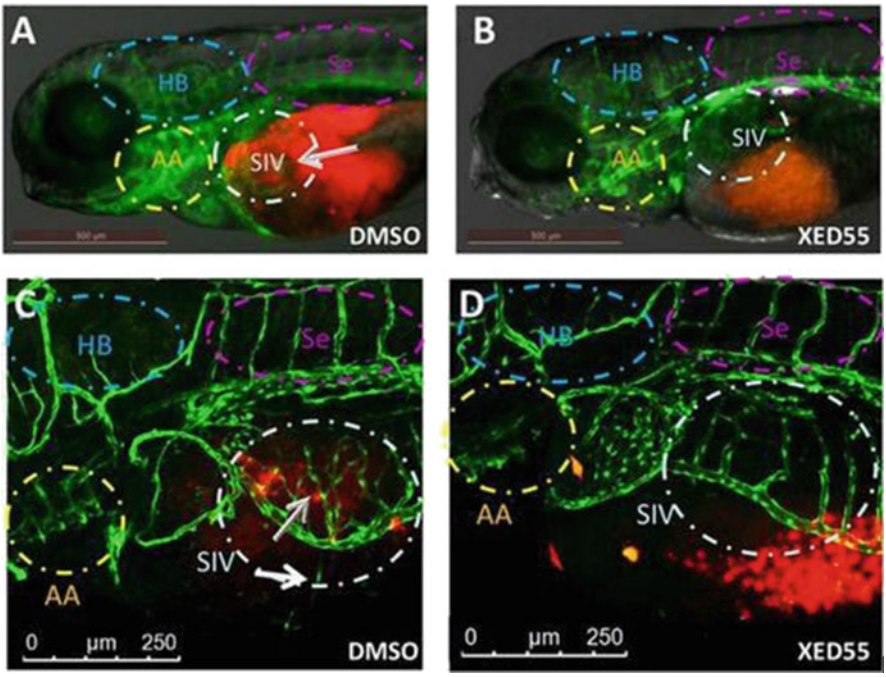

Fig. 2.7 Angiogenesis is negatively affected by XED55 in zebrafish embryo xenograft assays. (**a**) and (**b**) images show xenografted MA2 cancer cells (MA2) expressing red fluorescent protein (RFP) in Tg(Kdrl:EGFP)-s843 embryos (96 h post fertilization). After implanting tumor cells, the embryos were treated with DMSO or XED55. The green fluorescent proteins (GFP) in the tumor represent the vascular network, and the red fluorescent proteins (RFP) represent the tumor cell mass. Figures (**c**) and (**d**), respectively, demonstrate confocal analysis of (**a**) and (**b**) embryos. DMSO exposure led to the development of new vessels in the subintestinal vessels (arrows). XED55 exposure affects the normal embryonic vasculature (ISV) but has no effect on tumor blood vessels. (Figures obtained from Santoro 2014)

endothelial cells (EC). Angiogenic signaling deficiencies have become more severe. ECs will no longer be able to react to these stimulatory signals as they block the tumor's own release of excess stimulatory ligands. CDP diacylglycerol synthase 2 inadequate ECs are extraordinarily receptive to expanded VEGF incitement because of lower phosphoinositide re-amalgamation capacity, including phosphatidylinositol-(4,5)-bisphosphate (PIP2), bringing about VEGF-exacerbated angiogenic flagging deformities, as per examine utilizing in vitro zebrafish and human ECs (Stratman et al. 2020). The zebrafish efficacy-toxicity model supports the clinical trial outcomes of sorafenib, sunitinib (Cui et al. 2016), pazopanib, vandetanib, thalidomide, and tivozanib (Chimole et al. 2014).

Relapse, which is typically accompanied by fast rebound angiogenesis, is prevalent, and tumor regrowth is frequently more aggressive than before anti-angiogenic treatment (Georganaki et al. 2018). Proposed mechanisms for anti-angiogenic treatment resistance include co-opting normal tissue vessels, recruiting pro-angiogenic

myeloid cells, and activating alternative pro-angiogenic factors (Bergers and Benjamin 2003). Notably, anti-angiogenic therapy in experimental cancer models can increase invasiveness and facilitate the development of metastases (Ebos et al. 2009; Pàez-Ribes et al. 2009). While clinical trials have not proven that anti-angiogenic therapy causes metastasis, preclinical research has cautioned about how to properly deliver anti-angiogenic therapy (De Bock et al. 2011). According to Kickingereder et al. a radiomic-based super pc signature might be utilized as an imaging biomarker to identify individuals who could benefit from anti-angiogenic treatment (Muschelli et al. 2016).

2.8 Conclusion

The zebrafish model offers the chance to gain further insight into in vivo cancer angiogenesis and metastasis. Zebrafish is also a high-throughput model for anti-angiogenesis drug screening, so it can be used in preclinical studies to obtain useful results. This will pave the way for further study of anti-angiogenic drugs that can be used in the care of cancer patients with chemotherapy.

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Authors' Contribution K.G.H, R.Y, and S.V collected the literature and drafted manuscript. S.V secured the funding, designed the work, and approved the final submitted manuscript.

Declaration Authors declare no conflict of interest.

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Zebrafish as a Xenotransplantation Model for Studying Cancer Biology and Cancer Drug Discovery

3

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Abstract

Various malignancies have been successfully recapitulated in mammalian animal models, where mimicking the systemic and local environment of the developing tumor has contributed to our understanding related to the mechanisms of cancer, their diagnosis and possible therapeutic interventions to contain the growth and survival of the cancer. Advances in research have facilitated generation of these animal models of cancer by application of sophisticated technologies with integration of clinical information. Until now, rodent models especially mouse models have been the traditional models of choice for cancer investigation. Over the last decade, *Danio rerio*, commonly known as zebrafish, has emerged as a popular model to study many human disorders including cancer. Zebrafish offers several advantages over rodent models such as transparent embryos (allowing time-lapse noninvasive imaging using labeled cell tracking), high fecundity, external fertilization, and rapid development. Moreover, significant genomic similarity with humans especially in the disease-associated genes and their ease of maintenance and cost-effectiveness have made them a cancer model of choice today. Zebrafish has been used in several areas of cancer research including establishing cancer models by chemical and genetic manipulation or manipulation by xenotransplantation, investigating angiogenesis and metastasis, identifying new targets for therapeutics, anticancer drug screening, etc. This

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chapter is focused on the use of zebrafish as a xenotransplantation model to study cancer biology such as proliferation, metastasis, and angiogenesis. The xenotransplantation of human cancer cells into zebrafish also has a huge application in drug discovery and in identification of drug targets. In this chapter, we describe various zebrafish xenotransplantation cancer models that have been established in adult zebrafish, juveniles, and embryos of the zebrafish. Lastly, we discuss the use of zebrafish xenotransplantation models for precision oncotherapy.

Keywords

Zebrafish xenotransplantation · Cancer biology · Drug screening · Human xenografts · Precision oncotherapy

3.1 Introduction

The growth of cancer cells is influenced by the factors present in its microenvironment. Therefore, data obtained from in vitro cell and tissue culture must be validated and complemented by in vivo animal models because the complex microenvironment of whole animal is difficult to model in in vitro experiments. Transplantation of human tumors in animal models represents a pivotal preclinical tool in the field of cancer research. Traditionally, rodent models were the models of choice for studying cancer biology as, for long, rodent models have provided accurate modeling for many diseases. However, rodent models suffer from a notable limitation of being low-throughput. To bridge the gap between high-throughput in vitro studies and low-throughput rodent model studies, zebrafish has emerged as a relatively high-throughput vertebrate-animal model. In this chapter, we have restricted our discussion to the use of zebrafish xenotransplantation models for cancer research. Finally, we conclude that the zebrafish xenotransplantation model is an unparalleled and compelling tool for cancer research (Fig. 3.1).

3.2 Advantages of Zebrafish Xenotransplantation Model for Cancer Research

Zebrafish has gained attention due to the several advantages it offers over conventional rodent models. Zebrafish are small in size and therefore their maintenance is very cost-effective. They have high fecundity with optically transparent embryos. Under laboratory conditions, a single adult female zebrafish can spawn around 200–300 eggs with a frequency of 6–8 days. In zebrafish, embryogenesis is rapid with the entire body plan being established by 24 h post fertilization (hpf), and most of the internal organs like the heart, liver, kidney, and intestine are completely developed by 96 hpf. Minute size of the zebrafish embryos allows designing of high-throughput assays for chemical/drug screening with ease at significantly low costs. Optically transparent embryos allow easy developmental staging and imaging

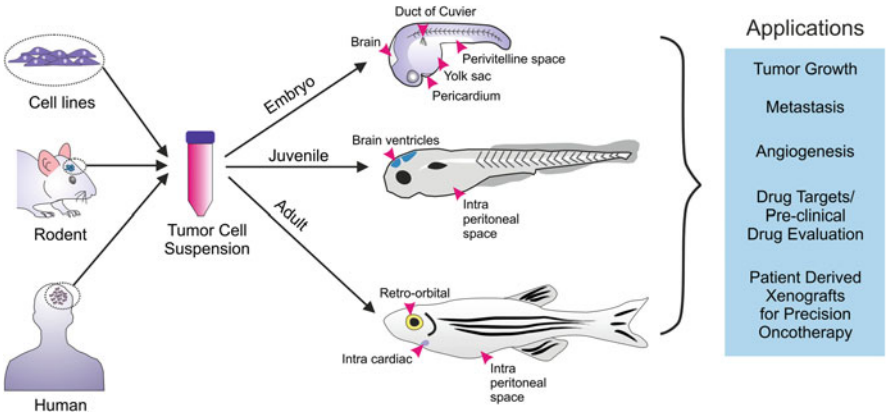

Fig. 3.1 Zebrafish as a xenotransplantation model to study cancer biology. Tumor cell suspension from cancer cell lines, rodent models, and human patients can be injected into the embryo, juvenile, or adult zebrafish to study the tumor growth, metastasis, and angiogenesis. Zebrafish xenotransplantation model is a great tool for drug discovery and precision medicine

to be performed noninvasively, which is particularly useful in the xenotransplantation as the tumor growth/engrafted cells can be imaged noninvasively. This advantage sets the zebrafish apart from rodent xenotransplantation models where imaging of engrafted cells/tumor growth requires complex imaging techniques and even surgery (Hason and Bartůněk 2019). Notably, morphology, biochemical processes, and physiology of all the stages of both sexes are known for the zebrafish. The knowledge of whole genome of the zebrafish along with the current molecular tools allow for easy genetic manipulation in the zebrafish (Hason and Bartůněk 2019). Humans and zebrafish also share a striking similarity in the cancer-related processes and gene expression patterns and exhibit remarkable conservation of cell cycle, tumor suppressor genes, protooncogenes, angiogenic factors, and extracellular matrix proteins (Zheng et al. 2014) which allows valid extrapolation of research outcomes obtained in zebrafish back to humans. Tumors in zebrafish were also found to be histologically similar to their mammalian equivalents (Zheng et al. 2014). Zebrafish embryos are ideally suited for transplanting cells from primary human patient tumors as relatively low cell numbers are required for xenotransplantation (approximately 50–300 cells), although a lot of studies have also used cancer cell lines for xenotransplantation to understand mechanisms of cancer and drug screening. Requirement of lesser cells also means that the host numbers can be scaled up easily, improving the accuracy of statistical tests. Remarkably, the adaptive immune system is absent in zebrafish embryos eliminating the need of any immunosuppression (Langenau et al. 2004).

3.3 Stages of Zebrafish for Xenotransplantation

3.3.1 Embryo Stage

So far zebrafish embryos remain the most popular stage of zebrafish for xenotransplantation due to their optical transparency, compatibility with high-throughput imaging, or the absence of a fully mature immune system. Zebrafish embryos have been used for xenotransplantation of patient-derived tumor cells or cells from tumor cell lines. A glass capillary is used for injecting the tumor cell suspension (usually single cells) into the embryos using a microinjection system. This microinjection system is now available from many commercial suppliers, for example, Eppendorf or World Precision Instruments (WPI). This process of microinjection is similar to the injection of molecules like mRNA and therefore is adaptable to automation in high-throughput screens. The tumor cell preparation for injection is obtained by enzymatically dissociating tissue samples or monolayers of cells in culture, and the cells are chemically or genetically labeled for tracking in zebrafish embryos after transplantation (DeRose et al. 2013). Several sites of injection in zebrafish embryos are now validated, e.g., near the blastodisc and blastocyst of the blastula stage, the yolk sac, perivitelline space, duct of Cuvier, hindbrain ventricle, and posterior cardinal vein of 6 hpf to 5-day-old embryos (Konantz et al. 2012; Mizgirev and Revskoy 2010). A unique feature of the zebrafish yolk sac is that it is not well vascularized and has been confirmed as a hypoxic site that may mimic the microenvironment of some human tumors (Pringle et al. 2020). Details on the transplant procedures in embryos can be referred to in Table 2 in reference Konantz et al. (2012). Xenotransplantation can be performed in wild-type embryos or transgenic embryos to facilitate visualization of transplanted cells in the vasculature. One such transgenic embryo line is Tg(fli1-eGFP) in which the fli1 promoter drives the expression of eGFP in the entire vasculature throughout the embryogenesis and thus it can be conveniently used for analyzing tumor-induced angiogenesis (Lawson and Weinstein 2002). Several other transgenic lines labeling vasculature have been developed to facilitate the tracking and/or analysis of transplanted cells. Some of these are Tg(kdrl:eGFP)^{la116} (endothelial cells express GFP driven by kdrl promoter) (Choi et al. 2007), Tg(flk1:mCherry) (endothelial cells and blood vessels express mCherry driven by flk promoter), or Tg(VEGFR2:g-rCFP) (blood vessels express green reef coral fluorescent protein driven by vascular endothelial growth factor receptor 2 gene promoter (VEGFR2)) (Konantz et al. 2012). Apart from labeling of the vasculature, other transgenic lines are also available that can label the components of the zebrafish immune system, e.g., Tg(mpeg1:eGFP) and Tg(cd41:eGFP) where macrophages and platelets are labeled, respectively (Ellett et al. 2011; Lin et al. 2005). Several studies have shown successful engraftment of varied types of tumor cells in zebrafish embryos following microinjection that include leukemia, melanoma, glioblastoma, fibrosarcoma, breast cancer, adenosarcoma, lung cancer, pancreatic cancer, retinoblastoma, prostate cancer, colon cancer, etc. (Stoletov and Klemke 2008). In zebrafish embryos, engrafted cells can be studied for only up to a week after transplantation due to the onset of the host innate immune response

(Stoletov and Klemke 2008). However, this time frame still allows researchers approximately a 72-h period to understand the mechanisms of cancer angiogenesis, metastasis and drug screening (Hill et al. 2018).

3.3.2 Juvenile Stage

Zebrafish larvae from 30 days post fertilization (dpf) till 89 dpf are called juveniles. Juveniles from casper fish, which is a transgenic mutant zebrafish line lacking all types of pigments, are commonly used for xenotransplantation (White et al. 2008). Lack of pigmentation increases the translucency in this line, and therefore, it is much easier to observe engraftment, metastasis, etc. An advantage of using juvenile fish over embryos is that all tissues and vasculature are fully developed. The most common site for tumor injection in juvenile fish is the peritoneal cavity (Stoletov et al. 2007). Also ventricle has been used as a site for tumor cell injection (Casey et al. 2017). Although one may argue that juveniles are more suited for orthotropic transplantation, there are not many studies which have used juveniles for xenotransplantation due to some limitations. Unlike embryos, the brain is not easily imaged by optical microscopy in juvenile zebrafish, even in casper strain, making it less suitable for in vivo imaging. Also, during the development of zebrafish embryos, immature T cells arise in the thymus by 3–4 dpf, and the complete immune system is functional at around 28 dpf (Langenau et al. 2004). Therefore, when zebrafish juveniles of 30 dpf are used for xenotransplantation, immunosuppression is required (Stoletov et al. 2007). Immunosuppression can be easily achieved in this stage by dexamethasone, a steroid. Juveniles easily absorb dexamethasone from the water and respond to immunosuppression within 3 days (Langenau et al. 2004). In juveniles, tumorigenesis and vascular remodeling can be studied for more than 2 weeks (Zhang et al. 2015). A remarkable feature of juvenile zebrafish is that the blood-brain barrier is functional in it and therefore it presents as an excellent model for studying gliomas. It has been shown to successfully recapitulate pediatric brain tumors where mouse gliomas retained the transcriptome of their parent tumor suggesting that juveniles could present as an excellent preclinical drug development tool.

3.3.3 Adult Stage

Zebrafish juveniles beyond 90 dpf are called adults. Casper fish is mostly used for adult zebrafish xenotransplantation. Similar to juveniles, xenotransplantation in adult zebrafish needs immunosuppression. Drugs such as dexamethasone or γ-radiation have been used where single doses of 20–25 Gy were found to be effective in providing immunosuppression. Tumors engraft at these doses, when these doses are given 2 days prior to transplantation (Patton et al. 2005). Efforts were also made to create adult immunocompromised zebrafish, similar to SCID mouse so as to allow the study of xenografts in the adult microenvironment without the need of

immunosuppression. Tang et al. have created the first immunocompromised zebrafish that has reduced numbers of functional T and B cells (Tang et al. 2014). Newer immunocompromised zebrafish strains with mutant DNA-dependent protein kinase (*prkdc*^{D3612fs}), or mutant Janus kinase 3 (*jak3*^{P369fs}) with affected T cells, B cells, and natural killer cells, have also been generated (Moore et al. 2016). These immunocompromised strains have also been crossed with casper fish to obtain better optical images during tumor imaging in these transgenic xenotransplanted zebrafish. A zebrafish strain lacking prkdc^{D3612fs}, il2rgaY91fs (prkdc−/−, il2rga−/−) on the casper background that lacks T cells, B cells, and natural killer cells is the most recent development in immunocompromised zebrafish. A unique feature of this strain is that the fish can survive at 37 °C and it can robustly engraft human cancers for more than 28 days (Yan et al. 2019). A prkdc-null severe combined immunodeficiency (SCID) zebrafish model also exists which provides an immune-deficient background required for xenotransplantation. However, as expected, it shows susceptibility to spontaneous infections, a well-known phenotype found in the SCID mutation (Jung et al. 2016). Yet another interesting xenotransplant model was developed by Zhang and colleagues where transplantation in the adult zebrafish was achieved without the use of immunosuppression. It was based on two rounds of cancer cell xenotransplantation where zebrafish achieved cancer-specific immunologic tolerance. This model seems useful as it can closely resemble human cancers without the need of immunosuppression (Zhang et al. 2016). Several transgenic fish with fluorescently labeled vasculature or internal organs were engineered to study tumor-host interactions in adult zebrafish (Zhang et al. 2015). For visualization of xenografts, Zhang and colleagues have developed an integrated optical system that combines a laser scanning confocal microscope and an in vivo flow cytometer for simultaneous visualization and cell quantification in adult zebrafish (Zhang et al. 2012). Several other techniques including micro-computerized axial tomography and micro-magnetic response imaging can also be used for tumor analysis following xenotransplantation in adult zebrafish (Spitsbergen 2007). The most common site for injection into adult zebrafish is also the intraperitoneal space like juveniles; however, other sites such as intracardiac and retro-orbital have also been reported (White et al. 2008; Guo et al. 2015). As compared to the zebrafish embryos, adult zebrafish is underutilized as the xenotransplantation model owing to many difficulties. Unlike embryos, transplantation into adult fish has to be performed manually. So far there is no automation on that aspect. Also, the success rate of engraftment is low. The tumor cell transplantation protocols may still need more optimization for engraftment in adult zebrafish.

3.4 Applications of the Zebrafish Xenotransplantation Model in Cancer Biology

Zebrafish as a xenotransplantation model has been widely exploited in the field of cancer biology for many purposes such as to study tumor proliferation, metastasis, and angiogenesis and to understand mechanisms of cancer manifestation in an

attempt to find new and specific targets. Lately, it has been the model of choice to screen compounds which may act as potential drugs. Potentially, patient-derived xenografts can be easily analyzed in zebrafish for precision oncotherapy which is the need of the hour. We have described these applications in the next few sections in detail. Please note that reviewing all such studies is not the goal of these sections, and we have described studies of interest in order to facilitate the understanding of our readers regarding the use of zebrafish xenotransplantation model in cancer biology. Further, readers can refer to Table 4 in the reference Hason and Bartůněk (2019) for zebrafish models of human cancer xenotransplantation.

3.4.1 Assessing the Tumor Growth, Metastasis and Angiogenesis

Zebrafish xenotransplantation model has been extensively used to study tumor growth, metastasis, and angiogenesis using several cancer cell lines and patient-derived primary tumor cells. The transparent zebrafish embryos can facilitate real-time in vivo visualization of the initial steps of neovascularization which is essential for tumor formation and its growth. An important step in the observation of engrafted tumor is the imaging and analysis of transplanted cells. For this purpose, the transplanted cells are labeled with fluorescent dyes such as CellTrace™ carboxyfluorescein succinimidyl ester, chloromethylbenzamido-DiI (CM-DiI) cell tracker or they can be tagged with fluorescent proteins. After injecting the labeled cells, embryos are usually incubated under gradually increasing temperature from 28 to 33–37 °C over 2 days and then kept at either 33 or 37 °C until the end of the experiment. Embryos are then optically imaged at regular intervals for measuring the fluorescent area of the engraft as an indicator of the tumor growth. From the images, cell proliferation rate can be determined by the percent change in fluorescent area over time. Light sheet fluorescence microscopy (LSFM) in combination with flow cytometry has been reported as a useful combination technique to assess tumor growth and proliferation in zebrafish embryo. Since LSFM is less phototoxic than conventional epifluorescence or confocal microscopy, it can be used for imaging over extended periods of time which can allow researchers to generate 3D reconstructions of the tumor directly from the in vivo sites without causing loss of viability of cells. With this combination technique, the researchers reported reliable changes in size and fluorescence intensity of transplanted cells using which tumor proliferation could be estimated in vivo (Vargas-Patron et al. 2019). A semiautomated, high-throughput, whole-organism noninvasive quantitative bio-imaging assay is also developed by Ghotra and colleagues for transplanted zebrafish embryos (Ghotra et al. 2012). In this study, the authors developed an algorithm to calculate tumor burden in transplanted embryos and calculated parameters like number of tumor foci, distance of tumor foci from the injection site, and cumulative distance traveled from the injection site (Ghotra et al. 2012). Cumulative distance can be used to study the tumor dissipation capacity as an indication of invasiveness and metastasis of the tumor. The assays to visualize pro-angiogenic potential of tumor xenografts involve imaging of the newly formed

vessels. Angiogenesis can be observed macroscopically and microscopically after whole-mount alkaline phosphatase staining of wild-type zebrafish embryos, or by fluorescence microscopy in embryos derived from transgenic zebrafish where endothelial cells express a reporter gene such as green fluorescent protein. Alkaline phosphatase staining will reveal any modifications of the subintestinal veins. In zebrafish, subintestinal veins originate from the duct of Cuvier at 48 hpf. The zebrafish angiogenesis assay is based on grafting of the tumor cells in the proximity of the developing subintestinal vein plexus at 48 hpf. Pro-angiogenic factors that are released from the tumor cells are anticipated to interfere with the normal developmental pattern of the subintestinal veins by stimulating the growth of new vessels toward the implant (Nicoli et al. 2009). Therefore, any deviation in the normal developmental pattern of the subintestinal veins after xenotransplantation indicates angiogenic potential of the tumor cells. Also, digitized images of the tumor xenografts can be used to quantify the microvessel density (Nicoli et al. 2009). Several studies have successfully demonstrated that human cancer cells can actively invade within the zebrafish and form metastatic tumors at secondary tissue sites, indicating that the mechanisms involved during metastasis are conserved between humans and zebrafish further supporting the use of zebrafish as an excellent model for cancer biology (see also below sections).

3.4.2 Investigating Mechanisms of Cancer in Search for Therapeutic Targets

Each cancer is peculiar in its pathology and specific therapies are limited. Therefore, there is a persistent search for new targets which may prove as specific and effective therapies. Also, understanding mechanisms is important from the point of view of detecting and distinguishing among the stages and pathology of different cancers. Zebrafish xenotransplantation has been widely used in search of therapeutic targets. Since metastasis is a very important risk factor in poor prognosis, several researchers have attempted to investigate the mechanisms of metastasis as well as to discover any early metastatic markers that could potentially help in early detection and alleviation of the severity of cancer pathology. MicroRNA-10a (miR-10a) was identified as an important mediator of metastasis in pancreatic tumor cells. Using miR-10a gene-specific morpholinos and xenotransplantation of pancreatic cell lines into zebrafish, it was observed that miR-10a expression alone can promote metastasis in pancreatic tumor cells and more importantly, inhibition of miR-10a is adequate to inhibit invasion and metastasis of pancreatic tumor cells (Weiss et al. 2009). This is an important lead owing to the lethality of the pancreatic cancer. Also, miRNAs have attracted enormous attention over the last decade due to their potential in diagnostics and therapeutics. Yet another microRNA-608 (miR-608) acts as a tumor suppressor in lung cancer cell lines (Othman and Nagoor 2017). MiR-608 was shown to regulate AKT2, suggesting that targeting of AKT2 via miR-608 could present as a potential therapeutic strategy for miRNA-based non-small cell lung cancer therapy (Othman and Nagoor 2017). Another cancer with very poor

prognosis is the esophageal cancer. Using zebrafish xenotransplantation, glucose-regulated protein 94 (GRP94) was identified as a potential target for suppressing the growth and metastasis of esophageal squamous cell carcinoma (Huang et al. 2018). GPR94 seems to target esophageal squamous cell carcinoma by interfering with the mitochondrial function (Huang et al. 2018). Cancer-associated fibroblasts are also being implicated in early metastasis of several types of cancer including hepatocellular carcinoma, lung cancer, squamous carcinoma, pancreatic cancer, and breast cancer (Liu et al. 2017). Cancer-associated fibroblasts can promote metastasis at the very early stage, and infiltration and activation of fibroblasts in these microscopic tumors is adequate to promote cancer metastasis (Liu et al. 2017). This finding suggests that cancer-associated fibroblasts may be targeted at the early stage of cancer to prevent metastasis. The findings in this study also provide some critical evidence challenging the general belief regarding cancer metastasis that the tumor size may be irrelevant to the metastatic potential and that it may depend on the infiltration and activation of fibroblasts in the microscopic tumor which may ultimately determine the fate of the tumor progression. Also, using zebrafish xenotransplantation, the study provided satisfactory evidence to show that the tiny microscopic tumor mass was not vascularized and that the tumor can disseminate through existing vasculature; therefore, angiogenesis may not be the first critical factor in metastasis (Liu et al. 2017). An attempt had already been made previously to exploit the potential of cancer-associated fibroblasts to inhibit oral squamous cell carcinoma in the zebrafish xenograft model. A triazine compound, S06, was identified as a novel inhibitor of oral squamous cell carcinoma (Jung et al. 2011). In vitro studies revealed that the mechanism of action of S06 involves inhibition of secretion of cancer-associated fibroblasts-derived proinvasive chemokines and targeting the N-terminus of heat shock protein, Hsp90. This study also identified Hsp90 as a novel target (Jung et al. 2011). Another pathway, SOX2-AKT pathway, has been recently indicated as a potential target for a subset of breast cancer patients that are SOX2-positive (Schaefer et al. 2015). Direct interaction of kinase AKT with SOX2 stabilizes SOX2 by promoting its nuclear localization. When breast cancer cells are SOX2-positive, SOX2 is detectable at the early stages of the disease as well as at the relapse. Using zebrafish xenotransplantation of SOX2-overexpressing breast cancer cell line T47D, it was observed that SOX2 overexpression augmented tumor formation and an AKT inhibitor was able to inhibit tumor formation (Schaefer et al. 2015). Therefore, inhibition of the AKT pathway may provide benefit to the SOX2-positive breast cancer patients, especially because SOX2 expression has not been reported in healthy tissues (Schaefer et al. 2015). Lately, a novel mechanism of communication through long membranous tunneling nanotubes (TNTs) has been identified between macrophages and cancer cells (Hanna et al. 2019). TNTs are long membranous structures connecting cells, which allows connected cells to act in an integrated manner over long distances (Hanna et al. 2019). Using a zebrafish xenotransplantation model that recreated macrophage-mediated breast tumor invasion, TNTs were observed that mediated macrophage-dependent tumor cell invasion (Hanna et al. 2019). In addition, the study provided in vitro evidence that reduction of M-Sec (TNFAIP2), a protein involved in TNT formation in macrophages,

blocked the ability of tumor cells to invade and also resulted in reduced macrophage-dependent long-distance tumor cell streaming (Hanna et al. 2019). We know that neovascularization and angiogenesis are important factors in tumor progression and metastasis. Vascular endothelial growth factor (VEGF) is a well-recognized mediator of angiogenesis that binds to two VEGF receptors (VEGFR-1 and VEGFR-2) expressed on vascular endothelial cells (Carmeliet 2005). Cancer cells release VEGF and other growth factors into the tumor microenvironment that may promote neovascularization and angiogenesis, and therefore VEGF and its receptors have been the subject of a number of studies in order to understand the mechanisms of angiogenesis. A transmembrane glycoprotein, MUC1, that is overexpressed in >80% of pancreatic carcinoma induces a pro-angiogenic tumor microenvironment by increasing the levels of neuropilin-1 (NRP1, a co-receptor of VEGF) and its ligand, VEGF. Using zebrafish xenotransplantation, it was shown that treatment with NRP1 antagonist A7R (a heptapeptide ATWLPPR), significantly blocked ectopic vessel formation and metastatic spread highlighting the interaction between NRP1 and VEGF as a critical factor in angiogenesis (Zhou et al. 2016). A zinc finger, MYND-type containing 8 (ZMYND8) protein which encodes a receptor for activated C-kinase protein has been shown to be upregulated in prostate cancer. Using zebrafish xenotransplantation of human prostate cancer cells and morpholinos-based approach, it was shown that downregulation of ZMYND8 results in the suppression of tumor angiogenesis without affecting the tumor size, thus indicating that ZMYND8 could be a target in prostate cancer (Kuroyanagi et al. 2014). Using zebrafish xenotransplantation, some novel targets such as ETS transcription factors, Etv2 and Fli1b, are also recently identified as novel critical regulators of tumor angiogenesis (Baltrunaite et al. 2017). Therefore, targeting cancer-specific novel regulators of angiogenesis may help in improving the prognosis of the disease.

3.4.3 Assessment of Cancer Treatment Drugs (Drug Screening)

An obvious need for the treatment of any type of cancer is the discovery of new and efficient drugs/chemicals for which drug screening systems are required. Animal models are preferred over toxicity screens as they provide native microenvironment to the cancer. Since zebrafish can absorb small molecular weight compounds directly from the surrounding medium (usually water supplemented with salts) in contrast to injection or feeding in rodents, it is fast becoming the model of choice for screening of drug candidates including drugs for cancer treatment. Automated handling, high-resolution imaging, high-content imaging, and quantitative data collection have significantly increased the applicability and throughput of zebrafish xenotransplantation model for drug screens in recent years. Since zebrafish has high fecundity, a large amount of embryos/larvae can be easily generated in the lab using commercially available husbandry systems. Even dispensing of embryos/larvae into multi-well plates can be automated. Usually, early stage embryos have been used in high-throughput screens, whereas adult zebrafish are used in the conventional drug

screens in the lab. Using zebrafish xenotransplantation models bearing either cancer cell lines or cells from patient-derived tumor tissue, a number of small molecules or chemicals have been identified as potential leads for cancer therapy. Even combination therapy has been tested using zebrafish xenotransplantation models. Evaluation and detailed investigations of the anti-cancerous drugs/peptides can also lead to the identification of new mechanisms operating in cancer cells as targets for inhibition of proliferation and metastasis of cancer cells. In a latest study, using breast cancer zebrafish xenotransplantation model, Wang and colleagues identified a synergistic effect between two existing breast cancer drugs, XIAOPI formula and chemotherapeutic drug Taxol. The authors showed that XIAOPI formula could chemosensitize breast cancer cells via autophagy inhibition (Wang et al. 2019). This study is also one of the studies implying the importance of autophagy in cancer therapeutics (Wang et al. 2019). Using zebrafish xenotransplantation of MDA-MB-231 and MDA-MB-453 breast cancer cell lines, Yang and colleagues showed that *Oldenlandia diffusa* could impede breast cancer metastasis by lowering the expression of caveolin-1 (an oncogenic membrane protein associated with endocytosis), pointing up the use of *Oldenlandia diffusa* as an auxiliary therapy for metastatic breast cancer patients (Yang et al. 2019). *Oldenlandia diffusa* has been used in traditional Chinese medicine with proven efficacy. Using zebrafish xenotransplantation of metastatic melanoma B16F10 cells expressing fluorescence resonance energy transfer-based caspase-3 sensors and a sensor-based platform, a pan-phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 was identified as an antiproliferative and pro-apoptotic compound for micrometastatic cancer cells (Fu et al. 2018). Peptides from venomous animals have long been identified as antimicrobial agents. One such peptide from spider venom is gomesin which has been reported to have antibacterial, antifungal, and anthelminthic activities (Ikonomopoulou et al. 2018). Recently, it was reported that gomesin could reduce the proliferation and invasive capacity of melanoma tumors in a zebrafish tumor model xenotransplanted with human melanoma (MM96L) cells (Ikonomopoulou et al. 2018). In the same study, the authors identified the mechanism of action of gomesin as activation of the p53/p21 cell cycle checkpoint axis and the Hippo signaling cascade, together with attenuation of the MAP kinase pathway which led to the reduction of proliferation of melanoma cells. Though the mechanism was identified in cell lines, together with the data from the zebrafish xenograft model, the study points toward a promising therapeutic role of gomesin as anti-cancerous agent (Ikonomopoulou et al. 2018). Tg(kdrl:HsHRAS-mCherry)^{s896} zebrafish embryos, which express Cherry fluorescent protein specifically in endothelial cells, injected with metastatic MDA-MB-231 breast cancer cells into the heart or perivitelline cavity showed a widespread tumor metastasis which was significantly reduced by 2-*O*-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-*O*-Bn-InsP5), a small molecular inhibitor of PDK1/PLCγ1 interaction (Raimondi et al. 2016). This interaction is important for the activation of PLCγ1 which is an important step in the tumor proliferation and metastasis. Another specific interaction between β-catenin and LEF-1 is surfacing as a promising strategy for combating cancer. The β-catenin/LEF-1 complex is a nuclear response transcription factor which plays an important

role in the tumorigenesis. Recently, a peptide (TAT-NLS-BLBD-6) that could interfere with the interaction of β-catenin and LEF-1 at the nuclei was shown to suppress breast tumor proliferation in the zebrafish xenotransplantation model of MCF-7-GFP and MDA-MB-231-GFP cells (Hsieh et al. 2016). Of note, the peptide did not show any general toxicity, therefore presenting a potential powerful therapeutic strategy for breast cancer. Zebrafish xenotransplantation model has also been used to study the progression and metastasis of transplanted human pancreatic cancer cells and to identify drugs that may inhibit pancreatic cancers. CM-DiI-labeled (fluorescent dye, Thermo Fisher Scientific) human pancreatic cancer cells were injected into both larval and adult zebrafish. Pancreatic cells with KRAS mutations are known to exhibit intense proliferation, migration, and invasion in the zebrafish vasculature (Guo et al. 2015). A known small-molecule inhibitor of the KRAS signaling pathway, U0126, significantly inhibited the proliferation and migration of Mia PaCa-2 cells (human pancreatic cell line) in zebrafish larvae. This study not only identified a potential therapeutic compound but reiterated the usefulness of zebrafish xenotransplantation as an efficient tool to screen drugs (Guo et al. 2015). The zebrafish brain cancer xenotransplantation model has been used to evaluate the feasibility of brain endothelial cell-derived exosomes to deliver anticancer drugs for the treatment of brain cancer (Yang et al. 2015). It is well-known that the blood-brain barrier restricts the penetration of drugs into the brain making it impossible for the chemotherapeutic drugs to treat brain cancer. In the brain cancer model, exosomes delivered anticancer drugs across blood-brain barrier and significantly decreased the proliferation of xenotransplanted cancer cells (Yang et al. 2015). As an alternate to conventional chemical drugs, receptor-specific adeno-associated virus (AAV)-based cytotoxic therapy has also been tried as cancer therapy. A receptor-specific AAV-based vector to target the CD33 antigen which is overexpressed in leukemic cells was created (Khan et al. 2019). This AAV6-CD33 vector expressed a suicide gene, the inducible caspase-9 (iCasp9) which upon action of the prodrug AP20187 on iCasp9 induced apoptosis in the cells that were modified. In the zebrafish xenotransplantation model of acute myeloid leukemia when AAV6-CD33 vectors were administered, a significantly higher survival of the treated zebrafish with more apoptotic cells were observed demonstrating the specificity and efficacy of the therapy (Khan et al. 2019).

3.5 The Potential of Zebrafish Xenotransplantation for Personalized Cancer Therapy

It is now well-known that cancer is a highly individualized disease and no two cancers are alike. In spite of good progress in identifying drug targets, there is still a lot of uncertainty in predicting individual patient responses, and therefore treatments are designed according to average response rates. This challenge is addressed by precision oncotherapy which focuses on a patient's unique cancer pathology and is not totally dependent on predictions based on group averages. The zebrafish xenotransplantation assay is ideal for designing precision oncotherapy for cancer patients.

The tumor analysis in zebrafish is faster (5–7 days in zebrafish embryos), and only a small amount of patient tissue is required for the xenografts. Several patient-derived tumors such as multiple myeloma, neuroendocrine tumor, leukemia, gastric cancer, breast cancer bone metastasis, etc. (Fazio et al. 2020) have been successfully transplanted into zebrafish for evaluation of tumor pathology and drug discovery. In most of these cases, the xenografted tumor recapitulated the patient's course of the disease and displayed the same metastatic and angiogenic potential further validating the zebrafish model for precision oncotherapy. Xenotransplantation of patient-derived tumors into large population of zebrafish permits testing of a large number of clinically available drugs, the response of which will help in making an informed clinical decision about the most suitable treatment for the tumor. These zebrafish avatars made it possible to increase the scale of drug testing which has not been possible so far with other models of xenografting such as rodent models. In these avatars, the cancer drug response is usually measured by imaging of xenografted cells which have been genetically or chemically labeled by fluorescent dyes. The number of cells and tumor surface area has been used as a tumor response measure using RECIST (Response Evaluation Criteria in Solid Tumors) criteria (Eisenhauer et al. 2009). The RECIST criterion defines standard measures to evaluate response to cancer drugs for overall evaluation of tumor burden. First reports of generation of zebrafish xenotransplantation avatars and drug testing using those avatars came around 2015–2016 (Fazio et al. 2020), and since then the field has steadily progressed. The first co-clinical trial in zebrafish larva with patient-derived xenografts is started in 2018 to observe if the zebrafish model is able to predict the therapeutic approach for each individual patient in the trial. This trial is an observational prospective co-clinical trial on patients operated for pancreatic and gastrointestinal cancers undergoing a chemotherapy treatment which will be run concurrently to the zebrafish larva with patient-derived xenografts (Fazio et al. 2020). Overall, these zebrafish models with patient-derived grafts seem to be important from clinical perspective where speed and short-term responses are the goal. However, the full potential of these zebrafish avatars is yet to be explored in terms of whether they are able to provide data on survival outcome and toxic effects or if they can help in preventing dangerous administration of ineffective treatments to the patients, etc.

3.6 Current Limitations of the Zebrafish as a Xenotransplantation Model

There are several concerns that have surfaced from time to time among researchers regarding the use of zebrafish model for xenotransplantation. The very first pertains to the differences in the microenvironment that the transplanted cells may encounter. This becomes even more critical when heterotopic transplantation is used because of the lack of corresponding organs in zebrafish such as prostrate, breast, and lung cancer. However, one can argue that several studies have not only shown the successful heterotopic transplantation but also have shown that the physiology of

cancer cells remain similar to the transplanted tumor cells (Konantz et al. 2012). The difference in the standard rearing temperature of zebrafish (28 °C) and human cells (37 °C) is yet another concern. However, several groups have now shown that human cells are able to proliferate at lower temperatures such as 31 °C (Geiger et al. 2008). In addition, zebrafish embryos develop normally at higher temperatures such as 35 °C where xenotransplanted human cells can still proliferate (Haldi et al. 2006). Thus, one can use higher zebrafish maintenance temperatures of around 34–35 °C when using zebrafish as a xenotransplantation model. Zebrafish cellular size poses another limitation. Zebrafish cells, vessels, and other anatomic structures are smaller than the corresponding human structures (Konantz et al. 2012). Nevertheless, circulation of leukemic cells and migration of larger human solid tumor cells has been time and again shown in zebrafish indicating that movement of human cells is possible through zebrafish vasculature (Konantz et al. 2012). Using a nuclear stain, DRAQ5, it was also demonstrated that the human cells in circulation are not damaged due to injection or passage through the zebrafish vasculature (Konantz et al. 2012). Thus, it seems that small size of the zebrafish vessels is not a limiting factor. Whether this is due to unusual elastic properties or any other reason remains to be determined. As mentioned earlier in this chapter, zebrafish embryos are the most popular stage of choice for xenotransplantation due to several advantages, but at the same time, their developmental immaturity can be problematic. For example, myelin axonal sheaths are not present in the zebrafish central nervous system until 4–7 dpf, which in turn may affect the invasion of transplanted glioma cells. Also particularly affecting glioma chemical drug screening is the absence of blood-brain barrier in early embryo stages (Fleming et al. 2013). Differences are reported in the cytokine expression between zebrafish and humans. Therefore, several groups are trying to generate human cytokine knock-in zebrafish which may improve the accuracy of results obtained with zebrafish xenotransplantation model. In this context, the first humanized zebrafish that expresses human hematopoietic-specific cytokines (GM-CSF, *S*CF, and SDF1α), termed as GSS fish, is already developed. This transgenic fish resembles human microenvironment more closely and has been shown to have enhanced proliferation and hematopoietic niche-specific homing of primary human leukemia cells, thus recapitulating human context (Rajan et al. 2019).

Overall, we think that zebrafish represents a powerful model for xenotransplantation despite these limitations. Careful choice of zebrafish strain together with careful analysis of results may allow to generate accurate results.

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Zebrafish Models for Screening of Metabolic Diseases

4

S. Arulmozhi

Abstract

Metabolic diseases such as type 2 diabetes, coronary heart disease, and obesity are the leading cause of morbidity and mortality. In the drug discovery process, many preclinical screening models exist. However, in silico approaches result in increased number of molecules which amplifies a substantial load on preclinical screening. Furthermore, newer targets of metabolic diseases are evolving. This prompts for use of high throughput in vitro screening and alternative models which can mimic lipid metabolism, pancreas structure, and glucose homeostasis.

Zebrafish is one such alternative model for diseases with a high degree of genetic, anatomical, and physiological similarities to humans. The ease of accessibility to embryonic and genetic manipulations, optical transparency of its embryos, relative short generation time, and facile maintenance make zebrafish an ideal model for screening of metabolic diseases.

Obesity is the root cause of many metabolic diseases. Overnutrition of zebrafish larvae and juveniles activates mTOR, suppresses notch signaling, and increases β-cell mass in tune with the increased requirement of insulin resembling changes in human obesity.

Diabetes is one of the prominent metabolic diseases with impaired glucose homeostasis. The morphology and cellular architecture, signaling pathways, and mechanisms of zebrafish pancreas with distinctive exocrine and endocrine compartments mimic the mammalian pancreas. The chief organ systems brain, liver, adipose tissue, and skeletal muscles, which are the systems of glucose homeostasis, are also intact in zebrafish which makes it an ideal model for screening of metabolic diseases. Transgenic models of insulin resistance in

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skeletal muscles and knockdown of liver-specific insulin receptors are available to screen type 2 diabetes. Though chemically induced models of type 1 diabetes are reported, the innate ability of zebrafish to regenerate β-cell is interfered and hence is a challenge. Zebrafish models to screen maturity-onset diabetes of young (MODY) are also achievable with targeted single gene mutation. Repeated blood collection to measure glucose, IGTT, fluorescent protein expression, and β-cell regeneration research are precisely established. The models to screen diabetic complications such as diabetic retinopathy, neuropathy, nephropathy, microvascular complications, impaired wound healing, and bone mineralization are developed in zebrafish models.

Chronic increased serum cholesterol and triacylglycerol are hallmarks of atherosclerosis. A specialized system of absorption, transportation, synthesis, and storage of lipids is present in humans. Presence of LDL receptor, lipid trafficking genes, lipoprotein-modifying enzymes, β-dominant fasting lipoprotein profile, molecular and cellular mechanisms similar to human lipoprotein biology makes zebrafish a relevant model to mimic hyperlipidemia. High cholesterol diets, deletion of apoc2 gene, and liver x receptor (LXR) deletion are few established models of hyperlipidemia. In addition to hyperlipidemia, formation of granulomas from macrophages as immune-induced inflammatory host response in zebrafish is promising in the development of atherosclerosis.

The optical transparency, ease of genetic manipulations, mimicking human structures and functions, high throughput, ability to develop diabetic complications in days are the advantages of using zebrafish for metabolic models. The limitation of zebrafish is the scientific validation of some of the models with respect to reversal upon treatment with drugs, which will be achieved in due course of time.

Keywords

Zebrafish · Obesity · Diabetes mellitus · Diabetic complications · Hyperlipidemia · Atherosclerosis

4.1 Epidemiology of Metabolic Diseases

Metabolic diseases are the leading cause of morbidity and mortality throughout the world. Four important metabolic diseases—increased blood pressure, overweight or obesity, hyperglycemia, and hyperlipidemia—are the leading cause of noncommunicable diseases. The prevalence of metabolic diseases like type 2 diabetes and coronary heart disease is increasing parallel to obesity, which is also considered the pulpit for cancer of various organs. According to the World Health Organization, abnormal/excessive fat is a health risk, where a person's body mass index above 30 is considered as obese while above 25 is considered as overweight. Obesity was considered as a problem in high-income countries earlier. However, the present scenario derives obesity to increase drastically in urban population of low-

and middle-income countries also. In 2019, around 38.2 million children below the age of 5 years were overweight. Overweight and obesity has increased throughout the world not only hereditarily but also due to the increase in food consumption and sedentary lifestyle. The food consumed in the recent years are energy-dense food with high fat and sugars combined with increase in physical inactivity due to societal and environmental changes (WHO 2019) https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight.

Long-term obesity predisposes to type 2 diabetes mellitus, a chronic metabolic disease caused due to insulin resistance, characterized by elevated blood glucose. The macro- and microvascular complications of diabetes can lead to coronary complications, neuropathy, nephropathy, and retinopathy. The prevalence of type 2 diabetes has increased dramatically in the last three decades in countries of all income levels (WHO 2019). Hyperlipidemia is another metabolic disease which increases the risk of heart diseases and stroke. Overall 2.6 million deaths are attributed to hyperlipidemia.

4.2 Need of Drug Discovery

The pathogenesis behind obesity, diabetes, and coronary heart diseases has been well understood. Though there are numerous drugs available to treat these health conditions, a plethora of associated adverse effects and paucity of addressing multiple pathogenic pathways render a need for new drugs. There is enormous research ongoing on this arena. There are many animal models of obesity, diabetes, and coronary heart diseases for preclinical screening. However, the drug discovery process has advanced into in silico approaches, resulting in tremendous increase in the number of molecules synthesized which amplifies a substantial load on preclinical screening. Furthermore, recent genetic sequencing studies direct multiple phenotypes to be causatives of the metabolic diseases, and involvement of more alleles in these metabolic diseases is likely. This prompts for use of high-throughput in vitro screening and alternative models which can mimic lipid metabolism, adipose biology, pancreas structure, and glucose homeostasis.

4.3 Need of Alternative Animal Models

Zebrafish is one such well-established alternative model for developmental biology, human genetics, and human diseases with a high degree of genetic, anatomical, and physiological similarities to humans. The glucose homeostasis, structure of the pancreas, presence of exocrine and endocrine cells of the pancreas, functional maintenance of lipid metabolism, and adipose biology make zebrafish a sensible model for studying metabolic diseases. Considering the physiology of glucose and lipid metabolism, it can also be used for identification of novel targets for treatment of obesity and metabolic diseases in humans. The ease of accessibility to embryonic and genetic manipulations, optical transparency of its embryos, relative short

generation time, and facile maintenance make zebrafish an ideal model for screening of metabolic diseases. Zebrafish can be used to produce a huge number of externally fertilized embryos rapidly. Furthermore, an enormous number of genome editing tools have been researched in the last decade including knock-in models, instrumental in embryological, forward genetic, and pharmacological research. These elaborations are made possible working with late larvae, juvenile, and adult zebrafish models for many diseases. And more than 65-molecule screens in zebrafish have been reported. Some of these screening have discovered novel compounds for diseases, while few have been identified repurposing possibilities of existing drugs.

Obesity is the root cause of many metabolic diseases. Overnutrition of zebrafish larvae and juveniles activated mTOR which suppressed the notch signaling, and beta cells increased their mass in tune with the increased requirement of insulin and adapted itself transiently.

Diabetes is one of the prominent metabolic diseases with impaired glucose homeostasis. The morphology and cellular architecture, signaling pathways, and mechanisms of zebrafish pancreas with distinctive exocrine and endocrine compartments mimic the mammalian pancreas. The chief organ systems brain, liver, adipose tissue, and skeletal muscles, which the systems of glucose homeostasis are also intact in zebrafish which makes it an ideal model for screening of metabolic diseases. Two transgenic models of insulin resistance—transgenic expression of dominant-negative IGF-I receptor (IGF-IR) for inducing insulin resistance in skeletal muscles and liver-specific knockdown of the insulin receptors using CRISPR/Cas9—are available to screen type 2 diabetes. Though chemically induced models of type 1 diabetes are reported, the innate ability of zebrafish to regenerate β-cell is interfered and hence is a challenge. Zebrafish models to screen maturity-onset diabetes of young (MODY) is also achievable with targeted single gene ablation. The parameters of evaluation of diabetes like repeated blood collection to measure fasting, postprandial glucose, intraperitoneal glucose tolerance tests, fluorescent protein expression, and β-cell regeneration research are precisely established. The models to screen diabetic complications such as diabetic retinopathy, neuropathy, nephropathy, microvascular complications, impaired wound healing, and bone mineralization are developed in zebrafish models.

Chronic increased serum cholesterol and triacylglycerol are hallmarks of atherosclerosis. A specialized system of absorption, transportation, synthesis, and storage of lipids is present in human. Presence of LDL receptor, lipid trafficking genes, lipoprotein-modifying enzymes, β-dominant fasting lipoprotein profile, and molecular and cellular mechanisms similar to human lipoprotein biology makes zebrafish a relevant model to mimic hyperlipidemia. High-cholesterol diets, deletion of apoc2 gene, and liver x receptor (LXR) deletion are few established models of hyperlipidemia. In the future, such models can be progressed from lipid accumulation to macrophages and other immune cell accumulation in plaques forming complex lesions. In addition to hyperlipidemia, mycobacterial infection and the resultant host response to mycobacteria formation of granulomas from macrophages in zebrafish directs that atherosclerosis can be developed with inflammatory and immune mechanisms in zebrafish.

The complex pathophysiology and multiple secondary messenger pathways of these metabolic diseases demand robust and high-throughput animal models for screening of potential drugs. Late larvae, juvenile, and adult zebrafish models have been developed for preclinical screening of many diseases, which can target the multiple pathways and serve as precise screening models.

4.4 Zebrafish Models for Obesity

The metabolism and regulation of energy homeostasis of whole human body is governed by multiple endocrine signals and multiple organs which play a role in complex balance on energy intake, utilization, and storage. The appetite circuits of the hypothalamus, pancreas, insulin-sensitive tissues, and white adipose tissues are the organs which control metabolism in humans. Obesity is characterized by excessive deposition of fat, in which adipocytes undergo both hypertrophy and hyperplasia. These changes in adipocytes are more prominent in the regional distribution of fat especially visceral adipose tissue (VAT) and the ectopic deposition of fats. These fats, in turn, elevate the plasma free fatty acids leading to increased influx of fats into the liver and muscle and promote lipotoxicity, increase insulin secretion, trigger insulin resistance, and develop steatosis, steatohepatitis, cirrhosis, and hepatocellular carcinoma in obese individuals. Hypertrophy and hyperplasia of adipose tissue cause alterations in the endocrine and metabolic functions. They increase the inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) through activation of proinflammatory adipocytokines. They also downregulate the anti-inflammatory adipocytokines, which develop metabolic diseases involving various organs. The regulatory mechanism of energy balance-hypothalamic circuit which consists of leptin receptor and melanocortin system is present in zebrafish similar to humans. Furthermore, stimulation of food intake upon administration of neuropeptide Y, ghrelin, and agouti-related peptide (AgRP) and inhibition of food intake upon administration of melanocortin, cocaine- and amphetamine-regulated transcript (CART) peptide melanocortin, and corticotropin releasing factors (CRP) were observed in zebrafish. The drivers of adipocyte gene induction are peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein α (C/EBPα), C/EBPβ, and C/EBPγ. Fatty acid binding protein 4 (FABP4), glucose transporter 4 (GLUT4), leptin, and adiponectin are terminal differentiation markers. The zebrafish larvae, adult, mutants, and transgenic are some of the available models for screening for obesity. Excess nutrients are converted to form large unilocular lipid droplets in white adipocytes and stored in the zebrafish which is the most essential feature to study the adipose depot.

4.4.1 Larval Models of Obesity

The larval zebrafish is one of the best models to study the sequential effect in lipid absorption, intestinal processing of the fat by enterocytes, storing of the fat by adipocytes and hepatocyctes, and the response of liver and acinar cells of the pancreas to these accumulations. The similarity between the cellular structures of zebrafish adipocyte and mammalian adipocyte tissue renders it an ideal model. The transparent zebrafish larvae develop outside the mother's body making it accessible to observe biochemical and microscopic changes due to obesity. There are various dyes available to observe the adipocytes of the larvae. Sudanophilic dye Oil Red O (ORO) is used to observe the larval triacyl glycerol. The zebrafish larvae start to eat 5–6 days post-fertilization (dpf) during which they absorb triacylglycerol, cholesterol, and fat-soluble vitamins through a maternally derived yolk sac. The lipids are visible in hepatocytes at this stage. The larvae are incubated in a 4% solution of fat which demonstrates the extent of dietary lipid absorption after a high-fat liquid feeding to the larvae. This can be observed in the anterior intestine and intersegmental vessels upon staining with ORO where the extent of ORO staining corresponds to the larval triacyl glycerol. The fatty acid mobilization and transport can be visualized by injecting the zebrafish yolk with a lipophilic BODIPY® fluorophore, which diffuses into the circulatory system within 3 h.

The microsomal triglyceride transfer protein (MTP) of zebrafish has 54% identity to that of human MTP. This MTP forms a complex ApoB-MTP lipoprotein by assembling into nascent ApoB. This complex prevents proteasome-mediated degradation of lipids, thus increasing the plasma lipid levels. The regulators of adipogenesis are well identified. The fatty acid binding protein 11a (*Fabp11a*), expressed in preadipocytes and adult adipocytes of zebrafish, is an ortholog of FABP4, which makes it possible to trace the effect of test compounds on FABP4. The fatty acid binding protein 11a (FABP11A) is involved in maintenance of glucose, lipid homeostasis, and angiogenesis. The peroxisome proliferator-activated receptor gamma (PPARG) is responsible for lipid storage, adipogenesis, regulation of insulin resistance, and glycemic control. The role of CEBP-α, leptin, and adiponectin in adipogenesis is also established. In zebrafish, the adiponectin is selectively found in adult adipose tissue and is not adipose-specific, found in higher levels in the liver. The mRNA levels of MTP in the intestine and liver are increased as a result of a single high-fat meal without altering the MTP protein level. The ApoB levels also increase in response to feeding. These increase the white adipose tissue distribution in zebrafish larvae, which is similar to the molecular mechanism of humans. The PPARG mRNA is expressed in early developmental stage of zebrafish at 5–10 h post-fertilization (hpf). Adipogenesis is visible first in the 8 dpf larvae in the visceral cavity close to the pancreas. These adipocytes can be clearly visible upon staining with Nile Red at 15 dpf. The CEBP-α mRNA is expressed in unfertilized eggs and also in pancreatic and visceral white adipose tissues, which are observed from 17 dpf. The larvae at 20–22 dpf develop a size-dependent subcutaneous and cranial adipocytes which are more than 8.2 mm and

9.4 mm, respectively. The transcriptor factor SOX6 is an activator of adipogenesis, which is common in humans and larvae of zebrafish.

4.4.2 Transgenic and Mutants as Model of Obesity

Incorporating transgenes with fluorescent protein on gene of interest makes it possible to monitor the live changes undergoing in the tissues of interest, which can be observed to study the effect of test drug in obesity. This combination of transparent ex utero developing zebrafish with fluorescent reporters has provided an opportunity to design fluorescent reporter system.

PPAR-γ is a key regulator of adipocyte differentiation, which is associated with the pathology of obesity, diabetes, atherosclerosis, and cancer. A green fluorescent protein (GFP) under regulatory control of PPAR-γ can be made using transgenic zebrafish, Tg(hPPARγ-eGFP). This model has been used to record the changes observed in different tissues to endogenous hormones and cofactors for evaluation of lipid accumulation. Using this model, studies have also proved the obesogenic effect of chemicals and their capacity to activate PPARG in zebrafish larvae. AKT1 gene acts upstream of PPAR-γ and CEBPα in modulating adipogenesis, and its expression in adipocytes is responsible for obese phenotypes. Adult *Tg(krt4:Has. myrAkt1)Cy18* zebrafish showed severe obese phenotype with increased triglyceride; glucose intolerance; fat tissue accumulation in dorsal muscles, gills, and tail; and neutrophil infiltration. The phenotype also demonstrated decreased activity in swimming behavior assay and lesser life span.

Zebrafish (*Tg(fabp10a:TetON;TRE:eGFP-kras)v12*) conditionally activates Kras and exhibited excessive accumulation of lipid droplets in hepatocytes and increased triglycerides.

The role of macrophages in adipocyte tissue inflammation and the different molecules involved in the process have been reported but with a limited clarity on the underlying mechanism. The macrophage infiltration can be studied with zebrafish macrophage reporter line Tg(lyz: Ds Red). The macrophage compartment on the larvae yolk can be specifically marked by lysozyme C expression. This in combination with fluorescent reporters in zebrafish can lead to unraveling the inflammatory triggers and process of transformation to a pathogenic adipose tissue.

The formation of transient knockdown of gene expression using morpholinos (MOs) allows quick and effective study of gene function in the early stages of development. Apolipoprotein (ApoC2) is essential for lipoprotein assembly in humans and is found in the larvae of zebrafish. Larvae when injected with apolipoprotein C2 (MO-ApoC2) reveal an unabsorbed yolk phenotype.

Similar to humans, starvation for 4 days resulted in decrease of neutral lipid depots in all locations in zebrafish, and on 7 days of starvation, all fat materials were dissolved. When dietary carbohydrates are low, the cytosolic phosphoenolpyruvate carboxykinase (PCK1) promoter is induced. PCK1 is under the control of insulin during feeding, while during fasting, it is under the control of glucagon, glucocorticoid, and adrenaline. The zebrafish transgenic bioluminescence reporter Tg(pck1:

Luc2) can be used to study the effect of drugs in the process of gluconeogenesis in response to feeding-fasting transition.

Mutant zebrafish that carry mutations in genes of AT storage and mobilization are used to study cellular and molecular mechanisms of fat accumulation and identify molecular targets and approaches on treatment of obesity. *GH1* gene encodes for the endocrine factor involved in growth and regulation of lipid metabolism. The *vizzini* mutant zebrafish carries a null mutation in GH1 leading to increase in AT and inability to mobilize stored fats. CYP2R1 mutant zebrafish also caused increase in number of adipocytes and increase in total lipids leading to excessive VAT accumulation. The *lepr* and *mc4r* genes are involved in feeding and metabolism, mutations of which in zebrafish caused an increase in body weight and fat percentage and impaired glucose tolerance, leading to obesity. Leptin deficiency in humans due to mutation in leptin or leptin receptor genes is a well-known cause of obesity. A leptin receptor mutant for zebrafish (leprsal 1508/sa1508) has been developed for preclinical screening, which increased the levels of insulin mRNA, caused alterations in glucose homeostasis, and increased β-cell mass without any significant change in adiposity or insulin resistance. Leptin receptor knockout was developed as *lepr-l-medaka*. This *lepr-l-medaka* mutant showed increased food intake and higher growth rate till it reached adult stage, and excessive visceral deposition of fat, which suggest it to be a good model to study the effect of neuroendocrine modulators in obesity. Plexin D1 (PLXND1) is a protein involved in body fat distribution, inhibition of which led to obesity in zebrafish. PLXND1-null zebrafish exhibits a preferential expansion of subcutaneous adipose tissue (SAT) in high-fat feed. PLXDN1 deficiency caused altered fat distribution, decreased the VAT/SAT ratio, and protected the zebrafish from insulin resistance and glucose intolerance.

4.4.3 Diet Models of Obesity

The prime significant factor for development of steatosis is obesity. The high-fat and high lipid diet containing cholesterol is considered to be the key reason of development of steatosis and hepatic lipid accumulation. Studies have proven the development of steatosis in zebrafish larvae upon high-fat diet containing cholesterol, which produced prominent, severe degree of production of steatosis. This model can be used for screening of drugs with anti-steatosis potential. The series of disorders which lead to liver inflammation, fibrosis, and cancer can be observed in zebrafish starting from the formation of liver primordium and differentiation of hepatocytes and cholangiocytes which can be observed from 48 hpf. The metabolic pathways in the liver are activated when there is depletion of the yolk in between Days 4 and 6, where the zebrafish liver is functional for metabolism from Day 6. The uncoupling protein 2 gene, pck1, and carnitine palmitoyltransferase 1A genes, which are key markers of liver metabolism, can be detected by quantitative reverse transcription PCR (qRT-PCR). A fatty liver can be detected in zebrafish by staining with Oil Red O. There are many biological processes which require metabolic enzyme *s*-adenosyl homocysteine hydrolase (ahcy), which produce methyl donors. Zebrafish "ducttrip"

line with mutations in achy can be developed to induce hepatic steatosis and liver degeneration, which is similar to that of human AHCY.

Zebrafish overfed with common live feed *Artemia nauplii* in high quantity caused a significant increase in BMI, hypertriglyceridemia, and hepatic steatosis along with NAFLD, and the gene expression also resembles human NAFLD. This diet-induced obesity was observed to be reversed on caloric restrictions or treatment with natural compounds, making it a suitable model to screen anti-obesity drugs. Adult zebrafish on high-cholesterol diet became obese with deposition of abdominal fat. In zebrafish fed with HFD, elevated blood glucose, triglyceride, and cholesterol levels were observed. High calorie (408 calories/day) normal diet also increased the BMI, adipose tissue volume, and plasma triglyceride, impaired glucose tolerance with high insulin, and increased β-cell mass.

Alterations in gut microbiota cause an increase in endotoxins-lipopolysaccharides leading to activation of inflammatory pathway resulting in obesity. A genetically manipulated zebrafish was developed to study the host-bacterial relation in the digestive tract. Dietary lipid content was found to alter gut microbiome in these zebrafish affecting the transcription genes of appetite control and in cholesterol metabolism leading to obesity. Treatment with probiotics could reverse these changes, which can be a suitable model to further understand the role of microbiota in obesity.

4.5 Zebrafish Models for Diabetes Mellitus

The pancreas of zebrafish structurally and functionally mimic the human pancreas. Zebrafish is a suitable model for screening antidiabetic drugs due to its conservation of the pancreas and glucose homeostasis system. Pancreatic islets in β-cell include single primary islets in the head of the pancreas that can be identified at 24 hpf and secondary islets dispersed throughout the organ that can be visible from 5 dpf. Several genes influence β-cell development which, when altered, can establish human diabetes. The blood glucose can be quantitatively determined by whole-blood analysis in mature zebrafish as well as using handheld glucometers; however, due to the small size of embryos and juvenile fish, absolute glucose levels can be determined from embryo extracts using dual-enzyme fluorescent assay. Repeated blood collection from the same adult zebrafish and glucose tolerance test are also possible. The levels of insulin and its function can be studied using surrogate indicators—*insulin* mRNA (qPCR), semiquantitative dot-blot, immunostaining with insulin antibody, insulin function by phosphorylation of Akt, and insulin promoter activity by determining EGFP signal intensity in Tg(-1.0ins:EGPF)^{sc1} zebrafish. The zebrafish can also be used to study the process of β-cell neogenesis. Activation of the nutrient sensors mammalian target of rapamycin (mTOR) and insulin pathways results in β-cell neogenesis.

4.5.1 Zebrafish Models of Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is an autoimmune disease caused by destruction of insulin producing pancreatic β-cells. The pancreatic β-cells can be destructed to develop T1DM by surgical removal and chemically induced and genetic ablation. Though pancreatectomy is possible under microscope in islet-specific GFP zebrafish, the method is difficult and hence not commonly used.

Chemically induced method like intraperitoneal injection of streptozotocin (six times in 4 weeks) is an effective method to induce T1DM, resulting in stable hyperglycemia. Injection of alloxan also results in T1DM. However, both these compounds also exhibit toxicity.

Genetic models of T1DM are more promising. Stable expression of diphtheria toxin A chain (DTA) destructed all β-cells. Exposure of transgenic zebrafish *(Tg (insa:NTR-CFP))* to metronidazole (MTZ) causes conversion of MTZ to β-cell-specific bacterial nitroreductase (NTR) enzyme resulting in a β-cell-specific cytotoxic compound leading to β-cell apoptosis. This model can be used to screen drugs which can cause β-cell regeneration. A combinatorial approach of inducible transgene with the insulin promoter which drives the expression of a doxycycline-/ecdysone-dependent transcription factor and TetOR-based promoter to express activated human Bid triggers apoptosis. β-Cell recovers completely within a few days of removal of ablation mechanism, which is a limitation.

4.5.2 Zebrafish Models of Type 2 Diabetes Mellitus

Transgenic zebrafish lines have been used to study glucose homeostasis. Zebrafish line *Tg(pck1:Luc2)* reiterates the endogenous regulation of *pck1* expression in mammals. The bioluminescence in *Tg(pck1:Luc2)* can exhibit the changes that are undergone during fasting-feeding transitions and gluconeogenesis.

Overnutrition of zebrafish larvae and juveniles causes suppression of Notch signaling in endocrine progenitors. When the Notch signaling is suppressed, the progenitor cells become activated and become β-cells. The increased demand of insulin to increase glucose levels is met transiently by increasing the number of β-cells of zebrafish pancreas. Lipid-rich and glucose trigger compensates β-cell expansion based on activation of insulin/IGF1 signaling and mTOR signaling, respectively. This has been developed in models *Tg(ins:Cre)x* and *Tg(ins:loxp:BFPloxp:DTA)*.

A zebrafish line *Tg(actc 1b:dnigf1ra-EGFP)* with insulin resistance in skeletal muscle has been reported. Overnutrition of these causes plasticity of β-cells, thereby increasing β-cell numbers. Skeletal muscle insulin resistance and blunted glucose uptake take place. This results in increased glucose level.

4.5.3 Zebrafish Models of Maturity-Onset Diabetes of the Young

Human maturity-onset diabetes of the young (MODY) can be developed in zebrafish by mutation in Hnf1 β, which results in underdevelopment of the pancreas with formation of kidney cysts. A line which fails to express the transcription factor gene PDX1 required for the induction of insulin gene by causing mutation in homeobox gene VHNF1 has been identified. This is in line with the VHNF1 being indicated in MODY type V and type IV for PDX1. Deletion of PDX1 caused decrease in zebrafish.

4.5.4 Transgenic Zebrafish Lines

The PAX6B-positive progenitor cells within the intrapacreatic duct can be visualized by double transgenic line TG(TP1:HMGB1-MCHERRY), TG(PAX6B:GFP). The TG(TP1:HMGB1-MCHERRY) zebrafish carry a Notch activity reporter which marks the cells of intrapancreatic duct by PAX6B:GFP expression. This model can be used to screen for drugs that can induce occurrence of secondary islets.

Zebrafish *Tg(fabp10a:foxn3, EGFP)* has been reported to have an increased hepatic gluconeogenesis and thereby an increased fasting levels. This model has been developed by overexpression of a gene *FOXN3* associated with human fasting glucose levels.

4.5.5 Zebrafish Models for Complications of Diabetes Mellitus

Methylglyoxal is a by-product of glycolysis and elevated methylglyoxal levels are observed in diabetics. Methylglyoxal reacts with lysine and guanidine groups of arginine and induces the formation of advanced glycation end products (AGE). AGE are the proinflammatory molecules which play a vital role in vascular complications of diabetes mellitus. Incubation of PDX1 knockdown embryo or larvae in 55 mmol/L of glucose medium resulted in malformation and uncoordinated growth of small intersegmental blood vessels and increased methylglyoxal levels. Incubation of GLYOXALASE 1 (GLO1) knockdown embryo/larvae caused malformation and uncoordinated growth of small intersegmental blood vessels and increased phosphorylation of VEGF receptor-2 and Akt/PKB. Incubation with aminoguanidine prevented the aberrations of blood vessel formation, which indicates its suitability as a screening model to study the effect of potential drugs.

The microvascular complications of diabetes mellitus include retinopathy, neuropathy, and nephropathy. Retinopathy has been developed by incubating the larvae in 130 mmol/L of glucose medium for 3 days. This resulted in enlarged and defected retinal vessels and increase in the concentrations of nitric oxide (NO) and vascular epithelial growth factor (VEGF). Incubation of adult zebrafish in high-glucose media (10%) resulted in retinal thinning. Incubation of adult zebrafish in 4% glucose medium for 28 days caused thickened, frail blood vessels and aneurysm-like

structures. The prevention of retinopathy by inhibitors of VEGF receptor tyrosine kinase or NO synthase or VEGF-A antibody—ranibizumab—has been observed in zebrafish models, illustrating a potential screening method.

Neuropathy has been developed in adult zebrafish by inducing acute hyperglycemia upon intraperitoneal injection of 2.5 g/kg of D-glucose, and chronic hyperglycemia was induced by incubation in a 111 mmol/L glucose medium for 14 days. This resulted in impaired regeneration and de novo formation of neuronal cells.

Morpholino-mediated PDX1 knockdown caused an enlargement of the pronephric glomeruli, disruption of filtration barrier, and defective podocyte development. Streptozotocin-induced diabetes in zebrafish caused thickening of the glomerular basement membrane. Overexpression of CIN85/RukL in adult zebrafish resulted in disruption of filtration barrier of whole fish resulting in edema.

Impaired wound healing is a typical problem of diabetic patients. In zebrafish upon induction of hyperglycemia with injection of streptozotocin, impaired caudal fin regeneration was observed. Hyperglycemia also altered the bone metabolism resulting in lower rate of bone mineralization, higher rate of bone resorption, and activation of osteoclasts in zebrafish resulting in osteopenia as observed in diabetic patients. Treatment with a vitamin D analogue could improve the regenerative capabilities in hyperglycemic zebrafish. These models can be used to screen the potential drugs to prevent complications of diabetes mellitus.

4.6 Zebrafish Models of Hyperlipidemia

The key drivers for atherosclerosis are elevated serum cholesterol and non-fasting triacylglycerol (TG). The challenge in developing more efficient and novel therapies for atherosclerosis is based on understanding of lipid consumption and its metabolism and modification in tissues. The main carriers of the bulk of absorbed and resynthesized neutral lipids are apolipoprotein B (APOB)-coated particles made by the intestine (chylomicrons) and liver (very-low-density lipoprotein (VLDL) particles, cholesteryl esters (CE), and triacylglycerol (TG)). The absorbable organisms like free cholesterol, free fatty acids, and monoacylglycerol ingested lipids are hydrolyzed in the lumen of the intestines. These molecules are re-esterified and packed into chylomicrons, the hallmark of which is coat protein Apob48 in humans, into triacylglycerol (TG), cholesterol esters (CE), and phospholipids (one molecule per particle). This particle reaches the vasculature and is made from an HDL particle by an Apoc2 molecule (not shown). Apoc2, an enzyme tied to the apical surfaces of capillary bed cells in muscle and adipose tissues, is a necessary binding partner in lipoprotein lipase (LPL). LPL activates these tissue free fatty acids. The remaining chylomicron, partly lipid-deepened, is rapidly eliminated by the liver by the Apoe-binding LRP receptors and Apob-binding lipoprotein recipients. In amniotes, two different protein products are encoded by a single APOB locus. In enterocytes of reptiles, birds, and mammals, the APOB pre-mRNA undergoes cytosine deamination (catalyzed by APOBEC) to generate a transcript encoding a truncated protein (APOB48) that is found

exclusively on chylomicrons. Both the liver and intestine will translate the full-length APOB transcript (encoding APOB100). The remaining APOB48-coated liver chypoprotein residues [intermediate-density lipoprotein (IDL) particles] will mature in the long-lived and atherogenic low-density lipoprotein (LDL) particles. APOB48-coated chylomicron residues are vulnerable to rapid postprandial clearance by the liver. It is therefore important to recognize that Apob-coated zebrafish chylomicrons most likely are not rapidly removed. This absence of Apob48 could lead to rapid dyslipidemia and atherogenesis seen in zebrafish dietary and genetic studies that will be addressed in subsequent sections. There are finally two paralogues of zebrafish Apob (two Apob genes on different chromosomes). The contribution to circulating β-lipoproteins and atherogenesis of these Apob paralogues, i.e., the expression and incorporation into the chylomicrons and VLDL, cannot be seen, but the patterns of larval expression are distinct, and the proteins encoded structurally different.

Zebrafish use highly conserved β-lipoprotein assembly proteins and transport pathways immediately beyond these essential problems of Apob biology. Gene expression survey and knockdown approaches verified that in the zebrafish yolk cell layer, the central Apob-coated lipoprotein particle-producing enzyme microsomal triglyceride transfer protein encoded by mtp and with orthologs in all organisms ranging from insects to mammals is present and functional in hepatitis and bowel. More recently, studies on intracellular trade in nascent chylomicrons have confirmed the zebrafish model to be useful for investigating the molecular and cellular machinery of nutritional energy collection: enterocyte undergoes stereotypical changes in the ultrastructure in the absorption of fat. Finally, the LDL receptor (Ldlr) has retained its role in zebrafish, the main determinant of removing LDL particles from the circulation. Briefly, zebrafish has a complement to preserve lipid trafficking genes which makes the analysis of lipid transport important to human physiology in this model organism. The next section will consider one additional protein, which makes the biology of the lipoprotein zebrafish particularly useful when modeling the biology of human lipoprotein.

After release into circulation, enzymatic devices alter lipoproteins in the blood of zebrafish similar to that of humans. Zebrafish, in fact, bears a human CETP gene orthology. CETP encrypts a circulating protein which, in exchange for TG, transfers CE from HDL particles to LDL particles. When loaded with TG and changed, HDL is more likely to clear rapidly, decreasing its "capacity" for athero-protective processes such as transport of reverse cholesterol (i.e., retrieving cholesterol from tissue macrophages to delivery to the liver and intestine for elimination). Increased CE charge and degradation of LDL-based TG often contribute to atherogenesis by easily modifiable (oxidizable) small dense particles which are capable of entering subintimal space and of contributing to atherogenesis.

4.6.1 High-Cholesterol Diet (HCD) Paradigm

Zebrafish are now recognized for their nutritional requirements. This has helped to develop criteria for altering standard diets to induce metabolic stress. Zebrafish are vulnerable to obesity, hyperglycemia, and dyslipidemia from high-fat diet (HFD).

High-cholesterol diet (HCD) is the greatest advance in the use of zebrafish for dyslipidemia analysis. In the analysis of dyslipidemia and atherogenesis, not only large and adult animals are able to consume such diets, but also display a series of response choices that have been firmly found in this organism. β-Dominant hypercholesterolemia in adults and accumulation of vascular intimate lipids in larvae upon exposure to HCD were observed. Furthermore, these accumulated lipids attracted circulating monocytes followed by oxidation of LDL due to HCD.

4.6.2 Zebrafish APOC2 Deficiency

Zebrafish apoc2−/− genome editing methods were used to produce mutants. These mutants showed that human APOC2 deficiency of reduced lipase activity in plasma and extreme hypertriglyceridemia can be developed. In apoc2−/− mutants, the buildup of lipids and lipid-loaded macrophages, the two distinctive characteristics of atherosclerotic plaques, was observed. In studying LDL extravasation, oxidation, and absorption steps via vascular wall macrophages, this powerful dyslipidemia model may prove especially useful.

4.6.3 Zebrafish Liver X Receptor (LXR) Deletion

Lxrs are the key inducers of catabolism cholesterol. These nuclear receptor transcription factors control metabolism through the use of oxysterol ligands and modify functionally integrated genes involved in the transport, alteration, absorption, and excretion of intestinal cholesterol, liver fatty acid and TG, bile, and immune and inflammatory signals. Two paralogs of Lxr in mammals are present. Lxrα, which originated from fish, is primarily expressed in tissue macrophages, the liver, and the intestine, while Lxrβ is more commonly expressed in amphibians. Lxrīm is upregulating and rising blood TG levels in lipogenic enzymes. The apparently self-defeating function of the Lxr therapeutics is that of driving cholesterol removal and triggering fatty acid synthesis. In fact, BMS-852927 is not only a reverse cholesterol tract for LXRβ selective agonist cholesterol transport in humans but also induces hepatic de novo lipogenesis and attendant hypertriglyceridemia; BMS-852927 also causes a rapid decrease in circulating neutrophil counts in humans, but not in cynomolgus monkeys, underscoring the challenge of drug development.

4.7 Zebrafish Models of Atherosclerosis

Atherosclerosis is narrowing of arteries due to formation of plaques as a consequence of high blood lipid levels. It starts as a simple accumulation of lipid below the vasculature which further develops complex histopathological changes. Zebrafish upon exposure to high-cholesterol diets developed these histopathological changes,

and the phases of developments can be observed using fluorescence proteins. The human CETP is conserved in zebrafish; thereby, the cholesteryl esters in zebrafish are prone to form LDL cholesterol, increasing the susceptibility for atherogenic events. The slow oxidation of LDL by malondialdehyde can be visualized by fluorescent reporters like GFP and live imaging. Feeding of transgenic zebrafish line *flil:EGFP* with high-cholesterol diet laced with a fluorescent lipid tracer also could be possible to view lipid accumulation in the intima of vasculature. The recruitment of macrophages to occurrence of vascular lesions can also be monitored. *Lyz:Ds Red* is a zebrafish line used for labeling macrophages and granulocytes allowing monitoring of myeloid cells in the vasculature. This zebrafish model also can be used for studying the effect of overfeeding in macrophage infiltration into adipose tissue. The pathogenic events as a result of inflammation in obesity and the resultant insulin resistance would be possible using the same technique utilizing lipid accumulation in the liver as a surrogate marker. Such studies clarify that the role of inflammatory activation is due to or a consequence of insulin resistance. It can also identify the pathological process of remodeling of adipose tissue to form hypertrophy and hyperplasia. The human response to mycobacteria is another interesting model developed in zebrafish. This model reveals the conserved inflammatory and immune mechanisms due to the ability of zebrafish to form granulomas in response to mycobacterial infection. This confirms that zebrafish can also form lipid-laden "foam cells" as found in the atherosclerotic plaques addressing the complex set of events from recruiting additional cells to the plaque and driving inflammation. However, owing to the lower blood pressure of zebrafish, it will be challenging to develop plaque rupture models.

Studies direct the increase in adipose mass and adipocyte size in obesity causes an inadequate supply of oxygen resulting in hypoxia and pro-inflammation leading to insulin resistance. Zebrafish lines of hypoxia-inducible factor (HIF), the von Hippel-Lindau protein (p VHL) and isoforms of prolyl hydroxylase (PHD) are expressed. These are primary components of the mammalian oxygen-sensing signaling system. *Tg(phd3:EGFP)* is a transgenic zebrafish line with hypoxia-responsive transgenic reporter line. Another zebrafish line in which the color changes from yellow to red in the presence of H_2O_2 was developed to study the spatiotemporal gradients of H_2O_2 in wound healing.

The regulator of cholesterol homeostasis in mammals is encoded by liver X receptor (LXRA and LXRB), the function of which is conserved roles in zebrafish. Lxr activates an acyl-CoA synthetase and metabolizes absorbed lipids and is stored as lipid droplets. One *lxr* ortholog encoded by *lxra/nr1h3* exists in zebrafish, which will directly assess the gain or loss of functions of its physiological role. *Lxra−/−* knockout zebrafish are viable and imitate cholesterol intolerance.

4.8 Advantages, Limitations, and Conclusion

4.8.1 Advantages

1. The opacity, fecundity, rapid development, and ex utero development of zebrafish larvae are distinct advantages for studying their stages of pathogenesis.
2. The manipulation of gene expression is simple and efficient in zebrafish models with a plethora of readily available genetic tools.
3. Expression of fluorescent protein in transgenic larvae is a very useful tool to monitor the development and progression of diseases like obesity.
4. Embryos, hatchlings, and mature fish readily absorb/take compounds from their aqueous environment and are also DMSO tolerant, which is a major advantage for potential drugs with limited aqueous solubility.
5. In zebrafish, the excess nutrients are converted to form large unilocular lipid droplets and stored as white adipocytes which are similar to humans, while in high-throughput models like *Drosophila* and *C. elegans*, the fat is stored in non-specialized cells.

4.8.2 Limitations

1. Zebrafish have a tremendous regenerative capacity, and hence restoration occurs in β-cell mass once the ablation mechanism is removed.
2. The small size of the zebrafish limits some metabolic investigations; although intraperitoneal and intracerebroventricular injections are possible in adult zebrafish, they are very challenging due to the size.
3. Blood sampling is a terminal process in zebrafish rendering the repeated sampling process impossible. This prevents the use of biochemical parameters to analyze disease progression.
4. The available ELISAs are for use with rodent samples and will cross-react with zebrafish serum. Need for zebrafish-compatible assays will overcome this limitation.
5. High genetic diversity between individual zebrafish genomes even within the same strain of fish is a major limitation. Inbred zebrafish strains (at least 20 times in-crossed) can decrease genetic diversity.

4.9 Conclusion

The metabolic diseases have been successfully developed in zebrafish. The small molecule screens can be effectively performed using these models for metabolic diseases. Zebrafish lines are very useful to study less understood metabolic process, interrelated biochemical pathways, and complex interaction of pathological sequences. The genetic tools for manipulation and fluorescent reporter lines help in study of development biology, and live automated imaging tools allow a high-throughput screening.

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Role of Zebrafish as an Experimental Model for Renal Disorders

5

Tejus Anantharamu

5.1 Introduction

The kidneys perform the vital function of maintaining body homeostasis by excreting metabolites in urine, conserving essential nutrients, and maintaining osmoregulation and acid-base balance. Renal disorders are a global health problem affecting more than 750 million persons globally. The practices of data collection and surveillance related to various renal disorders are lacking or inconsistent even in developed countries leading to difficulties in defining the global burden of renal disorders (Crews et al. 2019; McKee and Wingert 2015). The Kidney Disease Outcomes Quality Initiative (KDOQI) has classified kidney disorders into acute kidney injury (AKI), chronic kidney disorders (CKD), acute kidney disease and disorder (AKD) and no kidney disease based on levels of serum creatinine, glomerular filtration rate (GFR) and the presence or absence of structural damage to kidneys (Barry and James 2015).

According to KDOQI guidelines, AKI is defined as an increase in serum creatinine by 50% within 7 days or an increase by 0.3 mg/dL within 2 days or the presence of oliguria with no structural damage to kidneys (e.g. prerenal causes like decreased perfusion either due to hypovolaemia/hypotension, intrinsic kidney diseases like acute tubular necrosis and acute interstitial nephritis or postrenal causes including urinary tract obstruction). AKD is defined as the presence of AKI or glomerular filtration rate (GFR) less than 60 mL/min/1.73 m^2 or decrease in GFR by $\geq$35% or increase in serum creatinine by more than 50% for less than 3 months with associated structural kidney damage (e.g. acute and rapidly progressing glomerulonephritis, acute nephritic syndrome, acute pyelonephritis and partial urinary tract obstruction) (Barry and James 2015; Levey et al. 2015).

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CKD is defined as GFR of less than 60 mL/min/1.73 m^2 with structural kidney damage for more than 3 months and is often irreversible (e.g. diabetic kidney diseases, hypertensive nephropathy, chronic glomerulonephritis, chronic interstitial nephritis, chronic pyelonephritis, polycystic kidney diseases, chronic heart failure and chronic liver diseases). CKD is further staged based on the levels of albuminuria and GFR. Both acute and chronic kidney disorders ultimately lead to development of renal failure or end-stage renal disease (ESRD) necessitating renal replacement therapy (Barry and James 2015; Levey et al. 2015).

The animal models have contributed greatly in understanding most aspects of renal biology and renal manifestation of systemic diseases. Most of the studies have been conducted on rodents (rats/mice), but in recent years, there has been a progressive increase in mouse models of renal disease compared to rats due to its easy maintenance, availability of mouse-specific reagents and the development of genetic models in mice. Few disadvantages associated with mouse models of renal diseases are difficulties in performing surgical procedures, estimation of blood pressure and availability of relatively lesser tissue sample in comparison to rat models (Becker and Hewitson 2013).

Zebrafish is becoming increasingly popular as a model for study of renal diseases because its embryos experience little or no pain making less of ethical concerns and on pairings can produce large number of offspring in a short span of time (approx. 3 months). Moreover, the embryonic pronephros with two nephrons and adult mesonephros with hundreds of nephrons are simple to study and almost similar to mammalian kidneys anatomically. As the zebrafish possess both pro- and mesonephros, both of them can be studied for nephrogenesis during homeostasis and as a response to injury (Becker and Hewitson 2013). The differences between mammalian and zebrafish kidneys are tabulated [Table 5.1].

The most important advantage with zebrafish is it allows easier observation of in vivo growth of nephrons as their embryos are transparent in nature and with development of functional pronephros within 48 h provides a great opportunity to study the complete development of glomerulus, tubules and other aspects of cellular differentiation. Chemical treatment can be used to reduce the pigmentation in adult zebrafish, and moreover albino strains (genetically modified) are being preferred these days for phenotypic analysis (Outtandy et al. 2017; McCampbell et al. 2014; Gerlach and Wingert 2013). They are increasingly being preferred to study renal genetic diseases because of their high amenability to manipulation of genes via microinjections of DNA and RNA (Gerlach and Wingert 2013).

In addition, there is high conservation of functional domains, with 70% zebrafish proteins having human orthologue and up to 47% human genes possessing one-to-one association with orthologue of zebrafish making them a suitable model for studying nephrogenesis. The epithelium of differentiated nephrons shares many cellular and molecular characteristics (Outtandy et al. 2017; McCampbell et al. 2014). Zebrafish embryos and adult larvae up to 7 days of life can absorb low molecular weight substances from the skin and gills offering an advantage of determining dose-dependent and time-dependent effects on the development of various organs and tissues (Outtandy et al. 2017; McCampbell et al. 2014).

Table 5.1 Comparison of characteristics of kidneys of humans, mice, rats and zebrafish (McCampbell et al. 2014; Gerlach and Wingert 2013)

S. No	Characteristics	Human kidneys	Mouse/rat kidneys	Zebrafish kidney
1.	Location	Retroperitoneal	Retroperitoneal	Dorsal body wall
2.	Basic functional unit	Nephrons (> one million per kidney)	Nephrons Mice: 8000–10,000/kidney	Nephrons (in hundreds)
			Rats: 30,000/kidney	
3.	Development of the kidney	Intermediate mesoderm → pronephros → mesonephros → metanephros	Intermediate mesoderm → pronephros → mesonephros → metanephros	Intermediate mesoderm → pronephros → mesonephros
4.	Pronephros	Vestigial	Vestigial	Pair of nephrons with each nephron having two proximal segments (proximal convoluted and proximal straight tubules) and two distal segments (distal early and distal late)
				Between two distal segments, there is corpuscle of Stannius and following distal late segment, there is pronephric duct and cloaca
5.	Mesonephros	Functions transiently (degrades upon metanephros completion)	Functions transiently (degrades upon metanephros completion)	Single organ consisting of bilaterally symmetrically regions—head, trunk, and tail—consisting of several hundred nephrons (develops into adult kidney) [at 6 months possess around 450 nephrons]
6.	Metanephros	Develops into adult kidney	Develops into adult kidney	Absent
7.	Parts of nephrons with function	Glomerulus/renal corpuscles (filtration)	Glomerulus/renal corpuscles (filtration)	Renal corpuscles (filtration of blood)
		Proximal convoluted tubules (reabsorption of filtered solutes especially glucose and amino acids)	Proximal convoluted tubules	Proximal tubule (similar function)
		Intermediate tubules/loop of Henle (regulation of salt and water)	Intermediate tubules/loop of Henle	Absent
		Distal convoluted tubules (fine-tuning of solutes)	Distal convoluted tubules	Distal tubule (similar function)
		Collecting duct (fine-tuning of solutes)	Collecting duct	Collecting duct

5.2 Zebrafish Models of Acute Kidney Disorders (AKI and AKD)

Acute kidney disorders primarily affect the epithelial cells of tubules of nephrons although damage to other parts like glomerulus and vascular components can progress to AKI (McKee and Wingert 2015). The response of innate and adaptive immune responses to an injury can also induce damage to renal tissues. This damage to epithelial cells leads to accumulation of debris and casts of proteins in the lumen leading to obstruction of flow of fluid (McKee and Wingert 2015). AKI is often reversible but can also progress to end-stage renal disease (ESRD) necessitating requirement of dialysis or even transplantation. AKI is often associated with significant mortality especially in critically ill patients (McKee and Wingert 2015).

Although various mammalian animal models have provided valuable insight into the pathological process of acute kidney disorders, still many questions remain unanswered. Hence, the zebrafish models can prove to be valuable in better understanding of pathogenesis of acute kidney disorders, screening and discovery of novel therapeutic drugs (McKee and Wingert 2015; Gerlach and Wingert 2013).

1. **Nephrotoxic agent (gentamicin)-induced model of AKI**: The administration of nephrotoxic agents like gentamicin, an aminoglycoside antibiotic, is the most commonly used model of AKI in zebrafish (McKee and Wingert 2016; Datta et al. 2017). In a cultured stock solution of E3 (contains 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$), zebrafish embryos at 24 h post fertilisation (hpf) were dechlorinated (removal of chorion) and treated with fungicide 0.05% methylene blue and pigmentation blocking solution containing 0.003% 1-phenyl-2-thiourea (PTU). Two percent methylcellulose solution is used for imaging purpose; every day the PTU solution is decanted and replaced with fresh media. At 72 hpf, embryos will be anaesthetised using 0.2% tricaine and transferred to injection mould (McKee and Wingert 2016; Datta et al. 2017). The embryos in the mould will be divided into three groups that will be either mock treated or treated with saline (vehicle control) or microinjected with 2.5 mg/mL of gentamicin into the cardiac venous sinus at the rate of 15–20 fish/h. After reviving the embryos in fresh media, observe the embryos for 1 (96 hpf) to 2 (120 hpf) days post injection for analysis and acquisition of image. Gentamicin injected embryos demonstrate development of oedema, morphological disruption of tubules and loss of cell polarity in the proximal tubular epithelium in comparison to saline/mock microinjected embryos (Datta et al. 2017).

   The greatest advantage of this model in comparison to mouse/rat models is the provision of direct real-time observation of cellular and molecular changes in nephrons post microinjection avoiding the need to sacrifice the experimental animal post administration of nephrotoxic agent to observe the same. The disadvantage associated with this model is gentamicin has been found to be extremely lethal to the embryo of zebrafish as it can cause irreversible tubular damage making the study of regeneration post acute kidney damage difficult (McKee and Wingert 2016; Datta et al. 2017).

Few regenerative agents have been tested to prevent the damage induced by gentamicin including histone deacetylase inhibitors and hydrogen peroxide which plays a dual role as a protective factor during renal repair and as an initial signal generator during regeneration of kidneys post AKI (Chen et al. 2019; Cosentino et al. 2013).

2. **Precise cellular laser ablation-based model of AKI**: In order to overcome the drawback of gentamicin-induced AKI model, the embryos of transgenic zebrafish with specific green fluorescent protein (GFP) expressing nephron segments were dechlorinated, immobilised on a stage of confocal imaging system set at 40× magnification, under water dipping lens of 0.8 NA and dichroic mirror that allows both 488 and 405 nm illumination on a 35 mm dish. To this add 3 mL of E3-PTU imaging solution along with 0.2% mg/mL tricaine. The GFP is activated using 488 nm laser at low intensity of approx. 1%, and damage to specific kidney cells is induced using high-intensity (100%) 405 nm violet laser (Datta et al. 2017).
   The cellular death can be assessed using difference in GFP fluorescence between ablated segment and segments anterior/posterior to it. The measurement of photoablation is based on the percentage of GFP fluorescence reduction initially post ablation to the decline in the same 5 h post ablation. The advantage with this model is the achievement of injury to spatiotemporal location of the kidney that is precisely controlled in comparison to generalised toxicity with gentamicin-induced AKI. The drawback with this model is it does not consider all the factors leading to AKI in humans (Datta et al. 2017).
3. **Cisplatin-induced nephrotoxicity**: Cisplatin is the chemotherapeutic agent commonly used in the treatment of various cancers. All the steps are similar to gentamicin-induced AKI model except for microinjection of cisplatin 1.5 mg/mL to embryos at 48 hpf, and gentamicin can be used as active control. The visible damages to pronephros in larval zebrafish at 96 hpf include vacuolisation of cells due to fluid retention, lost/flattened brush border of the epithelium and decreased height of proximal tubules. These changes are similar to histopathological changes seen in mammalian models which can be utilised to test various pharmacotherapy agents. An important feature of cisplatin toxicity—apoptosis—is difficult to capture in this model (Hentschel et al. 2005). Recently, it has been demonstrated that pharmacological inhibition of poly-ADP-ribose-polymerase (PARP) which is an enzyme implicated in cell death induced by cisplatin could offer a novel pharmacotherapeutics option to prevent renal damage (Kim et al. 2020).
4. **Acetaminophen-induced nephrotoxicity**: 12–60 hpf zebrafish embryos with green fluorescent kidney line Tg (w1b: GFP) were treated with 0 nM (mock treated) to 45 mM (highest dose) doses of acetaminophen over 12–60-h time periods. Zebrafish treated with 22.5 and 45 mM of acetaminophen demonstrated various malformations like curved/cystic pronephric tubes and ducts and cystic/atrophic glomerulus. The severity of damage was more with earlier developmental stage of zebrafish (12–24 hpf), and the more the time duration of

exposure, the more severe was the damage with no survival beyond 72 hpf (Peng et al. 2010).

5. **Citrinin- and patulin-induced nephrotoxicity**: They are the secondary metabolites of fungus found in food that can induce developmental toxicity in embryonic kidneys. The zebrafish with green fluorescent kidneys (w1b: GFP) were treated with citrinin and patulin and showed no morphological changes but altered the functional ability of dextran clearance in embryos, partly reversed by pentoxifylline administration. Histological changes include cystic lesions of glomerulus and tubular lesions with citrinin and disorganised arrangement of renal cells in patulin-treated groups. As the proinflammatory genes COX2a, TNF-α and IL-1β were overexpressed, it can be concluded that both agents cause nephrotoxicity through inflammatory pathways and by a reduction of blood flow to the glomerulus (Wu et al. 2012).
6. **Aristolochic acid-induced nephropathy**: Aristolochic acid is found in the herbal remedies commonly used in Taiwan and has been implicated by epidemiological studies to be associated with long-term risk of development of urothelial cancer and renal failure. Twenty-four to 31 hpf transgenic zebrafish Tg (wt1b: EGFP) which allows easy visualisation of the glomerulus, pronephric tube and ducts and pancreas were treated with 3 ppm of aristolochic acid. The malformed kidneys demonstrate curved and dilated pronephric tubules and unfused and swollen glomerulus in comparison to mock-treated zebrafish. These changes were attenuated in those zebrafish treated with nephroprotective agents like resveratrol and ursolic acid (Ding et al. 2015).
7. **Sepsis-associated AKI (S-AKI)**: The systemic response that is triggered by an infection is often referred to as sepsis that contributes to nearly 10% of hospital admissions, and the main cause of death in sepsis is the development of multi-organ dysfunction syndrome (MODS). The innate immune response plays a very important role in AKI developed during sepsis, but the exact mechanism still remains to be elucidated. As the larvae of zebrafish possess only the innate immune response until maturation to juvenile stage (4 weeks post fertilisation) offers a great platform to unravel various mechanisms in development of S-AKI (Wen et al. 2018).

   It has been observed that zebrafish does not respond to administration of typical pathogens (*Escherichia coli*/lipopolysaccharide) used in mammalian models. The gram-negative aquatic pathogen *Edwardsiella tarda* (*E. tarda*) is often used to induce systemic infection in zebrafish. The graded doses of 100 colony-forming units (CFU), 300 and 600 CFU are administered into the blood stream of 3-day postfertilisation larvae through its duct of Cuvier. Similarly, 6×10^6 and 6×10^7 CFU were administered into the adult zebrafish and are observed at 24, 48 and 72 h post infection. The results demonstrates a dose-dependent mortality in larvae and adult zebrafish with doses of $\geq$300 CFU in larvae and 6×10^7 CFU in adults demonstrating significant mortality in comparison to control group. Significant difference was noted for the expression of biomarkers of kidney injury (igfbp7, timp2, kim1) in test group in comparison to control groups (Wen et al. 2018).

8. **Anticancer antibiotics-induced glomerular damage**: Adriamycin an anthracycline antibiotic is used for treating various neoplastic disorders (e.g. leukaemia, multiple myeloma, lymphomas and sarcomas). Low concentration of Adriamycin (10–20 μM) is dissolved in medium containing 9 hpf embryo of zebrafish and removed after 48 h. Adriamycin interferes with development of podocytes and alters the GFR. This model mimics the congenital nephrotic syndrome developed in mammals (Zennaro et al. 2014).
   Doxorubicin hydrochloride at various concentrations (0–100 mg/L) added to larvae of zebrafish produces developmental defects. Puromycin 250–350 mg/kg microinjected into zebrafish larvae at 80 hpf produces podocyte damage leading to altered GFR and proteinuria (Zennaro et al. 2014).
9. **Model of renal fibrosis**: Most of the CKD are characterised by an accumulation of extracellular matrix leading to fibrosis and altered architecture of the kidneys. Although injury to endothelial cells and activation of the myofibroblasts play a key role in development of fibrosis, the exact mechanisms are still unknown. Hence, various animal models have been designed including chemical (adenine, folic acid, aristolochic acid), and overexpression of transforming growth factor (TGF-β1) induced fibrosis in mice to understand the same and to develop antifibrotic compounds.
   As explained previously, aristolochic acid has been used to develop model of acute nephropathy. Fifteen days post fertilisation larvae of zebrafish were exposed to low doses of 0.5 μm aristolochic acid for a prolonged period of 11 days, but there were no signs of collagen deposition. Hence, the zebrafish lacking reverse transcriptase of telomerase ($tert^{-/-}$ zebrafish) were exposed to aristolochic acid, which demonstrates mild collagen deposition (Elmonem et al. 2018).
10. **Models of mechanical obstruction of pronephros tubules**: Mechanical obstruction of tubules of pronephros using physical compress or fine tweezers has been tested as a model for development of AKI. Sudden occlusion of tubules often leads to distension of tubules within 30 min and was associated with increased beating rate of cilia of tubular epithelium and upregulation of gene expression like foxjla regulating ciliogenic genes (McKee and Wingert 2015).

5.3 Genetic Models of Renal Diseases

Since the earlier days of genome sequencing of zebrafish in the 1990s, it has been commonly employed to study genetic abnormalities known to cause disease in humans. The latest complete reference genome of zebrafish GRCz11 was released in 2017. The approaches often employed include forward and reverse genetics. Forward genetics include induction of random DNA mutation either through gamma irradiation/chemical mutagens (*N*-ethyl-*N*-nitrosourea)/insertional mutagenesis involving use of transposons (DNA elements) or retroviral vectors into blastula followed by mating with females (wild-type), and offspring are propagated through

inbreeding producing required mutants and those with phenotype of interest are isolated (Cirio et al. 2015; Outtandy et al. 2019).

Reverse genetics approach involves identification of interested gene and targets it specifically (knockdown/knockout/knock-in alleles). The technique most often employed includes use of morpholino (MO) antisense oligonucleotides that are resistant to nuclease degradation that are injected into embryos and bind to complementary mRNA molecules, thereby blocking translation of target gene efficiently or disrupting its splicing. This method offers a simple, low cost, acute downregulation of target genes and can produce severe phenotypes, but is transient and can produce off-target effect (activation of pro-apoptotic pathway) needing validation studies (Cirio et al. 2015). The latest advance in reverse genetics is the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology. It offers genetic models that are permanent, highly efficient, affordable, specific, and with possibility of both knock-in and knockout models in comparison to other techniques like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Cirio et al. 2015; Outtandy et al. 2019; Thomasova et al. 2016).

The various genetic models of kidney disease in zebrafish include the following:

1. **Tubular disorders**: The tubules reabsorb the filtered solutes (proteins, vitamins, hormones and others) through proximal tubular epithelial cells by multi-ligand transmembrane receptor (megalin, cubilin)-mediated endocytosis. The targeted genes include ctns (cystinosis—altered megalin protein), lrp2a, b (Donnai-Barrow syndrome—loss of megalin protein), ocrl (Lowe syndrome—altered megalin protein), sec61al (autosomal dominant tubulointerstitial kidney disease), slc30a10 (hypomagnesaemia with dystonia type 1), kcnj10a (SeSAME syndrome), slc4α4 (proximal RTA with ocular anomalies), casr (familial hypocalciuric hypercalcaemia type I) and arll5b which maintains calcium and magnesium homeostasis (hypomagnesaemia) (Thomasova et al. 2016).
   Knockdown of murine double minute-2 (Mdm2) negative regulator of p53 gene (tumour suppressor) by SiRNA produces swelling of proximal tubular cells (Yin et al. 2016). Tamoxifen-induced kidney injury molecule-1 (KIM-1) overexpression in zebrafish models demonstrates the involvement of mammalian target of rapamycin (mTOR) in progression from AKI to CKD. KIM-1 (phagocytic receptor) is overexpressed in tubules during acute and chronic injuries to the kidney. Although it produces antiinflammatory effects, long-term expression leads to mammalian target of rapamycin (mTOR)-mediated damage to tubules (chronic tubular damage with loss of brush borders, reduction in GFR, pericardial oedema and an increase in mortality) ultimately progressing to CKD (Gehrig et al. 2018).
2. **Glomerular disorders**: Glomerulus of the kidney typically performs the task of ultrafiltration of various molecules from the blood. The barrier of glomerulus is well established in an embryo of zebrafish by 72 hpf, hence proteinuria to be measured only after 4 days post fertilisation (dpf). The functionality and integrity of glomerulus can be assessed using dextran, a complex polysaccharide. The advantages of dextran are it is inert, doesn't induce immunological reactions, is

available in various molecular weights (low molecular weight dextran 3–10 kDa can be used to measure GFR as it is freely filtered, and higher molecular weight dextran 70–500 kDa can assess the integrity of barrier of glomerulus as it doesn't get filtered) and can be labelled with fluorescent tags to provide visualisation of vasculature and tissues (Cirio et al. 2015).

The alternate polysaccharide that can be used in place of dextran is inulin. The drawback is the tedious and labour-intensive injection of fluorescent tagged dextran/inulin into vasculature of zebrafish. To circumvent the same, the transgenic mice that can express plasma protein that are fluorescent tagged were developed. The commonly used plasma protein in humans is albumin (50% of plasma proteins), but the gene encoding the same is absent in zebrafish. Hence, the GFP fused vitamin D binding protein (VDBP) which has an almost similar molecular weight and electric charge to that of albumin is preferred to develop in transgenic zebrafish (Elmonem et al. 2018).

The targeted genes include nphs1 causing steroid-resistant nephrotic syndrome (SRNS) 1—Finish type, nphs2 (SRNS2), plce1 (SRNS3), cd2ap (SRNS4), wt1a, b (Denys-Drash syndrome), Lmx1b (nail-patella syndrome), smarrcal1 (Schimke immune-osseous dysplasia), apol1 (focal segmental glomerulosclerosis—FSGS4), inf2 (FSGS5), myole (FSGS6), anln (FSGS8), crb2b (FSGS9), vhl (Von Hippel-Lindau disease), shroom3 and fat1 (glomerulopathy) (Cirio et al. 2015; Outtandy et al. 2019; Thomasova et al. 2016; Choksi et al. 2014).

3. **Models of renal ciliopathies**: The dysfunction of the cilia which are microtubule-based hairlike organelles on the cell surface often leads to the development of monogenic cystic kidney diseases. It is characterised by the development of cysts which are fluid-filled cavities that are lined by epithelial cells. The most common mechanism of cyst formation is increased epithelial proliferation and abnormal accumulation of fluid. It is suggested that the cilia present on the cells of the kidney act as a sensor which stops the cellular proliferation once the flow of fluid is optimal in tubules. Hence, any derangement in the same would lead to development of cystic diseases (Choksi et al. 2014; Shi et al. 2017).

   The targeted genes include pkdla/b; pkd2 (autosomal dominant polycystic kidney disease), dzip1l (autosomal recessive polycystic kidney disease), nphp1 often leading to nephronophthisis1 inherited as an autosomal recessive disorder, producing cystic kidney disease and is the most common genetic cause of ESRD in first three decades of life; invs (nephronophthisis2); nphp3 (nephronophthisis3); nphp4 (nephronophthisis4); iqcb1 (nephronophthisis5); cep290 (nephronophthisis6); glis2 (nephronophthisis7); nek8 (nephronophthisis9); sdccag8 (nephronophthisis10); wdr19 (nephronophthisis13); and cep164 (nephronophthisis15) (Cirio et al. 2015; Choksi et al. 2014; Shi et al. 2017).

   The other targeted genes include traf3ipl (Senior Loken syndrome), inpp5e (Joubert syndrome1), tmem216 (Joubert syndrome2), ahi1 (Joubert syndrome3), tmem67 (Joubert syndrome6), rpgrip1l (Joubert syndrome7), arl13b (Joubert syndrome8), cc2d2a (Joubert syndrome9), ofd1 (Joubert syndrome10), ttc21b (Joubert syndrome11), bbs1 (Bardet-Biedl syndrome1), tscla (tuberous

sclerosis1), tsc2 (tuberous sclerosis2), ift172, ift80, tekt1 (short-rib thoracic dysplasia with or without polydactyly), dcdc2 (renal-hepatic ciliopathy), cep120 (Jeune thoracic dystrophy) and pik3r4 (ciliopathy) (Shi et al. 2017; Sanna-Cherchi et al. 2018).

4. **Models of congenital anomalies of the kidney and urinary tract (CAKUT)**: CAKUT accounts for around 23% of the birth defects and around 50% of the paediatric ESRD worldwide. The spectrum of developmental malformations covered under CAKUT includes renal agenesis, multicystic dysplastic kidney, obstruction of ureteropelvic junction, megaureter, vesicoureteral reflux and obstruction of posterior urethral valves (Wang et al. 2020). The targeted genes for development of genetic model in zebrafish include pax2a (papillorenal syndrome), crkl, aifm3, snap29 (DiGeorge syndrome), wtla (Denys-Drash syndrome), hnflba, b (renal cysts and diabetes syndrome) and six2 (renal hypodysplasia) (Cirio et al. 2015; Wang et al. 2020).
5. **Limitations of zebrafish models**: Although considered a very suitable alternative to mammalian models, it has its own limitations. The most important is the duplicity of genes during evolution, thereby being unable to produce the desired phenotype. Zebrafish is not a suitable alternative for modelling few human diseases like hereditary nephrogenic diabetes insipidus caused by defect in AVPR2 or AQP2 (aquaporin2) gene defects because of the absence of true orthologue. The absence of genes especially those coding for the subunits of epithelial sodium channels (SCNN1A, SCNN1B, SCNN1G and SCNN1D) whose mutation causes Liddle syndrome and type 1 pseudohypoaldosteronism in humans making the genetic model for the same in zebrafish difficult. The other generalised limitations include derivation of human doses from these models is difficult because of the varied physiology including haemodynamics and mode of administration of drugs. Moreover, the amount taken up by the larvae is difficult to estimate, and handling of drugs by few organs (lungs, mammary gland) cannot be predicted as they are absent in zebrafish (Elmonem et al. 2018).

5.4 Conclusion

Zebrafish modelling of renal diseases is a very valuable model not only for better understanding of the pathophysiological mechanism but also for evaluating newer pharmacotherapy options. The significant advantage of visualising the development of various organs in zebrafish without sacrificing the animal is a welcome step to practice the replacement, reduction and refinement principles in animal experimentation. The zebrafish models also have their share of limitations, but recent advances in overcoming the same especially the development of genetic models have revolutionalised the research in zebrafish models.

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Evolution of Zebrafish as a Novel Pharmacological Model in Endocrine Research

6

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Abstract

Zebrafish is a powerful platform in the modern era of phenotype-based drug discovery and eminent vertebrate model to study disease progression and its pathophysiology. Zebrafish possess several advantages over rodent model including low cost, females that lay up to 300 eggs per week, the optical clarity of embryo, external fertilization, and highly amenable to transgenic modifications

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using various genetic toolkits. Zebrafish have almost 70% genetic homology with humans, and 82% of disease-causing human proteins are orthologue to zebrafish. The bottleneck in drug discovery is high cost, laborious, and time taking processes to generate hits. Zebrafish provide a novel option to overcome this bottleneck and have enabled rapid drug discovery in the area of cancer, cardiovascular diseases, endocrine diseases, and many more. However, zebrafish cannot completely replace the mammalian model in drug discovery, but it can form a bridge between cell-based assays and mammalian models, thus reducing the overall cost and time in lead generation. Therefore, in this chapter, we have discussed the role of zebrafish as an emerging vertebrate model in the area of endocrinology disorders.

Keywords

Zebrafish · Mutant · Transgenic · Diabetes · Obesity · Thyroid · Osteoporosis · Gonadal hormone

6.1 Introduction

Discovering a new drug entity is a very complex long process that involves testing of lead compounds in different animal models for toxicity evaluation and their efficacy. However, it is now an unprecedented challenge for researchers to use mammalian models for drug discovery because of high cost, laborious procedures, requirement of a huge amount of chemical, and logistic challenges (Forum on Neuroscience and Nervous System Disorders 2014). Further, another limitation is stringent guidelines regarding the usage of animals, and a significant number of animals would not be available to reproduce the same data. Hence, the authenticity of data is on stake and thus halting the process for producing drug candidates. Thousands of lead compounds are generated and their testing is done during the early phase of drug discovery, but a large number of the compounds do not progress toward the next step because of their safety and toxicity concerns usually because of off-targets which causes heavy revenue loss (Kari et al. 2007). Hence, exploring alternative methods for testing is highly appreciable which can potentially reduce the cost of expensive preclinical as well as clinical trials. The alternative trial methods include in silico studies, in vitro evaluation, 3D tissue models, organ-on-chip, machine learning, and nonmammalian models such as *Drosophila*, *Caenorhabditis elegans*, cockroach, and zebrafish (Peterson et al. 2008; Cassar et al. 2020). Since the 1970s, the zebrafish (*Danio rerio)* is used as an animal model in a large number of research centers to study developmental biology and embryogenesis (Detrich et al. 1999; Gemberling et al. 2013). George Streisinger is the first scientist from the Oregon University who used the zebrafish as a tool for studying the nervous system and asserted that this vertebrate animal is less complex as compared to rodents (Gemberling et al. 2013). Now zebrafish is extensively utilized as an experimental model for various biological disorders and in drug development especially in preclinical evaluation

of new chemical entities (NCEs) because of its morphological and physiological similarity to mammals. Genome sequence analysis of zebrafish showed an approximate 70% genetic homology with humans and 82% of disease-causing human proteins are orthologue to zebrafish (Howe et al. 2013). Zebrafish protein is 54% similar to the glucocorticoid receptor and 91% to thyroid receptor of humans (MacRae and Peterson 2015). Zebrafish possess highly conserved integrative physiology with complete organ systems such as the heart, liver, kidney, pancreas, etc., for example, human islet is very similar to zebrafish pancreatic islet comprising α-, β-, δ-, and ε-cells which regulate glucose homeostasis, by modulating the release of insulin, glucagon, somatostatin, and ghrelin (MacRae and Peterson 2015; Tiso et al. 2009; Gut et al. 2013). Similarly, cardiovascular, nervous, digestive, and hematopoietic systems possess high similarity with the humans in the composition of blood cells (Jagannathan-Bogdan and Zon 2013; Li et al. 2019). Besides, zebrafish share common genes and proteins with humans in disease pathology such as interleukins in inflammation, apolipoprotein H in clotting pathway and leptin, peroxisome proliferator-activated receptors α/γ, nuclear receptor subfamily 1 group H member 3, and sterol regulatory element binding transcription factor 1in lipid metabolism (Oka et al. 2010).

Zebrafish offers various advantages over the mammalian model, and just like any other preclinical model, certain disadvantages do exist. Zebrafish is a small freshwater minnow, an adult grows around 5 cm in length that can live up to 5 years, and females lay up to 300 eggs per week (Epstein and Epstein 2005; Chico et al. 2008). Fertilization of egg occurs externally and the development of the embryo is readily observed from the earliest stages. The optical transparency of zebrafish embryo and early adults facilitates direct observation of internal organs by light microscopy. Zebrafish embryogenesis is finished in 72 h post-fertilization (hpf); considered as larva and organs are fully developed as soon as completion of 96 hpf (Kari et al. 2007; Delvecchio et al. 2011; Rocke et al. 2009; Chakraborty et al. 2009). Due to its small size embryo, it is very convenient for the researcher to study its entire embryonic development inside multi-well plates. Zebrafish maintenance is very easy and extremely economical and it breeds well under laboratory condition as compared to the rodents. Sexual maturation is touched in around the third month, and it is tolerant to dimethyl sulfoxide up to a maximum of 1% concentration (Delvecchio et al. 2011). Zebrafish is highly tenable to genetic manipulation like knockdown, and transposon-mediated gene insertion and zinc-finger-mediated recombination can be done with ease as compared to rodents (Kawakami 2005; Moens et al. 2008; Amacher 2008). However, some of the disadvantages which include tissue-like breasts, lungs, and prostate are absent. Further, adult large size zebrafish are not suitable for high-throughput screening (HTS) of bioactive compounds and also have two copies of many mammalian genes (Delvecchio et al. 2011). This chapter describes the use of zebrafish as an emerging model in various endocrinology disorders including diabetes, obesity, bone-related disorders, and infertility.

6.2 Zebrafish as a Preclinical Model for Diabetes Drug Discovery and Development Research

Diabetes mellitus (DM) is a group of metabolic disorders described by high glucose and disturbances in the metabolism of carbohydrates, fat, and protein that results from defect in either insulin secretion, insulin action, or both (Cattin 2016; Kahn 2003). DM is a major health concern as it reduces the life expectancy due to its secondary complications such as microvascular and macrovascular disorders (Brownlee 2005). In 1980, there were 108 million cases of diabetes and in 2014 it reached 422 million cases worldwide. It is estimated that by the year 2040, 642 million people will be suffering from DM globally (Mirzaei et al. 2020). The Food and Drug Administration (FDA) approved antidiabetic drugs to control glucose levels including insulin therapy, thiazolidinediones, biguanides, sulfonylurea, SGLT2 inhibitors, GLP-1 analogs, and so on (Bailey and Turner 1996; Tan et al. 2019). Various animal models are available for screening of potential antidiabetic candidates, but the major disadvantages of these models include high cost, extreme labor work, ethical concerns, and a small number of animals that are ethically cleared for experimental purposes, which is a big hurdle for drug discovery (Al-Awar et al. 2016; Brito-Casillas et al. 2016). Hence, alternative models are emerging such as the zebrafish, which offer multiple advantages to overcome these limitations and provide certain superior features over rodent models for drug discovery. Further, there are studies available that show zebrafish could be utilized for screening of lead compounds in various metabolic disorders such as nonalcoholic steatohepatitis (NASH), coronary artery diseases, visceral adiposity, and DM (Alexandre-Heymann et al. 2019). It has been observed that morphogenesis and basic cellular structure showed high similarity with the human pancreas, and this makes a zebrafish an emerging model for induction of diabetes (Kari et al. 2007). The pancreas has both exocrine and endocrine functions; exocrine plays an important role in the digestion process, whereas an endocrine part is involved in the regulation of glucose homeostasis. The endocrine is ductless glands and consists of α-cell, which produces glucagon, β-cell which secretes insulin, δ-cell which produces somatostatin, and ε-cell which produces ghrelin and pancreatic polypeptide cells (Alexandre-Heymann et al. 2019; Argenton et al. 1999). The normal blood glucose range for zebrafish is 50–75 mg/dL which is very close to the human blood glucose (70–110 mg/dL) (Zang et al. 2015). Moreover, zebrafish model is well established to study pancreatic β-cell regeneration from non-β-cell (Moss et al. 2009; Pisharath et al. 2007; Anderson et al. 2009). The most common reason for diabetes mellitus is the impaired function of the pancreatic β-cells and its failure.The study reveals that exposure of diet rich in high fat and calories in zebrafish induces alteration in metabolic pathways that resemble to humans up to a great extent (Williams and Watts 2019). In rodents apart from various genetic toxins, drugs and diet rich in high fat and calories are used for the induction of diabetes. Similarly, diabetes can also be induced in zebrafish. There are multiple ways to induce hyperglycemia in adult zebrafish as well as in embryo which effectively mimics type 1 and type 2 DM. These are summarized in Table 6.1.

Table 6.1 Zebrafish as a model of diabetes mellitus

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
Induced models				
Type 1 diabetes mellitus	Immersion in 1% water glucose solution for 30 min, followed by immersion in water for 1 h, and subsequent immersion in 300 mg alloxan/ 100 mL water for 30 min	Adult	Increased blood glucose level, β-cell necrosis	Shin et al. (2012)
	β-Cell ablation by alloxan intraperitoneal injection or immersion of alloxan	Larvae, adult	Increased blood glucose level, β-cell necrosis with reduced neuro-mast cell number	Moss et al. (2009), Nam et al. (2015), Benchoula et al. (2019b) and Castañeda et al. (2017)
	Pancreas removal	Adult	Increased glucose level	Moss et al. (2009), Heckler and Kroll (2017) and Delaspre et al. (2015)
	Intraperitoneal injection of streptozotocin	Adult, larvae	Hyperglycemia, β-Cell apoptosis and destruction	Moss et al. (2009), Benchoula et al. (2019b), Wang et al. (2020b) and Olsen et al. (2010)
Type 2 diabetes mellitus	Glucose immersion	Adult	Increased glucose level, glucose intolerance	Gleeson et al. (2007), Alvarez et al. (2010) and Capiotti et al. (2014a)
	Immersion in 25% glucose	Adult	Increased glucose level, impaired glucose tolerance	Elo et al. (2007)
	Overnutrition	Adult	Increased glucose level, glucose intolerance, hyperinsulinemia	Zang et al. (2017)
Transgenic models				
Type 2 diabetes mellitus	Tg(acta1: dnIGF1REGFP) is transgenic expression of a dominant-negative IGF-I receptor in skeletal muscle	Adult	Increased glucose level	Zang et al. (2017)
	Tg(actb2:cas9; (U6x: sgRNA(insra/b) is knockdown of the insulin receptor a and b in the liver	Larvae	Hyperglycemia, insulin resistance, hyperinsulinemia	Yin et al. (2015)

(continued)

Table 6.1 (continued)

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
MODY10	Tg(C43G-GFP) is transgenic expression of C43G human proinsulin	Larvae, adult	Normal glucose homeostasis, without loss in β-cell mass	Eames et al. (2013)
Targeted genetic ablation				
Type 1 diabetes mellitus	Nitroreductase expressing transgenic lines exposed to metronidazole induces cell ablation	Larvae, adult	Destruction of islet tissue, increased blood glucose levels	Moss et al. (2009), Pisharath et al. (2007) and Curado et al. (2007)
	Apoptosis through expression of activated Bid using transgenic line Tg(1.2ins:htBid;LR) induced by doxycycline and tebufenozide	Larvae	β-Cell ablation, increased glucose levels	Li et al. (2014)
Mutant lines model				
MODY5	Liger^{s430}(hnf1ba^{s430}) (hnf1ba mutation)	Larvae	Pancreatic hypoplasia with decreased β-cell numbers	Lancman et al. (2013)
MODY6	Mutation in neurod1 using CRISPR induced gene deficiency	Larvae	Failure of endocrine cell differentiation, increased glucose levels	Dalgin and Prince (2015)
MODY4	Mutation in pdx1 (mutant pdx1^{sa280})	Larvae, adult	Reduced β-cell numbers, impaired glucose homeostasis	Kimmel et al. (2015)

6.2.1 Type 1 Diabetes Mellitus Models of Zebrafish

Type 1 DM is insulin-dependent that involves autoimmune destruction of pancreatic β-cell, but in reality, the autoimmune model for T1DM is not available in zebrafish. However, destructing the β-cell through toxins, chemical-genetic ablation of β-cell, and surgical removal of β-cells from the islet of the pancreas is a sophisticated approach to generate model of T1DM in zebrafish. Pancreatectomy can be performed in transgenic zebrafish under a microscope, but this method is very difficult to perform and not commonly used in zebrafish. Currently, there are very few models that mimic T1DM including intraperitoneal injection of toxin

streptozotocin (STZ), a glucosamine-nitrosourea compound which was initially approved for pancreatic cancer but is now widely utilized as an experimental tool for induction of diabetes in rodents (Furman 2015; Shibuya et al. 2018). The STZ administration to zebrafish causes significant ablation of β-cells leading to hyperglycemia and insulin deficiency (Intine et al. 2013; Oyelaja-Akinsipo et al. 2020). Multiple injections of STZ in zebrafish represent stable hyperglycemia with the development of other secondary complications such as retinopathy, nephropathy, impaired wound healing, angiogenesis, and fin regeneration (Sarras Jr et al. 2014). Besides STZ, alloxan is a toxic glucose analog which selectively damages the β-cell in larvae of zebrafish and mimics the symptoms of T1DM (Nam et al. 2015; Wang et al. 2020a). A study reported the concept of metabolic memory, which occurs after the termination of STZ administration. They observed that after 14 days of STZ termination, the blood glucose and insulin levels reached normal levels possibly due to regeneration of β-cells; however, the molecular level alteration is persisting (Olsen et al. 2012). Hence, the genetic ablation of β-cells is preferred for the modeling of T1DM. Pisharath et al. removed β-cell from the islet of the pancreas using metronidazole (Pisharath et al. 2007). They expressed an *Escherichia coli gene* called nfsB that encodes for nitroreductase enzyme in the β-cell of embryonic zebrafish, which activates the prodrug metronidazole into cytotoxic that damages β-cell. Through the fusion of two proteins, *nfsB* to *mCherry*, they were able to make β-cell susceptible to the metronidazole-dependent cell ablation. Further, this methodology showed β-cell-specific ablation without interfering with the function of other cells of islet of the pancreas (α- and δ-cell) (Pisharath et al. 2007). Another approach has also been used in which inducible transgene where the insulin promoter drives the expression of a doxycycline/ecdysone-dependent transcription factor and the TetOR-based promoter to express activated human Bid that triggers apoptosis. However, the β-cell ablation model is not so conclusive because of the recovery of β-cells after discontinuation of the drug that was used for the ablation process (Li et al. 2014; Zang et al. 2018). Besides, gene manipulation can also be done by using the CRISPR/Cas9 system and morpholinos that can halt the β-cell development process by altering the function of PDX1 leading to diabetic phenotype and development of hyperglycemia (Moulton 2017; Heckler and Kroll 2017). Moreover, Kimmel et al. developed a stable PDX 1 mutant zebrafish line with hypoinsulinemia (Kimmel et al. 2015).

6.2.2 Type 2 Diabetes Mellitus (T2DM) Models of Zebrafish

T2DM is characterized as an insulin resistance condition or pancreatic β-cells do not produce enough insulin leading to impaired glucose homeostasis (Kao and Sabin 2016; Navik et al. 2019). There are various methods to induce T2DM in zebrafish including immersion of zebrafish into high glucose solution (0 and 2%) for 28–30 days or continuous exposure of 2% glucose/111 mM of glucose for 14 days mimics diabetes phenotypes with impaired insulin function and hyperglycemia (Gleeson et al. 2007; Alvarez et al. 2010; Capiotti et al. 2014a). Hyperglycemia also leads to memory impairment due to increased activity of acetylcholinesterase

(Capiotti et al. 2014b). Further, the genetic, nutritional, and dietary modifications have also been utilized to develop a model of T2DM in zebrafish that resembles with the pathological features of other mammals (Zang et al. 2017). Zebrafish on high-fat diet leads to the development of hyperinsulinemia, hyperglycemia, glucose intolerance, and steatosis in the liver, and these alterations were alleviated after the treatment with antidiabetic, metformin, and glimepiride. Further, overfeeding of zebrafish with high glucose diet showed hyperinsulinemia, increased glucagon level, and upregulated activity of phosphoenolpyruvate carboxykinase (Pck1), which can be directly linked with T2DM (Zang et al. 2017).

Further, leptin is an important hormone that regulates energy homeostasis and reduces fat storage in adipocytes and hunger. Zebrafish deficient in leptin fed with diet induces hyperinsulinemia and impaired wound healing which is common among diabetic patients but without hyperglycemia, obesity, and adiposity in zebrafish (Michel et al. 2016). Similarly, Maddison et al. developed insulin resistance in the skeletal muscle of zebrafish using a transgenic methodology which represents impaired glucose after overfeeding. In other models, insulin resistance was achieved by CRISPR/Cas9 to knock down the liver-specific insulin receptor that causes fasting hypoglycemia and postprandial hyperglycemia (Yin et al. 2015). Moreover, Marin-Juez et al. injected human recombinant insulin to induce hyperinsulinemia in larvae of zebrafish that significantly upregulated the negative immune modulator PTPN6 (protein tyrosine phosphatase non-receptor type 6) in the insulin resistance larvae (Marín-Juez et al. 2014). Yang et al. reported that mutant zebrafish underwent knockout of insulin receptor genes along with high-carbohydrate diet representing similar phenotypic changes as observed in humans (Yang et al. 2018). Also, Gut et al. developed a fluorescent reporter and transgenic bioluminescent zebrafish by using the phosphoenolpyruvate carboxykinase (Pck1) promoter [Tg(pck1:Luc2)]. Pck1 regulates gluconeogenesis in fasting conditions and breaks oxaloacetate to form phosphoenolpyruvate with the production of carbon dioxide and GDP (Gut et al. 2013; Yang et al. 2009).

Maturity-onset diabetes of the young (MODY) is a rare kind of diabetes having several hereditary forms due to mutations in an autosomal dominant gene disrupting insulin production from the pancreatic β-cell that usually developed in adolescent or early adulthood (Anık et al. 2015). This rare diabetes condition occurs due to mutation in the single gene, so MODY models can be established in zebrafish by targeting gene ablation. For example, removal of hepatocyte nuclear factor-1β gene leads to reduced β-cell numbers mimicking MODY5 (Lancman et al. 2013). The mutation in NEUROD1 genes results in the development of MODY6 that represents hyperglycemia, impaired endocrine cell differentiation, and increased generation of reactive oxygen species (ROS) in larvae of zebrafish (Malecki et al. 1999). Similarly, MODY4 due to PDX1 mutation and MODY10 results from a mutation in the INS gene (Zang et al. 2018; Stoffers et al. 1997). Altogether, zebrafish could be used as a model of diabetes due to similar phenotypic features that are comparable with the mammalian model.

6.2.3 Zebrafish Diabetes Models to Study Glucose Homeostasis

As like humans, zebrafish also utilizes the glucose transporter (GLUT) for the uptake of glucose into the cells. In zebrafish, GLUT 1–3, 6, and 8 are expressed in the gills and GLUT 5 and 9 in the intestine, respectively (Tseng et al. 2009).

Due to the small size of zebrafish (typically <4 cm in length) and its embryo, it presents a challenge for both collection of blood samples and for measuring glucose. Traditionally, fish biologists used glucose oxidase assay for large fish in which sample collection is very easy and sufficient quantity of samples can be collected, whereas performing the same assay in zebrafish is a big challenge. In such situations, blood from multiple individuals is combined or blood can be diluted for further estimation process (Eames et al. 2010). Several other methods have also been established for blood collection in zebrafish like tail ablation, incision of dorsal aorta in the lateral region, decapitation, and estimation of glucose level using handheld glucose meters. Moreover, glucose tolerance methodology also has been developed for zebrafish that is a widely used method for the diagnosis of insulin sensitivity, insulin resistance, and pancreatic β-cell function in diabetic patients (Kari et al. 2007). However, multiple sampling from small fish is still a challenging task.

Hence, developing a new method to check glucose is highly appreciable, in the view of this microsampling of whole blood and plasma which has been developed for the estimation of blood glucose level in zebrafish (Eames et al. 2010). Jurczyk et al. established a fluorescent, dual enzyme assay for the estimation of glucose in the embryo lysates of zebrafish. At early embryo stage, glucose is not detected, but the level is increased with an increase in post-fertilization time, i.e., 16 and 24 hpf, and the highest level of glucose was observed during the differentiation of pancreatic endocrine cell and initial stages of islet morphogenesis (Jurczyk et al. 2011). Further, in vertebrates, a gene PDX1 (pancreatic and duodenal homeobox 1) is an important transcription factor involved in β-cell maturation, which is orthologous to mammals (Brooke et al. 1998; Mahdipour et al. 2019). Hence, targeting a gene PDX1 of zebrafish causes islet hypoplasia and impaired β-cell maturation process leading to the development of hyperglycemia which is well correlated with impaired PDX1-induced altered pancreatic function in humans (Clocquet et al. 2000; Tabassum et al. 2015).

Shreds of evidence reveal that genes regulating carbohydrate metabolism are the same in both mammals and zebrafish and perform the same functions in the regulation of glucose homeostasis. The genes involved in gluconeogenesis and lipolysis are phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, fatty acid synthase, acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, glycogen synthase, and glycogen phosphorylase transcribed after 96 hpf in zebrafish (Kimmel and Meyer 2016; Benchoula et al. 2019a). Drug altering glucose levels like corticosteroids and cAMP activates the phosphoenolpyruvate carboxykinase of zebrafish after 96 hpf, and this enzyme is inhibited by glipizide, metformin, and rosiglitazone in larvae of zebrafish. Hence, this study indicates that antidiabetic drug screening can be done using zebrafish larvae in 96-well plates (Lee et al. 2013; Yuan et al. 2002; Elo et al. 2007).

Impaired glucose homeostasis is a hallmark alteration in metabolic disorders such as obesity and diabetes; hence, direct visualization of glucose flux in vivo could be an ideal approach for the discovery of new drugs. There are glucose bioprobes used in vivo such as [^{18}F]-2-fluoro-2-deoxy-D-glucose, [^{14}C] 2-deoxy-D-glucose, and [^{14}C] or [^{3}H]3-omethyl-D-glucose technique, but its radioactivity limits applications in drug discovery (Conti et al. 1996; Sokoloff et al. 1977; Dienel et al. 1997). Therefore, fluorescent-tagged glucose bioprobes2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose(2-NBDG) have been developed to visualize glucose uptake in cells. The fluorescent glucose bioprobes comprise three parts: a glucose, a linker, and a fluorescent dye (Park et al. 2014). Glucose flux in zebrafish is measured using 2-NBDG after 72 hpf of larvae by utilizing a fluorescent microscope in the zebrafish eye because in the eye the abundance of glucose transporter (GLUT) proteins is high (Tabassum et al. 2015). Therefore, natural compounds or new chemical entities, drugs having antidiabetic potential, can enhance the glucose uptake in the cells, and this could be easily quantified by lysing the larvae and measuring 2-NBDG fluorescence in a microplate reader (Tabassum et al. 2015). However, for visualizing glucose homeostasis in zebrafish, the concentration of 2-NBDG required is high (600 μM) which causes photobleaching and also has low sensitivity. Hence, alternative glucose probes need to be developed, and recently, Cy3 and GB2-Cy3 stronger fluorophores are devoid of these disadvantages which have been reported with 2-NBDG (Kim et al. 2012; Park et al. 2007). It has been reported that GB2-Cy3 has tenfold more sensitivity for monitoring glucose flux and works even at very low concentrations (5 μM) in zebrafish larvae. Later, various natural products like emoting and antidiabetic drugs, ampkinone and rosiglitazone, were evaluated for glucose uptake to validate and visualize glucose homeostasis using GB2-Cy3 probe in this zebrafish system, and the result was more robust as compared to screening based on the 2-NBDG probe. Unlike 2-NBDG, GB2-Cy3 is not available commercially and the guided fractionation approach cannot be done by using GB2-Cy3 probe (Park et al. 2014; Oh et al. 2010). Further, glucose homeostasis as well as pancreas development can also be studied in transgenic zebrafish strains with a fluorescent protein in live vertebrate (Prince et al. 2017; Kinkel and Prince 2009). The transgenic zebrafish line Tg(−1.2ins:EGFP) and Tg(gcga:GFP) where GFP is driven by zebrafish pre-proinsulin promoter and pre-proglucagon promoter which marks pancreatic β-cell and α-cell number and area that can be correlated to predict glucose homeostasis (Xu et al. 2010; Zecchin et al. 2007; Maddison et al. 2015). In addition, insulin mRNA expression, insulin antibody, and dot-blot technique can be used to determine glucose homeostasis (Olsen et al. 2012; Kimmel et al. 2015; Michel et al. 2016). Table 6.1 summarizes the different zebrafish-based models used in diabetes research.

6.3 Study of Pancreas Development

The endocrine compartment of the pancreas plays an important role in the maintenance of glucose homeostasis and regulates the production of insulin, glucagon, somatostatin, and ghrelin. Hence, this conserved pancreas structure and glucose

homeostasis system in zebrafish enables to study pancreas-related disorders and diabetes (Kinkel and Prince 2009). In both mammals and zebrafish, the pancreatic development commences from both ventral and dorsal pancreatic buds where PDX1 is expressed. In zebrafish after 14 hpf, the PDX1-positive cells seem to be developed from pancreatic primordia, and after 24 hpf, the dorsal bud appears from PDX1-positive pancreatic primordia (Tiso et al. 2009; Herrera et al. 2002; Yee et al. 2001; Field et al. 2003). In the embryo of zebrafish, the early forming dorsal bud forms a small islet by 24 hpf, and anteriorly located ventral bud forms by 32 hpf which gives rise to acinar, ductal, and the second wave of endocrine cell types (Field et al. 2003; Biemar et al. 2001). At 35 hpf, the pancreas transcription factor1a (ptf1a) which is a master regulator of pancreatic exocrine cells is found to be expressed in the ventral bud of zebrafish, and its knockdown leads to complete loss of acinar cells. Later, at 48 hpf, ventral bud fuses with the dorsal bud. Dorsal bud of zebrafish produces only endocrine cells, whereas ventral bud produces both exocrine and endocrine cell populations (Tiso et al. 2009; Field et al. 2003; Afelik et al. 2006; Lin et al. 2004). The morphological reorganization of the gut brings the dorsal and ventral bud tissues together at 52 hpf, enlarging the principal islet (Field et al. 2003). However, in mammals during early pancreatic embryogenesis, the multipotent progenitor cells (MPCs) are differentiated to form ductal, exocrine, and endocrine cells which are developed from both dorsal and ventral buds. However, in mice notch signaling regulates the cell fates of MPCs, and increased expression of *ptf1a* induces exocrine differentiation in notch-off MPCs mice, while the notch-on bipotent progenitor cells differentiate into ductal cells (Afelik and Jensen 2013). Interestingly, the ventral bud of mice is similar to the zebrafish ventral bud consisting of notch-off, $Ptf1a^{positive}$ cells which develop the exocrine pancreas and notch-on, $Ptf1a^{negative}$ cells which differentiate into the ductal system and endocrine cells (Ghaye et al. 2015; Parsons et al. 2009; Wang et al. 2011, 2015). Additionally, as the development of larvae continues, the secondary islet cells arise along the length of the intrapancreatic duct. The organization of the mature zebrafish pancreas with multiple islets distributed throughout the organ is similar to mammalian pancreas (Ninov et al. 2012).

6.4 Zebrafish as a Tool for Research and Development in Obesity

Obesity is defined as excessive fat accumulation in the body, which is a major risk factor for developing T2DM, hypertension, fatty liver disorder, and coronary artery disease (Mandviwala et al. 2016). Body mass index (BMI) is used to determine overweight and obesity in adults (Keys et al. 1972). The extra fat accumulation leads to lipotoxicity, insulin resistance, and impaired glucose homeostasis. It can also increase the secretions of pro-inflammatory molecules such as tumor necrosis factor-α, interleukin-6, and monocyte chemoattractant protein-1 and altered numerous molecular pathways that regulate lipid metabolism (Saltiel and Olefsky 2017; Vekic et al. 2019; Kang et al. 2016). Still, pathways are unexplored and for better understanding, a relevant model of obesity needs to be developed. Zebrafish is

emerging as an excellent model for studying obesity pathology and screening of potential anti-obesity compounds. Further, zebrafish is an ideal vertebrate to develop obesity (compiled in Table 6.2) due to the resemblance in the anatomy of zebrafish adipocytes and organs that regulates lipid metabolism with the mammals (Seth et al. 2013; Faillaci et al. 2018).

6.4.1 Nutritional Modulatory Models of Obesity in Zebrafish

The primary features of obesity are impaired adipocyte function with hypertrophy and hyperplasia. Oka et al. first used the zebrafish and developed the obesity model through overfeeding of *Artemia nauplii*. The feeding of *Artemia* represents key features of obesity such as increased body mass index, hepatic steatosis, and deregulation of genes involved in lipid metabolism. Interestingly, this model shares common pathophysiological pathways with mammalian obesity (Oka et al. 2010). Later, several other methods have been developed to induce obesity. Chen et al. developed an obesity model by feeding a high cholesterol diet leading to increased body weight, hypertriglyceridemia, and hepatic steatosis (Chen et al. 2018). Likewise, Forn-Cuní et al. fed zebrafish with a high-fat diet leading to the development of the same alterations as reported by Chen et al. (Forn-Cuní et al. 2015). Further, overfeeding of normal fat diet and high-fat diet to zebrafish for the short term increases adiposity and obesity, but metabolic alteration was observed only in the case of zebrafish fed with high-fat diet (Landgraf et al. 2017).

6.4.2 Mutant and Transgenic Model for Obesity

Further, there is an altered function of genes involved in the pathophysiology of obesity. Hence, genetic and mutant models of obesity have been characterized in zebrafish through genetic transgenic lines expressing obesogenic genes and genetic modifications. Currently, mutant fish were developed to study obesity and its related disease. A zebrafish mutant, *vizzini*, exhibits a severe defect in growth and increased adipose tissue mass. The *vizzini* mutant carries a null mutation in the growth hormone gene (*GH1)* produced from the anterior pituitary, which regulates growth and lipid metabolism. Further, the positional cloning of *vizzini* exposed a premature stop codon in gh1. Similarly, in children growth hormone (GH) deficiency represents short stature and mild obesity, whereas GH supplementation decreases adipose mass. Likewise in rodents, GH gene inactivation leads to increased obesity and adipose tissue mass which is independent of feed intake in animals (McMenamin et al. 2013).

Peng et al. generated a cyp2r1-deficient zebrafish lines to develop obesity in zebrafish. Cyp2r1$^{-/-}$ has a premature stop codon in the CYP2R1 gene (Peng et al. 2017). CYP2R1 gene is a vitamin D 25-hydroxylase-related gene, which is a member of cytochrome P450 superfamily of enzymes that catalyzes the conversion of vitamin D into 25-hydroxyvitamin D, which is the major circulatory form of

Table 6.2 Zebrafish as model of obesity

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
Induced models				
High-fat diet	Heavy whipping cream	Larvae	Increased lipid, triglyceride and apolipoprotein B level	Schlegel and Stainier (2006)
	Chicken egg yolk	Larvae, juvenile, adult	Hypertrophy of adipose tissue area and increased triglyceride levels	Tingaud-Sequeira et al. (2011) and Zhou et al. (2015)
	Corn oil and lard	Adult	Increased body fat	Oka et al. (2010), Hasumura et al. (2012) and Meguro and Hasumura (2018)
	High cholesterol diet	Adult	Vascular lipid accumulation, increased IL-1β expression	Yoon et al. (2013)
	High-fat diet and high fat plus high cholesterol	Larvae	Increased free cholesterol, total cholesterol, and hepatic steatosis	Dai et al. (2015)
	Otohime B2 (commercial fish food)		Insulin resistant, glucose intolerance	Zang et al. (2017)
Overnutrition	Artemia	Adult	Obesity, increased triglyceride levels with hepatic steatosis	Oka et al. (2010)
	Artemia and egg yolk powder	Adult	Adipocyte hypertrophy, increased body weight and triglyceride levels with hepatosteatosis	Landgraf et al. (2017)
Transgenic line-based models				
g(b-actin:AgRP)	Overexpression of *agrp* (melanocortin antagonist agouti-related protein)	All stages	Obesity, increased linear growth, adipocyte hypertrophy	Song and Cone (2007)
Tg(-2.5β-Act: mCherry-miR-27b-SP)	MiR-27b depletion	All stages	Increased weight gain, larger fat pad, hyperlipidemia, adipocyte hyperplasia	Hsu et al. (2017)

(continued)

Table 6.2 (continued)

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
Tg(krt4Hsa.myrAkt1)^{cy18}	Overexpression of *akt1*	Adult	Increased body weight, adipocyte hyperplasia, increased adipogenesis, and lipoma formation	Chu et al. (2012)
Tg (hPPARγ-eGFP)	Activate the PPARγ	Larvae	Increased lipid accumulation, adipocyte differentiation, late onset weight gain	Tiefenbach et al. (2010) and Riu et al. (2014)
Mutant lines model				
oie gras (hi^{1532B})	Mutation in *trappc11*	Larvae	Hepatic steatosis and hepatomegaly	Sadler et al. (2005) and DeRossi et al. (2016)
cdipt$^{hi559Tg/+}$ (hi^{559})	Mutation in *cdipt*	Larvae	Fat accumulation in the liver	Thakur et al. (2011)
harvest moon (hmn^{z110})	Mutation in *gfpt1*	Larvae	Increased triglyceride and hepatic steatosis	Hugo and Schlegel (2017)
vmp1 (7466^{mu110})	Mutation in *vmp1*	Larvae	Hepatic steatosis	Kim et al. (2015)
ducttrip (dtp^{p14nb})	Mutation in *achy*	Larvae	Hepatic steatosis, destructed exocrine pancreas and mitochondrial function	Yee et al. (2005)
red moon (rmn)	Mutation in *slc16a6a*	Larvae	Hepatic steatosis	Hugo et al. (2012)
vizzini	Mutation in *gh1*	Larvae, adult	Increased adiposity and reduced somatic growth	McMenamin et al. (2013)
cyp2r1 mutants	*cyp2r1* mutations	Adult	Diminished growth and increased adiposity	Peng et al. (2017)
plxnd1^{fov01b}	Mutation in *plxnd1*	Adult	Impaired body fat distribution *Plxnd1*-deficient zebrafish protected from high-fat-induced insulin resistance	Minchin et al. (2015)

vitamin D (Ramos-Lopez et al. 2007). Zebrafish mutant with Cyp2r1$^{-/-}$ gene represents excessive visceral adipose tissue mass and lipid accumulation due to an increased number of adipocytes, while the supplementation of 1,25(OH)2D3 reversed these alterations indicating vitamin D3 is an important regulator of lipid metabolism (Peng et al. 2017). Minchin et al. reported that zebrafish deficient with Plxnd1 developed hyperplastic morphology in visceral adipose tissue (VAT) and

reduced lipid storage, but the subcutaneous adipose tissue (SAT) growth and morphology were unaffected. This leads to impaired body fat distribution and a reduced VAT to SAT ratio in zebrafish. Consequently, the Plxnd1-deficient zebrafish fed with high-fat diet stored lipids in SAT are protected from developing insulin resistance (Minchin et al. 2015). Further, there are several mutant zebrafish that have been developed through the mutation of a gene such as *trappc11*, *cdipt*, *gfpt1*, *vmp1*, *achy*, and *slc16a6a* in the larvae of zebrafish to induce hepatic steatosis (Sadler et al. 2005; Thakur et al. 2011; Hugo and Schlegel 2017; Kim et al. 2015; Yee et al. 2005; Hugo et al. 2012).

Further, Fei et al. developed a leptin receptor (*lepr*) and melanocortin receptor 4 (mc4r) mutant zebrafish through the CRISPR/Cas9 technology. The adult $lepr^{-/-}$ and $mc4r^{-/-}$zebrafish represents key phenotypic features of obesity such as increased food intake and increased weight, with higher body fat percentage, but these alterations were not found in post-juvenile fishes. The lepr and mc4r proteins play a pivotal role in feeding and metabolism (Fei et al. 2017). Like humans, teleosts also possess leptin receptor, agouti-related peptide (AgRP), and melanocortin system in the hypothalamus regulating energy homeostasis. The blockade of the central melanocortin system disrupts the action of leptin leading to increased adiposity. Ollman et al. have identified endogenous melanocortin antagonist agouti-related protein (AgRP) and its transgenic overexpression in the brain leading to the development of obesity. Song et al. developed a transgenic zebrafish, which has overexpressed endogenous melanocortin antagonist AgRP [Tg(b-actin:AgRP] representing obesity with increased linear growth, body weight, visceral adipose accumulation, adipocyte hypertrophy, and hyperplasia (Song and Cone 2007). Further, reports showed that microRNA miR-27b regulates the expression of genes that involves in lipid metabolism (Vickers et al. 2013; Karbiener et al. 2009). In the line of these reports, Hsu et al. developed transgenic zebrafish deficient with miR-27b by using microRNA-sponge (miR-SP) technology. This technique generated zebrafish expressing transgenic miR-27b-SP (C27bSPs), which alters the activity of miR-27b leading to hyperlipidemia, lipid accumulation, increased weight, larger fat pads, and adipocyte hyperplasia (Karbiener et al. 2009). Chu et al. generated adult stable zebrafish transgenic of Tg(krt4Hsa.myrAkt1)(cy18) which was initially used to study skin cancer; but it also presents obese phenotypes, with increased BMI, fat deposition, adipocyte hyperplasia, and impaired glucose homeostasis with upregulation of genes that promote adipogenesis, adipocytokine, and inflammation but do not display adipocyte hypertrophy. The Tg(krt4:Hsa.myrAkt1)(cy18) is the expression of exogenous human constitutively active Akt1 (myrAkt1) that can activate mTOR, GSK-3α/β, and 70S6K signaling and also exhibits ectopic lipoma-like adipose tissue in the dorsal muscle, gill arches, and tail bone tissues (Chu et al. 2012). In addition, zebrafish transgene line Tg(hPPARγ-eGFP) incorporating green fluorescent protein (GFP) has been used to monitor PPARγ in live fish. This transgenic line is tested for obesogenic potential through environmental toxin bisphenol A analogs and evaluated for lipid accumulation in correlation with their capacity to activate PPARγ in zebrafish larvae (Tiefenbach et al. 2010; Riu et al. 2014). Hence, producing stable transgenic models could be a promising tool to

discover the molecular pathway involved in metabolic disorders. Altogether, diet-induced obesity; mutant and transgenic zebrafish as a model of obesity could be utilized to understand the disease in the context of systematic obesity, and it also mimics the most common process occurring in humans affected by this condition.

6.4.3 Methods to Study Adiposity in Zebrafish

There have been numerous techniques developed to study adiposity in humans such as body mass index (BMI), quantitative computed tomography, magnetic resonance imaging (MRI), bioelectrical impedance analysis, dual-energy X-ray absorptiometry, and air displacement plethysmography (Shuster et al. 2012). However, extrapolating these techniques to zebrafish is very difficult; hence, various other methods were developed to study adiposity in fish. Adipocytes and lipids can be visualized in developing larvae and adult zebrafish by using lipophilic dyes such as Nile red, Oil red O, and Sudan black B. Zebrafish has many advantages over other mammalian models because of its optical transparency of larvae and its feasibility to study adiposity, lipid metabolism, or digestive physiology through live imaging and fluorescence-based screens. Nile red dye has been utilized for live imaging and quantitative evaluation of intracellular neutral lipid droplets as well as purification of adipose tissues (Oka et al. 2010; Flynn et al. 2009; Greenspan et al. 1985; Jones et al. 2008). Further, to check the fatty acid mobilization and transport in the live zebrafish, various fluorescent lipid analogs and tracers are used including boron dipyrromethene (BODIPY) fatty acid analogs, BODIPY-cholesterol analogs, and fluorescence reporters like PED6. The fluorophore is directly injected into the yolk of zebrafish and it gets rapidly diffused in the circulatory system within 3 h (Lee et al. 2011; Hölttä-Vuori et al. 2010; Anderson et al. 2011). Moreover, 3D microCT scan, MRI, and Echo MRI were used to measure body fat volume and total adipocytes in zebrafish with accurate measurement (Landgraf et al. 2017; Hasumura et al. 2012). Table 6.2 summarizes the different zebrafish-based models used in obesity research.

6.5 Zebrafish as a Tool to Study Osteoporosis

Osteoporosis is a metabolic bone disorder characterized by the loss of bone mass and bone strength and decreased bone mineral density and trabecular volume of long bones that leads to fragile fractures (Raisz 2005). Generally, this condition occurs due to an imbalance between bone catabolism and anabolism process. Since the first osteoporosis case in humans showed a link between bone mass, fragility, and *vitamin D receptor (VDR)* gene polymorphism and later more than 30 genes have been identified that affect skeletal mass and fragility (Liu et al. 2003). There are many reasons for osteoporosis including hormone deficiencies in postmenopausal women, nutritional deficiencies, excessive use of steroid drugs, reduced exposure to sun, and vitamin D deficiency. The pathogenesis of skeletal fragility involves extreme bone resorption, decreased skeletal mass, altered bone remodeling process,

and impaired osteoblast and osteoclast activity (Kanis et al. 2019). Hence, regulation of these alterations could be the therapeutic approach (Baron and Hesse 2012; Baldock and Eisman 2004). The bottlenecks are limited affordable testing models and a long time of testing (Bergen et al. 2019; Huang et al. 2018). Further, similar to humans, the bone development mechanism, signaling pathways, and related genes are highly conserved in zebrafish. Various transcription factors and hormones including osterix, runx2a/b, col10a1, and osteonectin, which are similar to mammals, control bone formation in lower vertebrates. Hence, these common developmental features enable the use of zebrafish as a model for bone-related disorders (Huang et al. 2007; Li et al. 2009). The embryo of zebrafish showed the presence of mineralization after 5 days post fertilization of eggs, and thus it is utilized as a powerful model to study osteogenesis (Apschner et al. 2011; Ofer et al. 2019). Further, adult zebrafish bone showed the presence of osteocytes and thus provides additional insights into important osteocyte-related skeletal diseases that develop in adult humans, such as osteoporosis and osteopenia (Witten and Huysseune 2009). In the fish, the active transepithelial calcium transport channels are conserved in skin cells, whereas in mammals, it is expressed in the kidney and intestines (Lin and Hwang 2016). Moreover, human bones store 99% of calcium and essential compounds, but it is not the same in case of fish because fish live in a calcium-rich environment and fish under low calcium environment represent bone resorption and stimulate calcium uptake in gills, indicating calcium homeostasis maintenance through osmoregulation (Blaine et al. 2015; Glowacki et al. 1986; Lleras-Forero et al. 2020). Moreover, zebrafish are genetically highly amenable and their genome can be manipulated using the genetic toolkit. Further, there is a high similarity in bone morphology and function between human and fish. Hence, the mutation in the orthologue gene of zebrafish for bone development could be exploited for drug development and translational medicine studies, and zebrafish models for osteoporosis research are summarized in Table 6.3.

6.5.1 Zebrafish as a Model to Study Impaired Iron Homeostasis

Iron is an essential trace element and its deficiency can lead to bone loss and osteoporosis. However, iron overload in the body leads to impaired balance between bone resorption and bone formation leading to low bone mass and development of osteoporosis, osteopenia, and bone fractures in patients (Cheng et al. 2017; Jeney 2017). In mice, high iron content augments osteoclast differentiation, and deferoxamine treatment promotes osteogenesis in the larvae of zebrafish. This evidence indicates iron overload is a crucial factor in the loss of bone mass. Cheng et al. developed a rapid model of osteoporosis by using high iron stress (ferric ammonium citrate) in both zebrafish larvae and adults that represents osteoporosis phenotype. High iron stress leads to decreased bone mineralization and cartilage defects with downregulated osteoblast and cartilage-specific mRNA expression levels (Zhang et al. 2018a). Bo et al. reported that weh (tp85c) mutant larvae present altered vertebral mineralization with impaired osteoblast-specific gene expression

Table 6.3 Zebrafish as a tool in osteoporosis

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
Induced models				
Iron overload	High iron stress	Larvae, adults	Low bone mass, osteoporosis	Zhang et al. (2018a)
Corticosteroids	Prednisolone	Larvae, adult	Impaired osteoclast, osteoblast activity, decreases bone mineral density	de Vrieze et al. (2014), He et al. (2018) and Pasqualetti et al. (2015)
Toxin	Alloxan-induced diabetic osteoporosis	Larvae	AGE accumulation, modulates RAGE/MAPK signaling pathways in osteoblast	Wang et al. (2020a)
Transgenic lines model				
Tg(5xBMPRE-Xla.Id3:GFP)	Downregulating BMP pathway	Embryo	Defects in the mandibular and hyoid arches, as well as disrupting the trabecular cartilages of the palate	Alexander et al. (2011)
TgBAC (col10a1a: Citrine)	Mutation in collagen 10a1a	Embryo and larvae	Low bone mass, microarchitectural deterioration of bone tissue and an increased risk of fracture	Stewart and Ralston (2000)
TgBAC(ctsk: Citrine)	Mutation in ctsk	Embryo	Decreases bone resorption and increases the bone formation rate (BFR)	Bussmann and Schulte-Merker (2011)
Tg (Col2a1aBAC: mCherry)	Mutation in Col2a1a	Embryo and larvae	Osteosarcoma, chordoma, chondromyxoid fibroma, chondroblastoma	Stewart and Ralston (2000)
Tg(Gli-d:Gfp/mCherry)	Hedgehog pathway (increase the expression of Runx2 and Osx and inhibit MSCs)	Embryo and early larval development	Progressive osseous heteroplasia and proliferation of osteoblast	Schwend et al. (2010)
Tg(7xTCF.XlaSiam: nlsGFP)	Alteration of WNT-β-catenin pathway	Embryo	High bone mass	Moro et al. (2012)

(continued)

Table 6.3 (continued)

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
Tg(−4.9Sox10: EGFP)	Mutation in sox10	Embryo	Human craniofacial syndromes	Wada et al. (2005)
Tg(Ola. Osteocalcin: EGFP)	Downregulation of osteocalcin	Embryo	Progressive osseous heteroplasia and proliferation of osteoblast	Knopf et al. (2011)
Tg(Hsa. RUNX2- Mmu. Fos:EGFP)	Upregulation of runx2	Embryo	Progressive osseous heteroplasia and proliferation of osteoblast	Knopf et al. (2011)
Tg(sp7:EGFP)	Downregulation of sp7 (osx)	Embryo	Progressive osseous heteroplasia and proliferation of osteoblast	DeLaurier et al. (2010)
Tg(rankl:HSE: CFP)	Misexpression of rankl	Embryo	Osteoporotic bone loss	To et al. (2012)
Tg(Ola.sp7: luciferase)	Mutation in sp7 (osx)	Embryo	Progressive osseous heteroplasia and proliferation of osteoblast	de Vrieze et al. (2015)
Tg(sp7:nuGFP/ mCherry) or Tg (Ola.sp7:NLS-GFP)	Alteration in sp7 (osx) (by impaired level of retinoic acid and Cyp26b1)	Embryo	The severity of bone defects increases over time with the older mutants exhibiting the broadest arches, protruding jaw, swimming problems	Spoorendonk et al. (2008)
Tg(osterix: mCherryNTRo) pd46	Downregulation of osterix sp7 (osx)	Embryo	Problems in ossification of bones and cartilage and low bone mass	Singh et al. (2012) and Kronenberg (2003)
Mutant lines model				
gräte	Mutation in ABCC6	Embryo	Increased mineralization in spine and soft tissues	Mackay et al. (2015) and Li et al. (2010)
atp6v1h	Mutation in ATP6V1H	Embryo	Increased osteoclast activity by upregulated MMP9 and MMP13	Zhang et al. (2017)

(continued)

Table 6.3 (continued)

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
panther, csfr1a	Mutation in C-FMS (CSF1R/CD115)	Embryo	Reduced osteoclast number and immune cell mobility causing stenosis	Charles et al. (2017), Herbomel et al. (2001) and Chatani et al. (2011)
frilly fins, welded	Mutation in BMP1	Embryo	Affecting bone matrix integrity by fibrillar collagen process	Charles et al. (2017), Asharani et al. (2012) and Bowen et al. (2012)
dmh21 (?), dmh27, dmh28, dmh30 (?)	Mutation in COL2A1	Embryo	Notochord and vertebra deformations	Henke et al. (2017)
col11a2	Mutations in the genes encoding type XI collagen and COL11A2	Larvae	Dominant effect	Lawrence et al. (2018)
ctsk*U	Mutation in M-CSF and CTSK gene	Embryo	Decreased level of immature and mature osteoclasts	To et al. (2015)
nackt (eda), finless (edar), fang (edar), topless (edar)	Mutation in EDA and EDAR	Embryo	Lacking and deformation of dermal skeleton bone structures such as lepidotrichia, elasmoid scales, and skull	Harris et al. (2008)
chihuahua, microwaved, dmh13, dmh14, dmh15, dmh29	Mutation in COL1A1	Embryo	Dominant effect leading to brittle bones in axial and fin skeleton	Gistelinck et al. (2018), Gioia et al. (2017), Asharani et al. (2012), Henke et al. (2017), Fisher et al. (2003) and Fiedler et al. (2018)
stoepsel, short-of-fin	Mutation in stp and CX43 (GJA1)	Embryo	Loss of Ca^{2+} channel activity	Zhang et al. (2002) and Misu et al. (2016)
hs:dkk*	Eda and Fgf mutation DKK1 (DICKKOPF)	Embryo	Heat shock, inhibits the Wnt/beta-catenin signaling, and dkk1 is expressed	Aman et al. (2018)

(continued)

Table 6.3 (continued)

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
stocksteif, dolphin, cyp26b1	Mutation in CYP26B1	Embryo	Altered retinoic acid metabolism	Laue et al. (2011), Spoorendonk et al. (2008) and Laue et al. (2008)
itga10,itgbl1	Downregulated ITGA10 ITGBL1	Larvae	Prednisolone larvae downregulated	Huo et al. (2018)
golgb1	Downregulation of GOLGB1 (giantin)	Embryo	Ectopic mineralization in spine and soft tissues	Bergen et al. (2017)
Dragonfish	Mutation in ENPP1	Embryo	Altered phosphate metabolism	Apschner et al. (2014) and Huitema et al. (2012)
No bone	No bone involvement	Embryo	Altered phosphate metabolism	Huitema et al. (2012)
Ihha	IHH and osteoblast no. decreases	Embryo	Inhibition of osteoblast differentiation	Apschner et al. (2014), Iwasaki et al. (2018), Huycke et al. (2012) and Paul et al. (2016)
gba1	GBA1 deficiency	Embryo	Altered Wnt signaling	Zancan et al. (2015)
hs:gli2-DR	GLI2	Embryo	Impaired scale calcification	Aman et al. (2018)
lgmn	LGMN inhibited osteoblast differentiation	Embryo	Osteoblast activity inhibited by legumain	Jafari et al. (2017)
mef2ca	Mutation in MEF2C	Embryo	Altered DNA methylation	DeLaurier et al. (2014) and Nichols et al. (2016)
panx3 MO	Mutation in PANX3 causes delayed osteoblast proliferation	Embryo	Low endochondral ossification because of altered Ca2+ channel activity	Oh et al. (2015)
lrp4 MO	Mutation in LRP4	Embryo	Deformed craniofacial skeleton with kidney cyst	Tian et al. (2019)
Bone calcification slow	N/A	Embryo and larvae	Late ossification and increased Cyp26b1 expression	Xi et al. (2013)

(continued)

Table 6.3 (continued)

Type of model	Dietary/genetic manipulation	Age	Phenotype	References
ptch1 (ptc2), ptch2 (ptc1)	Activation of PTCH1, PTCH2	Embryo and larvae	High mineralization in endochondral bone	Hammond and Schulte-Merker (2009)
pthlha/pthlhb MOs	PTHrP/PTHLH/ PTH3 are necessary for embryonic growth	Embryo	Premature ossification	Yan et al. (2012)
sp7 (osx, osterix) U	Mutation in SP7 (OSX, osterix)	Embryo	Low osteoblast number	Azetsu et al. (2017) and Yu et al. (2017)
rapunzel	Missense mutation in RPZ	Embryo	Increased BMD	Green et al. (2009)
pth4	Antisense mutation in PTH4	Embryo	Neuronal maintenance of phosphate metabolism	Suarez-Bregua et al. (2017)
pls3 MO	Mutation in PLS3	Larvae	Low larval operculum mineralization	van Dijk et al. (2013)
rankl U	Altered expression of RANKL	Embryo	Activate osteoclast activity	To et al. (2012)
slc10a7 MO	Mutation and complete loss of SLC10A7	Embryo	Secretory pathway defect	Ashikov et al. (2018)
sp7 (osx, osterix)	Mutation in SP7 (OSX, osterix)	Embryo	Low mineralization, high proliferation of osteoblast	Kague et al. (2016, 2018)
twist1b and tcf12	Mutation in TWIST and TCF12	Embryo	Normal mineralization and increased proliferation of osteoblasts	Teng et al. (2018)
tgfb3 MO	Mutation in TGFB3	Embryo	Low calcification of young bone	Cheah et al. (2010)
spp1 (osteopontin)	Knockdown of SPP1	Embryo	Lacking in whale shark genome	Venkatesh et al. (2014)

alpl, runx2a, and col1a1a and BMPs signaling genes in osteoblast differentiation, bmp2a and bmp2b. These mutant fish are defective in ferroportin 1 gene leading to impaired iron transport with severe hypochromic anemia indicating an involvement of iron deficiency in impaired bone formation and homeostasis (Bo et al. 2016).

6.5.2 Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (*Enpp1*) Gene Mutation and Generalized Arterial Calcification

Generalized arterial calcification of infancy and pseudoxanthoma elasticum is a fatal human disease caused by mutations in the gene Enpp1 (Apschner et al. 2014; Nitschke and Rutsch 2012). The Enpp1 gene mutation in patients leads to ectopic mineralization of soft tissues and arteries and increases the risk of cardiovascular disorders including atherosclerosis. Likewise, zebrafish mutant with *enpp1* presents ectopic calcifications in soft tissues and notochord mineralization (Spranger 1998).

6.5.3 Zebrafish as a Model to Study Collagenopathies, Osteogenesis Imperfecta, and Scoliosis

Collagenopathy is a genetic defect of collagen formation characterized as a rare disorder that affects the connective tissue that supports the body's joints and organs. Collagenopathies are caused by defects in type I, type II, or type XI collagen (Spranger 1998; Gistelinck et al. 2018). Generally, the mutation in type I collagen gene causes type I collagenopathies, and it is well related to human bone disorders such as osteogenesis imperfecta and the Ehlers-Danlos syndrome. Further, there are several collagen mutant zebrafish that have been developed to enable the etiology of bone-related disorders and fragility (Gistelinck et al. 2018). Further, several other zebrafish mutants such as bone morphogenetic protein 1 (bmp1) gene, membrane-bound transcription factor peptidase site 1 (MBTPS1), plastin-3 (PLS3), Sec24d, and *Chihuahua* mutant embryos were developed to study bone-related disorders, osteogenesis imperfecta, and bone mineralization in larvae (Schlombs et al. 2003; van Dijk et al. 2013; Garbes et al. 2015; Gioia et al. 2017).

Further recessive mutations on collagen type VIII alpha1a (col8a1a) gene in zebrafish leviathan mutant reduce the production of collagen which impairs the embryonic notochord development with a defective vertebral column at the adult stage of zebrafish (Gray et al. 2014). Similar to human *scoliosis*, zebrafish mutant with *kif6* gene showed alteration in spinal curvature (Grimes et al. 2016; Buchan et al. 2014). Hayes et al. reported that zygotic protein tyrosine kinase 7 (*Zptk7*) mutant zebrafish showed dysregulated Wnt signaling with impaired spinal curvature at late larval and early juvenile stage indicating the development of congenital and idiopathic scoliosis phenotype (Hayes et al. 2014). Moreover, Kou et al. reported that G-protein-coupled receptor 126 (GPR126) is abundantly expressed in the cartilage and chondrocytes, and its knockdown using morpholino causes delayed ossification of the developing spine mimicking the phenotype of adolescent idiopathic scoliosis (Kou et al. 2013). Further Guo et al. reported adolescent idiopathic scoliosis phenotype due to increased expression of ladybird homeobox 1 (llbx1) gene in the embryo of zebrafish presents deformation of the embryonic body axial development with defective body curvature (Guo et al. 2016). Further, the increased expression of *basonuclin-2* gene (BNC2) is linked with spine curvature in

developing zebrafish and implicated in the etiology of adolescent idiopathic scoliosis (Ogura et al. 2015).

Raine syndrome is a rare genetic disease associated with exophthalmos, choanal atresia, generalized osteosclerosis, cerebral calcifications, and facial dysmorphism (Vishwanath et al. 2014). Eames et al. reported that zebrafish homozygous mutant *fam20b gene* showed impaired cartilage matrix production, altered chondrocyte maturation, and development of the skeleton that represents similar morphological alterations in Raine syndrome patients (Eames et al. 2011). Further, mutant CYP26B1 and TALEN mediated mutations in transcription factor 12 (TCF12) or twist-related protein 1 gene in zebrafish embryos causing loss of the coronal suture with impaired skull development mimicking human craniosynostosis and Saethre-Chotzen syndrome phenotype (Governale 2015; Teng et al. 2018; Laue et al. 2011). Several other mutants were developed such as mutant *PTDSS1* (codes for phosphatidylserine synthase 1) RNA in zebrafish embryo leading to developmental defects, craniofacial anomalies, trunk angulation, and small or absent eyes with abnormal cartilage (Sousa et al. 2014). The *endothelin-1* (*edn1*) gene disruption leads to loss of identity of neural crest cells with misdifferentiation of lower jaw structures into maxillary-like structures (Clouthier et al. 2013). Zebrafish mutant with col11a2 showed the impaired formation of joint cartilage affects joint function, and the hyperactive $RIPK2^{104Asp}$ gene expressed in the embryo of zebrafish results in upregulation of osteoarthritis-related gene (Lawrence et al. 2018; Jurynec et al. 2018).

Further studies also showed that gut dysbiosis in zebrafish leads to altered bone homeostasis and administration of *Lactobacillus rhamnosus*, with the diet to zebrafish embryo showing increased calcification. This also stimulates the expression of genes that are involved in osteogenesis such as runt-related transcription factor 2 (runx2), Sp7 transcription factor (sp7), matrix Gla protein (mgp), and bone gamma-carboxyglutamate (gla) protein (bglap) and inhibits the expression of sclerostin (sost), a bone formation inhibitor (Charles et al. 2015; Maradonna et al. 2013).

Dexamethasone treatment induces apoptosis in osteoblast through the activation of caspase-3 and GSK-3β signaling in bone (Yun et al. 2009). In 2006, glucocorticoid-induced osteoporosis model has been established in zebrafish (Barrett et al. 2006). Prednisolone treatment to adult zebrafish induced the glucocorticoid-induced osteoporosis phenotype in regenerating scales, with impaired expression of osteoblasts and osteoclasts with increased matrix breakdown, and these alterations recovered by alendronate treatment (de Vrieze et al. 2014; He et al. 2018; Pasqualetti et al. 2015). Moreover, He Hanliang et al. established osteoporosis model in zebrafish 15 days post fertilization using prednisolone presenting key alterations such as reduced bone mass, upregulation of MMP9 and MMP13, and downregulation of entpd5a, acp5a, and sost (He et al. 2018). Diabetic osteoporosis is caused due to chronic hyperglycemia, deficiency of insulin or impaired function of insulin, and other factors such as hormone imbalance and calcium metabolism disorders. Reports showed that the accumulation of advanced glycation end products plays an important role in the development of diabetic osteoporosis and induces

osteoblast apoptosis (Asadipooya and Uy 2019; Lecka-Czernik 2017; Zhang et al. 2018b). Alloxan exposure to zebrafish larvae induces osteoporosis through the modulation of RAGE/MAPK signaling pathways in osteoblast and has been suggested as a good fit model to study diabetes-associated osteoporosis (Wang et al. 2020a).

6.5.4 Genetic Manipulation in Zebrafish for Studying Osteoporosis

The embryo is developed externally, so it is amenable to do genetic manipulations such as knockout, knockin, and DNA insertion. Zebrafish mutant lines are developed by using mutagens N-ethyl-N-nitrosourea (ENU), and in 1919, ENU was synthesized by reacting N-ethylurea with nitrous acid (Knapik 2000). This mutagen induces random point mutations in offspring and uncovered a huge number of variants related to major aspects of skeletogenesis, morphological evaluation, and human skeletal diseases (Schilling et al. 1996; Neuhauss et al. 1996; Busse et al. 2020; Salinger and Justice 2008). Presently, there are various genetic tools used such as zinc-finger nucleases, TALEN, and CRISPR/Cas9 which induce targeted gene mutation in zebrafish.

Many transgenic zebrafish lines have been developed like Tg(ola.sp7:nlsGFP) that express an enhanced green fluorescent protein (eGFP) in the bone which is used to study the development of axial skeleton. The eGFP signal area and integral optical density (IOD) are checked which represent bone mineralization and bone mineral density in the fish (Huang et al. 2018; Spoorendonk et al. 2008). Alexander et al. established zebrafish transgenic lines of bone morphogenetic proteins (BMPs) responsive gene Tg(Bre:GFP) and dominant-negative BMP receptor 1a [Tg(hs70I:dnBmpr1a-GFP)] to monitor BMPs signaling that plays an important role in craniofacial development. Like in mice, zebrafish possess gene *Bmp4* expressed in ventral arch ectoderm and *Bmp2* in cranial neural crest, and pharyngeal endoderm/ectoderm expresses bmp5 and bmp7a/b (Alexander et al. 2011). Similarly, other transgenic lines were developed to study the musculoskeletal system in small teleosts (Table 6.3).

6.5.5 Model to Study Bone Development and Skeleton Mineralization

Like in vertebrates, the skeletal cell type and bone regulation are conserved in zebrafish, but the bone of zebrafish is thin, with fewer embedded osteocytes with little trabeculation (Weigele and Franz-Odendaal 2016; Witten et al. 2017). The skeleton of fully developed zebrafish includes cranial skeleton, axial skeleton, caudal skeleton, unpaired fins (dorsal, anal, and caudal fins), paired fins (pectoral and pelvic fins), and elasmoid scales.

The craniofacial skeleton system forms early and at 2 dpf the first cartilaginous structures of the jaw are formed (Schilling and Kimmel 1997). At 3 dpf, the first

mobile skeletal joints are formed and at 5dpf hypertrophic chondrocytes, col10a1a, are observed. The first osteoblasts surrounding the cartilage and forming bone matrix appear by 7 dpf (Brunt et al. 2017; Hammond and Schulte-Merker 2009; Mitchell et al. 2013). At 72 hpf, the first intramembranous bones appear in the craniofacial skeleton, and the development of skeleton arises early through the remodeling of osteoblasts and osteoclasts activity does not commence until the second week of development (Schilling et al. 1996; Witten et al. 2017; Hammond and Schulte-Merker 2009).

Several mutants and transgenic and reporter lines are available to study bone homeostasis and mark musculoskeletal tissues in zebrafish (Hammond and Moro 2012; Borovina et al. 2010). In vivo osteoblast labeled with reporter sp7 and osteoclast, a reporter CTSK, is utilized to study bone homeostasis, which is a potential tool to study bone activity, location, and number in living bone tissue in response to toxins, environment, genetic mutation, and drug treatment. Further, zebrafish autosomal dominant mutations col1a1 and col1a2 genes altered glycine-X-Y repeat domains that cause defects in the maturation of collagen α1(I) and α2 (I) heterotrimer resulting in the formation of fragile bone with impaired mineralization (Bella et al. 2006; Eyre and Weis 2013). There are several chemicals and transgenic and mutant models developed (Table 6.3). There are various chemical staining methods to study and visualize bone mineralization in zebrafish. Staining agents such as calcein, quercetin, and alizarin are extensively used; however, this method has several drawbacks, such as tedious technical procedures, time-consuming steps, and unstable dye labeling (Barrett et al. 2006; de Vrieze et al. 2014; Du et al. 2001; Kimmel et al. 2010). Further, other approaches to study phenotypic features of zebrafish skeleton are histology, fluorescent reporter, radiography, microCT, and synchrotron radiation microcomputed tomography (Kwon et al. 2019). Further, the development of transgenic and mutant lines has an advantage over the conventional method because of improved convenience, efficiency, and stability and provides a powerful tool for HTS of anti-osteoporosis drugs.

6.5.6 As a Model to Study Bone Matrix Formation with Emphasis on Bone Regeneration and Fracture Repair

Skeleton system consists of different types of bone for the protection of organs and tissues in the body. Bones contain calcium phosphate and calcium carbonate in the form of salts. The organs like the heart, lens, bones, and pancreas are regenerated in zebrafish. Skeletal tissues also show regeneration after cutting off limb or removal of scale (Mariotti et al. 2015; Roehl 2018). Cutting off limb and any injury lead to the synthesis of epimorphic blastema, and this blastema helps in the regeneration of affected tissues and bone in a measured manner (Akimenko et al. 2003). During inflammation, osteoblasts get dedifferentiated and multiply for the formation of blastema (Sousa et al. 2011; Knopf et al. 2011). These immature osteoblasts release matrix and along these osteoblasts osteoclast cells too mature (Sousa et al. 2011;

Knopf et al. 2011). Regeneration process is helpful in osteoporosis. Different types of genes participate in the regeneration of caudal fins of zebrafish like RARγ, Wnt3a, Msxb, and Hoxd11. RARγ is a retinoic acid that is a communicating fragment which helps in vertebrate development. Its main function is to develop and regenerate the tissues (Kashyap et al. 2011). Wnt3a is a glycoprotein that helps in the body arrangement, tumor development, cell multiplication, etc. It helps in the regeneration of adult zebrafish epidermis fins. This protein also plays a major role in body arrangement in the primary embryogenesis (Shimizu et al. 2005; Poss et al. 2000). This protein is also necessary because after injury it passes the signals to the stem cells to repair the injured site (Shimizu et al. 2005). Table 6.3 presents zebrafish models for osteoporosis research.

6.6 Zebrafish as a Tool to Study Reproductive Biology

6.6.1 The Hormonal Signaling in Zebrafish and its Similarities to the Human Hormone Axis

Zebrafish is a relevant model to study the endocrine system because of the homology of genome, receptors, and hormones with their mammalian counterparts. The endocrinology of stress, growth, thyroid, peripheral hormones, and reproductive system of zebrafish is regulated by the hypothalamic-pituitary axis comparable to human forms with duplicate genes in some cases. The hypothalamus modulates anterior as well as posterior pituitary to release a hormone into the blood circulation. In humans, the posterior pituitary produces two hormones including oxytocin and vasopressin, but in zebrafish they are known as isotocin and vasotocin that are secreted from the anterior pituitary.

6.6.2 Hypothalamic-Pituitary-Gonadal (HPG) Axis

The hypothalamic-pituitary-gonadal axis (HPG), GnRH, and kisspeptin (Kiss) regulate the secretion of reproductive hormones, luteinizing hormone (LH), and follicular-stimulating hormone (FSH). In humans, three types of GnRH genes are identified, namely, herring GnRH (GNRH1), chicken GnRH 2 (cGNRH 2), and salmon GnRH (sGNRH/GNRH3). Gonadotropin's releasing factor is well studied in the fish and encoded by genes Gnrh. The zebrafish Gnrh2 is identical to human (GNRH2), whereas Gnrh3 is unique in teleost fishes, although with human (GNRH1) it showed 80% similarity. Gnrh2 and Gnrh3 proteins are also known as chicken GnRH2 and salmon GnRH, respectively. The expression of Gnrh2 was first observed in the midbrain area and remains located there for life, but Gnrh3 appeared in olfactory placode (outside of the brain) and then migrating to other neurons including the olfactory bulb, terminal nerve ganglion, ventral telencephalon, preoptic area, and hypothalamus. After 12 dpf, Gnrh3-GFP neurons reach the hypothalamus and are observed in the trigeminal nucleus sending axons down the

spinal cord to the tail region. Gnrh3 plays a key role in the release of gonadotropin hormones such as luteinizing hormone (lhb) but does not stimulate the production of follicle-stimulating hormone (fshb). After the release of gonadotropins, it binds to its Gnrh receptors, which is a G-protein-coupled receptor (GPCR) as in humans. Like humans, zebrafish fshb gene plays an important role in spermatogenesis in males, while in females it stimulates estrogen production and inhibin (inha) having inhibitory control on hormone release.

The transcripts for gnrh2 and gnrh3 in zebrafish were observed at 0.5–1.5 hpf using PCR, at 22–28 hpf by in situ hybridization, but not until 30–31 hpf using immunohistochemistry. The early expression of zebrafish gnrh mRNA indicates that gnrh mRNA is a maternal transcript in the eggs, as embryo-generated transcripts are only detected after 3–4 hpf.

Another important hormone that affects zebrafish reproduction is kisspeptin (Kiss) having duplicate copies of Kiss and its receptor. Likewise, human Kiss regulates the GNRH signaling through kisspeptin receptors. In zebrafish, expression of Kiss has been detected at 1, 3, 7, 30, and 45 dpf by using PCR and abundantly expressed in the diencephalic midbrain region of adult zebrafish. Further Kiss1 transcripts were also expressed in the pituitary, pancreas, and intestine, whereas Kitahashi et al. reported that Kiss 1 mRNA is found in the brain and testis and identified a duplicate kiss2 with transcripts in the brain, gonads, intestine, and kidney. The duplicate hormone expresses in the brain, kiss1 mRNA in the habenula, and kiss2 in the hypothalamus. Duplicate receptors for Kiss1 are kiss1ra transcripts located in the brain and testis, whereas kiss1rb transcripts in the brain, pituitary, spleen, gills, kidney, intestine, pancreas, and adipose tissue. Further, kisspeptins and Kiss1ra receptor are linked with the onset of puberty in zebrafish, and injection of Kiss2 increases the expression of fshb and lhb mRNA in the pituitary of adult females, indicating similarity with the other vertebrates.

6.6.3 Hypothalamic-Pituitary-Thyroid (HPT) Axis

HPT axis is also known as thyrotropic feedback regulation or thyroid homeostasis. This axis also regulates the reproductive system via the release of thyroid hormone (TH). HPT axis works opposite to HPG axis but the functions are the same in both axes (Rae et al. 2007). Both axes are correlated with each other but in a different way (Doufas and Mastorakos 2000). Hypothalamic-pituitary axis releases two distinct thyroid hormones named T_3 and T_4. The thyroid gland also has a function in the reproductive system to secrete the FSH and LH, thus taking part in the regulation of reproductive system as well (Manna et al. 2001).

Disruption in the thyroid hormone levels in the body causes reproductive disorders because the HPT axis is unable to regulate the sex steroids causing amenorrhea and oligomenorrhea. Impaired thyroid hormone regulation results in hypothyroidism and hyperthyroidism.

The physiology of zebrafish is quite similar to the mammalian thyroid system. Both zebrafish and mammals release T_3 and T_4 in the presence of TSH (thyroid-

stimulating hormone). The function of T_3 in male zebrafish is to regulate the Sertoli cell proliferation, spermatogenesis, and steroidogenesis (Holsberger and Cooke 2005). Gonadotrophin hormone 1 and gonadotrophin hormone 2 are essential for the activation of LH and FSH which help in the synthesis of sex steroids and gametes. Disruption of thyroid hormone also blocks the spermatogenesis and steroidogenesis process. An elevated level of T_3 hormone in hyperthyroidism causes the production of 11KT, a type II ketotestosterone which is not found in females and males.

6.6.4 Hypothalamic-Pituitary-Adrenal (HPA) Axis

HPA axis also shows a major part in the maintenance of reproductive system (Khan et al. 2016). In stress corticotrophin-releasing hormone and vasopressin are stimulated through the hypothalamus (Tsigos and Chrousos 2002). These two hormones stimulate the anterior pituitary to release the ACTH. This ACTH activates the adrenal gland which secretes cortisol to regulate the stress condition and also activates the gluconeogenesis process to meet the energy demand (Mouthaan et al. 2014). Cortisol is anti-inflammatory which blocks the cytokine release and also suppresses the overactivation of immune response (Coutinho and Chapman 2011). The whole process of release and binding of cortisol is similar in mammals as well as in zebrafish (Alsop et al. 2009).

During stress in zebrafish, the ACTH binds to the melanocortin receptor (MC2R) and releases cortisol (Alsop and Vijayan 2009). There are four isoforms of MC2R including two active and two inactive forms. MRAP-α (melanocortin 2 receptor accessory protein) and MRAP-β are two active isoforms of MC2R. MRAP-β is more effective than MRAP-α in terms of stress regulating mediators (Liu et al. 2013).

6.6.5 Reproductive Biology of Zebrafish

In the mammalian system, the development of mostly two sexes is seen because of dimorphic sex chromosomes. Mammals are comprised of a differentiating gene or factor, which differentiates the sexes on the basis of their testis-determining factor (TDF) (Kashimada and Koopman 2010; Piprek 2010). Other factors or genes along with TDF are SOX9, FGF9, Rspo1, Wnt4, Dax1, and Foxl2 (Piprek 2010). Expression of SOX9 and FGF9 has been observed in XY male gonads which are the precursor for Sertoli cells. There is a production of testosterone and AMH (anti-Mullerian hormone) with the expression of these genes and factors. Its function is to degenerate the Mullerian ducts. However, in female XX chromosomes, there is downregulation of FGF9 and upregulation of Rspo1 and Foxl2 genes (Grinspon and Rey 2019).

However, zebrafish have no sex defining factors because of the absence of dimorphic sex chromosomes. At the early stage of development of zebrafish, it comprises immature oocytes in the gonads (Maack and Segner 2003). In the

development of testes and ovaries, there is requirement of a specific expression of a gene in zebrafish. Genes for testes development are SOX9, cyp11b, and amh and for ovary development are cyp19a1a and Foxl2 (Maack and Segner 2003; Rodríguez-Marí et al. 2010). There is an activation of the apoptosis process in the oocytes after 30 days of post-fertilization and the male fish developed. If apoptosis is not activated and oocytes are present, that fish becomes a female (Wang et al. 2007). The environmental conditions like temperature and oxygen availability also affect the sex development and sex differentiation in zebrafish (Uchida et al. 2004; Shang et al. 2006). Amh and other genes are responsible for the development of testes via the inhibition of P450 aromatase enzyme, and these enzymes convert androgen to estrogen (Rodríguez-Marí et al. 2005). Zebrafish has a short generation time and it accomplishes reproductive maturity within a period of 3–6 months after fertilization. Reproductive organs of zebrafish and humans are anatomically similar. Male zebrafish also comprises paired testes which are existing inside the sac, with spermatogenic epithelium lining. LH hormone activates the Leydig cells which are present inside the interstitial space, and these cells are required for the production of testosterone (Van den Hurk and Resink 1992; Siegfried and Nüsslein-Volhard 2008; Schulz et al. 2018).

In contrast to female reproductive system, the organs are also structurally and functionally similar to humans. Location of zygomorphic ovaries is in between the abdominal wall and swim bladder with epithelium lining in the walls of ovaries (Gupta and Mullins 2010). Gametes are developed and produced in the ovaries that means the gametogenesis process occurs in the ovaries. Androgen production in female zebrafish is with the activation of theca cells, and androgen brings a reactant for estrogen production in the follicular cells (Huhtaniemi 2000).

6.6.6 Zebrafish as a Tool to Study Infertility

There are high morphological similarities in the reproductive axis of zebrafish as compared to humans, so zebrafish can be utilized as the model in the assessment of reproductive toxicity. Therefore, discovering novel compounds that improve fertility, we require a reliable, cost-effective, and fast infertility model to study infertility.

6.6.7 Genetic and Transgenic Model of Infertility

Hsu et al. developed male sterility in zebrafish by expressing an *Escherichia coli* nitroreductase (Ntr) gene in the germline (Hsu et al. 2010). Ntr catalyzes the conversion of prodrug metronidazole to cytotoxins (Curado et al. 2007). A fusion protein eGFP:Ntr (fusing Ntr to eGFP) was developed in the germline of male juvenile zebrafish, and expression is under control of ~2 kb putative promoters of the zebrafish testis-specific genes. A total of seven independent transgenic zebrafish lines were developed Tg(Asp-eGFP:Ntr), Tg(Odf-eGFP:Ntr), or Tg(Sam-eGFP:Ntr), and female carriers were fertile, whereas the males were with defective fertility.

The transgenic line exhibited depleted prospermatogonia after metronidazole (Met) treatment in the testes of male zebrafish while normal in case of without Met treatment. These triple transgenic lines present small testes size, devoid of germ cells, and after 2 weeks of Met exposure, the germ cells disappear indicating the development of sterility in male zebrafish (Hsu et al. 2010). Likewise, Hu et al. developed transgenic line Tg(ZP:NTR-EGFP) in the oocyte by a zona pellucida promoter, and exposure of Met from 28 to 42 days post-hatching results in the blockage of oogenesis, gonad atrophy, undeveloped oocyte, and oocyte death which has been observed due to dysregulation of pro-apoptotic protein leading to infertility (Hu et al. 2010). Lin et al., utilizing the Cre-loxP transgenic tool, developed a Cre transgenic zebrafish using a cytomegalovirus promoter and transgenic loxP expressed with apoptotic genes, Bax-1 and Bax-2, that degenerates the reproductive organs ovaries or testes in Cre zebrafish (Lin et al. 2013). Moreover, Abraham et al. established transgenic zebrafish line Tg(GnRH3:EGFP), and its ablation of gene gnrh3 induces infertility with underdeveloped gonad (Abraham et al. 2010). Similarly, Spicer et al. developed mutant gnrh3$^{-/-}$(62 bp deletion in gnrh3 gene) zebrafish line utilizing TALEN technology and confirmed that gnrh3$^{-/-}$mutant fish showed absence of Gnrh3 peptide in the whole brain by using immunohistochemistry. The author reported that deficiency of Gnrh3 has minor effects on the HPG axis due to activation of the compensatory mechanism as gnrh3$^{-/-}$ zebrafish remains fertile with the normal reproductive performance (Spicer et al. 2016).

6.6.8 Stress-Induced Infertility

Stress-induced release of hormones such as CRF, ACTH, and cortisol results in impaired function of reproductive hormones and affects gametogenesis ultimately leading to infertility (Alsop et al. 2009). The stress-induced high levels of ACTH and cortisol decrease the release of estradiol from ovarian follicles, causing oocyte DNA damage and vacuolization in ooplasm of zebrafish, similar to mammals (Alsop et al. 2009; Sousa et al. 2015; Volkova et al. 2015).

6.6.9 Chemical- and Environment-Induced Infertility

Other than stress, environmental toxin and chemicals are well associated with infertility in mammals (Pizzorno 2018). Glyphosate is a well-known herbicide that inhibits enzyme 5-enolpyruvylshikimate-3-phosphate synthase in plants and affects the reproductive system of aquatic life (Duke 2018; Uren Webster et al. 2014). Webster et al. reported that exposure to high glyphosate concentration in zebrafish disrupts steroidogenesis with increased expression of cyp19a1 gene, aromatase activity, and estrogen receptor (Uren Webster et al. 2014). Further, glyphosate exposure to male adult zebrafish results in oxidative damage in testes (Lopes et al. 2014). Low concentration of endosulfan exposure to adult male zebrafish induces testicular damage, sperm necrosis, and reproduction abnormalities (Han et al. 2011;

Velasco-Santamaría et al. 2011). Similarly, other toxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, an endocrine disruptor causes, and di(2-ethylhexyl) phthalate, a commonly used plasticizer, altered gonad development and reproductive toxicity and impaired spermatogenesis and oocyte maturation in zebrafish, respectively, and other toxins are compiled in Table 6.4 (Heiden et al. 2008; Uren-Webster et al. 2010).

The reproductive system is also affected by environmental factors such as oxygen, exogenous heat exposure, and high altitude (Figà-Talamanca et al. 1992; Kesari et al. 2018; Vargas et al. 2011). Low oxygen condition induces hypoxia that alters the circulating hormones testosterone and estradiol through the modulation of HPG-related genes and alters the hypoxia-inducible factor 1 and leptin activity with cellular lipids and steroid hormones. Further, hypoxia results in aberrant primordial germ cell migration, impaired gonadal development, and decreased germ cell quantity and quality with reduced fertilization and hatching success in zebrafish (Thomas et al. 2007; Yu et al. 2012; Martinovic et al. 2009; Lo et al. 2011).

Moreover, testicle temperature also determines the quality of sperm in humans and other mammals. Normally, the temperature of testicles is 2 to 4 °C lower than the body temperature, which is essential for testicular function (Bujan et al. 2000; Lue et al. 2000; Sheiner et al. 2003). Likewise, in zebrafish, the surrounding temperature also affects the gonadal functions. A high water temperature affects the virilization, induces oocyte apoptosis and differentiation of spermatogonia, and suppressed the activity of gonadal aromatase (Uchida et al. 2004; Luzio et al. 2016). In addition, the water temperature increase from 24 °C to 28 °C results in shorter hatching rate with impaired hatching rhythm (Villamizar et al. 2012).

6.7 Advantages and Limitations of Zebrafish as a Tool for Endocrinology Research

Zebrafish comes under the Chordata phylum which matches the mammal especially human (Herzog et al. 2003). Zebrafish length is very short from 2.5 to 4 cm (Martin et al. 2019). Initially, zebrafish was mainly used to study only vertebrate development but now helpful in endocrine research related to human because 80% of zebrafish genes are the same which are associated with human defects. Zebrafish development is very fast after the post-fertilization stage, and many of the endocrine systems develop within 5 days of post-fertilization (Herzog et al. 2003). In each generation, zebrafish develops approximately 200–300 offsprings, which is a much larger number in comparison to rodents. Zebrafish are commonly used to study early-stage development because at that time zebrafish become transparent and provide real-time observation, which is not possible in rodents (Martin et al. 2019). Administration of drugs is very easy in zebrafish than rodents because only adding of the drug into water therefore only water-soluble drugs are easily administered by the fish and oil-soluble drugs difficult to solubilize in water and have to employ another route of drug administration (Vogel 2000).

Table 6.4 Zebrafish as a model for reproductive disorder

Type of model	Toxin/genetic manipulation	Age	Phenotype	References
Induced models				
Chemicals	Glyphosate	Adult	Disrupts steroidogenesis, increased aromatase activity and estrogen receptor, oxidative damage in testes	Uren Webster (et al. 2014) and Lopes et al. (2014)
	Endosulfan	Adult	Testicular damage, sperm necrosis, and reproduction abnormalities	Han et al. (2011) and Velasco-Santamaría et al. (2011)
	2,3,7,8-Tetrachlorodibenzo-p-dioxin	Adult	Altered gonad development and reproductive toxicity	Uren-Webster et al. (2010)
	Di(2-Ethylhexyl) phthalate	Adult	Impaired spermatogenesis and oocyte maturation	Heiden et al. (2008)
	Bisphenols	Adult	Decreased egg production and sperm count, imbalance of steroid hormones, and embryonic malformation	Naderi et al. (2014)
	2,4,6-Tribromophenol	Adult, larvae	Decreased egg production, early post-hatching mortality, disturbed gonad morphology, and complete hatching failure	Kuiper et al. (2007)
	Cobalt	Adult, larvae	Reduced fertilization rate, embryo survival to hatching decreased, DNA damage in sperm	
	2,4-Dichlorophenol	Adult	Impaired expression of steroid gene and plasma sex hormone level with reduced number of eggs released and hatching rate	Ma et al. (2012)
Environmental	Increase temperature	Adult	Shorter hatching rate, oocyte apoptosis, reduced gonadal aromatase activity	Uchida et al. (2004) and Luzio et al. (2016)

(continued)

Table 6.4 (continued)

Type of model	Toxin/genetic manipulation	Age	Phenotype	References
	Stress		Impaired function of reproductive hormones, infertility, oocyte DNA damage	Alsop et al. (2009), Sousa et al. (2015) and Volkova et al. (2015)
	Low oxygen	Adult	Impaired gonadal development, decreased germ cell quantities and qualities, reduced fertilization and hatching success	Thomas et al. (2007), Yu et al. (2012), Martinovic et al. (2009) and Lo et al. (2011)
Transgenic lines model				
Transgenic line expressing eGFP:Ntr	Tg(Asp-eGFP:Ntr),	Juvenile	Depleted prospermatogonia, small testes size	Hsu et al. (2010)
Ntr fused with EGFP	Tg(ZP:NTR-EGFP)	Juvenile	Blocks oogenesis, oocyte cell death, gonadal dysgenesis	Hu et al. (2010)
Selective ablation of olfactory region GnRH3 neurons	Tg(GnRH3:EGFP)	Adult, larvae	Arrested oocyte development, reduced average oocyte diameter	Abraham et al. (2010)
Cre-loxP transgenic tool	Cre transgenic zebrafish	Adult	Damage of reproductive organs ovaries or testes	Lin et al. (2013)
Mutant lines model				
Mutant gnrh3$^{-/-}$	Talen	Larvae	Minor effects on the HPG axis	Spicer et al. (2016)

However, several limitations need to be considered during the planning of endocrinology experiments. Due to its small size, it is very difficult to administer drugs through intraperitoneal and intracerebroventricular injections but this problem does not appear in adult zebrafish (Kinkel et al. 2010; Yokobori et al. 2011). Multiple blood sampling cannot be possible from individual zebrafish, and very less amount of blood sample is collected after the terminal procedure for the estimation of hormone levels and metabolic parameters. Thus, it increases the number of fish requirements in the endocrinology research, and there is no robust method/technique available for monitoring food-water intake and energy expenditure in zebrafish that is very crucial in endocrinology research. In addition, zebrafish represents high genetic diversity between individual genomes, as well as within fish of the same strain (Seth et al. 2013). Further, cold-blooded zebrafish physiology does not match completely with humans because humans are warm-blooded. Alteration in the environment, temperature, lighting, and water quality affects zebrafish

physiology (Lahiri et al. 2005). Taken together, there is an urgent need to develop and establish robust methods and technical tools for conducting endocrine research in zebrafish so that it would make a smooth bridge between the mammalian systems to translate into the humans.

6.8 Future Perspectives and Summary

Zebrafish is mainly used for the developmental biology-related studies. However, currently the zebrafish is an emerging vertebrate model for the screening of pharmaceutical compounds for its toxicity and biological activities in a high-throughput manner. Due to the small size of zebrafish, it requires very little space and a very less amount of testing sample. Lead compounds can be validated rapidly in zebrafish in a cost-effective manner using various genetic toolkits such as TALEN, morpholino, and CRISPR/Cas9. Zebrafish have been magnificently modeled for endocrinology disorders such as DM, obesity, adiposity, osteoporosis, as well as fertility. These models are utilized with great success for live imaging and screening of potential novel chemical entities in endocrinology disorders. There are several transgenic, fluorescent reporter lines developed to enable mechanisms and pathways involved in the pathophysiology of diseases that would help in discovering the novel therapeutic targets in the field of endocrinology. Figure 6.1 provides the summary of various models based on zebrafish used in endocrinology research.

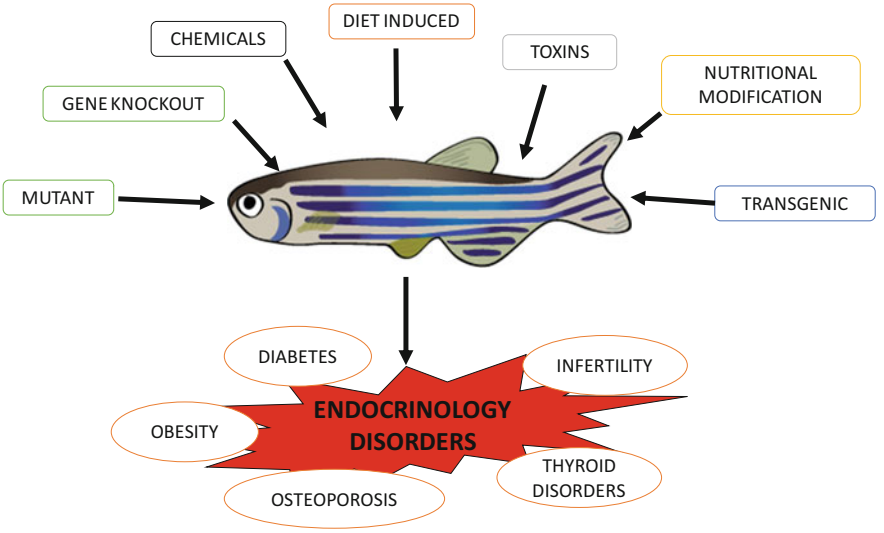

Fig. 6.1 Zebrafish as a model of endocrinology disorders including diabetes mellitus, obesity, osteoporosis, thyroid hormone disorder, and infertility. There are several approaches to produce endocrine-related disorders such as dietary-nutritional modification, by using various toxins and chemicals, as well as production of mutant and transgenic lines using genetic toolkit

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Zebrafish as a Versatile Model for Cancer Research

7

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Abstract

The utility of zebrafish as a laboratory model has only been realized recently. It has helped gain great insights in the field of developmental biology and is only beginning to be employed for understanding mechanisms of diseases and finding appropriate therapeutic solutions. High fecundity, ex utero development of embryos which allow scope for manipulations, and the transparent body of developing embryos allowing in vivo imaging of the developing tissues and biological processes are some of the great advantages of zebrafish as a model organism. Tumors can be spontaneously induced in zebrafish by mutagen

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exposure or transgene technology. Cancers induced in zebrafish greatly resemble the human cancer in terms of histology and genetic makeup. Zebrafish not only allows modeling of various cancers but enables their characterization as well. Live imaging and chemical and genetic screens are powerful tools in the study of zebrafish cancer models. Broadly speaking, the major applications of zebrafish in the study of cancer include developing human cancer models, assessing various tumor-related processes such as angiogenesis and tumor metastasis, and screening small molecules with anticancer potential. These areas will be the major focus of this chapter, which will help to appreciate versatility of zebrafish in cancer study.

Keywords

Zebrafish · Cancer · Screen · Model · Mutation · Genetic · Genes · Tumor

7.1 Introduction to Zebrafish in Cancer Discovery

Danio rerio or more popularly known as zebrafish has served as a useful animal model for quite a few decades now. Initially, zebrafish were extensively employed for studying various aspects of developmental biology. However, over time, researchers observed that zebrafish can develop a variety of diseases, including cancer (Kent et al. 2002; Matthews 2004). A large number of tumor types were found to spontaneously develop in zebrafish (Smolowitz et al. 2002).

It is well-known that cancer is a complex genetic disease. Accumulation of a large number of somatic mutations (and sometimes inherited mutations as well) induces the multistep process of tumor formation (Hanahan and Weinberg 2000). Gain-of-function mutation in oncogenes and loss-of-function mutation in tumor suppressor genes are the prominent genetic aberrations, responsible for cancer. Mouse models have yielded valuable information regarding the genetic events of tumorigenesis. For instance, numerous genes involved in the pathogenesis of lymphoma and leukemia have been identified using mating strategies of genetically manipulated mouse or the retroviral insertion screens (Fanidi et al. 1992; Alt et al. 2003; Haupt et al. 1991; Shinto et al. 1995; Scheijen et al. 1997; Strasser et al. 1990; Blyth et al. 2001). However, these methods have their own limitations. The retroviral insertion method was successfully applied for the oncogenes but was largely unsuccessful in identifying the tumor suppressor genes. Similarly, the gene knockout in mouse required prior information about the target gene. These limitations can be overcome by forward genetic screens in *Caenorhabditis elegans* and *Drosophila melanogaster* (which are also useful laboratory model organisms). However, these organisms are invertebrate and do not effectively model the pathophysiology of many human diseases. On the contrary, zebrafish being a vertebrate can better recapitulate the human diseases and underlying pathologies. Moreover, transgenic expression of fluorochromes, such as green fluorescent protein (GFP) in zebrafish, allows easy visualization of cancer formation in vivo in transparent zebrafish embryos (Long

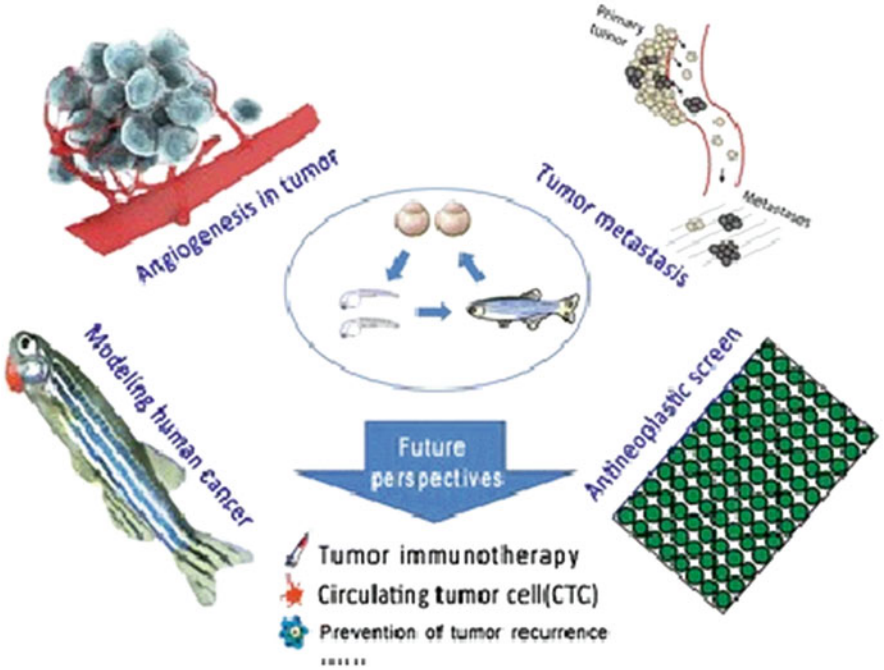

Fig. 7.1 Various applications of zebrafish cancer models. (Reprinted from Zhao et al. (2015) under Creative Commons license)

et al. 1997; Jessen et al. 1999, 2001; Ward et al. 2003; Hsu et al. 2004; Udvadia and Linney 2003).

In this chapter, we will discuss about various aspects and utility of zebrafish in cancer research (Fig. 7.1). This chapter will inform about various strategies of developing zebrafish tumor models as well as explain various cancer models already established in zebrafish. We will also discuss about the utility of zebrafish as a promising tool for exploring the genetics and biological pathways involved in human cancer as well its application in discovery of various anticancer drugs and therapies.

7.2 Various Cancer Models Developed in Zebrafish

Many human cancers have been successfully modeled in zebrafish using approaches such as genetic modifications, carcinogen treatment, or xenotransplantation of tumor cells (Feitsma and Cuppen 2008a) (Table 7.1). Treatment with carcinogenic compounds is the most common strategy used for developing tumor zebrafish models. Carcinogenic compounds such as N-methyl-N1-nitro-N-nitrosoguanidine (MNNG) (Shepard et al. 2005), N-ethyl-N-nitrosourea (ENU) (Basten et al. 2013),

diethylnitrosamine (DEN) (Mizgirev and Revskoy 2010), dimethylbenzanthracene (DMBA) (Mirbahai et al. 2011), and N-nitrosodimethylamine (NDMA) (Mizgireuv et al. 2004) are used to induce tumorigenesis. Canceration is induced in a variety of organs, covering a wide range of tumor types (Basten et al. 2013; Lam et al. 2006; Mizgireuv and Revskoy 2006; Spitsbergen et al. 2000a, b). Studies have reported that DMBA exposure to *vhl*$^{+/-}$ zebrafish increases the occurrence of tumorigenesis in the intestine, bile duct, and liver (Santhakumar et al. 2012). Exposure of zebrafish to DEN is reported to result in cholangiocarcinoma, pancreatic carcinoma, hepatoma, hepatoblastomas, and hepatocellular carcinomas (Mizgireuv and Revskoy 2006). NDMA exposure for 2 months induces hepatocellular tumors (adenomas and hepatocellular carcinomas) and cholangiolar tumors (cholangiomas and cholangiocarcinomas) (Mizgireuv et al. 2004). MNNG and ENU induce canceration in the liver and testis of zebrafish (Basten et al. 2013; Spitsbergen et al. 2000a).

The genetic tools which help in generating zebrafish cancer models include morpholinos, TALENs (transcription activator-like effector nucleases), engineered endonucleases such as ZFNs (zinc finger nucleases), and the CRISPR-Cas system. Injecting 1–4 cell stage zebrafish embryos with morpholinos results in transient knockdown of the target gene (Hurwitz et al. 2004). ZFNs, TALENs, and CRISPR-Cas disrupt the target genes by causing double-stranded breaks (Stern et al. 2005; Huang et al. 2012). Targeting Induced Local Lesions IN Genomes or TILLING is another important technology for targeted modification of genome (Kuroyanagi et al. 2013; Wienholds et al. 2003; Da Costa et al. 2014; Stemple 2004). Several zebrafish cancer models have been established by mutations in the tumor suppressor genes. For instance, mutation of zebrafish *APC* gene induces formation of colon adenoma (Phelps et al. 2009), mutation of *espl1*, *mybl2*, and *BRCA2* results in testicular neoplasias (Shive et al. 2010; Neumann et al. 2009); mutants of *bmyb*, *cds*, and *pen/lgl2* genes induce epidermal neoplasia (Shepard et al. 2005, 2007; Reischauer et al. 2009); malignant peripheral nerve sheath tumor (MPNST) is modeled by knockout of zebrafish *p53 (*Storer and Zon 2010*)*; *NF1* gene mutants develop MPNSTs and high-grade gliomas (Shin et al. 2012); *vhl* gene mutation is related to intestinal and hepatic cancers (Santhakumar et al. 2012); and deletion of *GSTT1* results in lymphoma (Yang et al. 2014). Due to similar hematopoietic and immune system of zebrafish and humans, both solid tumors and hematologic tumors can be modeled in zebrafish (Lieschke et al. 2001). Mutation of *pten* tumor suppressor gene helps in modeling hemangiosarcoma and T-cell acute lymphoblastic leukemia (T-ALL) in zebrafish (Gutierrez et al. 2011; Choorapoikayil et al. 2012). Tumor models can also be established by expression of transgenic human oncogenes in zebrafish, such as T-cell acute lymphoblastic leukemia which was induced in zebrafish by introduction of Myc transgenes (Langenau et al. 2003a). *Akt1* transgene helps in creating lipoma zebrafish models (Chu et al. 2012). KRAS$^{(G12D)}$ upregulation leads to rhabdomyosarcoma induction in zebrafish (Storer et al. 2013). Neuroblastoma was formed in zebrafish by transgenic expression of *MYCN* and *fgf8* genes (Zhu et al. 2012). Introduction of different oncogenes in *p53* mutant zebrafish induces formation of different tumors, such as *BRAF* oncogene expression leads to melanoma (Storer and Zon 2010), *scr* overexpression induces hepatoma

Table 7.1 Different methods to develop zebrafish cancer model

Technology	Treatment	Types of induced tumor	References
Chemical treatment	DMBA	Hepatoma, cholangiocarcinoma, and intestinal cancer	Mirbahai et al. (2011)
	DEN	Hepatoma, cholangiocarcinoma, and pancreatic carcinoma	Mizgirev and Revskoy (2010)
	NDMA	Hepatoma and cholangiocarcinoma	Mizgireuv et al. (2004)
	ENU	Hepatoma and testicular cancer	Basten et al. (2013)
	MNNG	Hepatoma and testicular cancer	Shepard et al. (2005)
Genetic technology			
Knockout	*P53*	Malignant peripheral nerve sheath tumors	Storer and Zon (2010)
	APC	Colon adenoma	Phelps et al. (2009)
	NF1	Gliomas and malignant peripheral nerve sheath tumors	Shin et al. (2012)
	BRCA2, MYBL2, esp11	Testicular cancer	Shive et al. (2010) and Neumann et al. (2009)
	pen/lgl2, bmyb, and cds gene	Epidermal cancer	Shepard et al. (2005), Reischauer et al. (2009) and Shepard et al. 2007)
	GSTT1	Lymphoma	Yang et al. (2014)
	vhl	Hepatoma and intestinal cancer	Santhakumar et al. (2012)
	pten	T-cell acute lymphoblastic leukemia and hemangiosarcoma	Gutierrez et al. (2011) and Choorapoikayil et al. (2012)
Overexpression	*Myc*	T-cell leukemia and hepatoma	Langenau et al. (2003a, 2013)
	xmrk and KRAS $^{(V12)}$	Hepatoma	Li et al. (2012), Nguyen et al. (2012) and Zheng et al. (2014)
	MYCN and fgf8	Neuroblastoma	Zhu et al. (2012)
	KRAS $^{(G12D)}$	Rhabdomyosarcoma	Storer et al. (2013)
	Akt1	Lipoma	Chu et al. (2012)
	Scr in p53 mutant background	Hepatoma	Lu et al. (2013)
	NRAS, BRAF in p53 mutant background	Melanoma	Storer and Zon (2010) and Dovey et al. (2009)

(continued)

Table 7.1 (continued)

Technology	Treatment	Types of induced tumor	References
	EWS-FIL1 in p53 mutant background	Ewing's sarcoma	Leacock et al. (2012)
Xenotransplantation	Transplant tumor cells in zebrafish	Melanoma, glioma, hepatoma, lung cancer, pancreatic cancer, ovarian carcinomas, breast cancer, prostate cancer, retinoblastoma, leukemia	Drabsch et al. (2013), Jo et al. (2013), Weiss et al. (2009), Moshal et al. (2011), Bellou et al. (2013), Yang et al. (2013), Lee et al. (2005), Hou et al. (2013), Latifi et al. (2011), Wagner et al. (2010) and Zhang et al. (2014)

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(Lu et al. 2013), *EWS-FIL1* overexpression helps in modeling Ewing's sarcoma (Leacock et al. 2012), and *NRAS* transgenic expression results in melanoma (Dovey et al. 2009).

The third approach of developing zebrafish cancer models is xenotransplantation wherein tumor cells are transplanted in zebrafish. The tumor cells can be fluorescently labeled to enable distinguishing them from normal cells allowing visualization of the process of tumor development (Smith et al. 2010). Different types of cancer cells are transplanted to create different zebrafish tumor models. Many different tumors such as breast cancer, retinoblastoma, pancreatic cancer, lung cancer, etc. have been modeled by this approach in zebrafish (Drabsch et al. 2013; Jo et al. 2013; Weiss et al. 2009; Moshal et al. 2011).

7.2.1 Various Tools and Techniques in Zebrafish as a Model for Cancer Screening

After inducing tumors in zebrafish, the process of tumor development is monitored using imaging tools. In case of xenotransplantation, the cancer cells are fluorescently labeled with protein-based reporters or membrane dyes such as DiD, DiO, DiR, and DiI, which allow real-time monitoring of tumor development at single cell level (Progatzky et al. 2013). Recently, many specific and more accurate imaging techniques have been developed for cancer screening in zebrafish. For instance, to observe in vivo cancer formation without the need of labeling molecules, Kumar et al. established 3D-fluorescence imaging technique. This involves angularly multiplexed optical projection tomography with compressive sensing (Kumar et al. 2016). Similarly, to analyze the dissemination of tumor cells in a xenograft assay, Ghotra et al. developed a quantitative imaging platform (Ghotra et al. 2012). Pardo-

Martin et al. reported vertebrate automated screening technology (VAST), which allows automatic image capturing (Pardo-Martin et al. 2010). The VAST platform is especially useful in screening assays which require a large number of images to be analyzed. This tool was further developed into a highly automated VAST BioImager system (Pulak 2016).

Some genetics-based tools also enable studying the process of cancer formation in zebrafish. The in vivo cell fate of transplanted ZMEL1 was easily visible in the transparent adult zebrafish *casper (*Benjamin and Hynes 2017*)*. This zebrafish was engineered to carry homozygous mutation in two loci responsible for pigmentation (White et al. 2008a). Transgenic zebrafish lines expressing luciferase allow tissue imaging even in free swimming zebrafish (Chen et al. 2013). Transgenic lines such as *flik-EGFP*, *mtie2-GFP*, and *fli-GFP* have fluorescently marked vasculature (Lawson and Weinstein 2002; Motoike et al. 2000; Cross et al. 2003).

Ex vivo approaches have also been utilized to study the tumor formation in zebrafish. To assess the xenotransplantation of tumor cells, the injected embryos can be dissociated into single-cell suspension and the fluorescence (corresponding to tumor cells) can be visualized by imaging (Corkery et al. 2011). Alternatively, real-time PCR of tumor-related genes or human housekeeping genes can also help analyze the xenotransplantation and tumor formation in zebrafish (Bentley et al. 2015; Xu et al. 2018).

7.2.2 Zebrafish Cancer and Human Cancers: How Much Is the Correlation?

An important aspect which makes zebrafish as a suitable cancer model is that fact that many zebrafish and human tumors are histologically similar (Amatruda et al. 2002). Many oncogenes and tumor suppressor genes are conserved between humans and zebrafish. Moreover, many of the general features of cancer such as invasiveness, cancer stem cells, transplantability, and genomic instability are observed in case of zebrafish also (Langenau et al. 2007). Nonetheless, some differences do exist between zebrafish and human cancers, in terms of tumor spectrum and cancer incidence, as discussed below.

The classification of zebrafish cancer is broad, whereas the human cancer classification is highly detailed with specific identified markers for the various cancer types. It is reported that the liver and testis are the common sites for spontaneous mutation in zebrafish. However, genetic mutations such as mismatch repair, genomic instability mutants, *p53* mutants, and ribosomal protein mutants lead to MPNSTs formation in zebrafish (Amsterdam et al. 2004a; Moore et al. 2006; Berghmans et al. 2005a). Similarly, human *MYCN* transgenic zebrafish also develop MPNSTs (Yang et al. 2004). In zebrafish, *apc* mutation induces liver and intestinal cancer which are comparable to the human tumor in these organs (Haramis et al. 2006). Since zebrafish has a small gut compared to that of humans, the possibility of modeling gut cancer is less. In humans, *pten* mutation leads to many different cancers, whereas

the same mutation in zebrafish results in only one tumor phenotype in the eye (Faucherre et al. 2008; Cristofano et al. 1998; Bonneau and Longy 2000).

Comparison of gene expression profiles of human tumors with those of zebrafish tumors also led to interesting findings. For instance, in case of *bymb* mutation, the gene expression profile of human tumors and those of homozygous zebrafish embryos highly correlated with each other (Shepard et al. 2005). Likewise, the expression profile of zebrafish liver tumors (induced by 1,4-diphenyl-1,3-butadiene and 7,12-dimethylbenz[a]anthracene chemical treatment) had significant correlation with human liver cancer (Lam et al. 2006; Grabher and Look 2006; Lam and Gong 2006). The two forms of human rhabdomyosarcoma, namely, alveolar and embryonic rhabdomyosarcomas, have their signature molecules. However, the expression array of activated RAS transgenic fish matched well with the molecular signatures of human embryonic rhabdomyosarcoma but not the alveolar form of human rhabdomyosarcoma. The microarray profile of this transgenic fish line was also similar to human pancreatic adenocarcinoma, which is also a RAS-induced tumor (Langenau et al. 2007). These observations point to the fact that the pathways involved in fish and human cancer development are conserved.

7.2.3 Studying Tumor Angiogenesis and Metastasis in Zebrafish with Emphasis on Tumor Microenvironment

The tumor microenvironment (TME) influences several aspects of cancer formation and progression. For instance, tumor metastasis and angiogenesis are deeply linked to TME. Due to the complex cellular composition of TME, involving multiple cell types (such as cancer cells, fibroblasts, immune cells, and endothelial cells), TME studies are generally carried out in vivo. Recent findings have suggested that zebrafish is a suitable model for studying the interactions between cancer cells and immune cells. Zebrafish also possess immune cells which exert pro-tumor effects.

Angiogenesis—Angiogenesis plays a determining role in tumorigenesis as the blood vessels supply the necessary nutrients and oxygen to support tumor growth. Angiogenesis not only determines the malignancy of tumor but also influences the antitumor therapy and prognosis. Antiangiogenic therapy combined with chemotherapy has been generally found to improve outcome in cancer patients (Bellou et al. 2013). However, the visualization of vascularization in traditional mammalian models is difficult, as these models allow image capturing of the later stages of tumor. Since the earlier stages of tumor cannot be observed in these models, understanding the mechanism of vascularization is not possible with these models. In vitro assays and models have been utilized to understand some aspects of angiogenesis (Yue et al. 2011). However, owing to the fact that angiogenesis is greatly influenced by the TME, the in vitro observations may vary widely compared to the cancer patient results.

Efforts in the direction of utilizing zebrafish as an in vivo model for studying angiogenesis have been largely successful. Due to a strikingly similar tumor environment, the pathophysiology of tumor angiogenesis in zebrafish shares similarities

with that in case of human cancer (Tobia et al. 2011). Moreover, the vasculature development in zebrafish is fairly rapid. Also, the transparency of zebrafish embryos (which can be maintained for a greater number of days post-fertilization by treatment with melanin synthesis inhibitor PTU) also in vivo imaging of blood vessel formation (Tobia et al. 2011; Goldsmith and Jobin 2012). The transgenic fish line *casper* is engineered to possess a transparent body, allowing visualization of vasculature throughout the body (White et al. 2008a). The vessels can also be observed in real time by microinjection of either suitable dyes or transgenic zebrafish lines expressing fluorescent green fluorescent protein specifically in the vasculature tissues (such as Tg(*flk1: EGFP*) zebrafish) (Kamei et al. 2010; Nicoli et al. 2007).

Zebrafish are easily amenable to genetic manipulation in order to understand the genes and pathways involved in angiogenesis, helping in the discovery of novel targets for antiangiogenesis therapeutics. For instance, knockdown of TNFRSF1A gene in zebrafish prevented apoptosis of endothelial cells, while knockdown of TNFRSF1B gene promoted apoptosis, implying that TNFRSF1A and TNFRSF1B play a role in angiogenesis signaling pathway (Espín et al. 2013). Similarly, another study reported decrease in angiogenesis upon silencing of *LIM* kinases in zebrafish pancreatic tumor (Vlecken and Bagowski 2009). Other models for angiogenesis such as the chick embryo chorioallantoic membrane are also utilized; however, zebrafish are a superior model, as they allow easy visualization of the angiogenesis process, easy genetic manipulation, and an in vivo environment for studying tumor angiogenesis.

Metastasis—Metastasis is a complex and dynamic process which involves spread of tumor cells to distant body sites through the circulatory system followed by engraftment and tumor growth at the new site (Eccles and Welch 2007). Understanding tumor metastasis is very important for identifying more potent therapeutic targets for improving clinical treatments. Though mouse is used as in vivo model system for studying metastasis, it suffers from several drawbacks – residual antitumor immunity even in immunodeficient mouse models (Teng et al. 2013), inability to study early stages of metastasis, absence of any real-time imaging system to visualize tumor lesions in deep tissues, and longer time required for the process of metastasis in mice are some of the limitations.

Zebrafish cancer models do not have these limitations. Their adaptive immune system takes around 14 DPF to develop (Traver et al. 2003). During this time, tumor cells can easily metastasize. Also the process of metastasis is rapid in zebrafish. Metastasis can be easily observed after 2 days of tumor cell transplantation (Yang et al. 2013). Moreover, transparent body of zebrafish allows imaging the metastasis process in real time such as the transgenic line *casper*. The tumor cells can also be fluorescently labeled to track their movement inside the fish body. Transgenic Tg (*fli1*: EGFP) zebrafish with GFP-labeled vasculature can enable simultaneous visualization of RFP-labeled tumor cell metastasis along with changes in vasculature. Zebrafish is also a useful model for exploring signaling pathways involved in metastasis. Knockdown of protein targets has been the usual way of understanding signaling pathways for metastasis. Zebrafish metastasis model helped to decipher that TGF-β plays an important role in metastasis of human breast tumor. When this

zebrafish model was treated with TGF-β inhibitors, it led to successful inhibition of cancer metastasis and invasion (Drabsch et al. 2013).

7.2.4 Zebrafish as a Screening Model of Anticancer NCE

The anticancer effect of small molecules is generally assessed by in vitro methods such as cell line models. However, when administered to vertebrates, these drug molecules fail to exert therapeutic effect. Using whole animals as screens for identifying potent anticancer molecules will be more reliable and will also provide information regarding pharmacokinetic profile and organ toxicity of the drug molecules (Ghotra et al. 2012). Mice are not economically feasible for using as whole animal screens for testing anticancer drugs. However, zebrafish not only can be a good whole animal screen but also can enable high-throughput screening of anticancer molecules. A single mating session yields hundreds of zebrafish eggs and the hatched larvae can be easily maintained in 96-well plates. Also, the drugs to be tested can be simply administered by adding them to the aqueous fish environment. The effect of drug can be monitored in real time by virtue of the transparent fish body.

One popular example of zebrafish as a screening model is the anti-melanoma chemical genetic screen. It is known that melanoma progression is intimately linked to neural crest lineage. Thus, in this anti-melanoma zebrafish screen, around 2000 compounds were tested for their ability to inhibit neural crest lineage. The lead molecules identified were then tested for potential in melanoma. Among these, one compound was identified which served as an inhibitor of both melanoma and neural crest lineage. This compound was leflunomide, a dihydroorotate dehydrogenase inhibitor (White et al. 2011).

The development of zebrafish hematopoietic system is quite similar to that of human. Most of the blood lineages of human have a counterpart in cell line in zebrafish. Thus, hematopoietic drugs found effective in zebrafish may be useful in humans as well. For this reason, zebrafish was successfully employed as a screen for antileukemia compounds. When used to screen around 25,000 compounds, this zebrafish model helped to identify a compound lenaldekar (LDK) possessing therapeutic effect against T-cell acute lymphoblastic leukemia (T-ALL). Later this drug was found to show remission in other blood cancers such as CML and B-ALL (Clements and Traver 2012; Ridges et al. 2012a).

Zebrafish has also been used successfully for screening antiangiogenesis compounds. For instance, in a drug screen for 288 compounds, two kinase inhibitor molecules were identified as antiangiogenesis drugs (Camus et al. 2012). Similarly another drug rosuvastatin was found to possess antiangiogenic properties in zebrafish embryos (Wang et al. 2010a). Drug molecules capable of inhibiting signaling pathways in cancer have also been screened in zebrafish. Compound persynthamide was screened out of 16,000 compounds in a search for molecules able to inhibit *bymb* pathway-dependent mitotic defects (Stern et al. 2005). *Bymb* regulates mitotic checkpoint and is involved in cancer susceptibility (Shepard et al.

2005). Drug toxicity is an important factor in determining the clinical translation of anticancer molecules. Due to the rapid development of zebrafish, the toxic effects of drug molecules can be assessed in short time frame. In an investigation for drug toxicity on inner ear hair cells, 13 out of 88 screened antitumor drugs were identified as ototoxic (Hirose et al. 2011).

7.3 Zebrafish as a Tool for Chemical Genetic Manipulation for Cancer Discovery

Chemical genetics involves use of small molecules which hamper a biological pathway. There are two types of chemical genetics screens—target-based and phenotype-based. In the target-based screens, a library of molecules is first screened in vitro against a known target and then the lead molecules identified are validated in vivo. In the phenotype-driven screens, a library of molecules is tested for its ability to disrupt biological pathways leading to aberrant phenotypes, in vitro as well as in vivo. The components of the signaling pathways disrupted by the small molecules are then assessed by chemoinformatic and biochemical techniques.

Zebrafish has also been proved as a useful in vivo chemical genetics screen and is preferred over in vitro cell-based and biochemical screens. High fecundity of zebrafish, ex utero and fast development of embryos, transparency of embryos, and similar biological pathways compared to humans are some of the factors which make zebrafish a useful chemical genetic screen.

In the experimental workflow, first a small-scale trial screen is set for optimizing the experimental parameters such as choice of chemical library, concentration of chemicals to be tested, duration of chemical exposure, number of embryos to be tested, and the embryonic stages to be evaluated (Rennekamp and Peterson 2015; Tan and Zon 2011). This is followed by setting a full-scale chemical screen. For this thousands of embryos which are developmentally synchronous are collected either by pairwise mating (Adatto et al. 2011) or breeding the fishes *en masse* in specialized breeding tanks. The viable embryos are distributed into individual wells of multi-well plate and exposed to molecules of a chemical library (Fig. 7.2). Embryos are exposed to the molecules, when the specific biological pathway to be targeted becomes active. The compound libraries may consist of 10 to 50,000 molecules and are of three types – natural products, synthetic molecules, or commercial vendor. For example, LOPAC and Chembridge DIVERSetE are chemical libraries of bioactives and synthetic molecules, respectively. Embryos are then examined for morphological abnormalities or molecular defects due to chemical exposure. Chemical screens for studying oncogenic pathways utilize transgenic zebrafish embryos. The chemically exposed embryos are assessed for alterations in cell state which can be determined by examining changes in protein expression (by immunohistochemistry), alterations in RNA expression (by in situ hybridization or ISH), or expression of reporter genes (by fluorescence microscopy). The lead molecules are validated by rescreening, followed by identification of their target proteins. For instance, tagging the molecule of interest with magnetic bead, followed

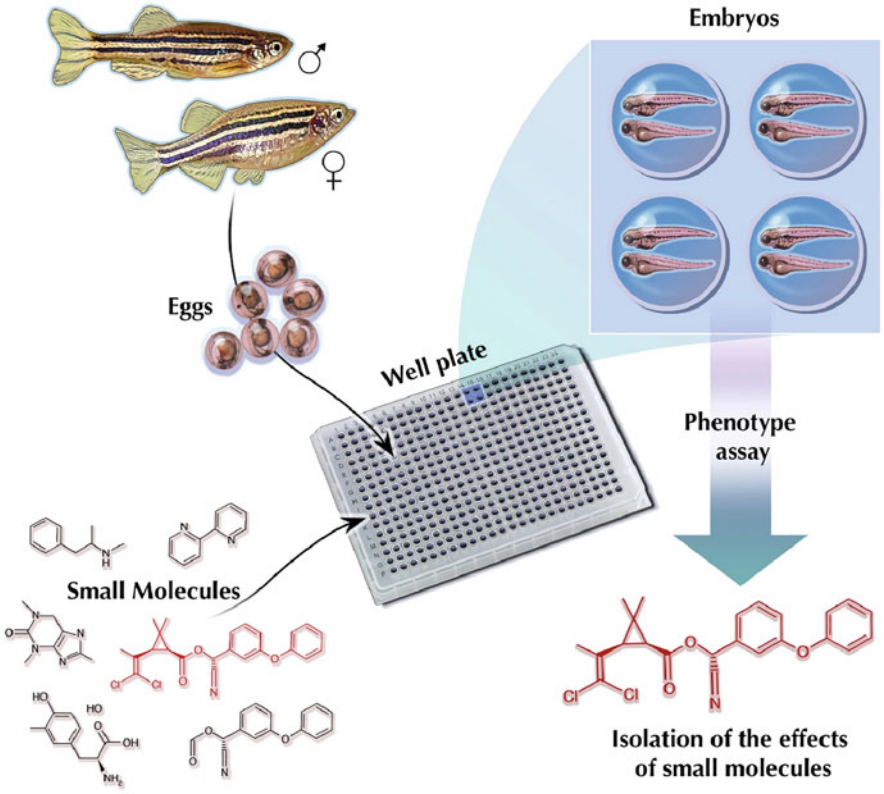

Fig. 7.2 Zebrafish as a tool for chemical genetics. (Reprinted from Kithcart and MacRae (2017) with permission from Elsevier)

by its isolation, will help to know its binding partners. Chemoinformatics can be employed in cases where the target protein of the compound is unknown. The activity of compounds is then analyzed by genetic studies such as real-time PCR and microarray analysis (Xie et al. 2015).

7.3.1 Chemical Screen for Identifying Novel Oncogenes

An understanding of the various oncogenes and other genes involved in tumorigenesis helps in development of more efficacious and targeted therapy for cancer. Zebrafish chemical screens have been employed for many cancer-related events such as angiogenesis and biological pathways like Hedgehog, Wnt/b-catenin, and Ras (Hao et al. 2013; Le et al. 2013; Tran et al. 2007). Chemical screens have also been successfully used for identifying therapeutic molecules for rhabdomyosarcoma, melanoma, prostate cancer, and leukemia (White et al. 2011; Gutierrez et al. 2014; Wang et al. 2010b).

In a zebrafish genetic screen for identifying novel genes involved in the process of tumorigenesis, Shepard et.al. identified a crash and burn (*crb*) mutant which results in mitotic arrest. The *crb* mutants also have a splice-donor mutation in the *bymb* gene due to which there is decreased expression of cyclin B1, resulting in mitotic arrest and genomic instability (Shepard et al. 2005). Later, Stern et al. screened 16,320 molecules of DIVERSetE library to identify chemical hits which interact with the *bymb* pathway. Embryos from heterozygous *crb* mutant parents were collected to set up the chemical screen. Immunohistochemistry staining of whole embryos to assay for mitotic cells was used to identify molecules which can suppress the mitotic arrest in *crb* mutants. One such lead molecule was identified and had no effect in wild-type embryos. This chemical was then resynthesized, and its chemical structure was validated by mass spectrometry, liquid chromatography, and nuclear magnetic resonance. The compound was named as persynthamide by Stern and colleagues (Stern et al. 2005).

Similarly, Le et al. set up a chemical screen to identify suppressors of RAS signaling, using embryos from transgenic *hsp70-HRAS*^{G12V} fish line. This screen resulted in identification of two compounds exhibiting anticancer efficacy against rhabdomyosarcoma, both in zebrafish and a human cell line model (Le et al. 2013). Alternately, Hao et al. used wild-type zebrafish embryos for chemical screening, to suppress oncogenic pathways active during normal embryo development. They identified small molecules exerting inhibitory effect on Wnt pathway, simply by assessing the morphological phenotype of chemically exposed embryos. A small molecule, Windorphen, was the outcome of this screen (Hao et al. 2013). Windorphen only affected tumor cells with perturbation in the Wnt pathway, such as the prostate cancer and colon cancer.

Though a small cell population in cancer tissues, cancer stem cells are responsible for tumor metastasis and relapse. Yeh et al. identified a small molecule inhibitor of AML1-ETO (AE), which is reported to exert oncogenic effects on multipotent hematopoietic progenitor cells. Using *Tg(hsp:AML1-ETO)* embryos, they identified nimesulide, which inhibited AE activity and prevented tumor formation of xenografted cancer (Yeh et al. 2009; Zhang et al. 2013).

7.3.2 Zebrafish as a Tool to Identify Melanoma Therapies

Zebrafish chemical screens allow simultaneous analysis of drug efficacy and toxicity, enabling faster clinical translational of the lead molecules identified. In a zebrafish chemical screen, White et al. identified a therapeutic molecule for melanoma (White et al. 2011). The molecule was already FDA approved.

Mutation in *BRAF(V600E)* oncogene is generally seen in melanoma (Davies et al. 2002). White and colleagues first studied the interaction between *BRAF(V600E)* and developmental transcriptional programs. In their study, they employed a transgenic zebrafish expressing *BRAF(V600E)* oncogene under the control of a melanocyte-specific promoter *mitf*. When this zebrafish line was crossed with *p53*$^{-/-}$ mutants, the resulting progeny developed melanoma. Neural crest progenitor *crestin* showed

aberrant expression in adult tumor cells. This implied that the tumor melanocyte cells were in the multipotent, neural crest progenitor state. Thus, this state of the melanoma tumor cells was maintained by BRAF(V600E) expression. Thus, the overlap between active neural crest transcriptional programs and BRAF expression, during Tg*(mitf:BRAF(V600E)); p53*$^{-/-}$ embryogenesis, mimicked the events of melanoma tumor initiation.

Assuming that inhibitors of neural crest progenitors would also exhibit anti-melanoma activity, a chemical screen using wild-type embryos was employed. Embryos exposed to chemicals were fixed for secondary ISH and the expression of *crestin* was observed. One molecule NSC210627, whose function was unknown, significantly downregulated *crestin* expression. Using chemoinformatic analysis, it was found that the structure of NSC210627 was similar to brequinar, which is an inhibitor of dihydroorotate dehydrogenase (DHODH) enzyme (McLean et al. 2001). Another DHODH inhibitor, leflunomide, showed similar effects in vivo as NSC210627 (Schiff et al. 2000). Leflunomide is an FDA-approved drug and was further characterized in vitro and in vivo for melanoma treatment. There was significant lack of melanocytes in leflunomide-treated zebrafish embryos at 36–48 hpf. Moreover, the treated embryos completely lacked melanocyte progenitors at 24 hpf. A771726 is the active ingredient of leflunomide. When tested in vitro in melanoma cell line model, A771726 was able to suppress tumor cell proliferation. In combination with PLX4720 (an inhibitor of BRAF(V600E)), A771726 showed synergistic reduction of tumor proliferation. Promising results were also seen in mouse xenograft model, wherein almost complete tumor regression was seen upon combination treatment of A771726 and PLX4720, in nearly 40% animals (White et al. 2011).

By modeling melanoma initiation in zebrafish embryos, White and colleagues were able to combine drug discovery and toxicity testing in a single platform (White et al. 2011). Combined with drug repurposing, the zebrafish-based chemical screen can help in rapid clinical translation of drugs. A short while after the zebrafish-based screening studies, leflunomide and PLX4720 entered clinical trial studies.

7.4 Genetic- and Transplantation-Based Cancer Models in Zebrafish

7.4.1 Liver Cancer

Liver cancer is considered as the second most emerging root of cancer-associated casualty. Most of the patients with hepatocellular carcinoma (HCC) are recognized at late stages when the curative treatments are almost impossible. The most widespread form of liver cancer initiates in hepatocytes which is termed as hepatocellular carcinoma. Out of all types of liver cancer cases, HCC contributes for 90–95%. Other types of liver cancer, like hepatoblastoma and intrahepatic cholangiocarcinoma, have less prevalence in comparison to HCC (Lu et al. 2015). Present animal models of metastasis are having different shortcomings that limit and

challenge our ability to be aware of the disease complexity. Zebrafish offers several advantages over other animal models like its small size, ability to generate hundreds of embryos from a single mating, low cost, and short life span, making them a highly acceptable experimental model for cancer research. Due to these advantages, zebrafish is progressively utilized for cancer modeling and drug discovery, especially xenografting cancer cell lines of humans, and may offer therapeutic and new scientific insights. Moreover, this model system remains sufficiently utilized (Yan et al. 2017; Spitsbergen and Kent 2003). Recently, an extensive model of liver cancer is developed by a group in which they developed a transgenic zebrafish cancer model specifically for liver cancer by inducing the oncogenic kras expression using mifepristone. For this approach, they used Cre-loxP system to make a permanent genomic recombination that will facilitate the experiments of liver cancer that originates from a small number of cells or a single cell through clonal expansion. In 20–40% cases of HCC, stimulating mutation in the gene encoding for β-catenin is reported. So moving forward to develop the same condition in zebrafish, Evason et al. designed a transgenic zebrafish cancer model that expresses the hepatocyte-specific activation of β-catenin. Extensively, they utilized same model to filter the druggable pathways that mediates liver cancer by modulating β-catenin activity and identified two antidepressants as promising therapeutics and two c-Jun N-terminal kinase (JNK) inhibitors for liver cancer (Nguyen et al. 2016; Evason et al. 2015). Hepatitis C virus (HCV) can directly confer to HCC by incorporating into the cancer-correlated genes like TERT and disrupt them. Transgenic zebrafish model exhibiting HCV core protein (HCP) generated the HCC two times more in contrast to wild-type controls when they were introduced to carcinogen thioacetamide. Modulation in the expression of Yes-associated protein (Yap) which is the transcriptional regulator central to the Hippo pathway currently comes out as one of the mechanisms through which cells counter to mechanical signals like substrate stiffness, even though the mutations in Hippo or Yap signaling pathway genes are less common in HCC (Farazi and DePinho 2006). In approximately 60% of HCC cases, immunohistochemical analysis of biopsies demonstrated the nuclear Yap staining, indicating functioning Yap signaling, implying transcriptional or posttranscriptional unregulation of Yap. To explore the impact of Yap on HCC, Cox AG et al. examined the Tg(-2.8fabp10a:yap1-1bS87A) transgenic line stating an activated form of Yap exclusively in hepatocytes. Overexpression of activated Yap specifically in hepatocytes induced hepatomegaly in larva and adult stage of zebrafish. Interestingly, after DMBA exposure to transgenic zebrafish causes tumor formation in the liver in an accelerated manner as compared to WT controls. Therefore, utilizing a transgenic zebrafish model in tumorigenesis accompanied by mechanistic investigation utilizing both larval and adult stage substitutes, the phenotypes recognized Yap signaling as a key player of HCC, instructive novel molecular and biochemical mechanisms, and validating novel chemical inhibition approaches (Cox and Goessling 2015; Cox et al. 2016).

7.4.2 Brain Cancer

Disorders of brain growth are known to root a broad variety of pathological and physiological symptoms that include the intractable intellectual disability, cognitive and motor impairment, autism, and different forms of epilepsies. Somatic mutations that activate the MAPK and PI3K signaling play a critical role in both brain developmental disorders and tumor initiation. Brain tumors comprise the most fatal forms of childhood cancer; still very little novel treatments of this disease have been advanced from the last 30 years. Gliomas are usual cause of primary malignant tumors of the CNS (central nervous system) affecting around 2000 people in Europe each year with a gross age-dependent frequency rate of 4.67–5.73 per 100,000 subjects (Ju et al. 2015). The World Health Organization classified gliomas according to their molecular and histopathological features in grades I to IV, where grades I and II correlate with slowly developing diffused gliomas which are slightly intrusive, and grades III–IV comprise extensively intrusive with highly malignant glioma state. Grade IV gliomas are also termed as glioblastoma multiforme (GBM); these are considered as maximally chronic and highly malignant gliomas, distinguished by an increased rate of proliferation, chemoresistance, and tumor angiogenesis (Kim et al. 2010). In vivo animal model using rats and mice to study brain tumor is very sophisticated and takes a long time that limits the disease understanding. In vivo tumor models using zebrafish provide an insight on how divergent genes, modified in a particular glioma context, interrelate to steer tumor progression. Approach to reproduce impulsive glioma progression in zebrafish using genetically engineered models is produced by somatic/germline alteration of endogenous key glioma genes, xenotransplantation, and transgenic models. Each of the zebrafish glioma models offers benefits for the assessment of infiltration in tumor, which is an attribute of all the tumors of the brain and facilitates prognostication; these alterations would demand costly and multifaceted sophisticated equipment in other systems. In vivo orthotopic grafting of human glioma cells in zebrafish is developed as a high impact technique to study cancer model. This approach presents the possibility to envisage tumor interactions and development of tumor concurrently within the microenvironment at single-cell level. For monitoring various features of glioma, zebrafish xenograft approaches are frequently employed together with transgenic models and drug therapeutics. In three distinct experimentations, it has been reported that how transplant of U87 or U251 glioblastoma cells in zebrafish transgenic model is utilized to see blood vessels *Tg(fli1:EGFP)*^{y1} can be a beneficial asset to assess the susceptibility to therapeutic intervention related to tumor invasiveness and angiogenesis (Idilli et al. 2017; Vittori et al. 2015). This model helps in understanding and evaluating proliferation of cells generating tumors, engaging blood vessels and the number of new vessel generation which offer supremacy to estimate the angiogenic ability of tumor cells and also the outcome of novel treatments. A comprehensive method described by Stewart and colleagues to spot the compounds that inhibit pediatric in vivo brain tumor growth, where they generated embryonic brain tumors through orthotopic transplantation of cells that are obtained from genetically engineered zebrafish models of the embryonic brain

tumors to tons of receiver embryos at 2 dpf. Native donors were originated by the expression of wild-type or oncogenic human NRAS that are steered by the *sox10* promoter together with $p53^{zdf1/zdf1}$ and $mitfa^{w2}$ background. The preliminary tumors generated in the CNS after 6 weeks revealed preservation of typical characteristics similar with human embryonic brain tumors in molecular and histological analysis. Stimulation of the RAS/MAPK pathway and elevated expression of *sox10/olig2* were also reported (Casey et al. 2017). Rapidly increasing development in reproducible xenotransplantation procedures of mouse and human glioblastoma cells has presented novel insights regarding the function of presumed glioma cancer stem cells in drug resistance and tumor deterioration. In this context, recently a primary brain cancer model has evolved after xenotransplantation to learn how to carefully transport siRNA through the blood-brain barrier (BBB), along with the purpose of knocking down a desired gene with good precision and less toxicity (Yang et al. 2017). Therefore, the zebrafish emerges as the model of diverse possibilities for the evolution of nanotechnological appliance to be utilized in vivo for genetic engineering. Moreover, zebrafish model of cancer can be employed for patient-tailored therapies in the future and could be helpful in predicting the cancer aggressiveness and disease progression (Fig. 7.3) (Kirchberger et al. 2017a).

7.4.3 Skin Cancer

Skin cancer, which most commonly includes squamous cell carcinoma (SCC) and melanoma, is a cutaneous malignancy around the globe, and the incidence rates are continuing to rise. Due to its high prevalence and mortality, melanoma is a leading worldwide health problem. The American Cancer Society, in 2018, estimated that around 91,270 people will be diagnosed with melanoma, and approximately 9320 (about 5990 men and 3330 women) citizens are expected to succumb of melanoma skin cancer (von Mässenhausen et al. 2016; Sarasamma et al. 2018). Generally, UV light persuades DNA damage in melanocytes and keratinocytes. Extreme UV exposure due to sunburn or other source leads to substantial loss of keratinocytes, but melanocytes can survive due to their efficient and well-regulated DNA damage repair response (Thompson et al. 2005; Maddodi and Setaluri 2008). Recently, in melanoma exome sequencing revealed high heterogeneity and identified a large number of recurrent somatic variants that were mostly found to be within the neuroblastoma RAS viral oncogene homolog (NRAS) and B-Raf proto-oncogene, serine/threonine kinase (BRAF) genes. However, nearly all of the recurrence cases are due to emerging multidrug resistance (MDR). Due to limited available experimental disease models, novel genes have not yet been exposed in human melanoma; therefore, a profound understanding of carcinogenesis in animal models is the need of the hour to tackle drug resistance in chemotherapy-based disease treatment (Berger et al. 2012). Hitherto, the existing animal models of melanoma have been Syrian hamster, Mongolian gerbil, mouse, platyfish opossum, medaka, and zebrafish. All of them have their own advantage and shortcomings in molecular delineation of melanoma and drug screening (Howe et al. 2013). Genetic zebrafish

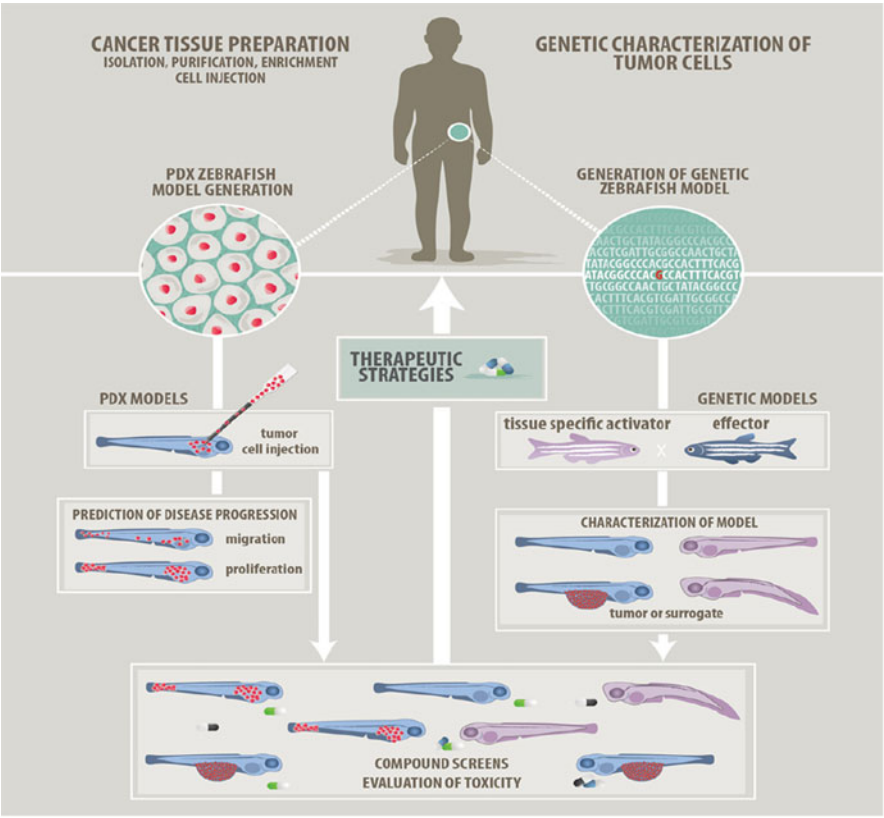

Fig. 7.3 Approaches to modeling cancer in zebrafish. (Reproduced from Kirchberger et al. (2017a) under Creative Commons license)

models are being exploited for the novel therapeutic targets and come out as narrative in vivo manifesto for identification of drug of melanoma. Melanoma is accountable for the fatality of above 70% of skin cancer patients, and surprisingly only 14% of patients having metastatic disease live for hardly 5 years. Contrary to various different types of tumors, rising patients and deaths related to melanoma are nevertheless increasing rapidly. Although some *NRAS*- and *BRAF*-related oncogenic driver mutations have been recognized in melanoma, the efficiency of treatment is restricted and the prediction of metastatic melanoma sufferer stands bad. Zebrafish offers some impetuous, oncogene-directed experimental melanoma models. Patton et al., in 2005, expressed $BRAF^{V600E}$ in melanocytes by utilizing microphthalmia-associated transcription factor a (*mitfa*) as a promoter. In the same way, human oncogenic $NRAS^{Q61K}$ expression guided by the monitoring of *mitfa* promoter emerged into a transgenic fish which need loss of p53 activity for the initiation of melanoma (Patton et al. 2005). Many scientists and researchers have concluded that zebrafish is the finest model to demonstrate studies related to melanoma. The nodal

inhibition advances the reprogramming of melanoma cells toward a melanocytic phenotype. Moreover, the nodal signaling plays a central role in the in vivo tumorigenicity and melanoma cell plasticity. The biosynthesis of melanin in vertebrates relies on the function of three distinct enzymes of tyrosinase family, dopachrome tautomerase (Dct or Tyrp2), tyrosinase, and tyrosinase related protein 1. The mutations in enzyme related to this tyrosinase family result in melanophore death, leading to semidominant phenotype in the zebrafish. Importantly, in terms of both molecular findings and histopathological signatures, zebrafish models for melanoma closely mimic the human cancer (Krauss et al. 2014).

7.4.4 Blood Cancer

Leukemia is the ninth most common type of cancer that usually involves defects or nonfunctioning of white blood cells (Miller et al. 2016). Majorly, leukemia has been studied via transgenesis. In the early 2000s, a zebrafish model of T-cell acute lymphoblastic leukemia (T-ALL), which is usual sort of leukemia in children, was developed by expliciting a mouse *c-Myc* transgene that is allowed to fuse with green fluorescent protein (GFP) under the controlled regulation of a zebrafish *rag2* promoter. Monitoring of GFP^+ leukemic cells has illustrated that leukemia arises in the thymus, metastasizes to the gill arches and adjoining retro-orbital soft tissue, and afterward extends to abdominal organs and skeletal muscle (Ridges et al. 2012b; Santoriello and Zon 2012). Feng et al. have upgraded this model by generating heat-inducible, conditional activation of the *c-Myc* oncogene that resulted in increased invasion of T-ALL and enhanced regulation of arrival of disease (Langenau et al. 2003b; Feng et al. 2007). In the same way, one more zebrafish model of T-ALL has been developed by the expression of the truncated human NOTCH1 protein fused to EGFP (ICN1-EGFP) under the regulation of the *rag2* promoter of zebrafish. By the age of 5 months, these transgenic zebrafish developed T-ALL; leukemia onset was significantly hastened up when crossed to zebrafish that overexpressed anti-apoptotic protein (Bcl2). In this model, the oncogenic synergy among NOTCH1 and Bcl2 reveals that genetic modifier screens might disclose other genes also that interrelate with NOTCH1 to stimulate T-ALL. These transgenic zebrafish models regulated by *rag2* promoter are compliant to genetic and drug screening to design individual specific therapeutic approach for lymphoma and leukemia patients (Tregnago and Manara 2016). In AML, cAMP response element binding protein is persistently overexpressed gene, though this is not clear whether overexpression solely is enough to cause leukemia. Tregnago C et al. developed a transgenic zebrafish model that was overexpressing cAMP response element binding protein in association with *spi1* myeloid promoter that led to deterioration of myelopoiesis in 79% of adult fish including 66% succeeding to monocytic leukemia (latency 9–14 months) almost the same as that of human disease pattern. These transgenic fish models illustrated a transcriptional indication with 20 distinctly expressed genes similar to pediatric AML, together with the CCAAT-enhancer-binding protein-δ (*c/ebpδ*). Induced *c/ebpδ* expression damages the myeloid differentiation that can be

inverted by silencing the *creb-c/ebpδ* axis; recognition of this *creb-c/ebpδ* axis in zebrafish AML resulted in characterizing *C/EBPδ* expression as a novel pediatric AML subgroup followed by justification in openly accessible patient databases by Tregnago et al. (Baeten and de Jong 2018; Walter and Kazianis 2001).

7.5 Genetic Predisposition to Cancer in Zebrafish

Fishes also are genetically predisposed to cancer, and this was first reported around 70 years ago, when hereditary melanoma was detected in hybrid *Xiphophorus* (platyfish) strain (Wittbrodt et al. 1992; Schartl et al. 1999; Driever et al. 1996). Later, in 1990, two ENU-based mutagenesis screens led to the identification of a large number of zebrafish mutants which exhibited a wide range of defects in developmental phenotypes. The two screens were simultaneously performed at Max Planck Institute (Tübingen, Germany) and Massachusetts General Hospital (Boston, MA) (Haffter et al. 1996; Nasevicius and Ekker 2000). These genetic screens were followed by a number of other zebrafish screens.

Overall, there are three approaches to identify cancer relevant gene mutations, namely, Targeting Induced Local Lesions IN Genomes (TILLING), insertional mutagenesis screens, and classic and gynogenetic haploid or diploid screens. TILLING is a reverse genetic-based screen, while the classic and gynogenetic haploid or diploid screens are based on forward genetics. The ENU-based screens performed at Boston and Tübingen were focused on the identification of recessive mutations in embryo of F_2 generation. This is because the genes which are required during early embryonic development are also essential in later life stages for regulation of cell growth and maintenance of tissue functions. Surprisingly, these same genes are most often found to be involved in cancer pathogenesis as well, emphasizing the role of developmental genes in cancer. Thus, such zebrafish screens which enable identification of genes in recessive embryonic screens are important for exploring novel genes which play a role in cancer biology as well.

7.5.1 Reverse Genetics and Insertional Mutagenesis Model

Reverse genetic approaches involve disruption of function of specific genes. The target gene can be either disrupted by transgenesis methods or knocked down by using antisense morpholinos. Morpholinos prevent translation of the target gene by binding to its mRNA. They do not cause RNaseH mediated degradation of the target mRNA, which is the case in RNA interference (RNAi). Gene knockdown by morpholinos is reported to be less toxic and at the same time more specific compared to other antisense approaches (Lee et al. 2002). One- or two-cell stage zebrafish embryos are injected with morpholinos to knock down a target gene. This helps to dissect and provide greater understanding of biological pathways involved in development. However, due to degradation of morpholinos, this method does not allow study of events beyond the initial few days of embryo development. Alternatively,

approaches for stable gene knockout have been recently developed. These involve direct microinjection into the embryo nuclei. However, these approaches require greater technical expertise (Ma et al. 2001; McCallum et al. 2000). Another reverse genetic strategy named TILLING, which was first described in plants (Oleykowski et al. 1998), is now being applied in zebrafish as well.

In TILLING, a male zebrafish is randomly mutagenized by ENU and then outcrossed (Fig. 7.4). The genomic library of the F_1 male fishes is screened for mutation in the gene of interest. The mutation can be screened either by sequencing or by using CEL I nuclease. This enzyme can cut DNA heteroduplexes, thus enabling identification of even single base pair differences between the mutant and wild-type alleles of the genes of interest (Golling et al. 2002). Since sperms can be cryopreserved, TILLING utilizes only the male zebrafish progenies. This allows repeated screening of the isolated genomic DNA while the sperms are cryopreserved. If the genomic DNA library harbors the mutation in desired gene, the particular sperms of the particular F_1 male progeny can be revived and fertilized in vitro with wild-type zebrafish eggs to recover the mutant zebrafish line. Using TILLING approach, Berghmans et al. successfully obtained two mutant zebrafish lines for tumor suppressor gene *tp53*, which is mutated in a large number of cancers (Berghmans et al. 2005a).

Another strategy of mutagenesis in zebrafish is insertional mutagenesis. In this approach, exogenous DNA is introduced using retroviral vectors (Amsterdam et al. 1999, 2004b). The retroviral insertional mutagenesis in zebrafish generally results in loss-of-function mutation due to DNA insertion into promoters. The DNA flanking the retroviral insert can be cloned using inverse PCR, followed by sequencing of cloned DNA. The DNA sequence can be compared with genetic sequences available in databases, to identify the disrupted gene. In a recent study, around 525 embryonic lethal mutations in 390 genes were identified with the help of insertional mutagenesis zebrafish screen (Goldsmith 2004). Initial identification of mutants was based on

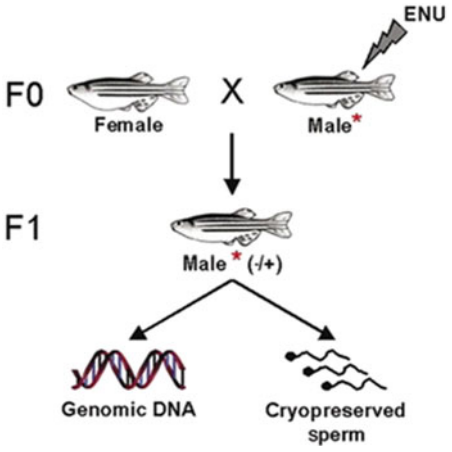

Fig. 7.4 TILLING, a reverse genetic approach for gene targeting in zebrafish. (Reprinted from Berghmans et al. (2005c) under Creative Commons license)

morphological assessment of embryos followed by examination of the genetic pathways affected.

7.5.2 Zebrafish for Chemical Modifier Screen

The zebrafish chemical modifier screens allow drug discovery (Peterson et al. 2004). For instance, Peterson et al. identified chemical inhibitor of *gridlock* mutation, using the chemical modifier screen in zebrafish (Druker et al. 1996). These mutants harbor a mutation in the *hey2* gene, which affects the aortic blood flow causing early embryonic lethality. Using this zebrafish, Peterson and colleagues were able to identify molecular therapeutics which are able to upregulate vascular endothelial growth factor or VEGF. These small molecular inhibitors were able to rescue the embryonic lethality and allowed normal heart development. The drug treated mutant embryos were able to grow into adult fishes. Similarly in case of cancer, *tp53* deficient embryos may be employed as chemical screens, and small molecules may be identified for their ability of tumor suppression and restoration of apoptosis. This approach may be helpful for identifying chemical hits for a wide range of cancers which involve loss of functional *tp53*. Likewise, transgenic zebrafish expressing oncogene can serve as a chemical screen for identifying small molecules which can inhibit the oncoprotein directly or target other effectors which function downstream of the oncoprotein. For example, transgenic *rag2-EGFP-mMyc* zebrafish chemical screens can help in identifying inhibitors for leukemia regression which can be monitored by the loss of GFP-labeled blasts.

There are many benefits of employing zebrafish for discovery of novel drug molecules. Many drug molecules need to be converted to their active form in order to exert their therapeutic effect. Such metabolic conversion is not possible in vitro and thus many drug molecules may be falsely regarded as ineffective. However, zebrafish chemical screens are a more fullproof platform for drug discovery as they allow metabolic conversion of drug to their active forms. Simple visual assessment of cancer phenotypes can help assess the effect of drug molecules in case of zebrafish chemical screens. Tumor initiation and progression can be easily tracked for GFP-labeled tumor cells. Also, in case of in vitro chemical screens, a prior knowledge of the protein, gene, or biological pathway to be targeted is required. For instance, in case of chronic myeloid leukemia, a prior knowledge or information about ABL kinase was important in order to design the drug imatinib (Gleevec®) (Lin et al. 2008). On the contrary, a rescue of the disease phenotype in mutant zebrafish is sufficient to identify a therapeutic molecule for a disease. This approach also allows discovery of novel drug targets or novel pathways which may play a role in disease pathophysiology. Furthermore, zebrafish-based chemical screens also allow the possibility of identifying two or more therapeutic molecules for the same disease but which act on different target proteins and signaling pathways. Thus, these drug combinations can then be explored further for circumventing drug resistance which might arise for the disease in study.

7.5.3 Zebrafish as a Tool to Study Oncogenomics

Genomic changes in human cancer have been identified by "The Cancer Genome Atlas" and other similar projects. However, the functional implications of these changes have not been analyzed yet. This can be carried out in zebrafish by two approaches – first, functional analysis of the candidate changes in vivo in transgenic zebrafish models and second is to compare the genomic alterations of human cancer and the zebrafish cancer model. Since a large number of zebrafish cancer models are now available, their genomes can be compared with human cancer genomes to find the common genomic alterations. The changes can be screened both in the DNA (such as mutations and copy number changes) and RNA (such as transcriptomic commonalities). This approach will filter the changes which are evolutionarily conserved across many years. Cell line models and zebrafish and mouse models can then be employed to test these abnormalities. The oncogenomic studies using zebrafish model has been conducted for many cancers such as melanoma (Ceol et al. 2011a), pancreatic cancer (Liu and Leach 2011), and T-cell acute lymphoblastic leukemia (T-ALL) (Rudner et al. 2011) and also to study the mechanism of drug resistance (Kansler et al. 2017; Heilmann et al. 2015).

7.5.4 Zebrafish as Tool to Study Cancer Drug Resistance and Epigenetic Modifications

Screening of anticancer drug molecules in phenotypic screens using zebrafish has provided researchers with a promising in vivo platform to trace epigenetic changes and effector molecules responsible for drug resistance.

Most cancer cells exhibit multiple drug resistance (MDR), arising as a consequence of drug efflux mechanisms, altered drug targets, drug metabolism, and enhanced DNA repair system, among others (Mansoori et al. 2017). Additionally, several combinations of these mechanisms might act in synergy to acquire drug resistance and even tumor relapse. Dissecting out the responsible modalities and the associated pathways using traditional mice models can be time-consuming and expensive. Zebrafish, on the other hand, provides a manageable and cost-effective stage to perform high-throughput drug screening. Due to its huge clutch size and ease of maintenance, many drug molecules can be screened altogether using drug-resistant patient-derived xenograft (PDX) models, and with regression of the tumor as a visual readout.

In a study, furadiene, a natural terpenoid, was shown to decrease levels of an efflux transporter, P-glycoprotein, thereby reversing MDR in a zebrafish model transplanted with cis-platinum-resistant human non-small cell lung cancer cells and Adriamycin-resistant human breast cancer cells (Zhu et al. 2019). Similarly, we can also identify multiple drugs with the same or different molecular targets in a single pathway to increase our chances of preventing multidrug resistance and tumor relapse (Berghmans et al. 2005b).

Epigenetic modifications in the cancer cell genome can also sometimes result in acquiring drug resistance. Mapping out these epigenetic modifications, like DNA modifications and histone modifications, remains a challenge in cancer biology. Zebrafish, with its traceable in vivo system and conserved epigenetic signatures, proves to be an excellent model organism for such studies (Chernyavskaya et al. 2016). It has been speculated that somatic mutation and change in certain epigenetic regulators' expression pattern is linked to the onset of certain cancers (Shih et al. 2012). In a screen for oncogenes in zebrafish, two histone methyltransferases (HMTs), SETDB1 and SUV39H1, with differential expression patterns in different cancer types, were experimentally shown to be involved in tumorigenesis (Ceol et al. 2011b; Albacker et al. 2013; Chiba et al. 2015). Furthermore, analyzing the cancer epigenome in zebrafish has been made possible by exploiting techniques like chromatin immunoprecipitation (ChIP), next-generation sequencing (NGS), and bisulfite sequencing PCR (BSP), with further improvements in progress (Chernyavskaya et al. 2016; White et al. 2013).

Although zebrafish as a research model comes with its limitations, it still proves to be a useful tool to carry out these studies. Its use in halting drug resistance and dissecting epigenetic modifications will have broad implications in our search for novel therapeutic targets in the future.

7.5.5 Screens Using Developmental Surrogate Markers

The zeal to identify novel anticancerous compounds using zebrafish has led to the development of several screens, with each of them employing a unique approach. One such strategy exploits the phenotypic similarities between early developmental processes and cancer metastasis, viz., epithelial-to-mesenchymal transition (EMT), migration, and many different orchestrated proliferative and anti-apoptotic pathways. Thus, analyzing the effect of small molecules on phenotypic screens with inhibition of developmental events as readout is an effective way to pool anti-metastatic compounds (Kirchberger et al. 2017b).

The posterior lateral line system has long been used to study migration and morphogenesis in zebrafish (Chitnis et al. 2012). In one study, a transgenic zebrafish line (*Tg(cldnb:EGFP)*) with GFP-labeled migrating posterior lateral line primordium (PLLp) was developed to screen for inhibitors of migration (Gallardo et al. 2015). A total of 2960 compounds were used to identify inhibitors of PLLp migration, out of which 165 compounds showed positive inhibition. To further verify the anti-metastatic property of these anti-migrating bioactive molecules, in vivo tumor implantation assays were performed in mice. A Src inhibitor SU6656, earlier identified in the phenotypic screen, was confirmed to have an anti-metastatic effect in mammary tumor cell lines. In another such experiment, Tenovin-6, a SIRT1 inhibitor, was found to block migration and inhibit the growth and spread of Ewing's sarcoma in a xenotransplanted transgenic zebrafish model (*Tg (fli1:EGFP)*) (Ban et al. 2014).

EMT is another developmental event tightly linked to metastasis. Use of EMT-based models in anti-metastatic screens has proved insightful in cancer research. In a study involving a double transgenic zebrafish model (*Tg(Fabp10A: mCherry-T2A-Twist1-ERT2)* × *Tg(fabp10a: TA; TRE:xmrk; krt4: GFP)*), 68 compounds were screened for their ability to block EMT and metastatic dissemination (Nakayama et al. 2020; Nakayama and Makinoshima 2020). Out of three hits, adrenosterone, a hydroxysteroid (11-beta) dehydrogenase 1 (HSD11b1) inhibitor, was identified to suppress both EMT and metastatic dissemination. In another research, TP-0903, a multi-kinase inhibitor, was found to inhibit EMT and dissemination in a transgenic zebrafish model (*Tg(snai1b:GFP)*) (Jimenez et al. 2016).

Researchers have also exploited the proliferative similarities between normal activated T-cells and T-cell acute lymphoblastic leukemia (T-ALL) cells to design antineoplastic screens. A transgenic zebrafish larvae model (*Tg(lck:GFP)*), highlighting the immature T-cells with GFP, was developed to screen 26400 small molecules for their ability to eliminate immature T-cells in the larvae and subsequently, prevention of T-ALL in adult models (Andrejeva and Rathmell 2017; Ridges et al. 2012c). Lenaldekar (LDK) was identified to be a positive hit with selectivity against cMYC-induced T-ALL in adult zebrafish.

Thus, it is clear that different developmental events can be used as surrogates for metastasis in various screens to identify novel anti-metastatic therapeutic leads.

7.6 Advantages of Zebrafish as a Tool for Cancer Research: Screen, Imaging, and Chemical Treatment in Search of Drug

Zebrafish as a model for cancer research has gained widespread attention, mainly due to its genetic susceptibility to cancer, and has embarked on an exciting journey in the field of cancer biology. Various methods employed to induce cancer in these vertebrates (transgenesis, carcinogen treatment, transplanting mammalian cancer cells, and reverse genetic methods like using morpholinos and TILLING (Berghmans et al. 2005b; Feitsma and Cuppen 2008b; Zhao et al. 2015)) have resulted in the successful generation of zebrafish cancer models. These models have eventually found their way in disease and chemical modifier screens and also in visualizing cancer progression.

The use of zebrafish in cancer modifier screens has shown a steady rise in its value. These modifier screens (like forward genetic screens and insertional mutagenesis screens) look for mutations that reverse or enhance the cancer phenotype and therefore help unravel novel tumor enhancer and suppressor modalities (Feitsma and Cuppen 2008b).

In addition to disease modifier screens, zebrafish are also vigorously used in chemical modifier screens in search of novel anticancer drug molecules. The amenability of zebrafish models for drug screening is due to its small body size and large clutch size aiding in a robust screening process (Zhao et al. 2015; Dang et al. 2016). As against in vitro chemical screens, in vivo chemical screens in zebrafish have

allowed researchers to identify chemicals that require in vivo metabolism to acquire its active form. More importantly, prior knowledge of the established disease-prone gene targets is not required (Dang et al. 2016). The screening focuses on a reciprocal approach that pins the reversion of disease phenotype by a drug molecule down to its corresponding gene equivalent. This approach allows researchers to discover drugs targeting novel pathways, or even multiple drugs acting on separate targets in the same or different pathways associated with cancer. These findings might help tackle drug resistance in patients by incorporating multiple drugs in disease treatment (Dang et al. 2016). Furthermore, mimicking patient-specific cancer mutations in zebrafish models and performing chemical screens might help achieve personalized medicine (Astone et al. 2017).

Perhaps, the most conspicuous advantage of zebrafish models over other cancer models is the production of optically clear embryos. This unique feature has given cancer research the power to visualize fluorescently labeled cancerous cells inside the embryo (Feitsma and Cuppen 2008b). In addition to conducting visual assays for specific cancers, it has also greatly aided in the direct visualization of the effect of a drug molecule on the tumor mass in chemical modifier screens. With the further development of pigment-free adult zebrafish (*casper* mutant), visualization of tumor progression and regression is now possible inside a live zebrafish using high-throughput fluorochrome-based techniques (White et al. 2008b).

7.7 Challenges and Limitations of Zebrafish Model in Cancer Research

Although the horizon of zebrafish as a cancer model is expanding, there are still many challenges preventing a smooth and full-fledged research experience. This section will encompass some of these obstacles. Zebrafish are poikilotherms and are maintained at 28°C as against homeothermic humans with a constant body temperature of 37°C. This temperature variation limits the use of zebrafish as cancer models in studies where there is a possible homeostatic temperature regulation of cancer phenotypes (Kirchberger et al. 2017b; Zhao et al. 2015; Astone et al. 2017).

In xenograft models, transplantation is preceded by immunosuppression treatment, to prevent rejection of the xenograft. Thus, the larva, with weak adaptive immunity, is used in most transplantation experiments that exclude the effect of adult tumor microenvironment in cancer progression (Kirchberger et al. 2017b; Zhao et al. 2015; Astone et al. 2017; White et al. 2008b; Potts and Bowman 2017).

Also, the organ system of zebrafish is much simpler than the mammalian system. Apart from the structural and functional simplicity in the existing organs, the absence of few organs like prostate, lungs, and breasts in zebrafish prevents study of human cancer phenotypes specific to these organs in zebrafish (White et al. 2013; Kirchberger et al. 2017b).

Furthermore, zebrafish have undergone an additional round of gene duplication, equipping them with an extra copy of a few genes complementary to its human equivalent (White et al. 2013; Kirchberger et al. 2017b). This genetic imbalance

makes loss-of-function studies of tumor suppressor genes difficult in these vertebrates. To counterbalance this obstacle, CRISPR-Cas9 technology for zebrafish is under development to ubiquitously delete all the gene copies of a particular gene from the genome to achieve the desired phenotype (Kirchberger et al. 2017b; Zhao et al. 2015; Potts and Bowman 2017). Besides, the zebrafish genome is approximately half the size of the human genome, thereby limiting gene studies to only the available genomic regions (White et al. 2013; Kirchberger et al. 2017b).

Another limitation of zebrafish lies in chemical screening studies. The insolubility of most drug molecules in water and their poor absorption by zebrafish via their skin and gills eliminate the possibility of water-insoluble drug molecules as potential hits. Additionally, the drug distribution across the body depends on the developmental stage, which, in turn, affects the chemical activity of the drug (Dang et al. 2016).

In the approach of introducing mutations to induce tumors, many of the homozygous mutations have proven to be lethal in the embryonic stage. The corresponding heterozygous mutants show a high predisposition to cancer only in the adult stage. This lethality constraint limits drug screening to heterozygous mutants in the adult stage which proves to be more expensive than the maintenance of zebrafish embryos or larva. In the case of homozygous mutants (where both the alleles of the gene need to be mutated), the production of conditional knockout models is under development (Kirchberger et al. 2017b). Although research is underway to eliminate these limitations, it might take a few decades until most of the barriers dissolve.

7.8 Future Perspectives and Summary

Zebrafish in mammalian cancer modeling has seen significant advances in the past few decades. Its small size, high fecundity, transparent embryos, high genetic homology with humans, and ease of genetic manipulation have made it an excellent tool for primary cancer studies (Gallardo et al. 2015). However, its limitations, as mentioned in the above section, restrict its full exploitation in elucidating the molecular and cellular players in tumorigenesis. Mentioned below are a few points that might help advance cancer research in zebrafish models.

- Generation of transgenic models capable of surviving at higher temperatures.
- A detailed study of the duplicated genes in zebrafish will help understand the functional role that these genes play in their development.
- Use of immunosuppressed mutant models, before xenotransplantation, to study cancer progression in adult zebrafish.
- Pharmacokinetic and pharmacodynamic studies of drug molecules in zebrafish models to study drug distribution and activity.
- Identification of embryonic and adult microenvironmental and cellular effects on cancer phenotype to understand the detailed regulation of tumorigenesis in zebrafish.

The field of zebrafish-based cancer research is up- and coming with a strong potential to carve out new paths in cancer treatment. With further improvisations in genetic manipulation and technological advances, this modality will conquer greater heights in cancer biology.

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Zebrafish as an Indispensable Tool for Infectious Diseases and Immune Modulatory Studies

8

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Abstract

Understanding the mechanism, host-pathogen interaction and spread of an infection is essential in order to design approaches to eradicate the infection and the causative agent. Animal models have been promising tools to analyse and study the immune reactions and other physiological changes that occur during and after an attack by pathogenic microorganisms. Nevertheless, animal models like rat, mice, rabbits, guinea pigs, etc. consume cost, well-trained labour and specialized housing for maintenance, which might hinder immediate evaluation of promising candidate compounds that were effective in vitro. *Danio rerio*, commonly called as zebrafish, has become an ideal model to study infectious diseases by virtue of its 70% genetic homology to humans, flexibility to genome editing and ease of maintenance. Transparent nature of embryonic/larval stage facilitates tracing the spread of pathogen and to study the innate immune responses which has garnered increased attention on zebrafish embryos. A broad range of bacterial species like *Mycobacterium marinum, Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Aeromonas hydrophila* and *Pseudomonas aeruginosa* has been studied using adult zebrafish and embryos. Mechanism of disease pathogenesis caused by herpes, chikungunya, SARS-CoV-2 viruses and fungal infections caused by *Cryptococcus neoformans* and *Candida albicans* has also been extensively studied using zebrafish model. Effectiveness of proposed treatments and

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alteration in expression levels of IFN-γ, IL-1β, IL-6, IL-15, TNF-α, COX-2a, and TLR-4a can be analysed to decipher any successive immunomodulatory changes. Thus, zebrafish proves to be an exceptional tool to explore mechanisms of various infectious diseases and for immunomodulatory studies and is equally reliable to other animal models owing to its unique advantages.

Keywords

Zebrafish · Animal model · Infectious disease · Immunomodulation · Transparent embryo · Host-pathogen interaction

8.1 Suitability of Zebrafish in Infectious Diseases Research

Danio rerio, commonly known as zebrafish, has emerged as a prime model in biomedical research, encompassing areas that are directly relevant to human diseases. One of the major advantages of zebrafish is its similarities to humans that has paved the way for considering zebrafish as a model for understanding host-pathogen interactions (Sullivan and Kim 2008). Zebrafish genetics has opened up novel possibilities to its research applications. What makes zebrafish a go-to laboratory model is the ease of use and culturing and inexpensive techniques required for its rearing. Added to this are the transparent embryos that can be easily monitored for a variety of applications. Emergence and more importantly re-emergence of infectious agents have necessitated a continuous research into the discovery and development of novel antimicrobials, and zebrafish, including all its life stages, has tremendously aided our understanding of host-pathogen interaction, disease mechanism, effectiveness of antimicrobials and drug toxicities. Due to conserved genetic homology and function (Howe et al. 2013), zebrafish has become an ideal model to study infectious processes and immune responses. Also, it is easy to apply forward and reverse genetics and newer genome-editing techniques (Gaj et al. 2013), resulting in the production of a variety of transgenic models (Mathias et al. 2006) that are well suited for studying various innate immune mechanisms (Novoa and Figueras 2012). Gene knockouts with high frequency (75–99%) can be achieved using CRSPR/Cas system in zebrafish (Hwang et al. 2013). In fact, it was also successfully shown that targeted knock-ins in zebrafish, to induce single nucleotide polymorphism (SNP) changes by homology directed repair using CRISPR/Cas system, were indeed possible, which highlighted the versatility and suitability of these models for genetic research.

Innate immunity is the first line of defence in zebrafish post-fertilization (Herbomel et al. 1999), and acquired immunity comes into effect around 5 week post-fertilization (Lieschke and Currie 2007). Thus, this separation of the immune system post-fertilization allows for better delineation and understanding of these systems when challenged with infectious agents. Owing to the similarities with mammalian immune system in general and humans in particular, a lot of studies on infectious diseases have utilized zebrafish as model for deciphering the nature and

role of innate and adaptive immune components. Zebrafish innate immune cells are mainly comprised of neutrophils (Renshaw et al. 2006), macrophages (Herbomel et al. 1999), eosinophils and mast cells (Dobson et al. 2008; Balla et al. 2010). All these cells represent potent defence against infectious agents and are well studied in humans too. Under inflammatory conditions, these cells are known to migrate to the wound site as seen with higher animals. Similarly, varieties of protein and genes that are part of the innate immune responses in humans and other mammals have been identified in zebrafish too. Immune components such as toll-like signalling pathway, interferon signalling pathway, etc. have been identified in zebrafish (Stein et al. 2007). Other innate immune molecules such as complement (Sun et al. 2010) and mannan-binding lectin (Wang et al. 2009) too have been described in zebrafish. Antimicrobial proteins (Zou et al. 2007) and pattern-recognition receptors (Chang et al. 2007) are also similar to that observed in mammals. Such similarities with mammals have facilitated the emergence of zebrafish as a viable alternate to other models of infectious diseases that are more expensive and complicated. Indeed, there are numerous studies that have used zebrafish as a model for studying viral, fungal and bacterial pathogens that are of clinical importance.

As far as viral pathogens are concerned, most studies have looked at virus affecting aquaculture systems (Novoa and Figueras 2012), while fungal pathogens such as *Candida albicans* (Prasath et al. 2021), *Aspergillus* (Thrikawala and Rosowski 2020) and *Cryptococcus neoformans (*Rossi et al. 2020*)* and fungus causing mucormycosis (López-Muñoz et al. 2018) too have been studied. When compared to these two groups, bacterial infections have been extensively studied in zebrafish, and there is a vast amount of literature on bacterial infectious diseases, which will be discussed in the later sections.

Further, as mentioned before, genetic techniques can be easily applied on zebrafish enabling the rapid generation of 'knockouts' (Deiters and Yoder 2006), which can then specifically be used for studying a particular infection. Use of microarrays post-infection has enabled clearer understanding of the host-pathogen interactions earlier (Ordas et al. 2011), as has been described with higher animals. After the advent of RNA seq, microarrays have been widely replaced, and by using a combination of histology techniques, low input RNA sequencing and mathematical reconstruction, a high-resolution genome-wide 3D RNA tomography was successfully obtained in zebrafish embryos (Junker et al. 2014). Zebrafish as an infectious disease model also allows for easy creation of transgenics, and this, together with the application of siRNA, has vastly improved molecular changes and their characterization during infections (Levraud et al. 2008; Chang and Nie 2008). The use of embryonic stages of zebrafish for chemical screens (Bowman and Zon 2010) has enabled quicker selection and development of antimicrobials. Live imaging is a common strategy employed for zebrafish due to its 'transparent' body (Singer et al. 2010), and zebrafish have been used for studying a wide variety of infectious agents that are known to cause serious and often fatal infections in humans such as lung infections (Renshaw et al. 2007; Balkrishna et al. 2020), cardiomyopathy (Friedrichs et al. 2009; Santiago et al. 2021), septic shock (Walters et al. 2010; Wang et al. 2020) and those affecting the gastrointestinal tract (Fleming et al. 2010; Kaser et al. 2010;

Tonon et al. 2020). Putting together all these evidence clearly shows the usefulness and importance of zebrafish as a crucial model for not only studying human infectious diseases but also for finding possible cure.

8.1.1 Bacterial Infections

A wide variety of Gram-positive and Gram-negative pathogens have been studied using zebrafish as a model system, mainly to understand host-pathogen interactions, and there are numerous reports available on using zebrafish as model for bacterial infections. *Mycobacterium marinum*, a natural pathogen to zebrafish, is the closest relative to *Mycobacterium tuberculosis* genetically and can cause tuberculosis-like disease in adult zebrafish similar to that caused in humans (Ramakrishnan 2012). Attempts to study mycobacterial pathogenesis using zebrafish larva revealed the recruitment of macrophage to the site of infection, and transfer of the infected macrophages to deeper tissue, which causes granuloma formation (Clay et al. 2007). Zebrafish model of infection with *M. marinum* improved our understanding of granuloma; using zebrafish larva, Prof. Lalita Ramakrishnan's group had shown that *Mycobacterium* spp. use granuloma as a bait to attract macrophages by chemotaxis and infect them, thereby easily disseminating the pathogen rather than the previously held notion that immune cells of granuloma effectively quarantines the pathogen (Ramakrishnan 2012). Evaluation of pathogenesis and antimicrobial efficiency against *M. marinum* can be studied by infecting zebrafish larva by microinjection of fluorescent protein expressing *M. marinum* either to the hindbrain ventricle or to the caudal vein and can be analysed using high-throughput fluorescence microscopy or calculating intracellular bacterial burden (Takaki et al. 2013). In order to take advantage of optical transparency and to perform a broad-based genetic screen, zebrafish embryos were injected with *M. marinum*. The model revealed that regardless of lack of lymphocytes in embryonic state, macrophage aggregates were formed that initiate granuloma formation and granuloma-specific genes were found to be activated. These observations exposed the sole involvement of innate immunity in granuloma formation (Davis et al. 2002). *A. hydrophila*, a causative bacteria for gastroenteritis and tissue infections, was injected through different routes of infection – duct of Cuvier, caudal vein, notochord, muscle and bath infection to healthy and injured zebrafish larva (4 dpf) (Saraceni et al. 2016). Localized infections were seen in injections of organs and systemic infection in case of duct of Cuvier injection, whereas natural means of infection were obtained by bath infection. Higher expression levels of IL1-β and TNF-α were observed in infected injured larva compared to uninfected injured larva, and hence increased recruitment of neutrophils to wounds of injured larva was noticed (Saraceni et al. 2016). Pathogenicity and host resistance of *Staphylococcus aureus* have also been studied using zebrafish embryo model (Prajsnar et al. 2008). *S. aureus* injected to the yolk of the embryo showed a logarithmic increase of CFU/embryo, which reached a plateau around 18 h and all embryo were dead by 42 h, whereas systemic injection of the bacteria made the host to defend the infectious dose and hence the embryos

survived until 44 h (Prajsnar et al. 2008). In addition, bacterial mutants of *perR* and *pheP*, corresponding to peroxide regulon repressor and phenylalanine permease, respectively, were unable to grow in vivo, and a significant tolerance was observed in the embryo. A recent study reported the development of humanized zebrafish model to study host specificity of the virulence factors in vivo. It was found that the neutrophil-specific expression of human C5a receptor confers susceptibility to PVL and HIgCB toxins of *S. aureus*, thus reducing the neutrophil counts at the site of infection leading to increased mortality (Buchan et al. 2021). Pathogenesis of *Streptococcus* was studied in zebrafish using *Streptococcus iniae* and *Streptococcus pyogenes* by injecting it into the dorsal muscle. The pattern of pathogenesis of the former was found to be similar to that of human streptococcal infections, viz. initial focal necrotic lesion progressing to other organs including the brain, and the disease by the latter was observed by absence of inflammation with early extensive myonecrosis (Neely et al. 2002). Another group found that the lack of *ptpn6* (corresponds to Src homology region 2 domain-containing phosphatase 1—SHP1, a tight regulator of innate immune response) hyperactivated innate immune system and weakened the control of bacterial infections in zebrafish embryos, when infected with *Salmonella typhimurium* or *M. marinum* (Herman Spaink et al. 2013). A recent report focused on the plasmid transfer among the zebrafish microbiome in vitro and in vivo, as an attempt to understand the transfer of antibiotic resistance genes (Loftie-Eaton et al. 2021). *Escherichia coli* containing pB10::*gfp* was mixed with the food of zebrafish, and they found that plasmid was transferred only to *Achromobacter* sp., a rare gut microbe (Loftie-Eaton et al. 2021). Involvement of adhesion factors like F pili in emergence of probiotic bacteria (*E. coli*) even after infecting with virulent *Edwardsiella ictaluri* in zebrafish larvae has been identified using oro-intestinal route model (Rendueles et al. 2012). Expression of virulence in *Klebsiella pneumoniae* has been studied in zebrafish embryo model (Anes et al. 2020; Marcoleta et al. 2018). A study evaluated the correlation between bacterial colistin resistance and zebrafish embryo survival upon infection (Leber 2019). They found that infection by both colistin resistant and colistin susceptible *K. pneumoniae* neither altered bacterial growth nor affected embryo survival. Though multiple bacterial species have been studied using zebrafish model, they are resistant to certain infections. A recent study revealed that intravitreal injection of *S. aureus* did not induce endophthalmitis, and it was found that the bacterial burden increased significantly 8 h post-infection but reduced drastically thereafter, depicting clearance of the pathogen by mounting monocyte/macrophage response (Mei et al. 2019). Apart from host-pathogen interactions and evaluation of bacterial infections, zebrafish have also been used in studying the microbiota-gut-brain axis (MGBA), which is a bidirectional signalling pathway that mediates the interaction of the microbiota, intestine and central nervous system (Lee et al. 2021). In addition, zebrafish has also been utilized as an in vivo model to analyse the effectiveness of a treatment against bacterial infection. β-Lactamase inhibiting activity against *S. aureus* infection by Kalafungin, isolated from *Streptomyces* sp. derived from marine sponge, was proved using zebrafish model (Jabila Mary et al. 2021). Effectiveness of phage therapy against *Pseudomonas aeruginosa* has been validated

by establishing cystic fibrosis model in zebrafish embryos, and a combination of phage with streptomycin against *K. pneumoniae* has been evaluated in adult zebrafish (Cafora et al. 2019). Combination of colistin + salicylate + curcumin was able to curtail multidrug resistant (MDR) and colistin resistant *Enterobacteriaceae* in adult zebrafish infection, as evidenced by reduction in bacterial bioburden (Sundaramoorthy et al. 2020). Similar studies have been performed by various other groups using zebrafish as a model to validate different treatment combinations in curtailing bacterial infections of MDR *E. coli*, *K. pneumoniae*, *S. aureus*, *Vibrio cholerae* and *Pseudomonas aeruginosa* (Sundaramoorthy et al. 2019a, b; Lowrence et al. 2016; Clatworthy et al. 2009; Ravindran 2016).

8.1.2 Viral Infections

Many viral diseases of fish have been extensively studied using zebrafish as a model. Reports have shown upregulation of *tlr3* in zebrafish in response to snakehead rhabdovirus (SHRV), viral haemorrhagic septicaemia virus (VHSV), cyprinid herpesvirus (CyHV) and spring viremia of carp virus (SVCV) (Varela et al. 2017; Zou and Nie 2017; Rakus et al. 2019). Apart from natural fish viruses, human viruses like influenza A virus (IAV), herpes simplex virus (HSV), Chikungunya virus (CHIKV) and vesicular stomatitis virus (VSV) have been studied in adult and embryo of zebrafish (Guerra-Varela et al. 2018; Antoine et al. 2014). Injection of IAV (H1N1 or H3N2) to bloodstream of embryos 2 days post-fertilization (dpf) increased viral load and mortality (Gabor et al. 2014). Moreover, the pathology of viral infected zebrafish reiterated that of human clinical symptoms, and the mortality of zebrafish was reduced by administering an anti-influenza compound, zanamivir (Gabor et al. 2014). Human noroviruses (HuNoVs) are one of the major contributors to foodborne diseases. Zebrafish larvae were able to support the replication of these viruses reaching a peak 2 days after infection and were detectable until 6 days. The virus was detected in haematopoietic lineage and in the intestine, which was eradicated by a viral polymerase inhibitor 2′-C-methylcytidine (2CMC) (Van Dycke et al. 2019). CHIKV was injected in zebrafish larva 72 hpf through the dorsal aorta or caudal vein and live imaging was performed (Palha et al. 2013). Viral load was detected in various organs 14 h post-infection, and as time progressed, the infection was found to shift towards the brain. Type I interferon expression was triggered by CHIKV infection, a condition similar to mammals, and neutrophils were found to play a dominant role in CHIKV repression (Palha et al. 2013). Another study performed imaging of neuroinvasion of Sindbis virus (SINV) and CHIKV, which are associated with neuropathies, in zebrafish and found that both viruses access the central nervous system (CNS) in macrophage-independent manner (Passoni et al. 2017). CHIKV was found to infect the endothelial cells of brain vasculature, whereas SINV efficiently utilized the axonal transport from periphery to CNS and between neural tissues. Thus, zebrafish model has helped in discerning the mechanism of neuroinvasion by two related human viruses. Another interesting study revealed that zebrafish lacking adaptive immunity ($ragl^{-/-}$) was successful in preventing

viral infection by rapid response to viral infection, increased survival, NK cell and macrophage mediated response and enhanced apoptosis by overexpression of CASPASE-3 against SVCV (García-Valtanen et al. 2017). They found that the resistance to viral infection in adaptive immunity lacking zebrafish increased with their age. Zebrafish model to study hepatitis C virus (HCV), a major risk factor for hepatocellular cancer, has also been developed (Ding et al. 2015). Briefly, a HCV sub-replicon gene construct was developed with the four essential genes of HCV replication—5′ untranslated region (UTR), the core protein, the non-structural protein 5B (NS5B) and 3′UTR, with a reporter green fluorescent protein (GFP) gene – and was transcribed using a mouse promoter. This vector was microinjected to zebrafish larvae and the virus in the liver was visualized by fluorescence. Amplification of the sub-replicon showed gene expression changes similar to that of humans (Ding et al. 2015). This model can be used to study the mechanisms of HCV and to screen anti-HCV drugs by selecting drugs that can inhibit sub-replicon amplification (Ding et al. 2011). Zebrafish was also recommended as a model to screen for drugs against the virus causing the pandemic—SARS-CoV-2 (Galindo-Villegas 2020). Exposing zebrafish larva to SARS-CoV-2 spike receptor binding domain (RBD) was able to elevate heart rate, and captopril treatment, an angiotensin-converting enzyme inhibitor, overturned this effect (Kraus et al. 2020). The group also found that SARS-CoV-2 administered intranasally induced severe olfactory and renal histopathology, and the fish became hyposmic to anosmic from few minutes to a day to a spectrum of odorants. RNA seq revealed loss of olfactory receptor expression and inflammatory response in endothelial and myeloid cell cluster. In addition, SARS-CoV-2 exposure in water to zebrafish larvae did not support active replication; nevertheless, inhibition of *ace2* was observed (Kraus et al. 2020). Xenotransplantation of human alveolar epithelial cells was performed by intramuscular injection at the posterior lobe of zebrafish swim bladder to develop a humanized zebrafish (Balkrishna et al. 2020). Seven days post transplantation, the fish were injected with SARS-CoV-2 spike protein and tested if Coronil, a tri-herbal medicine (*Withania somnifera* L. Dunai, *Tinospora cordifolia* (Wild) and *Ocimum sanctum* L.), could protect from infection. They found that Coronil possessed immunomodulatory property by weakening IL-1β induced IL-6 and TNF-α cytokine and TNF-α induced NF-κB/AP-1 transcriptional activity (Balkrishna et al. 2020).

8.1.3 Fungal Infections

Though fungal infections have not been extensively studied using zebrafish as bacterial infections, pathogenesis and infection by few of the common fungal pathogens have been reported. *Cryptococcus neoformans*, a substantial human fungal pathogen in immunocompromised patients, is known to cross the blood-brain barrier (BBB) and can potentially cause meningitis. Zebrafish embryos 2 dpf were injected with *C. neoformans* (with mCherry) into the caudal vein, and a time-lapse microscopy was performed (Tenor et al. 2015). The pathogen was able to replicate in the larvae and also intracellularly in the macrophages. The colonization

was observed in the cranial vasculature, brain tissues and ventricles by 4 dpi. *C. neoformans* lacking a putative *fnx1* gene was not able to permeabilize BBB in zebrafish, which can be correlated to an early finding from a mice model of cryptococcal meningoencephalitis (Tenor et al. 2015; Tseng et al. 2012). Another study was performed with a transgenic zebrafish containing fluorescently (mCherry) labelled macrophage membrane, and the embryo 2 dpf was injected in the yolk sac circulation valley with GFP expressing and capsule induced *C. neoformans* and visualized using time-lapse microscopy (Bojarczuk et al. 2016). The authors observed that the macrophages phagocytose cryptococci with smaller capsules, whose size increases 24 hpi and thus restraining further phagocytosis. Involvement of endothelial cells, macrophages and neutrophils in sustained fungemia by cryptococcal infection was discerned using a zebrafish model (Davis et al. 2016). The harvested spores or yeast (with nuclear localized GFP expression) was injected intravenously and in the hindbrain of embryos of transgenic zebrafish (with mCherry) 28 hpf. Both inoculum types survived and were able to replicate intracellularly with a sustained low-level fungemia, which followed a pattern of repeated escape and uptake by macrophages. Endothelial cells have shown to be an important forte for the pathogen survival, and the circulating yeast gets collected in brain vasculature, thus invading CNS, whereas neutrophils do not play a dominant role in fungemia (Davis et al. 2016). *Candida albicans* was able to colonize and invade zebrafish at different anatomical sites—the liver, muscles, gastrointestinal tract and connective tissue—when injected into peritoneal cavities (Chao et al. 2010). The increase in *C. albicans* was rapid between 15 and 23 hpi, and the *C. albicans* transition from unicellular yeast to filamentous hyphae was observed, which is one of the dominant virulence factors. At 2 hpi, the yeast form was noticed in the liver surface and at 8 hpi it switched to hyphae and started liver invasion. In addition, the infected fish showed an upregulation of IL-1β, TNF-α and iNOS expression, whereas a downregulation of IFN-γ was seen and the results went in hand with that of mice model. Aspergillosis is another fungal infection which can affect lungs and can spread to other parts of the body. Zebrafish has been found susceptible to *Aspergillus* sp. and disease progression can be studied (Thrikawala and Rosowski 2020). Non-invasive real-time imaging of zebrafish embryo infected with *Aspergillus fumigatus* and *Aspergillus niger* was performed (Koch et al. 2019). The infection was provided by injecting the conidia to the hindbrain ventricle through the otic vesicle of embryos 30 hpf and was monitored for 5 dpf. The group found that *A. fumigatus* was significantly phagocytized than *A. niger*, but *A. fumigatus* conidia germinated and was able to form hyphae within the macrophages, leading to severe infection. It was discerned that *A. niger* was dependent on extracellular germination and hyphal growth, and lack of a cell wall component, galactofuranose, weakened the pathogenesis.

8.2 Immunomodulatory Studies

Apart from infectious studies, zebrafish has been used extensively to understand immunomodulational activities. This is due to the optical transparency during its early development, ability to inactivate genes, use of transgenic fish and haematopoietic lineage identification, and reports have shown that the results obtained are similar in response to murine models (Trede et al. 2004). Gene inactivation can be performed by injecting morpholinos (MOs—modified antisense DNA oligonucleotides), which will inactivate regulatory genes of immune system and hence can help us to study the effect of gene functions. Targeting-induced local lesions in genomes (TILLING) technique utilizes a reverse genetics approach that identifies induced point mutation by chemical mutagenesis, which alters the function of the gene. This technique has been used in different animal models including zebrafish to detect natural polymorphisms in human (Kuroyanagi et al. 2013). Immunizing the adult zebrafish with haemocyanin from *Concholepas concholepas* (CCH) has shown to protect the fish when infected with *Francisella noatunensis* subsp. *orientalis*, a causative agent of a chronic granulomatous disease—francisellosis (Lagos et al. 2017). Moreover, a higher transcriptional level of IFN-γ, IL12a and TNF-α at 1 dpi was observed in the kidney and spleen of the immunized fish. These studies show the immunoregulatory property of haemocyanin and its potential use as a vaccine. Excretory/secretory (ES) compounds were isolated from third stage larvae of the anisakid nematode *Contracaecum osculatum*, which parasitizes the liver and was evaluated for its immunomodulatory property in LPS-induced inflammation model of an adult zebrafish (Mehrdana et al. 2017). Intraperitoneal injection of LPS with ES altered the expression of genes affecting Th1, Th2, Th17 and innate immune responses. This study discerned the immune regulating ability of ES due to which the parasite becomes less susceptible to host immune responses. Studies in zebrafish have shown the immunomodulatory cell response to retinal neuron cell death can promote human eye repair (White et al. 2017). The retinal microglia is the macrophage that resides in the retina and can regulate responsiveness of retinal Muller glia, which has a potential to retain neuronal regeneration, thus controlling photoreceptor regeneration kinetics. The study was performed using an inducible cell-type-specific ablation system to zebrafish that makes it suitable for degenerative diseases by a cell-specific transgenic expression of bacterial prodrug converting enzyme nitroreductase (NTR) (White et al. 2017). Exposing larvae of such fish to prodrug like metronidazole (Mtz) specifically eliminated NTR expressing cells. Further, retinal punctures were made by inserting glass capillary through ventral conjunctiva and to the retina followed by intravital imaging. The evasion of immune host response by the bacteria has also been evaluated using zebrafish. A gnotobiotic larval zebrafish model was adopted and found that a protein AimA, secreted by *Aeromonas*, had an immunomodulatory activity (Rolig et al. 2018). AimA was found to be required during colonization in order to prevent intestinal inflammation that is mutually essential for survival of host and the bacteria. When AimA was administered exogenously, it prevented excessive neutrophil accumulation in the intestine and protected against septic shock in

induced intestinal inflammation. Structural analysis of AimA revealed that it was similar to mammalian immunomodulatory protein – lipocalin 2. Rice husk silica (RHS) has shown to enhance innate immunity in zebrafish by increasing expression of IL-1β, IL-6, IL-15, TNF-α, COX-2a, TLR-4a, lysozyme and complement C3b and improved resistance against *Streptococcus iniae* and *A. hydrophila* (Hong et al. 2019). Another recent study showed that fucoidan, fucose-rich sulphated polysaccharides from brown seaweeds, decreased the expression levels of IL-1β in the intestine of adult zebrafish and altered the intestinal microbiota composition (Ikeda-Ohtsubo et al. 2020). The exopolysaccharides of lactic acid bacteria, was observed to protect the host from gastrointestinal stress and against pathogenic infections. The probiotic and immunomodulatory property of 2-substituted (1,3)-β-D-glucan produced by *Pediococcus parvulus* 2.6 was studied using a gnotobiotic and transgenic zebrafish model (Pérez-Ramos et al. 2018). Treatment of larvae with purified β-glucan showed positive effect in competition with *Vibrio anguillarum*. It also altered the expression of pro-inflammatory cytokines and possessed anti-inflammatory effect in induced zebrafish inflammation model.

8.3 Conclusion

Zebrafish by virtue of 70% homology with human genes and 84% homology with disease-associated genes in humans, ease of maintenance, amenability for genetic screen and similarity of innate and adaptive immune response with humans serves as an indispensable model for studying infectious diseases. Although its utility in bacterial infections is evident, its use as a model for viral infections, especially those that infect humans, is really interesting and underscores the importance of zebrafish as a model for infection studies. Relative to other animal models, transparency of the embryonic/larval stages permits tracking of pathogen dissemination and also allows visualization of interaction of pathogen with fluorescently labelled immune cells which enhances our understanding of microbial pathogenesis and host-pathogen interaction, which is absent in other animal models, and this advantage catapults zebrafish as a very vital and indispensable model organism for exploring microbial mediated infectious diseases.

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Zebrafish: Promising Model for Cancer Research

9

Mayuri Gurav and Vaishali Undale

Abstract

The evolutionary conservation between human and zebrafish genomes has resulted in emergence of zebrafish model as a valuable human cancer model. Extensive molecular pathways for onset as well as progression of human cancer can be developed and studied using zebrafish model. The transparency of the zebrafish permits direct evaluation of cancer development and progression. Small molecules can also be screened as compounds can be directly added to fish water and administered to adult fish.

The zebrafish embryo is a valid animal model, to achieve strong and wide support of vitro and in vivo research in tumour formation. Neuroendocrine tumour patient-derived xenograft (PDX) model developed in zebrafish embryos will help for development of novel compounds. Melanoma cell lines which are specific for zebrafish can be labelled with fluorescent indicator, and cancer progression can be viewed easily by in vivo imaging. Haematopoietic cancers and its pathogenesis can also be studied using zebrafish. Zebrafish livers function similarly as humans and development of hepatocellular carcinoma is also found to be same; thus, using this advantage, novel anticancer treatments can be developed. Osteosarcoma study can be performed by inserting human osteosarcoma cell into zebrafish embryos and study the behaviour of tumour cell in vivo. Zebrafish paediatric cancer models promise to address many of the current needs for childhood cancers. Antitumour effect of various drugs like ramucirumab and apatinib has been studied using zebrafish.

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Thus zebrafish presents an exciting whole-organism system that is suitable both for assessing signalling pathways in malignancy and developing novel therapeutic approaches.

Keywords

Zebrafish · Cancer · Xenograft · Melanoma

9.1 Introduction

Cancer is termed as a disorder in which cell proliferation is uncontrollable. There are more than 200 different types of cancer at present and each progressing in its specific manner. Also it is observed that all of them are activated in the same manner: an alteration in internal cellular structures. Similar features are observed in various types of cancers: extreme cell growth signalling, no response to anti-growth signals, cell death which is not prior scheduled, uncontrolled cell proliferation, angiogenesis and tissue invasion and spread of cancer via the bloodstream or lymph to different body parts (Hanahan and Weinberg 2000, 2011). Cancer has become the second major cause of death in the world (Khalili and Rezai 2019). In spite of the worldwide research, there is still no effective therapy or preventive medicine against this fatal disease (Ferlay et al. 2015).

Treatments such as chemotherapy, radiotherapy and surgery are presently used which are highly expensive and yet do not eradicate cancer completely. Moreover, these treatments produce adverse effects, which also produce negative effect on the quality of life of patients. In recent times, zebrafish has emerged as an invaluable model for study of cancers in humans. Using zebrafish includes advantages like the evolutionary conservation of genetic pathways, which are associated with cancer and are common in zebrafish and humans. Zebrafish is an excellent tool for studying human disease (Fig. 9.1) and investigating the cellular processes involved. Over the past few years, a wide range of models of human cancer have been developed in zebrafish mainly through well-established transgenic methodologies, and their identification is done using gene-specific mutations. These include models for haematologic malignancies, melanoma and rhabdomyosarcoma, like tumours. This chapter will present a subset of the currently published zebrafish cancer models and highlight several that demonstrate recent advances. Recently, pigment-deficient "Casper" zebrafish line has been developed which allows direct observation of cancer development (White et al. 2008). The optical clarity of zebrafish can be exploited further by the use of fluorescent tags to label specific cell lineages to visualize tumour processes including initiation, progression and regression. Recently, the power of zebrafish has been underscored by the remarkable progress and utility of tumour transplantation methodologies performed in this model organism. Xenotransplantation studies have also been carried out using zebrafish.

In recent years, many zebrafish cancer models have been generated that reflect human haematologic tumours. Technological advances have expressively improved

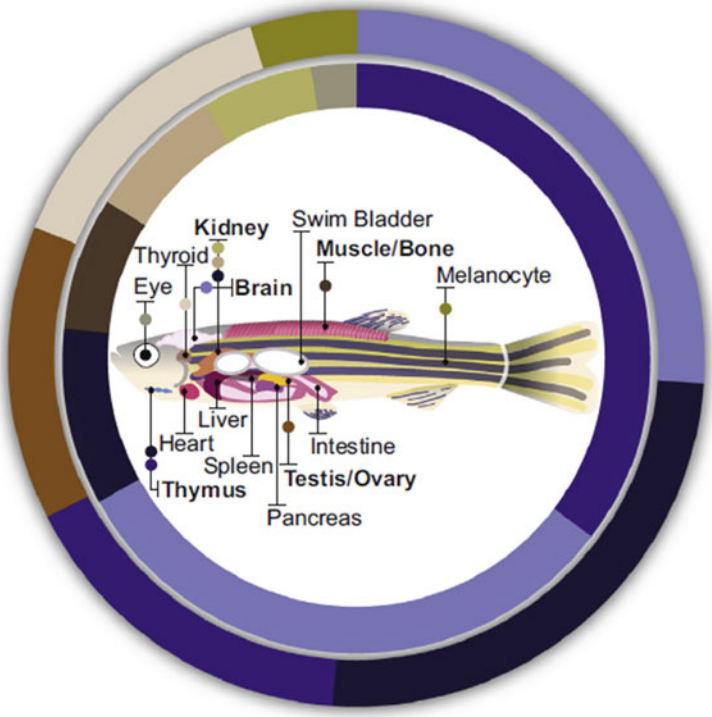

Fig. 9.1 Anatomical regions of zebrafish

allowing genetic tractability and in vivo imaging in zebrafish, leading to discovery of molecular pathways of tumour initiation, progression and metastasis. The zebrafish allows easy breeding and production of large number of offspring. The embryo is small size and transparent making whole-organism screening easier. This chapter provides description of various zebrafish models used for cancer and briefs about the benefits of the zebrafish for exploring novel therapeutic strategies (Sailo et al. 2018; Constantinou et al. 2020).

Selection of the zebrafish as an experimental model allows easy examination of in vivo pathways related to the pathogenesis of tumour (Howe et al. 2013). Zebrafish cancer models have advantages like fertilization is external, development is rapid, fertility is high and size of the adult zebrafish is small which can be handled easily. Furthermore, all major organs are completely developed within 5 days post fertilization (dpf). Stable transgenic lines can be developed easily in 6 months. The easy availability of fluorescent lines to mark organ leads makes it possible to observe disease processes in real time (White et al. 2008). If genomic conservation is considered, 71.4% of human genes contain minimum one zebrafish orthologue (Veinotte et al. 2014).

Using transgenic technology specific types of tumour can be developed by the human oncogene expression and tissue-specific promoters. Alternatively,

xenotransplantation of tumour cells of mammalian origin inside zebrafish larvae will be an excellent model for investigating in vivo human tumour, angiogenesis and inflammation (Veinotte et al. 2014). Large-scale chemical screens are possible due to the low maintenance cost of this model (Wiley et al. 2017), and simultaneous evaluation of drug efficacy and toxicity in vivo is also possible (Idilli et al. 2017).

9.2 Zebrafish Models Used in Cancer Research

9.2.1 Models for Tumour Angiogenesis

Tumours survive and invade due to angiogenesis (Carmeliet and Jain 2000), and the molecular mechanisms observed in humans and zebrafish are similar (Tobia et al. 2011). Zebrafish embryo has the distinctive capacity to persist without a completely formed or functional blood circulation (Sehnert et al. 2002); processes that interrupt blood vessels can be performed without having any lethal effect on embryo. Knockdown of a specific gene expression is possible by injection of specific oligonucleotide (Nasevicius and Ekker 2000), thus making it easy to study the genes involved in biological processes, like vascular development. Furthermore, adaptive immune system is lacking in embryos; thus, murine and human tumour cells can be easily injected with no rejection (Guerra et al. 2020).

9.2.1.1 Zebrafish Xenograft Model

The zebrafish embryo tumour/xenograft model was established to study the angiogenic response of cancer cells (Nicoli and Presta 2007). In this assay, a limited number of cancer cells were suspended in Matrigel or PBS and injected in the perivitelline space of 48 hpf embryos. New blood vessel originating from the SIV (sub-intestinal venous plexus) was investigated after 24 h post injection (Fig. 9.2). Using this technique, 25 to 30 embryos can be injected in 1 h. Due to the large number of samples in each experiment, the statistical strength of each experiment is increased. The zebrafish embryo tumour/xenograft assay is thus useful to investigate

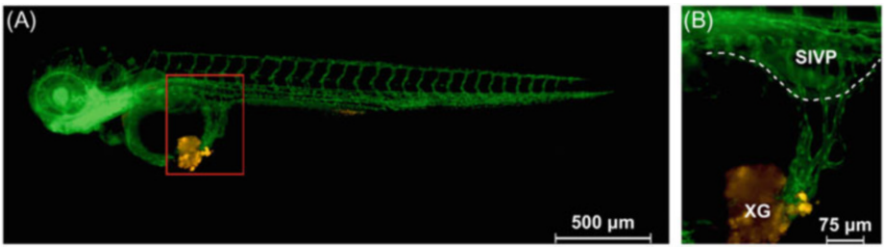

Fig. 9.2 Xenotransplantation of tumour cells within the perivitelline space induces an angiogenetic response in the SIVP of zebrafish embryos. (**a**) Whole-mount fluorescence image of a 72 hpf tg (fli1a:EGFP) embryo at 24 h post injection (hpi) and (**b**) magnification of the highlighted region. *EGFP* enhanced green fluorescent protein, *XG* xenograft, *SIVP* sub-intestinal venous plexus

the angiogenic potential of genetically modified cancer cells (Vlecken and Bagowski 2009).

On the other hand, the likelihood to change the host vasculature via chemical inhibitors, morpholino knockdown, CRISPR/Cas9-mediated knockout or knock-in may help to explain the role of specific genes of the host during the angiogenic process triggered by cancer cell implantation. The neovascularization of localized tumour is supported by host myeloid cells mass generated into the bloodstream of zebrafish embryo through injection of cancer cells (Koenig et al. 2016). Moreover, tumour dissemination is enhanced by co-injection in the tumour-associated macrophages (TAMs) and perivitelline space of cancer cells (Wang et al. 2015).

Thus, the zebrafish embryo tumour/xenograft assay may be used to inject tumour cells such as cancer-associated fibroblasts and TAMs (M1- vs. M2-polarized macrophages) (Lee et al. 2009) and perform detail study.

9.2.2 Zebrafish Models for Neuroendocrine Tumours

Neuroendocrine tumours (NETs) are a class of rare and heterogeneous neoplasms derived from the neuroendocrine system. The "neuroendocrine" term is used because of the presence and wide dispersal of cells having "neuro" and "endocrine" properties. Due to the wide distribution of these cells, NETs can arise at several sites of body. Primary tumours are commonly observed in the gastro-enteropancreatic tract and lungs (Oronsky et al. 2017). The clinical characteristics and biological behaviour of NETs vary significantly. Moreover, drug resistance develops by most of the standard treatments leading to the development of unwanted adverse effects and limited success rates. The selection of optimal treatment for tumour patients represents a clinically difficult challenge (Mazziotti et al. 2017; Pedraza-Arévalo et al. 2018).

9.2.2.1 NET Transplantable Model in Zebrafish Embryos

For the study of NETs, a system was developed by injecting numerous human NET cell lines in the sub-peridermal cavity of zebrafish embryos tg(fli1a:EGFP)^{y1}(Lawson and Weinstein 2002). The tg(fli1a:EGFP)^{y1}transgene consisted of the EGFP cDNA under control of the fli1 promoter which expressed EGFP in the entire blood vessel under the control of the endothelial fli1a promoter. Primary cultures were generated using post-surgical samples of several NET patients. NET cells were stained with a fluorescent dye. The embryos in control group did not show major changes in vasculature, but new vessels sprouted from the sub-intestinal vein plexus towards the tumour mass in the grafted embryos after only 24 hpi (hours post infection). A strong invasive behaviour was observed by the grafted NET cells, and they started migrating out from the tumour mass and invading different parts of the embryo (Fig. 9.3). Nuclear morphology and the expression of specific molecular markers were preserved in the injected NET cells (Gaudenzi et al. 2017; Würth et al. 2017).

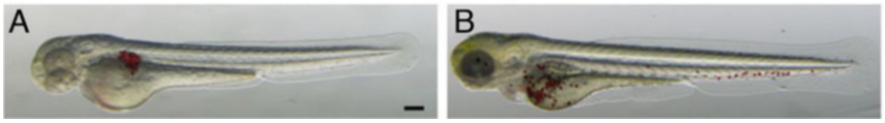

Fig. 9.3 Tumour-induced angiogenesis and tumour cell migration in zebrafish embryos grafted with primary culture cells derived from a patient with NET. (**a**) Tumour cells were well-confined at the injection site, and 24 hpi, (**b**) when cells were detected in distant areas, such as the head or the posterior caudal vein plexus. All images are oriented so that rostral is to the left and dorsal is at the top. *hpi* hours post implantation, *NET* neuroendocrine tumour

The post-surgical availability of NET cells is often limited; thus, the platform resulted particularly suitable for NETs wherein small tumour implants (100 cells/embryo) can be studied easily (Morton and Houghton 2007). Also tumour host microenvironment, angiogenesis and invasiveness can be investigated. New anti-cancer drugs can be tested due to the versatility of zebrafish embryos. Many compounds can be added directly to the embryo water as zebrafish embryos are permeable to small molecules. The larger or non-water-soluble molecules can be injected into the blood circulation (Vitale et al. 2014). Quick analysation of the in vivo pro-angiogenic potential of implanted NET cell lines is possible along with evaluation of two major factors of tumour progression, which are tumour-induced angiogenesis and invasiveness (Gaudenzi et al. 2017).

Zebrafish xenograft model thus is an innovative tool in the field of NETs, where only few preclinical models are currently available. The development of new precise medication and its application and also prediction of the individual clinical response to novel compounds are possible by use of PDXs (patient-derived xenografts) in zebrafish embryos (Gaudenzi and Vitale 2019).

9.2.2.2 MYCN Model

This model of pancreatic neuroendocrine carcinoma was based on the targeted expression of the human MYCN oncogene (Yang et al. 2004). The MYCN transgenic zebrafish was developed by injecting zebrafish embryos with a construct carrying the MYCN oncogene under the control of both zebrafish myod1 and human zebrafish hybrid MyoD promoter sequences. Expression of MYCN was detected in the muscle, hindbrain and spinal cord and ectopically in pancreatic islet cells. Increasing the levels of MYCN in these islet cells of the pancreas promoted the development of neuroendocrine pancreatic carcinoma (Etchin et al. 2011).

Thus, zebrafish models could be beneficial to identify best suited treatment in individual NET patient, with increased efficacy and lesser toxicity which would thus result in improved survival rate and increased quality of life of NET patients.

9.2.3 Zebrafish Melanoma Models

Melanoma is the deadliest and most aggressive type of skin cancer. Either single agents or combined therapies are given, depending on the health of the patient and location and stage of the tumour. But the resistance develops early which results in failure of given therapy (Domingues et al. 2018).

9.2.3.1 The $BRAF^{V600E}$ Zebrafish Melanoma Model

In melanoma, the BRAF is the most frequently mutated gene (Davies et al. 2002). A BRAF mutation is observed in over 50% of melanomas, from which over 80% carry a hyperactivating mutation $BRAF^{V600E}$, resulting in abnormal activation of the MAPK signalling pathway. $BRAF^{V600E}$ mutations are also observed in nevi (moles) signifying that $BRAF^{V600E}$ has an important role in proliferation of melanocytes but alone cannot stimulate melanoma formation. For the purpose of direct testing of the $BRAF^{V600E}$ function using animal model, the human $BRAF^{V600E}$ was expressed under the mitfa promoter to allow the expression of $BRAF^{V600E}$in zebrafish melanocytes (Patton et al. 2005). Mosaic $BRAF^{V600E}$ expression produced large ectopic melanocytic spots on the adult zebrafish which were composed of increased numbers of melanocytes and were similar to nevi. When a p53 mutation was also combined, melanoma was developed from the fish-nevi, thereby giving direct confirmation that melanoma was promoted by $BRAF^{V600E}$ along with tumour suppressor mutations (White et al. 2011).

9.2.3.2 The Mitf Zebrafish Melanoma Model

Microphthalmia-associated transcription factor (Mitf) is the key transcription factor for melanocytes and is required for their specification and development from the neural crest (Tsao et al. 2012). There are two mitf genes (a, b) in zebrafish. The mitfa gene is essential for the development of neural crest-derived melanocytes and mitfb is expressed in the eye (Lister et al. 2001). Germline mutations in MITF gene of mouse and human lead to attack of melanocytes (Waardenburg syndrome type 2 and Tietze syndrome) (Tsao et al. 2012); likewise, mutations in mitfa gene of zebrafish result in development of white fish (nacre) phenotype (Tsao et al. 2012). The regulation of MITF gene is highly complex, and MITF is responsible for the expression of genes involved in the melanocyte cell differentiation, cell cycle, DNA damage response and tanning (Cheli et al. 2010). Both low and high MITF levels have been reported in human melanoma (Konieczkowski et al. 2014).

9.2.3.3 Genetic Model of Melanoma in Zebrafish

In vitro studies have established the relationship between $BRAF^{V600E}$ and MITF, showing that MITF activity must be maintained to support tumour growth while preventing cell cycle arrest. (Gray-Schopfer et al. 2007). The assessment of the impact of critically maintaining MITF activity on melanocyte growth and melanoma formation in living animals has become easily possible now due to the isolation and characterization of a mitfa temperature-sensitive allele in zebrafish (Johnson et al. 2011).

The time-lapse imaging of mitfa mutant zebrafish has shown that mitfa is necessary for the pairing of cell cycle arrest and melanocyte differentiation (Taylor et al. 2011).

The association of mitfa with $BRAF^{V600E}$ in animals has been proved in animals. It has been reported that mitfa in collaboration with $BRAF^{V600E}$ promoted nevi and melanoma progression in adult fish, while mitfa levels sustained at intermediate levels rarely lead to nevi on their own (Roider and Fisher 2014). Importantly, in spite of having the same oncogenic BRAFV600E driver mutation, $BRAF^{V600E}$p53 and $BRAF^{V600E}$ mitfa melanomas have distinct histopathological features presenting discrete subtypes. Because of the temperature sensitivity of the allele once the $BRAF^{V600E}$ mitfa melanoma is formed, the temperature of the water can be raised to efficiently shut off mitfa activity, causing melanoma to regress dramatically. On the contrary, the cooperation of Mitf with $BRAF^{V600E}$ supported melanoma formation and survival (Domingues et al. 2018).

9.2.4 Models for Leukaemia

9.2.4.1 T-ALL Zebrafish Transgenic Model

T-cell acute lymphoblastic leukaemia (T-ALL) is a high-risk haematologic cancer characterized by immature T-cell progenitors infiltrating the bone marrow (Durinck et al. 2015; Palomero and Ferrando 2009). By expressing mouse *c-Myc* complexed with an enhanced green fluorescent protein (*EGFP*) (*EGFP-mMyc*) under the direct action of the zebrafish *rag2* promoter (Langenau et al. 2003), the T-ALL zebrafish transgenic model was first time produced. Also first time the dissemination of EGFP-labelled leukemic cells was observed under a fluorescent microscope. The onset of leukaemia in the fish of age 30 days was found to be very rapid and could only be controlled by in vitro fertilization.

This problem was prevailed by generating a transgene having the EGFP-mMyc oncogene preceded by a loxed *dsRED2* gene, and also the expression of Myc was managed by Cre-mediated recombination of the LoxP-dsRED2-loxP cassette upon Cre mRNA injection (Langenau et al. 2005). The characteristics of the fish developed by this method are presence of red fluorescent thymocytes and development of leukaemia *upon injection of Cre* mRNA recombinase. By injecting *Cre* mRNA into one cell stage embryos, T-ALL could be developed in transgenic progeny. This model has generated significant insight into the pathogenesis of the disease. Screening of various chemicals and transplantation experiments have been conducted by using this model with an aim of better understanding of T-ALL biology (Idilli et al. 2017).

9.2.5 Hepatocellular Carcinoma Models

Worldwide hepatocellular carcinoma (HCC) has been the fifth most usually diagnosed and second leading reason behind deaths as well. Aggregation of multiple

genetic mutations leads to arousal of HCC. Active mutations in the TERT (telomerase reverse transcriptase) promoter, directing to hyper-activation of TERT and genomic instability, have been reported in 47% and 60% of HCC cases (Nault and Zucman-Rossi 2016). Typically, in somatic cells including hepatocytes, TERT expression is reserved, curbing the total number of cell divisions. However, in self-regenerating cells such as stem cells, TERT expression is normally high, and during homeostasis and even after liver injury, high levels of TERT can progenerate the liver cells (Lin et al. 2018).

Cancer cells capture this stem cell machinery to avoid the Hayflick limit and propagate uncontrollably. In more than 30% of cases of HCC, the second most common mutation found is in TP53 (tumour protein 53), an influential tumour suppressor (Zucman-Rossi et al. 2015). TP53 safeguards the genome by commencing cell cycle arrest or apoptosis in response to diverse cellular stressors along with DNA damage. Frequent mutations leading to activation of the Wingless (Wnt) pathway, especially b-catenin (CTNNB1) and axin (Gao et al. 2012), and Ras pathways (Newell et al. 2009) are also seen in HCC (Mudbhary et al. 2014).

9.2.5.1 Wnt-Activated Hepatocellular Carcinoma Model

A large subgroup of HCC cases is classified by activation of the Wnt signalling pathway. In the process of normal liver organogenesis, crucial Wnt signalling has revealed a crucial role in developmental studies carried out in zebrafish (Goessling et al. 2008). Induction of spontaneous formation of intestinal, pancreatic and liver tumours has been reported due to inactivating mutation of zebrafish adenomatous polyposis coli (apc) activated Wnt/b-catenin signalling pathway (Haramis et al. 2006). With the combination of activation of Wnt signalling pathway with the chemical carcinogen DMBA 7, 12-dimethylbenz[a]anthracene, extent of tumorigenesis was increased. Another transgenic model of Wnt-activated HCC has used hepatocyte-specific promoter to impel an activated form of b-catenin. Enlarged liver phenotype during larval and spontaneous development of liver tumours by 3 months post fertilization is observed in this fish model. Using this substitute phenotype, substances that might avert Wnt-driven HCC were tested. The c-Jun N-terminal kinase (JNK) inhibitors and serotonin reuptake inhibitors were also discovered by using the fish model (Evason et al. 2015). These studies on zebrafish have enlightened molecular and cellular mechanisms by which the Wnt/b-catenin signalling pathway plays a vital role in normal development and HCC progression and has led to findings of prospective treatment possibilities.

9.2.6 Bone Cancer Models

9.2.6.1 Miscode

MSC codes for the mouse mesenchymal stem cell (MSC) in this model. It was revealed that early-passage non-transformed mouse MSCs stay in the yolk sac after injection, while late passage MSCs transform and have been observed to be tumorigenic in mice. They induced proliferation, migration and angiogenesis in embryos

produced from a cross of two transgenic strains, Caspar, which is transparent, and Tg (fli1:egfp), having green vasculature due to specific EGFP expression in blood vessels within 2 days of injection (Lawson and Weinstein 2002). The sub-intestinal veins developed 48 h post fertilization (hpf) showed angiogenesis in this study. In the donor cells, genes involved in migration and angiogenesis were upregulated, and immune response-related genes get significantly downregulated after the injection with transformed tumorigenic MSCs (Mohseny et al. 2012). To study tumour cell behaviour in vivo, injecting human osteosarcoma cell lines into zebrafish embryos had proven to be an extremely effective model (Lawson and Weinstein 2002; White et al. 2008).

9.2.6.2 CSC Model

In heterogeneous prostate tumours, a small subpopulation of cancer cells is found which are known as CSCs (cancer stem/progenitor-like cells). The capabilities of self-renewal, high motility, tumorigenicity and chemo-resistance are shown by these cells, and their role has also been indicated in initiation of bone metastasis, one of the key reasons behind deaths in several kinds of cancer (Reya et al. 2001). In spite of numerous studies conducted in diverse types of cancer over the past decades, tracking small numbers of CSCs during the onset of metastasis has been challenging. Recently, a zebrafish CSC-xenograft model has been put forth to envisage and evaluate the interactions with the cellular microenvironment and the operative role of CSCs at the inception of metastasis. The DoC, also termed as the embryonic common cardinal vein, connects the heart to the trunk vasculature on the embryonic yolk sac and provides an extensive circulation channel. To take off metastatic colonization and development in a short time window [normally within 3–6 days post implantation (dpi)], cancer cells can be directly transplanted into the blood circulation via the DoC. Transplantation of 100–500 cancer cells into DoC at 2 days post fertilization (2 dpf) rapidly instantly circulates most of the cells, while others are retained at the site of injection forming a solid primary-like tumour mass (He et al. 2014; Tulotta et al. 2016). The zebrafish CSC-xenograft model can be considered as a complete platform to study human CSCs and their mutual interaction with micro-environment during metastatic development. The mechanisms of the early steps of bone metastasis in patients has been revealed with help of studies on the zebrafish CSC-xenograft model.

9.2.7 Paediatric Model

9.2.7.1 Rhabdomyosarcoma Model

Rhabdomyosarcoma is the most common childhood soft-tissue tumour which appears in two forms: alveolar rhabdomyosarcoma (ARMS) and embryonic rhabdomyosarcoma (ERMS). Recent work in zebrafish has uncovered a previously unsuspected role for the small GTPase RAS in ERMS (Langenau et al. 2007).

The constitutively active kRASG12D mutant from the zebrafish rag2 promoter was developed expecting to model a role for RAS in leukaemia. Rag2-kRASG12D

fish developed early, aggressive muscle tumours resembling RMS by both histological and gene expression criteria. In related work, to gain temporal control of kRASG12D expression, a transgenic line expressing a fluxed kRASG12D allele driven by a ubiquitous promoter was crossed to a line expressing heat-shock inducible CRE (Le et al. 2007). This strategy resulted not only in RMS but also in malignant peripheral nerve sheath tumours, intestinal cell hyperplasia and a myeloproliferative disorder. This last condition could be induced in recipient animals by ex vivo heat shock and transplantation of the kidney marrow, neatly circumventing the early lethality due to RMS and the potent effect of RAS on development. This kind of manipulation of transgene expression and tumour transplantation will no doubt prove to be invaluable transgenic zebrafish cancer models (Amatruda and Patton 2008).

9.2.8 Techniques Used for Anticancer Research Using Zebrafish Model

Ramucirumab, apatinib, regorafenib and cabozantinib were screened for anticancer activity by evaluating their effect on the in vivo cell proliferation in cell line-derived tumour xenograft (CDX) model based on Tg (*fli*-1: EGFP) zebrafish embryos.

9.2.8.1 Cell Xenograft and Quantification

Adenocarcinoma gastric cell line (AGC cell lines) and human gastric cancer cell line (SGC-7901) were labelled with a fluorescent dye CM-DiI. Yolk sac of anaesthetized 48 hpf larvae was observed under the stereoscope after injecting approximately 200–300 CM-DiI-labelled cells with a microinjector. The embryos then were incubated for 1 h for 28.5 °C and then transferred to 32 °C till the end of experiment. At 24 h post injection (hpi), the injected larvae were sorted according to tumour size. About 15–20 embryos are sacrificed and suspended into single-cell suspension, and the number of CM-DiI-labelled cancer cells was counted (Gaudenzi et al. 2017).

9.2.8.2 Drug Administration by Soaking and Microinjection

The anti-angiogenic and antitumour effect of ramucirumab was compared by administration of the drug by microinjection technique. The 1–10 mg/mL dilutions were prepared in PBS for microinjection in yolk sac. The embryos were anaesthetized with 0.0003% tricaine, and then 1–10 mg/mL solutions of ramucirumab in PBS in volume approximately 10 nL/embryo were injected into the yolk sac using a microinjector. The vehicle control group of embryos was injected with 10 nL PBS. All the injected embryos were shifted to a 6-well plate in 3 mL fresh embryo medium and set aside at 28.5 or 32 °C till the test was completed. At the end of experiment, the embryos were euthanized by overexposure to tricaine for evaluation of effects.

9.2.8.3 Anti-Proliferation Determination in Zebrafish Tumour Model

Zebrafish xenograft tumour model was explored for evaluation of anti-proliferative activity of ramucirumab, apatinib, regorafenib and cabozantinib on gastric cancer cells. 48 hpf embryonic yolk sacs were injected with CM-DiI-labelled AGS cells. The predetermined doses of above drugs from teratogenic doses (maximum tolerated dose) were selected for the treatment of embryos at 1 dpi dispersed into 24-well plate. 0.1% DMSO was taken as positive control group. Cell proliferation was observed at 4 dpi (Wu et al. 2020).

9.2.9 Cancer Imaging in Zebrafish Model

Numerous fluorescent protein variants are currently available and can be used in imaging cancer in zebrafish.

9.2.9.1 Macroscopic Observation of Tumour Growth

For many applications, imaging fluorescent-labelled cancers in whole zebrafish is advantageous. For example, whole animal imaging can be used to assess growth kinetics and overall dissemination. Cell transplantation can also be used to assess if fluorescent-labelled cell populations are fully transformed—a hallmark of cancer (Smith et al. 2010).

9.2.9.2 Stereomicroscopy to Image Tumour Growth

Most zebrafish laboratories are equipped with a fluorescent dissecting microscope capable of imaging embryos, larvae and adult fish. For macroscopic imaging of embryos and larvae, stereomicroscopy is the preferred method for screening and imaging fish. Stereomicroscope used is fitted with a fluorescence emission setup and a colour camera. Stereomicroscopy can be used to image single adult animals; however, it is usually not possible to capture the whole fish within the viewing field (Ignatius and Langenau 2011).

9.2.9.3 LED Fluorescence Macroscope Imaging

A novel method for imaging a whole plate of adult zebrafish using the LED fluorescence microscope has been developed. This machine comprises LED fluorescent lights that illuminate a 10 by 10 cm viewing area, a series of cameras that interface with a computer and various filter sets permitting only emitted light of an exact wavelength to be captured and evaluated.

The LED fluorescence macroscope is inexpensive, can image up to 30 animals simultaneously, is capable of real-time imaging and can capture live, time-lapse dual-fluorescence images using two cameras. Additionally, small focal tumours will be hard to image; however, as tumours continue to grow, they can be imaged by the macroscope (Ignatius and Langenau 2011).

9.2.9.4 Microscopic Observation in Tumorigenesis

One of the unique advantages of the zebrafish model is the capability to image individual cells using confocal, spinning disc and two-photon microscopy. Although sophisticatedly used to image important progression in tumor development with axonal guidance (Kucenas et al. 2008), vascular development (Nicoli et al. 2010), cell movements in embryogenesis and the emergence of hematopoietic stem cells (Kissa and Herbomel 2010); cellular microscopic observation of the hallmarks of cancer are just beginning to be explored the types of technologies that can be utilized to image at the single-cell level in live zebrafish.

9.3 Conclusion

The zebrafish has proven as an excellent model organism for cancer research. As complementary to murine model system, it has been demonstrated effective assessment of tumour cells, malignancy, progression and evaluation of drugs to subsequent xenotransplantation. As (proto-) oncogenes and tumour suppressor genes are highly conserved between zebrafish and humans, the zebrafish model has been ideal for identifying clinically relevant genes and compounds. Furthermore, zebrafish tumours have similar histopathological and gene profiling characters to human tumours; thus, xenotransplantation with human cancer cells is feasible, and translational information for human cancer can be gained. Zebrafish tumour has been a perfect platform to acquire imperative in vivo information in nanomedicine, nanoparticle toxicity, biodistribution, stability, effective targeting and pharmacological functions of compounds. The experiments have succeeded to set up patient-derived xenografts in zebrafish with an aim to develop personalized medicine approach to cancer therapy. Requirement of less number of cells for each transplantation and development of the rapid angiogenic response of the host after cell injection have made the zebrafish embryo tumour/xenograft assay a powerful and promising tool in cancer research.

Although the zebrafish model has established many advantages, it represents certain constraints. Presence of many duplicate genes make genetic manipulations difficult and also temperature required for raising them is lesser (28 °C) than the optimal temperature needed for growth and survival of mammalian cells, i.e. 37 °C. Proteomic studies with zebrafish model are also difficult as many commercially available antibodies cannot recognize zebrafish proteins.

The zebrafish model has been utilized widely in cancer research for numerous types of cancer with various methodologies such as transgenesis, transplantation, treatment with carcinogens, etc. in the last decade though associated with a number of limitations.

Recent genomic techniques have offered better analyses of zebrafish cancer but it entails careful application and interpretation. Development of systematic and scalable methods of functional gene interrogation needs to be prioritized to maximize the potential of zebrafish in cancer research. The focused efforts will surely make zebrafish a more vital and productive model in cancer research.

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Zebrafish Model and Cardiovascular System for Novel Therapies

10

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Abstract

Cardiovascular disease (CVD) is a health burden to our society. Globally, 17.9 million people die from various cardiovascular diseases each year. In the last decades, the zebrafish has become an important model for cardiovascular research. It possesses a host of advantages in comparison to other experimental model such as ease of genetic manipulation, transparency of the embryo, small size, and cost-effectiveness. With real-time studies and fluorescent markers, it has become possible to analyze various cells and their locations and migration during morphogenesis of zebrafish heart. Even though there were challenges to overcome, future advancement in technology and experimental designs would help us to advance our cardiovascular disease knowledge and improve treatment strategies.

Keywords

Cardiovascular disease · Zebrafish · Model · Nobel therapies

10.1 Introduction

Cardiovascular disease, a noncommunicable disease, includes coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis, which is one of the leading causes of death in India. Non-modifiable risk factors such as hypertension, diabetes, and hyperlipidemia and modifiable risk factors like unhealthy diet, obesity, physical

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inactivity, harmful use of alcohol, and smoking are some of the etiologies of CVDs. It was estimated that India had a prevalence of 54.5 million cases of CVDs in 2016 (Prabhakaran et al. 2018). CVDs death in India accounts for one in four deaths, with >80% death responsible for ischemic heart disease and stroke. Globally, 17.9 million people die of CVD each year, representing 31% of all global death (World Health Organization n.d.). Various breakthrough researches based on molecular pharmacology in cardiac function and metabolism have significantly reduced cardiovascular diseases' mortality and morbidity. Nevertheless, still more research in cardiovascular biology is needed (Ohlstein 2010). The difference in human pathology and experimentally induced pathology in animal models sometimes made it difficult to rephrase CVD complexities and hence becomes a challenging process in translation to humans. Small animal models such as mice, rats, rabbits, cats, etc. were widely used in cardiovascular research mainly because of their genetic manipulation and easy handling and housing and low maintenance cost. However, due to distinct differences in these species and humans' physiology, there is uncertainty of the obtained experimental results (Milani-Nejad and Janssen 2014). Since last decades vertebrate model, zebrafish has gain momentum for in vivo large-scale small compound screening during the early phase of drug discovery (Beffagna 2019). New editing technologies such as TALENs (transcription activator-like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas have been applied in zebrafish model for obtaining mutagenesis as zebrafish genome can be easily and rapidly manipulated (Bedell et al. 2012). Hence, zebrafish has many advantages over other experimental models such as genetic manipulation, ease of screening mutation due to translucent embryo, smaller size, ability to house thousands of fishes, and cost-effectiveness.

Danio rerio (zebrafish) has provided a valuable tool for various research fields, including cardiovascular research, over the last few decades. George Streisinger first identified the qualities of zebrafish, and he was called the father of zebrafish research (George Streisinger Award 2020). Despite anatomical differences between human and zebrafish heart, real-time studies using fluorescent markers furthered our concept that progenitor cells of both species' cardiomyocytes are similar (Buckingham et al. 2005). The human heartbeat is somewhat similar to the zebrafish heartbeat which is 120–180 beats per minute (Baker et al. 1997). Moreover, zebrafish larvae are useful for cardiovascular disease study as their heart and vasculature can be easily visualized. Even the formation of heart chambers, cardiac contractions, blood flow, and vessels can be easily studied in vivo in zebrafish (Pelster and Burggren 1996). In the first few days of development, the zebrafish embryo does not depend upon a functional cardiovascular system as these lab animals get sufficient oxygen from passive diffusion. So, severe phenotypes concerning cardiac defects can be studied in these animals (Kalogirou 2020; Steed et al. 2016). Genetic manipulation to stimulate human cardiac disease can also be done easily in zebrafish (Howe et al. 2013). Several characteristics will be discussed below how the zebrafish model has been approached to observe and experiment with various cardiovascular diseases and develop phenotype-driven new drugs for the cardiovascular system.

10.2 Genetic Approach in Zebrafish Cardiovascular Research

In Online Mendelian Inheritance in Man (OMIM) database, 82% of human morbid genes were related to one zebrafish orthologue, and after genome-wide association (GWAS) studies, it was identified that 72% of zebrafish genes are related as orthologue to human genes (Howe et al. 2013). Because of amenability to genetic manipulation, zebrafish gained success as a transgenic model in various cardiovascular disease researches. To facilitate the human disease model, standard tools used to manipulate the zebrafish genome are forward and reverse genetics.

10.2.1 Forward Genetics

Thomas Hunt Morgan (1866–1945) was the first researcher who separated the spontaneous phenotypes and organized them on a genetic map in a population of inbred fruit flies. In forward genetics, zebrafish are randomly induced by mutation by using chemical mutagens like *N*-ethyl-*N*-nitrosourea (ENU) and produce a phenotype (Cokkinos 2015). The cells of zebrafish were mutagenized with ENU to induce mutations. Then the mutagenized zebrafish were allowed to bred and cross-bred for progeny, which carries the mutant gene (Driever et al. 1996). In this process, mutant phenotypes relevant to various aspects of development and function of the heart and vasculature were identified (Stainier et al. 1996). One such finding of forward genetics is the zebrafish mutant main squeeze (*msq*), which possesses a mutation in the gene encoding ILK (integrin-linked kinase). *Msq* mutant gene exhibits progressive loss of ventricular contractility leading to heart failure. Since zebrafish *msq* mutant embryos survive for days to the late larval stage, it permits to analyze the role of ILK in vertebrate cardiac functions in detail. Another zebrafish mutant, lost-control (*loc*), shows a loss-of-function phenotype (reduced contractility and aberrant cardiomyocyte shape, dilated and ruptured blood vessels) that can be co-related with dilated cardiomyopathy (DCM) in human (Bendig et al. 2006). This example exhibits that zebrafish could be a good model for screening new candidate genes for heart failure. Despite forward genetic screening results in novel genes and pathways required to develop vertebrates, large size of zebrafish genome, requirement of space and personnel in laboratories, and redundancy inherent due to genome duplication in teleost fish made it impossible for forward genetics to identify all the relevant developmental genes. Hence, the reverse genetic approach has been established to identify the function of these genes (Lawson and Wolfe 2011).

10.2.2 Reverse Genetics

The reverse genetic approach targets the investigated gene of interest by increasing, reducing, or silencing its expression. Techniques used in reverse genetics are mRNA overexpression, transgenesis, morpholino-modified antisense oligonucleotide (MO)-mediated knockdown, or gene-editing techniques such as zing finger nucleases

(ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/Cas (CRISPR-associated) (CRISPR/Cas) systems (Paone et al. 2018).

10.2.2.1 mRNA Overexpression

mRNA overexpression of a target gene in the early stage of zebrafish embryo is used to analyze for gain-of-function or loss-of-function. For example, research on human dilated DCM, mRNA overexpression of *NEXN* (nexilin) gene in zebrafish creates DCM phenotype. Because of this technique's limited stability, the use of mRNA overexpression is restricted to early organ development and functions (Hassel et al. 2009).

10.2.2.2 Transgenesis

It involves the insertion of a transgene into the zebrafish genome to obtain a highly efficient generation of transgenic F_1 offspring. The most commonly used transgene is *Tol2* system derived from medaka fish (Kawakami et al. 1998). A study on transgenic zebrafish arrhythmia model bearing the pathogenic human cardiac sodium channel mutation *SCN5A-D1275N* is useful in differentiating functional from benign human arrhythmia genetic variant (Huttner et al. 2013).

10.2.2.3 Morpholino-Mediated Knockdown

Morpholino (MO) reagents are injected to one-cell stage of zebrafish embryo for gene knockdown approach (Stainier et al. 2017). Many MO gene knockdown studies have been done to determine whether knockdown of a specific transcription factor would provide protection against toxicity or mediate toxicity (Prasch et al. 2003; Jenny et al. 2009), but it became a concern for researchers because of the off-target effects of MOs. Hence, scientists published some guidelines, including control and rescue experiments to distinguish between specific phenotypes and off-target effects (Eisen and Smith 2008).

10.2.2.4 Genome Editing Techniques

As there was concern regarding MO gene knockdown techniques, genome editing techniques came into the picture. With the help of genome editing, genetic materials can be added, removed, or altered at a particular location in a genome (NIH 2020). Zing finger nucleases (ZFNs) induced deletion in the *gata2a* gene to analyze the loss-of-function gene's role in vascular morphogenesis. The study revealed that *gata2a*-deficient embryos displayed defects in the formation of the dorsal aorta (Zhu et al. 2011). Later, the CRISPR/Cas9 system's discovery has carried the reverse genetics into a new era because of its simplicity and adaptability compared to ZFN and TALEN (Doudna and Charpentier 2014). *PR130*, the most extensive transcription of *PPP2R3A* (protein phosphatase 2 regulatory subunit B, alpha), is known for its critical cardiovascular development role. For a better understanding of the cardiac function using CRISPR/Cas9 system, a *pr130*-deletion zebrafish lines were generated. The study concluded that *pr130* knockdown leads to interruption of heart development. It was hypothesized that heart development restriction might

be via defected dephosphorylation on cardiac contractile proteins due to decreased PP2A (protein phosphatase 2A) activity in *pr130* knockdown zebrafish heart (Yang et al. 2016). Recently, various new applications for CRISPR/Cas9 system have been developed to minimize the off-target effects. One such was evolved by Gaudelli and his colleagues, where they evolved a tRNA adenosine deaminase, which fuses to a catalytically impaired CRISPR/Cas9. They demonstrated point mutations more efficiently and cleanly in adenine base editors than the original Cas9 nuclease-based method and also induced less off-target genome modification. Henceforth, advanced genome editing enables the conversion of all known base pair conformations holding great potential for the correction of hereditary cardiac diseases (Gaudelli et al. 2017).

10.3 Similarities of Human and Zebrafish Cardiovascular Development and Role in Research

10.3.1 Cardiomyocyte Differentiation

Even though the zebrafish heart is two-chambered and mammalians are four-chambered, the cardiomyocytes of both originated from the same progenitor cell (Buckingham et al. 2005). To analyze cardiomyocyte differentiation development, de Pater et al. created a timeline with a double-transgenic zebrafish model. This model identified two distinct stages of cardiomyocyte differentiation using a cardiac-specific red-to-green photoconvertible fluorescent protein, namely, (1) the ventricle and the part of the atrium (venous pole) and (2) atrial pole. The author and his colleagues also demonstrated that the cardiomyocytes of the venous pole, i.e., the future ventricle, is the first to differentiate. They also identified islet 1 (*Isl 1*) gene, which initiates cardiomyocyte differentiation at the venous pole and fgf8 to regulate the arterial pole (de Pater et al. 2009). The second phase of cardiomyocyte differentiation was identified as an expression of *mef2cb* gene, which regulates the late myocardial cells and is an addition to the development of the ventricle (Lazic and Scott 2011).

Identifying these two phases of heart development in zebrafish is essential as many genes and regulatory pathways that are similar in both zebrafish and humans could provide insight into newer treatment strategies for congenital heart diseases. For example, in DiGeorge syndrome, many individuals manifest conotruncal heart defects due to heterozygous deletion of the chromosomal segment 22q11.2 (*TBX1* gene). *Tbx1*-null embryos of zebrafish also mimic conotruncal heart defects. Hence, in the future, *tbx1*-null embryos would better understand early markers of conotruncal heart defects and identify various opportunities for therapeutic interventions (Piotrowski et al. 2003).

10.3.2 Vasculogenesis and Angiogenesis

Origin of diseases such as pulmonary arterial hypertension, hereditary hemorrhagic telangiectasia, and preeclampsia in an adult can be traced back to the embryonic vascular developmental stage (Venkatesha et al. 2006). During embryonic development, bone morphogenetic protein (BMP) plays a vital role in developing the vascular system (Wagner et al. 2010). In various experimental models, it was examined that BMP pathway is regulated by BMP modulators such as chordin (Piccolo et al. 1996), noggin (Smith and Harland 1992), Drm/gremlin, twisted gastrulation (Tsg) (Oelgeschläger et al. 2000), and BMP endothelial cell precursor-derived regulator (BMPER) (Moser et al. 2007). In the transgenic *fli1*:eGFP zebrafish model, it was found that BMP modulators exert various effects on endothelial function and angiogenesis. Zebrafish transgenic model could be a potential model to study the growth and regeneration of blood vessels during the embryonic period and adult diseases (Heinke et al. 2013).

10.3.3 Valvulogenesis

Endocardial cushion malformations leading to atrioventricular valve defect are some of the common congenital dysmorphisms in newborn infants. Understanding the mechanism of genetics, epigenetics, and cellular pathway, which regulates the morphogenesis of the heart, would provide better medical aid to newborn and infants. Since zebrafish embryos provide unique opportunities to understand morphogenesis, experiments have been done on transgenic *fli1*: eGFP zebrafish larva (from 6 days postfertilization to 28 days postfertilization) to study the development of atrioventricular valve from the endocardial cushion. This study suggested that valve maturation in zebrafish was influenced by the size of the larva and hemodynamic forces modulating the valve structure.

Experiments in humans and zebrafish show that BMP signaling plays a significant role during the induction of endocardial cushion within the atrioventricular valve. Irregularities in BMP signaling lead to the downregulation of many pathways such as *Has2* (hyaluronic acid synthase 2) (Rivera-Feliciano and Tabin 2006). *Has2* is responsible for the production of hyaluronic acid, which is an essential component of endocardial cushion mesenchyme (Camenisch et al. 2000). Again, miR-23 is a nucleotide single-stranded noncoding RNA responsible for organogenesis. A study was done on *dicer* null mutant zebrafish (loss of miR-23) showing that there is a restriction of endocardial cushion formation by inhibiting *Has2* expression and hyaluronic acid formation (Lagendijk et al. 2011).

10.4 Cardiovascular Disease Model

Due to the development of uncomplicated approaches such as forward and reverse genetics, mutation identification in zebrafish models becomes more efficient and less expensive and provides new insight into human cardiac development research. Moreover, the transparency of the zebrafish embryo has allowed noninvasive in vivo imagining techniques for analysis of cardiac development (Pelster and Burggren 1996). Various zebrafish disease models have been developed in the past decade to study human cardiovascular diseases.

10.4.1 Arrhythmia Model

To understand cardiac valves and cardiac conducting system development, Neil C Chi et al. developed a multidisciplinary approach in zebrafish embryos. Transgenic zebrafish line *Tg*(*cmlc2*: *gCaMP*)^{s878} (myocardial-specific line that expresses *gCaMP*, a fluorescent calcium indicator protein) and *Tg*(*cmlc2*:*eGFP-ras*)^{s883} were created and observed various developmental stages of the cardiac conduction system. Furthermore, they performed a diploid ethylnitrosourea (ENU) mutagenesis screen and in vivo optical imaging techniques and identified novel cardiovascular conduction/rhythm mutants such as *hobgoblin* (*hob*) (Chi et al. 2008). *Hob* is an AV conduction block mutant that affects *Tcf2* (homeobox gene transcription factor) (Movahed et al. 2005). *Tcf2* gene regulates the expression of Na^+/K^+-*ATPase*, which is necessary for cardiomyocyte electrical polarization (Bagnat et al. 2007). Thus, further analysis and isolation of genes affecting the cardiac conduction system's mutations would improve and develop therapeutic options.

10.4.2 Cardiomyopathy Model

Several cardiomyopathy models, such as dilated cardiomyopathy and hypertrophic cardiomyopathy (HCM), were developed. Zebrafish embryo gets oxygenation through passive diffusion and does not require a functional cardiovascular system during the first few days, facilitating mutant phenotype analysis of cardiomyopathy (Pelster and Burggren 1996). With the forward genetic approach's help, some mutant genes responsible for severe cardiovascular compromise have been identified. Among them is *silent heart* (*sih*) mutant, which is responsible for the disruption of the *TNNT2* gene (Sehnert et al. 2002). *TNNT2* gene encodes for a protein called cardiac troponin T, which is a part of the sarcomere, a basic unit of muscle contraction (TNNT2 Gene 2020). *Silent heart* (*sih*) mutant zebrafish embryo displayed a reduction in α-tropomyosin and cardiac troponins C and I leading to severe sarcomere defects. This same gene mutation in humans shows hypertrophic and dilated cardiomyopathy (Sehnert et al. 2002).

Similarly, in in vivo zebrafish model of human amyloid-light chain (AL-LC)-induced cardiotoxicity, the model exhibits cardiomyopathy phenotype (pericardial

edema, cardiac dysfunction, and cardiomyocyte death). Cultured cardiomyocytes demonstrated activation of p38 mitogen-activated protein kinase (MAPK) via a noncanonical signaling pathway independent of the upstream MAPKK MKK3/6 after induction with AL-LC. SB203580, a selective p38 MAPK inhibitor, seems to reduce the AL-LC-induced oxidative stress, cellular dysfunction, and apoptosis (Mishra et al. 2013).

Lastly, doxorubicin, an anticancer drug, is used in breast cancer, lung cancer, leukemia, and lymphoma treatment. Doxorubicin causes dose-dependent cardiac toxicity (Hershman et al. 2008). Again, tyrosine kinase inhibitors, which are used as targeted cancer therapy, also cause cardiotoxicity (Chen et al. 2008). The zebrafish model would be useful in screening dose-dependent cardiac toxicity.

10.4.3 Cardiovascular Regeneration Model

Zebrafish has the unique capacity to regenerate its myocardial cells within months of myocardial injury (Poss et al. 2002). This morphological change in the endocardium was observed due to the retinoic acid (RA) expression-synthesizing enzyme *raldh2* (Kikuchi et al. 2011). Recently, genetic manipulation and cell linage labeling by CreER-loxP and Ga14-UAS system help in better understanding of cardiomyocyte regeneration. Immediately after the zebrafish heart's cryoinjury, the epicardium gets activated by developmental genes such as *aldh1a2* (also known as *raldh2*), *tbx18*, *wt1b*, and *notch* genes or their reporters (Schnabel et al. 2011). As a result, the activated epicardium sends paracrine signal for cardiomyocyte proliferation (e.g., retinoic acid and Nrg1) (Kikuchi et al. 2011). Targeted cardiac cell ablation by expression of the fusion protein cyan fluorescent protein-bacterial nitroreductase enzyme (CFR-NTR) leads to inhibition of cardiomyocyte proliferation and heart regeneration is delayed (Wang et al. 2015). Like the epicardium, the endocardium after heart injury activates other developmental genes, such as *hand2*, *gata5*, and *notch* (Kikuchi et al. 2011; Zhao et al. 2014).

Another experiment conducted on a transgenic zebrafish model with Cre/tamoxifen and bromodeoxyuridine (BrdU) labeling shows proliferation of preexisting cardiomyocytes that undergo dedifferentiation. Upregulation of *polo-like kinase1* (*plk1*) expression, a regulator of cell cycle progression, was observed in the regenerating heart (Jopling et al. 2010).

We have discussed above that dedifferentiation and proliferation of preexisting cardiomyocytes play a crucial role in the regeneration of zebrafish heart. Various molecular studies suggested that a chemokine ligand (Cxcl12)-receptor (Cxcr4) system helps regulate cell migration (Mizoguchi et al. 2008). Another study reported fibronectin (*fn1a* and *fn1b*), a significant component of the extracellular matrix, was found in the injured epicardial cells of zebrafish hearts. Disruption of regeneration was seen with *fn1* gene loss-of-function (Wang et al. 2013).

10.4.4 Complex Congenital Heart Disease Model

Developing a complex congenital heart disease model is sometimes tricky as zebrafish does not produce the specific phenotypes (septal defects or transposition of great vessels) even if it belongs to the same orthologous gene (Gut et al. 2017). An autosomal dominant disorder, Noonan syndrome (NS), is associated with missense germline mutations in *PTPN11* (protein tyrosine phosphatase, non-receptor type 11). Individuals with NS are characterized by congenital heart defects (atrial and ventricular septal defects, pulmonary stenosis, and hypertrophic cardiomyopathy). *PTPN11* gene encodes a protein called SHP-2, which is crucial for the heart's development and helps regulate the MAPK (mitogen-activated protein kinase) signaling pathway. In the zebrafish model, NS-Shp2-expressing embryos showed impaired leftward heart displacement and defect in ciliogenesis due to the MAPK signaling pathway's hyperactivation (Bonetti et al. 2014).

10.5 System Pharmacology and Zebrafish Model

System pharmacology approach is a combination of system biology (computational and mathematical analysis and modeling of a complex biological system) and pharmacometrics (quantifies drug, disease, and trial information for efficient drug development) (Sorger et al. 2011). Quantifying drug effects in the preclinical experimental model is necessary to detect potential side effects in therapeutic doses that might be detrimental to humans. Zebrafish model proves useful in testing cardiovascular toxicity as it can perform both phase I (oxidation, n-demethylation, o-demethylation, and n-methylation) and phase II (sulfation and glucuronidation). Moreover, transcript profiling by microarray and quantitative PCR analysis revealed that many zebrafish cytochrome P450 (CYP) genes are direct orthologues of human CYP genes crucial for activation or inactivation of endogenous and exogenous chemicals. Henceforth, zebrafish model provides a foundation for pharmacological and toxicological cardiovascular disease research (Diekmann and Hill 2013).

In cardiovascular drug toxicity research, the zebrafish model was commonly used to test QT prolongation during new drug development. Zebrafish KCNH2 knockdown model reported bradycardia as a result of inhibition of repolarizing potassium current (IKr). This same phenotype correlates in humans when treated with drugs causing QT prolongation (Milan et al. 2003).

Letamendia et al. demonstrated a cardiotoxicity screenings by developing an automated high-throughput platform for zebrafish embryos (transgenic zebrafish embryos expressing CopGFP under the cardiac-specific promoter Cmlc2). They selected drugs affecting calcium (nitrendipine, diltiazem, verapamil) or sodium channel (lidocaine) that inhibits the zebrafish Ether-a-go-go Related Gene (zERG) channel, similar to the human Ether-a-go-go Related Gene (hERG) channel in humans. Letamendia and his colleagues observed a 2:1 atrio/ventricular arrhythmia in the zebrafish larvae that resembles QT prolongation in humans (Letamendia et al. 2012). Thus, for early screening of drugs, both the zebrafish adult and larvae stage

could provide potential information regarding pharmacokinetics and pharmacodynamics during new drug development.

10.6 Challenges and Future Perspective of the Zebrafish Model

We have seen from studies that zebrafish found an important place in the world of research. Zebrafish is a small-sized animal that easily breeds; it has become an essential model for human cardiovascular disease research. Finding solutions for complex diseases that involve multiple factors of disease progression is quite challenging. Moreover, a rapid increase in the genome sequencing project has led to a challenge for defining 40,000–70,000 genes constituting the vertebrate genome (Driever et al. 1996). Knowing zebrafish genetics and applying to human disease phenotyping would provide an insight into practical information of genes. For example, the genetic mutation of L-type calcium channel (LTCC) results in arrhythmias and cardiac hypertrophy, which are among the causes of cardiac mortality (Santoro et al. 2019). Again, conducting preclinical research that correlates with human disease is a challenge. Future studies in functional genes with rigorous experimental design in the zebrafish model in conjugation with pharmacology would help us understand cardiovascular diseases and improve future therapies.

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Zebrafish Model for Drug Discovery and Screening

11

Shantanu Joshi

Abstract

The zebrafish as a preclinical drug development model is discussed considering different aspects. 3-Rs Replacement, Reduction and Refinement related to zebrafish for drug discovery are discussed. The strength of zebrafish as a model for preclinical drug development is highlighted. The different aspects of drug development considering the genetics of zebrafish are well covered. Phenotype-based drug development, structure-activity relationship, target-based drug development and toxicities related to zebrafish are elaborated with suitable examples. Organs like heart, liver, intestines, kidney, CNS, haemopoietic system, endocrine system and their drug development-related aspects like pharmacokinetics and drug toxicities in zebrafish are elaborated. Separate topic is dedicated to cancer drug development using zebrafish. Drug resistance related to P-glycoprotein and other related mechanisms is also covered. Practical limitations in use of zebrafish as preclinical drug development models are addressed.

Keywords

Zebrafish · Drug development · Drug toxicities and zebrafish · Drug resistance and zebrafish · SAR and zebrafish · Heart toxicity · Liver toxicity · DILI · CNS and zebrafish · Cancer and zebrafish · Limitations to zebrafish drug research

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11.1 Introduction

After the thalidomide disaster in 1960–1961 in Europe, both EMA and US FDA made it mandatory to test the molecule in vitro and in vivo in embryonic states to check the developmental defects and teratogenicity and to establish safety of the new molecule (Marathe and Thomas 1990).

In vivo mammal segment studies and in vitro rodent whole embryo culture test, embryonic stem cell test, etc.—these tests show poor correlation in detecting embryonic and foetal toxicity in human (Oberemm 2000). FETEX (Frog Embryo Teratogenesis Assay—Xenopus) is one more test but is having limitations because these embryos are not sensitive to halogenated compounds (Fort et al. 1988).

Mammalian embryonic testing models are considered as gold standards, but zebrafish is being increasingly used for the developmental studies and teratogenicity testing in the US and UK (Parng 2005).

Very few drugs enter in clinical testing phase and very few of them enter the actual clinic and remain as useful drugs. This is due to the preclinical safety and efficacy studies (Blomme and Will 2016; Hay et al. 2014; Munos 2009).

Safety is the major concern of such attrition (Cook et al. 2014; Waring et al. 2015). Though the preclinical studies are rigorous and very useful to detect the toxicity, still many drugs show toxicity when used in clinical settings.

Depending upon the data from preclinical to clinical testing, the specificity of toxicity testing is around 80% (means prediction of true negative results is good), but the sensitivity is >50% indicating that true positive results may not be reflected properly (Monticello et al. 2017). It ultimately means that high false negative results are the most important cause of failure of the drugs during human testing.

Advances in toxicological testing especially investigations in toxicology may reduce such loss of molecules in advance stages and may save efforts and money. Replacement, Reduction and Refinement are the 3R of philosophical animal research and stem cell research, cell cultures are part of it (Kim et al. 2019; Stummann and Bremer 2012) organ on chips (Weinhart et al. 2019), in silico prediction (Kar and Leszczynski 2019) humanized chimeric mouse (Bissig et al. 2018) are the important means of more transferable data.

Preclinical toxicological improvement is important, but the mammalian animal models are cost and labour-intensive and many times pose ethical questions (Rovida and Hartung 2009; Walker and Roberts 2018). The acceptance of data by government depends upon well-established predictivity of human toxicity in a particular animal model. Acceptance of data for a particular human system depends upon similarity of that particular system with the animal model and its sensitivity and specificity. The animal species may change from system to system or organ to organ or tissue to tissue.

As per the guidelines by the European Commission, the regulatory requirements are applicable for zebrafish only after 5 dpf or 120 hpf. Zebrafish starts independent feeding after that period. Before this period, no regulatory requirements are there for animal experimentation.

The 3R of philosophical and ethical animal experimentation (Russell and Burch 2009; MacArthur Clark 2018; Russell 1995)

Replacement—Larval zebrafish can replace animal toxicity testing especially related to genes, target identification, pathway mechanisms, etc. Such studies require prior validation.

Reduction—The use of zebrafish reduces the possibility of using toxic drug candidates for further testing. It traps and eliminates the toxic candidate in early stage of drug development, thus reducing the total number of animals used for toxicology experiments.

Refinement—The embryos are transparent and develop independently out of the zebrafish body. It is possible to observe the toxicity just by observation without dissection. This is a very good example of refinement.

11.2 Strengths of Zebrafish

1. It is a very small but a vertebrate animal showing much conservation of systems and functions with human being.
2. The zebrafish embryo grows in the same fashion as the human embryo, but its growth is about 20 times faster than the human embryo. It is important for speedy experimentation and can save the valuable time required for drug development.
3. The zebrafish shows high fecundity, at a time the female lays about 300 eggs. Feeding and taking care of zebrafish is easy as compared to other mammalian animals.
4. The adult zebrafish is about 4 cm; this small size makes the handling easier and requires less space. Hundreds of zebrafishes can be housed in a simple aquarium and automated water supply can be maintained easily.
5. Its development is very rapid. Most of its organ development is complete and starts functioning by 48 to 72 hpf. It is easy to observe the effect of any chemical and is less time-consuming. GIT and hepatobiliary systems take a little more time to develop, but by 96 hrs development of these systems is complete and starts functioning (Cook et al. 2014).
6. For toxicological studies, it is better to use zebrafish larvae as they are transparent and their toxicity can be detected without dissection. Toxicological studies can be conducted on larval zebrafish in the handheld platform because microplates are sufficient to maintain such larva at this stage of development.
7. Most of the zebrafish organs perform similar functions as seen in humans. Physiology is mostly conserved, but being an aquatic species, some physiologic and anatomic differences are present.
8. Clinical development is possible based on the data from zebrafish, seven molecules are in clinical stages of development, and the preclinical data are derived from zebrafish (Cutler et al. 2013; Li et al. 2019; Mandelbaum et al. 2018; North et al. 2007; Ryals et al. 2018; White et al. 2011; Yu et al. 2008a, b).

9. Zebrafish genome is completely sequenced and shows 70% genetic similarity overall and about 82% similarity in the genes which are related to disease processes (Howe et al. 2013). Moreover, the protein targets of most of the commonly prescribed drugs show close homology with the orthologue protein. It ranges from 54% for glucocorticoid receptors to 91% in case of thyroid receptors (III-10). Enzymes, channels and receptors retain their function and structure to a great extent in the process of evolution. Ligand binding domains are generally showing close homology to human counterparts.
10. Genes of zebrafish can be modified to produce transgenic animals. Modification is comparatively easy as the embryo grows outside the mother's body. In transgenic animals' disease like human can be produced by manipulating genes. This modification in genes helps us to understand complex processes in disease development and also counteract the disease process by using chemical, thus helping the process of drug development.
11. One of the approaches of drug development is the phenotype drug development. Zebrafish are the best models for such phenotype drug development. It is like you see the results of the drug in the experimental model, and by reverse pharmacology, we understand how the drug works (identify the target of the drug).
12. Endocrine signalling and cell-cell interactions cannot be studied in in vitro models but zebrafish models provide the intact biological system to study the drug effect and hence is preferred over conventional in vitro testing of the drugs.
13. In cell-based assays, limited data could be obtained on absorption, distribution, metabolism and excretion of the drug. On the other hand, zebrafish models provide good opportunity to analyse these functions while testing the compound for side effects and toxicities. The drug needs to reach the target to produce the effect, which means absorption and distribution are required, and it should escape from rapid metabolism and excretion to produce an effect. The liver, kidney and blood-brain barrier are all working.

11.3 Genetics and Zebrafish

The use of zebrafish as a tool has increased after showing it can be modified for forward genetic screens (Eisen 1996), meaning identification of genes based on expressed phenotype. This makes vertebrate-related development easier to understand. It also gives a good idea for the development of disease.

The zebrafish genome has been completely sequenced by the Sanger Centre. The Transnational Institute of Health and Zebrafish Genome Initiative are the major contributors. cDNA of full length is available for experiments. Using antisense technology, gene function can be properly studied in zebrafish (Nasevicius and Ekker 2000; Heasman 2002). Transgenic lines (Kurita et al. 2004) targeted mutations (Wienholds et al. 2002) are possible with zebrafish. Cloning by using nuclear transfer can also be done in zebrafish (Lee et al. 2002).

11.3.1 Identifying Novel Drug Targets

We know much of pathophysiology of the disease but still target identification is difficult. It is really important to define the druggable target, which means modifying the target should reverse the disease process. The validation of the target is the most important part of drug discovery. Morpholino oligonucleotide screens are important tools to identify the function of gene in disease processes. This is the way that leads to identification and validation of druggable target. The phenotypes that are similar to diseases are identified in zebrafish, and the genes responsible for them can be identified by forward genetic screens. Chemicals those lead to alteration in gene can lead to the drug.

11.3.2 Genes and Polycystic Kidney Disease

PKD in zebrafish is caused by mutations in several genes and is similar to human PKD. Pkd2 and transcription factor 2 (tcf2) are involved in human PKD (Sun et al. 2004; Otto et al. 2003). Many of the new genes identified are related to ciliary function, and hence it is now suspected that PKD might also be related to ciliary function.

11.3.3 Genes and Heart Diseases

There are orthologue genes identified in zebrafish and human that are responsible for some cardiac diseases like TTN gene that causes cardiomyopathy (Gerull et al. 2002), TBX5 gene that is responsible for congenital heart diseases (Li et al. 1997) and HERG gene, which is related to potassium ion channel (also known as KCNH2) mutations, that causes arrhythmia (Langheinrich et al. 2003).

11.3.4 Morpholino Oligonucleotide Screens

In the embryo of the zebrafish, we can introduce the morpholino oligonucleotides. These are the modified antisense oligonucleotides that can bind to mRNA (Nasevicius and Ekker 2000) and suppress further process of protein synthesis. In one way we identify the function of mRNA against which we have developed the morpholino oligonucleotide. mRNA will be developed from the gene, hence ultimately understanding the function of the gene. It is very easy to introduce morpholino oligonucleotides in zebrafish embryo because the development of the embryo is outside the mother's body and at the same time its transparent nature will help to see the effect on phenotype development and can be easily identified. Rapid development of zebrafish embryo can save a valuable time. It is also reproducible. Because of these benefits, the zebrafish is a more suitable model for studying the functions of genes.

Diseases can be produced by pharmacological/chemical interventions, by infectious agent or by genetic manipulation. In the disease state, many morpholino oligonucleotide screening for multiple genes/mRNAs can be done (knockdown of gene), and one can identify the genes that are responsible for slow progress of the disease or arrest of the disease process. The potential therapeutic agent can be developed for that particular gene/mRNA or the binding of protein produced by that mRNA. All this is possible in zebrafish embryo and hence this can be an important approach for development of new drugs.

11.3.5 Phenotype-Based Drug Discovery

Validated targets are known in only a small number of diseases, and development of drug is comparatively an easy process. For many diseases like myelogenous leukaemia, atherosclerosis disease-modifying targets are not yet been identified. In these cases, it becomes difficult to decide which proteins need to be modified to improve the health of the patient. The in vitro data suggesting some targets imposes a significant risk of failure in ADME of the drug or the side effect and ADR profile when actually used in vivo or in human trials (Bleicher et al. 2003; Armer and Morris 2004).

Instead of going in details of the target for a particular disease, one can select the molecule which modifies the disease, and such approach is known as phenotype-based drug discovery. In this approach, we identify the molecule which is known to modify the phenotype of the disease, but we do not know what exactly the target of a particular molecule is. In simple language, we know that the molecule works and produces results, but we do not know how does it produce results. To explain this story of ezetimibe would be good guide. To produce the cholesterol lowering agent, scientist proposed that blocking of acyl coenzyme A cholesterol acyltransferase (ACAT) would be an important target. The scientist used this target-based approach and did the high-throughput screens and developed many compounds that are effective in blocking of acyl coenzyme A cholesterol acyltransferase, but none of them was practically found to be effective in lowering the cholesterol levels in high-fat diet fed hamsters.

On the other hand, a by-product derived in the process of synthesis of other ACAT inhibitors, which is a molecule having no affinity for acyl coenzyme A cholesterol acyltransferase (ACAT), showed the cholesterol lowering activity in hamsters on high-fat diet. Guided by this pure phenotype, the cholesterol lowering activity of that compound and other derivatives was developed, and finally ezetimibe was approved as a cholesterol lowering agent in 2002 by the US FDA. When it was approved, its target and mechanism of action were not known. In the later studies, Niemann-Pick C1-like protein 1 (NPC1L1) was identified as the target of ezetimibe and later it was validated (Clader 2004; Stitziel et al. 2014) (success of phenotype-based approach and failure of target-based approach). The antitubercular drug isoniazid was also developed out of phenotype-based approach.

Another example is amiodarone, which is the antiarrhythmic drug of choice. It was discovered serendipitously. It does not act through one mechanism of action but acts on multiple targets like ion channels, beta adrenergic receptors and probably on the nuclear receptors—hormone pathway (Kodama et al. 1997). The compound screening for a phenotype has more chances of success than the compound obtained by in vitro screening because in phenotype screening side effects are screened simultaneously and compound having good effect with minimum side effect is selected for further advancement; hence, chances of failure in further development are comparatively less.

There are certain characteristics which cannot be studied in vitro, e.g. pain, tumour metastasis, vascular tone, gut motility, sedation, etc., and for these types of phenotypes, only in vivo screen is possible. Such types of screens are easily done in zebrafish. Zebrafish embryo and larvae represent integrated in vivo vertebrate system for phenotype drug screening.

This phenotype-based approach is having some limitations of space, and great resources and efforts will be required. In vivo studies will require a large number of animals. These are few important drawbacks that make it difficult to utilize this phenotype-based approach. The phenotype-based screens should be less expensive, consume less space, consume less chemicals and be faster. Phenotyping should be easily recognizable. Many of these requirements would be fulfilled by zebrafish screening. One example of zebrafish phenotype reversal is gridlock mutation which causes vascular defect. The screening of small molecule libraries to identify the molecule which reverses vascular defect had been identified. It is proposed that suppressors of gridlock mutation are related with vasculogenesis and would be of great value in diseases like AMI, stroke, cancer and DM. Though its exact mechanism of action is not clear, it seems to activate VEGF (vascular endothelial growth factor). The two compounds GS4012 and GS 3999 are identified as gridlock mutation suppressors and suppose to promote angiogenesis (Peterson et al. 2004).

11.4 Structure-Activity Relationship

In vitro studies are regularly done to study structure-activity relationship. Here we produce the derivatives of the parent compound and test them for the affinity to the target in vitro. Here we develop/select the derivative having high potency, but it might affect the process of absorption and may show toxicity and subsequent failure in further drug development process. The zebrafish model of SAR is the potency/effect of the drug to be measured in vivo, and at the same time, absorption or other ADME issues are also addressed because with incomplete absorption or high FPM, the drug would not have produced effect in vivo. In addition, its advantage is that if any side effect or adverse effect is present, that will be noticed immediately and chances of subsequent failure remain less as compared to conventional in vitro testing of SAR. So, zebrafish would be a better option for testing of SAR screen which provides improved potency, without loss of efficacy and/or increased toxicity. Its high-throughput screening is possible.

11.5 Screening in Adult Zebrafish

Majority of zebrafish screens are performed in developmental states because of transparency and tiny size which suits to multiple assay plates. Certain experiments and testing of the drugs are to be planned in adult zebrafish as some conditions like arrhythmias, heart failure and transplant-related immunological reactions can be seen in adult zebrafish. Bone marrow transplantation and related studies are possible in adult radiation-treated zebrafish. Zebrafish is well known for its regenerative capabilities like regeneration of the heart, spinal cord, retina, etc. To understand the exact process and to develop drugs that can regenerate similar tissue in human, studies should be planned in only adult zebrafish (Poss et al. 2002, 2003; Guo 2004). Psychological and behavioural studies can be done only in adult zebrafish.

11.6 Screening for DNA Toxicity: Mutagenesis

Amanuma and co-workers developed the test for mutagenesis by small molecules (Amanuma et al. 2000). The zebrafish embryo line produces the plasmid containing 30S ribosomal subunit protein S12 (rspl) gene when this embryo is exposed to the drug candidate, and then the plasmid is removed from zebrafish genome and reinserted in bacterial genome. If the drug is capable of producing mutations, then the colonies of bacteria will be resistant to kanamycin as well as streptomycin, but if the drug candidate is not able to produce mutations, the colonies of bacteria remain resistant to kanamycin but sensitive to streptomycin. This is the in vivo assay where all metabolic machinery and DNA repair or other physiological mechanisms are active to prevent or to revert the mutation. It would be a more reliable assay for drug-induced mutagenesis.

11.7 Target-Based Drug Discovery

Identification of 3-hydroxy 3-methyglutaryl coenzyme A reductase as a rate-limiting enzyme in cholesterol synthesis resulted in statins, and one after another, many statins came to market as this target was the validated target. Blocking of this target is responsible for reduced cholesterol synthesis and ultimately the blood levels of cholesterol decline.

Some drug development examples where zebrafish was used as model for drug development:

1. *Prohema*—It is a PGE2 derivative which is in phase II of drug development. It is the drug that is used in the patients of leukaemia or lymphoma who are undergoing umbilical cord blood (UCB) transplantation. The compound that affects the PGE2 synthesis was shown to affect the HSCs population. It was first discovered in zebrafish affecting the colony of haemopoietic stem cells (HSCs) screened around aorta-gonad-mesonephros region of the embryo.

(III-40) Prohema's potential was first discovered to enhance the number of HSCs in adult zebrafish where the bone marrow recovery was accelerated after the sublethal irradiation. In mice the Prohema-treated ex vivo bone marrow shows more spleen colony-forming units than the untreated ex vivo bone marrow. (III-40) Phase II clinical trials are going on for the humans who are being treated with umbilical cord blood (UCB) as a source of HSCs. Actually, UCB contain a smaller number of HSCs to treat an adult patient. Prohema in such patient may help by rising the colonies of HSCs in UCB and increase the effectiveness of UCB in treatment of leukaemia or lymphoma (Cutler et al. 2013; Hagedorn et al. 2014).

2. *Dorsomorphin*—Hyperactive BMP (bone morphogenetic protein)—Signalling is associated with various diseases including FOP (fibrodysplasia ossificans progressiva) and anaemia of inflammation. Dorsolization in zebrafish is the process where there is expansion of dorsal tissue at the expense of ventral tissue. The drug that produces dorsolization shows no tail or very small tail in zebrafish. Pyrazolopyrimidine is screened for its dorsolizing activity, and when zebrafish embryo is treated with it (Yu et al. 2008c), it shows no tail or small tail and now it is named as dorsomorphin. The mutation in activin-like kinase-8 (Alk-8) gene shows loss of tail (Lost-a-fin) and later identified as target of dorsomorphin. In simple language, dorsomorphin blocks Alk-8 protein in zebrafish. In humans, dorsomorphin also blocks similar molecule, i.e. ALK2. In humans, BMP is ALK2. In simple language, dorsomorphin blocks BMP and it might be useful in fibrodysplasia ossificans progressiva (FOP) and anaemia of inflammation. Dorsomorphin and its derivative LDN-193189 are used as probes to manipulate BMP pathway (Cuny et al. 2008). BMP is also related to inflammatory bowel diseases (IBD) and arterial calcification. Dorsomorphin may also be useful for these conditions. US-NIH has initiated preclinical development of dorsomorphin through TRND (Therapeutics for Rare and Neglected Diseases) and BrIDGs (Bridging Interventional Development Gaps) programmes.
3. *PROTO-1*—PROTO-1 and its closely related compound benzothiophene carboxamide are the compounds found to protect hair cells in the ear of zebrafish and can prevent antibiotic-induced ototoxicity (Owens et al. 2008). Aminoglycosides are known for their ototoxicity; thus, these compounds can be used as otoprotectants. Oricula Therapeutics, a company involved in development of benzothiophene carboxamide as otoprotectant, reported the development of a compound which is 100 times potent than PROTO-1. Clinical trial for development of the drug is in progress.
4. *COX inhibitors and leukaemia*—AML1-ETO is an oncogene related to acute myeloid leukaemia (AML) (Yeh et al. 2008). Zebrafish model of AML can be produced by transgenic expression of this oncogene. Leukaemia-like phenotype can be supressed by blocking cyclooxygenase enzyme, and cyclooxygenase inhibitors can supress WNT-beta-catenin signalling (Yeh et al. 2009). This signalling is required for self-renewal of leukaemia stem cells (Wang et al. 2010). Treatment of zebrafish and other murine AML models by COX inhibitors supressed leukaemia and also inhibited in vivo progression of transplanted human

leukaemia cells (Zhang et al. 2013). COX inhibitors can be clinically used and hence phase I clinical trial can be initiated for the same (Klimek et al. 2012).

5. *Glucocorticoid in long QT syndrome*—Genetic long QT syndrome can be produced in zebrafish by manipulating kcnh2 gene. It shows prolong action potential and 2:1 AV block (Peal et al. 2011). Flurandrenolide a glucocorticoid is found to supress this phenotype. Experimentally, it was shown that glucocorticoids shorten the QT interval in the patients of LQT syndrome (Ziegler et al. 2013). Glucocorticoids are already approved drugs; the clinical trial has started at Massachusetts General Hospital, USA. Early results are encouraging and consistent with the animal model results.
6. Identification of mechanism of action of the molecules investigated in zebrafish:
   (a) A-alpha subunit of PP2A (protein phosphatase 2A) is found to be the target of perphenazine which is an anti-psychotic drug found to kill MYC-overexpressing thymocytes in a zebrafish model of T-cell-acute lymphoblastic leukaemia (T-ALL) (Gutierrez et al. 2014).
   (b) Visnagin, a cardioprotective drug found in zebrafish model of heart failure, shows the blocking of mitochondrial malate dehydrogenase (Asimaki et al. 2014).

11.8 Heart in Drug Development and Drug Toxicity

Zebrafish has two-chambered heart atrium that receives the blood from periphery and ventricle that pumps the blood all over the body. These specialized chambers, the vasculature, valve system, and specialized conduction system are similar to the mammalian heart.

Zebrafish ECG shows similar waves as that of the mammalian or human heart. ECG can be made by using two electrodes and paralysing the gills temporarily by using conotoxin to avoid artefacts. Conotoxin does not affect the heart rate.

Zebrafish exhibits the expression of ion channels as early as 24 hpf. The transparent nature of the zebrafish embryo makes it suitable to observe the development and to count the heart rate and observe arrhythmia without any invasive method. Adult male zebrafish has 150 ± 30 heart beats.

Many drugs lost their regulatory status due to QT prolongation and chances of development of Torsades de pointes leading to sudden cardiac arrest and death. It is related to drug-induced repolarization toxicity. It may be seen because of mutations in genes that encode for the ion channel proteins. These are difficult to predict in human as routine tests are not done or available.

Similar toxicities of QT prolongation due to drug-drug interaction can be seen only after clinical use in large population with multiple drugs use due to comorbidities. The drugs that exhibit the QT prolongation effect are shown to inhibit the potassium channels responsible for rapid repolarization of the heart. Zebrafish exhibits atrioventricular block and bradycardia with the drugs that are known to produce QT prolongation in human (e.g. astemizole, haloperidol, pimozide and

terfenadine), and hence bradycardia and atrioventricular blocks should be considered as surrogate markers for QT prolongation and ultimately cardiotoxicity.

In vitro binding of the drug to hERG channel (human ether a-go-go related gene) is accepted as valid assay for potential of the drug to cause QT prolongation and is accepted by regulatory authorities. This in vitro binding only confirms the affinity of the drug for this ion channel. The similar target of hERG is present in the zebrafish and binding of the drug to this target produces 2:1 atrioventricular block, and 2:1 atrioventricular block is equivalent to QT prolongation in humans. The in vitro technique only identifies affinity of hERG ion channel, but the larval zebrafish assay will identify other ion channels which cause QT prolongation. (The drug not binding to hERG but showing 2:1 AV block confirms binding of drug to other ion channels and having potential of QT prolongation.) This can save valuable time and money in development of drug. In this way, zebrafish larval assay is more productive than in vitro hERG assay for QT prolongation (Letamendia et al. 2012).

Zebrafish can regenerate heart muscles; the mechanism is being studied by many research workers. This will help to identify the hurdles that are present in human cardiac muscle regeneration and develop methods/drugs to help the human cardiac muscles to regenerate and heal the infarct. One group identified the role of TGF beta signalling pathway in this regard (Chablais and Jazwinska 2012).

The drug-drug interaction like cisapride and erythromycin showed the similar effect in zebrafish. AV block and bradycardia are noted with increasing concentrations of erythromycin. Erythromycin is known to inhibit CYP 3A4 and retard the metabolism of cisapride increasing the concentrations of cisapride. Similar drug-drug interactions are noted with cimetidine and terfenadine where cimetidine is an enzyme inhibitor that retards the metabolism of terfenadine. The increasing levels of terfenadine are responsible for AV block and bradycardia (Milan et al. 2003) and ultimately confirms the prolongation of QT interval.

All the above examples suggests that the zebrafish embryonic model for QT assessment may be helpful to detect QT toxicity and drug-drug interaction in very early stages of drug development. This effect may be useful to study the genetic variation related to ion channels. This cost-effective and in vivo technique of cardiotoxicity is having limitation of water solubility of the drugs to be tested. Non-aqueous drugs are difficult to administrate in zebrafish.

11.9 Liver: Drug Development and Toxicity

Zebrafish liver is having close similarity with human liver (Goessling and Sadler 2015). Fully functional liver develops by 5 dpf and can be used for testing of hepatotoxicity of any drug candidate. Multi-well setup can be used to study the drug in whole organism (Wang et al. 2017). The metabolic capacity of zebrafish liver is comparable to that of human liver (de Souza Anselmo et al. 2018; Vliegenthart et al. 2014).

The gene expression studies after exposure to hepatotoxic substances show similar findings in zebrafish and human (Driessen et al. 2015). Zebrafish liver exhibits all the cell types as that of human liver but show less organized structure.

Fluorescent technique makes it possible to study the drug effect in a particular cell type in real time (Goessling and Sadler 2015; Poon et al. 2017; Zhang et al. 2017); thus, zebrafish is an attractive model to study drug effects and toxicology in the liver. Hepatotoxicity studies with known hepatotoxic drugs like paracetamol, aspirin, tetracycline, erythromycin, cyclosporin and amiodarone revealed similar cytological changes as those seen in human hepatocytes (Mesens et al. 2015; McGrath and Li 2008). The hepatotoxicity assay of zebrafish is used for determining toxicity in early stages of drug development.

Some studies for hepatoprotective drugs against common hepatotoxic drugs are based on zebrafish model (Huang et al. 2018). Zebrafish models are also used to improve formulation as well as therapeutic index of the drug (Calienni et al. 2018; Lin et al. 2019). Similar studies were planned to investigate efficacy of novel drug candidate in autoimmune hepatitis (Sunke et al. 2019).

Transparent larvae screening in microtitre plate with the minimum drug quantity like 1 mg makes zebrafish an ideal model. Physiological and histological similarity with human is the most important advantage seen in zebrafish liver. There is no perfect model available in vivo and in vitro that can predict hepatotoxicity perfectly in human. Actually, present regulatory models show poor correlation between animal toxicity and human toxicity leading to black box warning and sometimes withdrawal of the drug from the market, e.g. troglitazone or iproniazid (Fung et al. 2001).

N-Ethyl-*N*-nitrosourea (ENU) is a common mutagen used to produce mutations and study the disease phenotypes. hhex gene plays an important role in development of the liver in zebrafish embryo; it is confirmed by morpholino oligonucleotide insertion in 1–4-celled stage of embryo. By 50 hpf significant reduction in size of the liver is observed as compared to control group. It shows liver necrosis (Wallace et al. 2001).

One mutant of foie gras gene developed lipid-rich hepatocytes resembling fatty liver disease. This model of zebrafish larvae will be useful to study fatty liver disease seen in more than 25% of the American citizens (CDC-2007). Obesity and fatty liver disease are interrelated.

DILI (drug-induced liver injury) is the most important cause in acute liver injury and contributes about 13% of cases. It is also one of the most important causes that is responsible for restrictions or withdrawal of the drug from the market, e.g. rosiglitazone. The conventional toxicity assays related to hepatotoxicity are having low sensitivity of around 25%. This is why high-order organisms like monkeys are used for screening of liver toxicity with full histopathological assessment (O'Brien et al. 2003). From this discussion, it is clear that conventional methods of screening hepatotoxicity have limitation.

Zebrafish from its larval stage can serve as a good model for screening of hepatotoxic potential of drug. It can be started as early as in lead optimization stage. It is possible because very low concentrations are required. It is the model

that provides in vivo information of drug toxicity which also includes toxicity due to toxic metabolites. Such vivo screens can reduce the chances of drug failure due to hepatotoxicity at the minor cost added.

Major advantage of this larvae model of zebrafish is phenotypic-based screens and morphological end points without need for dissection. The three most commonly used phenotypic screens are liver abnormality (tissue degeneration), changes of liver size (hepatomegaly) and yolk retention (yolk is utilized by the liver, but if the liver is not working well, it will not be utilized).

There is good correlation between the hepatotoxicity data of mammals and zebrafish. There is 88% sensitivity (to detect false positive results) and 67% specificity (to detect false negative results). It is important to note that tamoxifen and danazol which need metabolic conversion for hepatotoxicity are correctly identified as hepatotoxic compounds in zebrafish. Troglitazone is also found to be hepatotoxic in zebrafish, indicating that zebrafish larvae must have similar metabolic pathways as that of human.

The various CYP enzyme systems are being analysed by different scientific groups—CYP 3A, CYP1A, CYP19 and CYP26 (Carney et al. 2004; Bresolin et al. 2005; Tseng et al. 2005; Rubinstein 2006). There are some drugs like ketoconazole and sodium valproate which are hepatotoxic to human but found to be nontoxic to zebrafish, but their concentrations are found to be very less (absorption is possible only when the drug is water soluble or you need to inject the drug). This indicates that physicochemical properties of the drugs are also very important in this case. No single criteria for physiochemical properties are available for screening in zebrafish (Doshna et al. 2009).

The commonly used drugs and their effect in human and zebrafish—good correlation is observed.

Sr. no.	Drug name	Mammalian/human toxicity	Zebrafish toxicity
1.	Amiodarone	Severe liver toxicity	Liver necrosis
2.	Aspirin	Hepatotoxic to human	Liver necrosis
3.	Danazol	After metabolism—Hepatotoxic	Liver necrosis and yolk retention
4.	Ibuprofen	Hepatotoxic	Liver necrosis
5.	Naproxen	Hepatotoxic	Liver necrosis
6.	Tamoxifen	Moderately hepatotoxic	Liver necrosis
7.	Troglitazone	Hepatotoxic and withdrawn from market	Liver necrosis
8.	Valproic acid	Hepatotoxic	Liver necrosis
9.	Ketoconazole	Hepatotoxic	Nontoxic to liver
10.	Sodium valproate	Hepatotoxic	Nontoxic to the liver
11.	Astemizole	Non-hepatotoxic	Nontoxic to the liver
12.	Metformin	Non-hepatotoxic	Nontoxic to the liver
13.	Paracetamol	Non-hepatotoxic in regular does but hepatotoxic at higher doses	Nontoxic to the liver

(continued)

Sr. no.	Drug name	Mammalian/human toxicity	Zebrafish toxicity
14.	Gentamycin	Non-hepatotoxic but nephrotoxic	Liver necrosis
15.	Praziquantel	Non-hepatotoxic—Anthelminthic	Liver necrosis

All the published data suggests that zebrafish screening for hepatotoxicity may be a great hope to identify toxic drugs and also the drug-drug interaction at the early stage of drug development.

11.9.1 Microsomal Enzymes and Drug Development

The drug-drug interactions depend upon inhibition or stimulation of cytochrome system. Zebrafish shows very close similarity to the human cytochrome system and hence can be considered as a promising predictive model for understanding drug metabolism and safety. About 50% of the human drug metabolism depends upon CYP3A4/5 and it is confirmed to be CYP3A65 in zebrafish. Microplate-based whole zebrafish CYP3A65-functional activity assay was developed and compared with human CYP3A4 inhibitors and inducers.

Enzyme inhibitors—Azamulin, disopyramide, erythromycin, fluvoxamine, omeprazole and cimetidine. Correct prediction in human to zebrafish was 6/6 = 100%.

Enzyme inducers—Rifampicin, dexamethasone, prednisone, hydrocortisone, phenytoin, carbamazepine, lovastatin and pregnenolone. Correct prediction in human to zebrafish was 6/8 = 75%.

Non-CYP3A4 inducer/inhibitor—16 alpha-naphthoflavone (ANF) do not show any effect in zebrafish CYP3A65 system. Correctly identified. Overall correct prediction was 13/15 = 87.5%.

The above example indicates very good correlation of zebrafish CYP system with that of human.

The US FDA requires CYP assessment prior to drug approval.

The CYP inducer drug increases the quantity of enzyme required for its own metabolism and also induces the metabolism of the other drug which depends on the same enzyme, thus reducing its therapeutic effect.

The CYP inhibitors reduce the drug-metabolizing enzyme and thus reduce the metabolism of the other drug. The other drug will be metabolized slowly, thus showing toxicity. CYP3A4, CYP2D6 and CYP2C9 are the major enzymes contributing to almost 70% of the drug metabolism.

Zebrafish induces xenobiotic enzymes and increases the reactive oxygen species as a reaction to toxic chemical exposure (Wiegand et al. 2000). Zebrafish exhibits many enzymes like CYP3A65, CYP1A1, A19, B19, 2K6, 3C1 and CYP26D1 which are similar to human enzymes. Their genes show close homology with human genes

encoding these enzymes (Bresolin et al. 2005; Miranda et al. 1993; Collodi et al. 1994; Trant et al. 2001).

11.9.2 Intestines

The digestive system consists of the oesophagus, intestine, pancreas, liver and gall bladder. The stomach is absent but the intestine has one dilated area in initial part called intestinal bulb, and it initially stores the food and has natural ph (Nalbant et al. 1999). The intestinal epithelium has villi, enteroendocrine cells, enterocytes and goblet cells. The epithelium is constantly undergoing cell multiplication, differentiation and replacement. The intestine does not have Peyer's patches and intestinal crypts. The movement of the intestine depends upon both circular and longitudinal smooth muscles, and the enteric nervous system controls its. Zebrafish serve as a model to study the effect of microbiome, inflammation, intestinal tumour development and control of enteric nervous system (Zhao and Pack 2017). The gut movement and gut transit time are important for absorption of the drugs but found to be inconstant and may affect the absorption of drugs. So far intestinal toxicity is a concern, and both false positive and false negative results were noted (Cassar et al. 2015). There are many differences in the basic structure of intestines but intestinal toxicity assays detect more than 50% toxicities.

11.9.3 Pancreas

It has both exocrine and endocrine functions in zebrafish and it works in similar manner as that of human. The exocrine pancreas secretes the enzymes required for digestion, and the endocrine pancreas is responsible for the secretion of insulin, glucagon, ghrelin and somatostatin having similar endocrine functions like human. Polypeptide-producing cells (PP cells) are not seen in zebrafish pancreas (Kinkel and Prince 2009). Zebrafish pancreas is studied for research on diabetes (Zang et al. 2018).To damage the endocrine pancreas, similar chemicals that are used in other animal studies are also used and show similar results (Ko et al. 2018; Krishnan and Rohner 2019; Matsuda 2018; Prince et al. 2017). Toxicity to exocrine pancreas is studied in relation to environmental factors (Jacobs et al. 2018; Sant et al. 2017).

11.9.4 Gall Bladder

The transparent nature of zebrafish makes it possible to visualize the transport of lipophilic dye and understand metabolism. The uptake of the lipids, transport and metabolism show close homology with human counterparts (Carten and Farber 2009; Quinlivan and Farber 2017). No biliary toxicity is yet tested in zebrafish as a part of drug development process.

11.10 Kidney: Drug Development and Drug Toxicity

The complex nature of human kidney cannot be directly compared with zebrafish kidney, but the larval pro-nephrons can be compared with meta-nephrons in developmental stages, and because of similarity in these two, some idea of nephrotoxicity can be obtained reliably from zebrafish models (Elmonem et al. 2018; Gehrig et al. 2018; Gorgulho et al. 2018; Schenk et al. 2017). The known nephrotoxic drugs like gentamicin, paracetamol and tenofovir have been shown to be tubulotoxic in human and had shown similar histopathological and functional changes in zebrafish larvae (Morello et al. 2018). The potential drug candidate to repair acute kidney injury is being investigated in larval zebrafish (Hukriede et al. 2017).

11.11 Nervous System: Drug Development and Drug Toxicity

There is important role of zebrafish in understanding of neurological diseases and behaviour and being increasingly used for the same purpose (Babin et al. 2014; Bandmann and Burton 2010; Lee and Freeman 2014). The basic organization of zebrafish brain is similar to other vertebrates and shows basic divisions of the brain (Mueller and Wullimann 2016; Kalueff et al. 2014). The cognitive function in human is played by the neocortex which is absent in zebrafish; hence, cognitive function cannot be studied in zebrafish. Neurotransmitters like dopamine, 5-HT, noradrenaline, glutamate, GABA and acetylcholine are present in zebrafish (Basnet et al. 2019; Cassar et al. 2017; Horzmann and Freeman 2016; Panula et al. 2010; Rico et al. 2011). These neurotransmitters can be used as pharmacological tools to study the drug effect on CNS. Pentylenetetrazol and picrotoxin like GABA antagonist show increase in locomotor activity (Li et al. 2018; Yang et al. 2017), and antiepileptic drugs like phenytoin and valproic acid show decrease in locomotor activity (Liu et al. 2016). Moreover, similar results are also observed in rodents.

Most of the neurological diseases can be studied in zebrafish as most of the genes related to neurological diseases are conserved in zebrafish. These genes help in identification of molecular targets related to neurological diseases (Fan et al. 2010; Lee et al. 2016; Xi et al. 2011).

The transparent nature of zebrafish embryo enables to use fluorescent technique to study the development related to the nervous system. Easy genetic manipulation is possible as the embryo grows outside the mother's body; hence, many transgenic models for neurological conditions are available. The study of neuropsychiatric disorders like depression, anxiety (Marcon et al. 2019; Peng et al. 2016) and other developmental disorder like autism (Ma et al. 2018) is possible because of transgenic models. The transgenic models for neurodegenerative disorders like Parkinson's disease and Alzheimer's disease are available and are important tools to study the disease and to identify the drug targets.

One More Phenotypic Approach—It may be difficult to produce typical neurological/psychological diseases in zebrafish, but the cells, proteins and neurotransmitters are conserved in evolution, so the pathology can be produced.

To understand it more clearly, we will take the example of schizophrenia. The symptoms of schizophrenia may not be produced in zebrafish, but the drugs which can produce schizophrenia in human will produce some behavioural changes in zebrafish and those changes should be considered as equivalent to schizophrenia in zebrafish. The molecules which can control or reduce the manifestation of those behavioural changes can be considered as drug candidates for schizophrenia. This is a phenotype-based approach of drug development for CNS/psychiatric diseases.

11.11.1 Pineal/Circadian Rhythm

Beta blockers, opioids, amphetamine and benzodiazepines are known to disturb sleep architecture. Disturbance of sleep is associated with diabetes, hormonal imbalance and neurological diseases. It may be acceptable in a life-threatening disease like cancer but not in the general condition like hyperacidity. Zebrafish is having diurnal sleep activity and is similar to human and is controlled by melatonin produced by pineal gland. The hypnotic drugs show similar effect zebrafish as that of human, same is the case of CNS stimulants except for dopamine D1 receptor. Stimulation of D1 receptors increases wakefulness in human and induces sleep in zebrafish (Rihel et al. 2010). This suggests that zebrafish is a good model to study the effect on drug candidate on sleep and also to study the hypnotic drugs side effect profile.

11.12 Haematopoietic System and Drug Development and Drug Toxicity

In evolution the zebrafish conserved the haemopoietic and vascular system.

Haematopoietic system in zebrafish shows close resemblance with human. Because of similar type of blood cells like neutrophils, erythrocytes, lymphocytes, eosinophils, macrophages, etc., drugs used for anaemia and drugs affecting haematopoiesis have similar effect in zebrafish. Iron haemostasis and globulin switching are through the hepcidin-ferroportin pathway (Ganis et al. 2012; Steinbicker et al. 2011) and is similar to human. The effects of drugs on these systems in zebra fish can be extrapolated to mice and other mammals. The data suggests that zebrafish can be a suitable model for studying haemopoietic and vascular toxicity of the drugs under development. Transparent development of zebrafish and high-throughput screening are important benefits of zebrafish model. Blood cells and vascular cells develop from mesoderm embryo and blood islands. Ventral mesoderm and cephalic mesoderm converts into blood islands. BMP (bone morphogenetic protein) is responsible for the mesoderm differentiation (Lengerke et al. 2008). Platelets are represented by thymocytes in zebrafish. Zebrafish also exhibits haematopoietic stem cells (HSCs) and those can be transplanted in irradiated zebrafish. The stability of such graft is good and remains there for about

1 year (Choudhuri et al. 2017). The HSCs can be identified by using GFP (green fluorescent protein).

11.13 Endocrine System: Drug Development and Drug Toxicity

The hypothalamic-pituitary axis controls most of the endocrine functions in vertebrates. There is portal circulation between the hypothalamus and pituitary gland. The hypothalamus stimulates the pituitary gland by secreting its hormones in the hypothalamic-pituitary portal circulation. The stimulated pituitary gland secretes hormones which act on the adrenal gland (HPA axis), thyroid gland (HPT axis) and gonads (HPG axis). Many functions of the endocrine system are conserved in zebrafish though some differences do exist. Hypothalamic-pituitary portal system is absent, but the neurons from the hypothalamus directly enter the pituitary and secrete neurotranmitters (hormones) over cells of pituitary gland (Zohar et al. 2010). There is no distinct adrenal gland but inter-renal equivalent is present inside the kidney. Like the adrenal gland in human is no separation of the cortex and medulla, but functionally it has both chromaffin cells and cells responsible for steroidogenesis. The development of the endocrine system completes by 5 dpf (Lohr and Hammerschmidt 2011). Pituitary hormone expression starts by 48 hpf (Herzog et al. 2003). Aromatase gene expression can be confirmed by 24 hpf (Lassiter and Linney 2007) and expression of oestrogen receptors is seen by 24 hpf (Mouriec et al. 2009).The gene expression of inter-renal organ starts by 2 dpf (To et al. 2007). Thyroxin can be detected by 3 dpf (Porazzi et al. 2009). Endocrine-Disrupting Chemicals (EDCs) are of great importance because the endocrine system is related to many physiological processes of growth, health, stress response, reproduction and hence population (Lohr and Hammerschmidt 2011). Studies are mainly focused on oestrogen, androgen, thyroid and steroidogenesis. It is related to the environmental risk assessment of new chemical substance.

11.14 Ototoxicity and Drug Development

Drugs like aminoglycosides used for severe life-threatening infections and cisplatin used for cancer are known to produce ototoxicity (Lanvers-Kaminsky et al. 2017), and they significantly affect the quality of life of patient. Ototoxicity is rarely done for the purpose of regulatory requirement (Gauvin et al. 2016). It is difficult to screen ototoxicity in mammalian preclinical models, but zebrafish can bridge this gap. The zebrafish and human auditory canals are similar and show similar hair cell physiology (McPherson 2018; Pickett and Raible 2019) and similar anatomy and neuronal sensing mechanism. The zebrafish has three semi-circular cannels in the ear which are positioned in three planes. They sense the speed and direction of movement. The ear has otoliths that transmit the vibrations to maculae. There are three maculae: (1) articular macula for balance, (2) saccular macula for hearing and (3) lagenar macula for both hearing and balance. All these maculae work together and stimulate

cristae and ultimately hair cells which transmit the signals to the brain. This anatomy and physiology is similar to human internal ear, but zebrafish does not have cochlea, outer ear and middle ear. The amplification function in zebrafish is carried out by weberian apparatus which consists of four pairs of bones. Sound is transmitted to the brain via mechanosensory hair cells. Hair cells transduce the water waves or sound into electrical nerve impulse and transmit them to the brain. Hair cell functions are specialized and are well developed in zebrafish by 5dpf. Easy assessment of hair cells in zebrafish is possible and hence zebrafish become a popular model to study ototoxicity and oto-protection. The known ototoxic drugs to human show ototoxicity to larval and adult zebrafish, e.g. aminoglycosides, cisplatin, metal contaminants and carbonic anhydrase inhibitors (Coffin and Ramcharitar 2016; Matsumoto et al. 2017; Rah et al. 2017; Uribe et al. 2013).

11.15 Ocular Toxicity and Drug Development

Zebrafish shows very close similarity with the human eye and hence is the best model for studying ocular drug effects and toxicity. The vision develops on 4 dpf (Easter Jr. and Nicola 1996). The zebrafish has diurnal vision and has cone-rich retina similar to the human retina. Other experimental animals like mice and rats have less cones and are having weak colour vision (Goldsmith and Harris 2003). The retina of zebrafish does not have cone concentrated area. The cones are sensitive to ultraviolet light. The double cones system that consists of a red-sensitive compartment and green-sensitive compartment is also present in zebrafish (Slijkerman et al. 2015). The retina in zebrafish can regenerate and this is the most important difference between zebrafish and other experimental animals. In response to tissue injury, the retinal progenitor cells can differentiate into primary retinal neurons (Wan and Goldman 2016). Because of ocular similarities with the human eye, the zebrafish eye is a rational model to study the ocular drug development and drug toxicity. The consistency of results related to ocular effects of the drugs like chlorpromazine, deferoxamine, quinine, cisplatin, gentamicin, thioridazine, vardenafil and minoxidil is observed in zebrafish and human (Berghmans et al. 2008; Deeti et al. 2014; Richards et al. 2008).

11.16 P-Glycoprotein and Drug Resistance

Pgp efflux pump—This is the transporter present in the gastrointestinal tract and endothelial cells of brain capillaries. It is from the ABC family of transporters. There are six well-characterized transporters of this family: ABCB1, ABCC1, ABCC3, ABCC4, ABCC5 and ABCG2 (Dean et al. 2001). They are well known for their role in drug resistance (Loscher and Potschka 2005). Out of the six well-characterized transporters, ABCB1 also known by the name MDR1 is the most extensively studied transporter. It is responsible for efflux of small molecules from the brain to blood. It has very broad specificity and hence a large number of drugs are eliminated using

this efflux pump. It is an ATP-dependent pump. Its inhibition leads to increase in absorption of drug and also accumulation in the brain. The Pgp knockout mouse shows hundred-fold increase in absorption of drug as well as drug accumulation in the brain (Schinkel 1999; van Asperen et al. 1996)

The presence of BBB in zebrafish had been confirmed (Cserr and Bundgaard 1984; Jeong et al. 2008). The activity of Pgp can be noted on 3 dpf and it became fully functional on 7 dpf. Drugs like verapamil, phenytoin, loperamide, cyclosporine, RU486 and quinidine shown to inhibit Pgp efflux in zebrafish brain and caffeine did not show any inhibitory effect. Similar results are observed in other mammalian models. Thus, zebrafish is considered as excellent predictive model for Pgp expression assessment. Zebrafish model is more convenient because of transparent brain tissue and fluorescent imaging is possible to study the efflux.

11.17 Zebrafish and Cancer Drug Development

Cancers can be developed in zebrafish by using chemicals or inducing mutations.

11.17.1 Chemical Carcinogenesis

1. *TP53*—is the most common tumour suppressor gene found in human. Mutations of this gene result in tumour generation. The morpholino oligonucleotide antisense sequences revealed the tumour suppressor function of tp53 gene. *N*-Ethyl *N*-nitrosourea treatment results in the mutations in tp53 gene, leading to tumorigenesis. These mutations make the fish deficient with apoptotic activity and not able to arrest cell cycle on DNA damage. The development of zebrafish was found to be normal but after 8.5 months the zebrafish develops peripheral nerve sheath tumours (Berghmans et al. 2005). This is how zebrafish can be used as a model for tumour research.
2. *APC* (Adenomatous Polyposis Coli)—is a tumour suppressor gene. Mutations in this gene result in activation of wnt-beta-catenin pathway showing sporadic colorectal cancers in human. APC mutant of zebrafish can be developed and separated by treating embryo with *N*-ethyl-*N*-nitrosourea (ENU) (Haramis et al. 2006), and they show intestinal, pancreatic and hepatic cancers. These cancers show accumulated beta-catenin and express wnt genes. The histopathological changes observed in these zebrafish tumours are similar to sporadic colorectal cancers in human. Another effect of APC is it supresses cyclooxygenase-2-enzyme (Eisinger et al. 2006), and its effects on retinoic acid synthesis and metabolism (Shelton et al. 2006) are similar in zebrafish and in human/mammals.

11.17.2 Transgenic Models in Zebrafish Neoplasia

Acute myeloid leukaemia (AML) can be produced in zebrafish by RUNX1-CBF2T1, a human gene construct produced by translocation between chromosomes (8:21). The phenotypic presentation of these embryos showed dysplastic immature blood cells, internal haemorrhages and disorganized circulation which is consistent with the transgenic mice produced by using the same human gene construct. This proves that zebrafish can be used as a model in leukaemogenesis.

In a similar way, T-cell leukaemia can be produced in zebrafish and is a good model.

Myeloid leukaemia-TEL-JAK2A fusion has been identified in lymphoblastic and myeloid leukaemia. It is responsible for activation of JAK2 kinase protein which is associated with cytokine-independent growth of haematopoietic stem cells. If the same mutation is inserted in the growing zebrafish embryo, the zebrafish can serve as a model for myeloid leukaemia.

11.18 Challenges in Zebrafish as a Model for Drug Development and Drug Toxicity

1. The dosing is in-water dosing—The embryo and larva are fed by solubilizing the drug in water in which the embryo or larva is living. This is how the water-insoluble drugs cannot be given by this in-water oral route, and water-insoluble drugs need to be injected to get sufficient blood concentration.
2. In this in-water dosing method, all the animals are exposed to the same concentration of the drug and are immersed in the water containing drug, but how much is really absorbed needs to be find out by blood levels.
3. The translation of the data obtained by this in-water dosing to human needs to be worked upon. It may vary from system to be investigated and the physiochemical properties of the drug candidate under investigation.
4. The strength of zebrafish larva is its size the same possess problem while determining plasma concentration and ADME determination. These types of experiments are in their early stages (Grech et al. 2019; Villacrez et al. 2018).
5. The distance between efficacious concentration and toxic concentration defines the therapeutic window. The toxic concentration obtained by in-water dosing cannot be reliably correlated with mammalian plasma concentrations, more work is needed in this field (Grech et al. 2019; Villacrez et al. 2018).
6. Phylogenetic proximity is closely related to the predictive value of animal model. Other mammals like mouse are closer to human than zebrafish, and hence zebrafish will not completely replace classical mammalian model but will be complementary to classical model in some aspects. Extrapolation of data from zebrafish to human is poor as compared to mammalian data.
7. The gene similarity is okay but more important is the similarity between the biological systems, cellular metabolism and response to toxic chemicals. These differ sometimes between zebrafish and human.

8. Zebrafish shows regeneration in the organs like the fin, brain, spinal cord, heart and retina. This is good to study this in relation to regenerative medicine, but the same may interfere with the toxicological effects of the drug candidate on these tissues and the data may be misleading due to regeneration in these organs. The same is seen in retina (IV-38).
9. Zebrafish do not have organs like the lungs, prostate, mammillary glad and skin. Hence, the drug development related to these tissues will not be possible in zebrafish; similarly adverse drug reactions affecting these organs cannot be predicted in zebrafish.

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12 Zebrafish as a Novel Pharmacological Screening Model for Drug Discovery and Development Against Hematological Disorders

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Abstract

Zebrafish is a novel pharmacological screening model for increasing the pace of drug discovery and development. It is an extensively used vertebrate model with

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the purpose for understanding disease development as well as its progression. Zebrafish offers a variety of advantages over the conventional pharmacological models including economical maintenance, high fecundity, transparent body, and ease of inducing genetic mutations using a variety of techniques. Zebrafish have high similarity with humans and offer an alternative strategy for high-throughput screening of new chemical entities. Blood disorders constitute a significant number of human pathologies ranging from anemia to leukemia. To this end, zebrafish offers a unique platform to study a plethora of human hematological disorders. In the current chapter, we provide the details of advantages and limitations of using zebrafish in hematology research. The use of zebrafish in studying various non-malignant disorders like anemia, bone marrow failure, coagulation disorders, and immunodeficiency has been covered. Further, the advantage of zebrafish serving as a model to understand various malignant blood disorders like chronic lymphoblastic leukemia (CLL), acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML) along with acute myeloid leukemia (AML) has been described. In a nutshell, this chapter accentuates the role of zebrafish as an emerging pharmacological model to evaluate the pathology of hematological disorders and development of drugs for the treatment thereof.

Keywords

Zebrafish · Hematopoiesis · Pharmacological model · Drug screening · Leukemia

12.1 Introduction to Zebrafish in Hematology

Zebrafish (*Danio rerio*) served as a popular model in the field of hematological analysis. This model has gained popularity due to its attractive features like possibility of ex vivo fertilization; ease of handling and manipulation of transparent embryos; cheap maintenance costs; and real-time monitoring of various pathologies due to optical coherence of developing zebrafish embryos as well as its larvae. Employing zebrafish model to study these conditions has its advantages like (1) structural and functional homology between zebrafish hematological factors and its mammalian orthologs, (2) conserved initiation and coagulation endpoints,

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(3) rapid confirmation of disease-causing variants, and (4) ease of performing high-throughput experiments in in vivo system (Weyand and Shavit 2014). Several genetics together with chemical are being applied to this vertebrate model for creating mutants analogous to various human pathological conditions. In addition to these, the recent advancements in genome engineering technology have facilitated the use of zebrafish in identifying candidate genes in various pathological conditions. All these aid in rapid screening of molecular pathways and identification of novel therapeutics for various hematological disorders through zebrafish models (Jing and Zon 2011; Lieschke and Currie 2007).

Zebrafish have been used in the field of hematological research for over six decades. Foremost descriptions of zebrafish blood cells were given in the 1970s. Modern approaches using zebrafish models emerged in the early twenty-first century beginning with the application of large-scale chemical mutagenesis screens for identifying mutants mostly with defects in hematopoiesis. These early works paved the pathway for understanding molecular mechanisms involved in normal and pathological vertebrate hematopoiesis (Carradice and Lieschke 2008; Ransom et al. 1996). The first human disease model in zebrafish was a blood disorder associated with defect in hemoglobin production. The gene responsible for this condition is ALAS2 where the sauternes (sau) causes the pathological condition. Following this, various other mutants having similarity with human diseases have been screened including various hematological and cardiovascular diseases (Chitramuthu 2013).

It has been evident that the genetic approaches via zebrafish models aid in understanding hematopoietic regulatory pathways. A close parallelism has been identified between mammalian and zebrafish systems regarding transcriptional regulation of hematopoiesis. Characterization and understanding transcription regulation of hematopoiesis in zebrafish may provide valuable insights about mammalian hematopoiesis. Hence, it can be used as an effective model for studying pathological transcription-associated mutations and fusion proteins in humans (Carradice and Lieschke 2008). In addition, zebrafish model has also contributed for better understanding of vascular development and remodeling. Angiogenesis is conserved in zebrafish, and various findings are found to be relevant to human system (Quaife et al. 2012).

Due to their small size, zebrafish have been successfully used for the identification and testing of novel therapeutic agents by using high-throughput chemical screens. Screening of a complete organism provides an all-round spectrum of effects of an agent on various cell and tissue types (Kaufman et al. 2009). Several strategies have been developed for creating zebrafish mutants to develop patient-specific therapies. In vivo xenotransplantation and transgenic approaches have been used to test several chemical compounds and for identifying new therapeutics (Zizioli et al. 2019). Owing to the anatomical and physiological similarities among vertebrates, zebrafish provide a perfect tool for understanding molecular pathways applicable to human systems. Hits from these approaches have also entered into clinical trials for use in treating human hematopoietic malignancies (White et al. 2011).

Advantages

(a) Smaller size, rapid embryonic development, and good fecundity of zebrafish aid in large-scale forward and reverse genetic screens. Screening approach based on phenotype provides unbiased understanding of molecular pathways.
(b) Convenient and economical for both in vivo and in vitro studies compared to other mammalian systems.
(c) Ease of manipulation of embryos and adult organisms for functional screening of genes with the help of transient genetic assays without any requirement for the production of transgenic animals.
(d) Mutants constructed in zebrafish are homologous to various human diseases, and these models can be used for screening of small molecules for their application as therapeutics.
(e) Transparent embryos allow the procedure of real-time imaging of the hematopoietic stem cell origin and growth.

Limitations

(a) Differences in morphology of blood cells and gross anatomy.
(b) Lack of markers or antibodies, hematopoietic cell lines, and cell culture assays and lack of inbred strains.
(c) Genetic divergence between fishes and humans.
(d) Limited options for conditional transgenesis.

12.2 The Hematological Features of Zebrafish

Although murine organisms happen to be the prime models for hematological research, zebrafish emerged to be a crucial model for the analysis of hematopoiesis as well as hematological ailments related to humans. Several factors like anatomical and biological similarities in hematopoiesis and blood-related disorders, high-throughput screening techniques, highly conserved molecular pathways, and ease of performing genetic engineering experiments contributed to the success of zebrafish models (Zizioli et al. 2019).

12.2.1 Zebrafish and Human Hematology: Similarities and Differences

Many zebrafish developmental mutants were recognized via forward genetic screens in the 1990s displaying a large degree of conservation in genes related to disease (Driever et al. 1996). In addition, there is a powerful protection of major growth events of hematological organization which led to the recognition and better understanding of various genes which are included in the human hematopoietic and other hematological ailments (Robertson et al. 2016).

Zebrafish hematopoiesis occurs in three different sequential waves which include the primitive wave, the erythromyeloid progenitor (EPM-derived) wave, as well as

definitive wave occurring in varying sites in the embryo (Zizioli et al. 2019). Genetic events that control every individual wave are largely conserved in between zebrafish and mammals (Ciau-Uitz et al. 2014). The hematopoiesis inside the zebrafish takes place at the site of intermediate cell mass (ICM), whereas it is highly erythropoietic and also takes place in the region outside of the embryo and more precisely in blood islands of the yolk sac.

Analysis of coagulation factors in zebrafish revealed conservation with mammalian factors at multiple levels. Molecular and biochemical evidences for factor X, protein C, fibrinogen, and prothrombin have been explained in various fish systems (Jagadeeswaran and Sheehan 1999). The coagulation cascade of zebrafish is comparable to mammals with similarities in thrombocytopoiesis, fibrinogen receptor GPlb expressed on thrombocytes, presence of thrombin and ADP receptors on thrombocytes of zebrafish, and high sequence homology with human coagulation and anti-coagulation factors (Meng et al. 2020). Despite certain unexpected differences in some factors, functional conservation has been established for both intrinsic and extrinsic factors as well as platelet surface receptors including F2, F7, vWF, fibrinogen, and Mpl (Weyand and Shavit 2014; Jagadeeswaran et al. 2016).

12.2.2 Zebrafish Biology and Relevance for Hematology Model Development

Zebrafish share many features with mammals including humans in several aspects like hematopoiesis, coagulation cascade, thrombus generation, hematological diseases, and their molecular pathways. The conserved genes between these organisms can be exploited for development of hematology models in zebrafish for better understanding of normal and pathological mechanisms involved in human hematology.

12.2.2.1 Zebrafish Model for Hematopoietic Development

Due to the highly conserved genetic events among zebrafish and mammals, zebrafish use in hematopoietic research yielded good results in steady-state hematopoiesis which are validated in mammalian model organisms and humans. These include the validation of role of adenosine in production of the hematopoietic stem cells (HSC) from aorta-gonad-mesonephros (AGM) area and induction of sclβ expression in hemogenic endothelium (Jing et al. 2015); live imaging of HSC emergence from AGM (Kissa et al. 2008; Bertrand et al. 2010); role of earliest macrophages in the emergence of HSC emergence (Travnickova et al. 2015); role of cadherin 5 in emergence of HSC (Anderson et al. 2015); and role of prostaglandin E2 (PGE2) in modulation of HSC homeostasis (North et al. 2007). These efforts continue to help in generation of HSCs in vitro.

12.2.2.2 Zebrafish Model for Studying Coagulation Factors and Coagulation-Related Disorders

Various crucial factors of vertebrate hematological system appear during the evolution of pisces, providing an evidence for the presence of a comparable system in vertebrates. Especially the key mediators of blood clotting are mostly conserved in between zebrafish and other mammalian systems. A great extent of closeness was observed in between zebrafish factor VII along with mammalian F7 protein (Sheehan et al. 2001). cDNA cloning of the zebrafish von Willebrand factor (vWF) revealed protection of sequences and a protein similarity of 46% (Ghosh et al. 2012). Along with coagulation-associated factors, certain anticoagulant factors are also conserved in zebrafish being ATIII. Sequence alignment revealed 55% identity and 70% similarity between zebrafish and human ATIII (Kumar et al. 2013).

In addition, laser injury model along with knockdown studies revealed the roles and importance of factor V, prothrombin, factor VIII, protein kinase C-α, Mlck1a, G6fl, and protein kinase C-β in the process of hemostasis (Weyand et al. 2019; Day et al. 2004; Williams et al. 2011) . Zebrafish provides an efficient in vivo testing model for studying human genetic sequences related to coagulation disorders where the circulatory system complexity can be unveiled. Gene knockdown generated models of zebrafish were helpful in understanding megakaryopoiesis, platelet activation, thrombocyte aggregation, and pathological pathways behind fibrinogen deficiencies, thrombocytopenia, and many more coagulation-related disorders (Hu et al. 2019; Johnson et al. 2009; Albers et al. 2011, 2012).

12.2.2.3 Zebrafish Model for Studying Blood Disease

The first animal model for studying hematological diseases in zebrafish was established to evaluate the role of δ-aminolevulinate synthase (ALAS2) in heme synthesis (Brownlie et al. 1998). Many other erythroid and myeloid disease models were generated in zebrafish like Diamond-Blackfan anemia (DBA) (Danilova et al. 2008; Uechi et al. 2008), congenital erythropoietic porphyria (CEP) (Childs et al. 2000), hepatoerythropoietic porphyria (HEP) (Wang et al. 1998), and systemic mastocytosis (Balci et al. 2014).

The initial zebrafish model for leukemia was developed in the year of 2003 (Langenau et al. 2004). Since then several models using zebrafish have been constructed for different types of leukemia like T-cell acute lymphoblastic leukemia (T-ALL), B-cell acute lymphoblastic leukemia (B-ALL), and acute myeloid leukemia (AML) (Konantz et al. 2019; He et al. 2017). Zebrafish models for studying primary immunodeficiencies include reticular dysgenesis (Pannicke et al. 2009; Rissone et al. 2015); Wiskott-Aldrich syndrome (WAS) (Cvejic et al. 2008); warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome (Walters et al. 2010); leukocyte adhesion deficiency (LAD) (Deng et al. 2011); chronic granulomatous disease (CGD); and 70 kDa (ZAP70) deficiency, zeta-chain-associated protein kinase 70 (Christie et al. 2010; Moore et al. 2016).

12.3 Zebrafish as a Tool for Studying Hematopoiesis

At the recent times, the zebrafish has been evolved as a versatile as well as useful vertebrate model, which is reducing the gap in between the invertebrates along with mammalian systems. Although having over 400 million years of the divergence in between humans and zebrafish, the latter has a largely conserved hematopoietic structure at the cellular, developmental, biochemical, genetic, as well as functional stages. This has contributed for several significant hematological discoveries, especially in the field of hematopoiesis and hematological disease modeling.

12.3.1 Conserved Biology of Hematopoiesis in Zebrafish

The capability to easily manipulate the genome of the zebrafish resulted into the recognition along with analysis of several foremost genes which play an important role in hematopoietic progression and related diseases. Due to which, maximum amount of the hematopoietic transcription factors genes noticed in the mammals contain orthologous in the zebrafish. Loss of function mutants has been generated for several hematopoietic transcription factor genes to study and validate their role in hematopoiesis. These include *scl*, *runx1*, *runx3*, *cdx4*, *hhex*, *tif1γ*, *spt/tbx16*, *c-myb*, *gata1*, and *klf4* (Davidson and Zon 2004).

Vertebrate hematopoiesis takes place in the two waves which includes that the first wave results into transient population of the earliest blood cells which give support to the embryo during early growth, providing the embryo with primitive erythrocytes for oxygen transporting along with microbial protection via primitive leucocytes that are majorly macrophages. However, this primary wave of hematopoiesis is usually generated from the unipotent progenitors, as compared to the multipotent stem cells which underpin the following wave of the definitive hematopoiesis called second wave. This second wave produces definitive HSC. Moreover, these different cells are known by their potential to self-renew as well as differentiate inside all the blood lineages. Hematopoietic growth is primarily regulated inside a spatial, temporal, as well as molecular way. Despite the difference in their emergence, the genetic programs which modulate each particular wave are usually largely conserved in between zebrafish and mammals.

12.3.2 In Vivo Models for Investigating Hematopoiesis and High-Throughput Screening in the Zebrafish

Hematopoietic disorders of both malignant and non-malignant nature have been modeled in zebrafish providing various useful insights about both normal and pathological hematopoiesis in humans. Several successful methods have been applied for studying and better understanding hematopoiesis and hematopoietic associated disorders in zebrafish. These include chemical and genetic screens; genome editing; real-time studies and optical imaging; proteomics and

metabolomics studies; gene expression analysis; and sequencing techniques (de Pater and Trompouki 2018). Figure 12.1 graphically summarizes the utility of zebrafish to study hematopoiesis.

12.3.2.1 Malignant Models of Hematopoietic Disorders

Several oncogenes when expressed in zebrafish generated malignancies similar to their counterparts in humans (Kwan and North 2017). A highly conserved hematopoietic system together with distinctive experimental vigor makes zebrafish best suited for the study of leukemia (Langenau et al. 2004). Since then several models using zebrafish have been constructed for different types of leukemia like myeloid neoplasms which include myeloproliferative neoplasms, myelodysplastic syndromes, AML, 5q-syndrome, as well as chronic myeloid leukemia (CML)-like disease and ALL of B-cell and T-cell origin (Konantz et al. 2019). Zebrafish models for studying malignant hematopoietic ailments are discussed in further sections.

12.3.2.2 Non-malignant Models of Hematopoietic Disorders

Primary Immunodeficiencies

Mutations in gene coding for WAS protein are responsible for Wiskott-Aldrich syndrome, and functionally deprived mutants have been generated in the zebrafish by selecting induced confined lesions in the genomes (TILLING) to acknowledge the molecular activities in the disorder (Cvejic et al. 2008). Similarly, TALE nuclease-mediated loss of function in *ZAP70* gene mutants was generated in zebrafish to study *ZAP70*-related combined immunodeficiency (CID) (Moore et al. 2016). Truncated version of the CXCR4 was overexpressed inside the neutrophils of zebrafish to study warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome which has been associated with overexpression of same gene in humans (Walters et al. 2010). Morpholino oligonucleotide (MO)-mediated mutants of rac2, ak2, ncf1, and cybb were generated in zebrafish to understand molecular events associated with leukocyte adhesion deficiency, reticular dysgenesis, and chronic granulomatous disease, respectively (Yang et al. 2012).

Inherited Bone Marrow Failure Syndromes

More than half of the total Diamond-Blackfan anemia (DBA) cases occur due to mutations inside ribosomal protein genes. MO-mediated knockdown of rsp19 in zebrafish resulted in DBA-like phenotype (Jia et al. 2013). Following these findings, several genes encoding ribosomal proteins were targeted in zebrafish by MO knockdown and TALENs to understand pathophysiology of DBA (Antunes et al. 2015; Chakraborty et al. 2018). It was also observed that L-leucine and L-arginine were effective in treating DBA through mTOR pathway (Payne et al. 2012) and are currently in clinical trials. In the same way, zebrafish mutant models were generated via MO-mediated sbds and srp54 knockdown; TALEN-mediated mpl mutation and CRISPR/Cas9-mediated *ptprja* ablation; MO-mediated cfs3 knockdown and CRISPR/Cas9 targeting of *csfr3*; MO-mediated *fancd2* knockdown and CRISPR/Cas9-mediated *rad51* loss of function; and mutations in *nop10*, *dkc1*, *nola1*, and *tert*

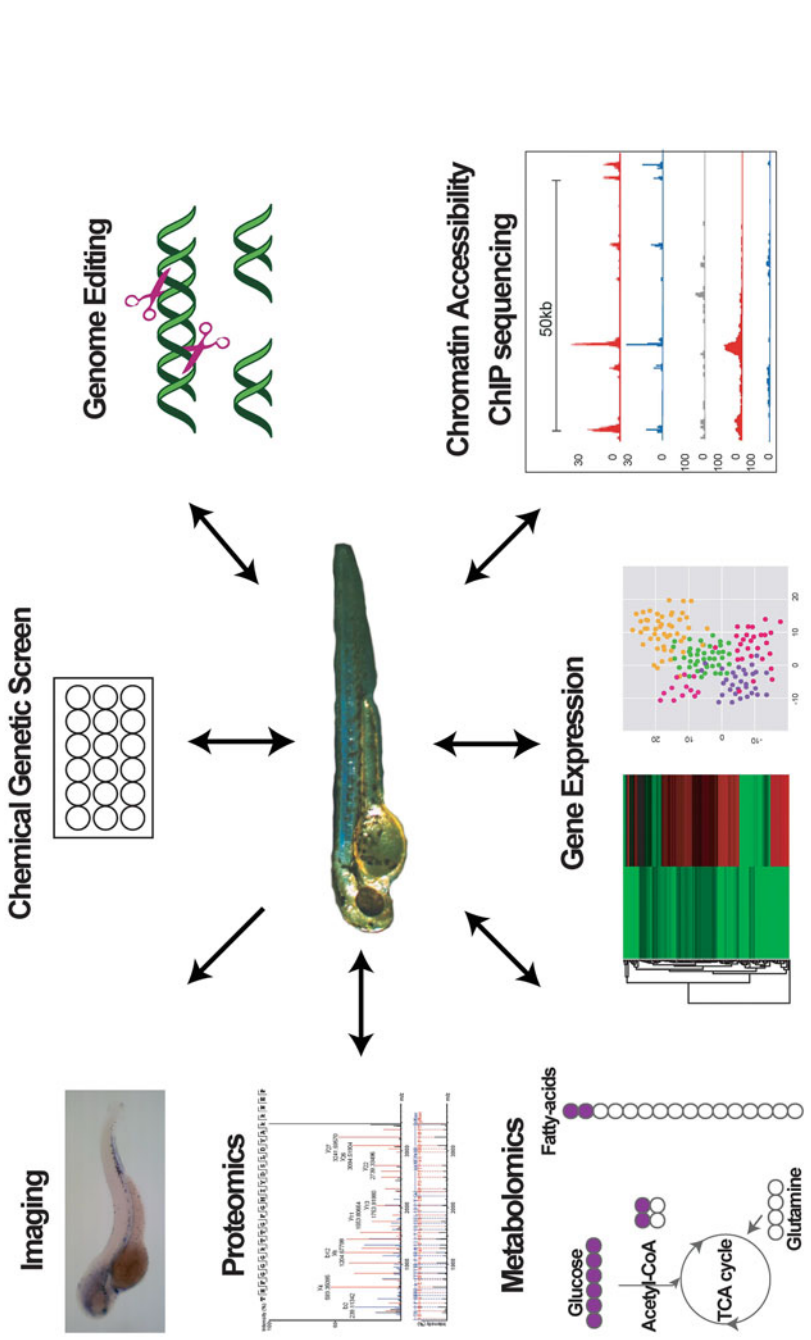

Fig. 12.1 Graph depicting varying approaches employed to analyze hematopoiesis in zebrafish. (The diagram was reproduced from de Pater and Trompouki (2018) under the Creative Commons Attribution License (CC BY))

genes for studying and validation of molecular events involved in Shwachman-Diamond syndrome, thrombocytopenia, severe congenital neutropenia, Fanconi anemia, and dyskeratosis congenita, respectively (Carapito et al. 2017; Lin et al. 2017; Griebel and Oller 2012).

Anemia

Several zebrafish mutants were generated to study various forms of anemia seen in human beings. *Merlot* and *chablis* strains and *riesling* mutants were generated to resemble hereditary elliptocytosis (HE) as well as hereditary spherocytosis (HS), respectively, both of which are different types of hemolytic anemia observed due to defects in erythrocyte membrane cytoskeleton (Liao et al. 2000). *slc4a1* gene mutant known as *retsina* was produced as a model for the dyserythropoietic anemia type II (Pollard et al. 2009). A hypochromic microcytic anemia model known as *zinfandel* was generated via abnormalities in embryonic globin synthesis (Brownlie et al. 2003). In the same way, *sauternes*, a congenital sideroblastic anemia mutant model, was generated by disrupting heme synthesis (Clark et al. 1998). Different mutant models for hemochromatosis were produced by targeting ferroportin 1 in weissherbst, transferring receptor 1 in *chianti* and *dmt1* in chardonnay models (Donovan et al. 2000). Dracula, the most anemic zebrafish mutant model, was observed in genetic screening and is an excellent representative for studying erythropoietic protoporphyria (Childs et al. 2000). Dracula gene encodes for ferrochelatase, an important enzyme in heme synthesis pathway.

12.3.3 Genetic Mutations to Study Hematopoiesis in the Zebrafish

12.3.3.1 Impact of Mutations on Embryonic Blood Cells in Zebrafish

In studies, impact of mutations on the blood cell enlargement were conducted for large-scale screen for the mutations impacting on the embryonic maturity in the zebrafish (Driever et al. 1996). The mutations are generally divided into four categories depending upon their cellular phenotype.

Group 1: Mutants defective in blood cell generation (bloodless mutants): vampire (vmp) m62, m262, vlad types (vlt) m651, m547 and m594 moonshine (mon) tb222b, tc239b, tc246b.

Group 2: Mutants defective in blood cell differentiation (decreasing blood counts): pale and wan (paw) m416, sticky blood (sti) m232 and clear blood (clb) m525, chablis (cha) tu242e, tu245, frascati (frs) tg221, tg280a, tm130d, tq223, merlot (mot) tm303c, tu275, retsina (ret) tr217, tr265, thunderbird (tbr) ty118b, riesling (ris) tb237, cabernet (cab) tl236, grenache (gre) tr219.

Group 3: Mutants with hypochromic blood (low hemoglobin): chianti (cia) tu25f, pinotage (pnt) tq209, sauternes (sau) tb223, ty121, zinfandel (zin) te207, chardonnay (cdy) te216, weißherbst (weh) th238, tp85c.

Group 4: Mutants having photosensitive blood cells: dracula (drc) m87, m159, m248 as well as desmodius (dsm) m534, freixenet (frx) tu271, yquem (yqe) tp61.

12.3.3.2 Mutations Affecting Blood Cell Generation (Bloodless Mutants)

These mutations are normally recessive in nature, with the deviation of m547. Besides, penetrance of the mutations seems to be based upon genetic background. Of note, Vmpm62 isolates in the form of a fully penetrant throughout various generations, but when crossed with AB hybrid, it shows reduced penetrance. Vmpm62 and vltm651 homozygotes are noticed by large decrement in the blood cell precursors along with the aggregation of the debris. Heme synthesis is unaffected in the mutant sibling. Additionally, a trophic factor produced by the marrow stroma is important for the endurance of pluripotent hematopoietic stem cells as well as receptors for trophic factor (Blume-Jensen et al. 1995). In the zebrafish cloche mutation, both blood and vascular lineages are reduced; these genes autonomously impact the hematopoietic along with endothelial lineages (Stainier et al. 1995). Furthermore, blood gathered from 2-day-old frstq223 mutant embryos depicted the cells at different levels of erythroblast development as well as none that involved the characteristics of the differentiated erythrocytes. However, the cells were substantial with the basophilic cytoplasm, and most of the nuclei were irregularly divided into the fragments of chromatin. Hence, this indicates that they are hindered in the erythroid cell differentiation. In another case, whole-mount o-dianisidine staining suggests that the spared blood cells of the ret. larvae depict hemoglobin alikeness to those of the frs mutants, depending on the reducing amount of the blood cells in this category of mutants together with the amount of hemoglobin in the spared circulating cells where these mutations might not instantly impact the management of the expression of globin gene whereas they impact the proliferation of erythroid cell along with differentiation. Moreover, embryonic endurance in mammals based upon blood, so it is very improbable that the mutations which damage earlier blood cell production would be observed in the humans, at least not in completely expressive state. However, aplastic anemia does found very infrequently as a congenital ailment in the humans (Saunders and Freedman 1978) but generally displays itself only later at the period of childhood and is usually induced by a significantly increased sensitivity of the cells towards DNA destruction (Bloom et al. 1966).

12.3.4 Real-Time Studies and Optical Imaging to Study Hematopoiesis in the Zebrafish

It is the transparency of zebrafish embryos and their accessibility that has played a key role in identifying the location of the emergence of hematopoietic stem and progenitor cells (HSPCs) in vivo (Kissa and Herbomel 2010). In addition to this, high-end imaging techniques unveiled the mechanisms of thymus development (Hess and Boehm 2012) and also aid in understanding the amplification and interaction of HSPCs with endothelial cells (Tamplin et al. 2015). It was also understood that the generation of HSC requires a neutrophil-mediated inflammatory signaling (Espín-Palazón et al. 2014). These unique features of zebrafish have aided in understanding the key role of macrophages in the mobilization of HSPC and

definitive hematopoiesis (Travnickova et al. 2015; Inoue and Inoue 1996). We can use light-sheet microscopy, selective plane illumination, or spinning disk microscopy to visualize transient and cellular interaction in vivo in embryos and adults as they record time-lapse 3D fluorescent images 100–1000 times faster than conventional methods (Huisken et al. 2004; Arrenberg et al. 2010). In addition to the works on transparent zebrafish embryos, transparent adult zebrafish models (White et al. 2008) have also been helpful in the imaging of adult hematopoiesis.

Several transgenic reporter lines expressing fluorescent proteins for studying hematopoietic genes of interest have been engineered due to the strong genetic conservation in zebrafish. These transgenic lines have been advantageous in quantification of changes in hematopoietic cell number and for real-time imaging of the emergence and migration of HSCs in vivo that is difficult in mammalian systems (Kissa and Herbomel 2010; Kalev-Zylinska et al. 2002). *Tg(−6.0itga2b (CD41): EGFP)* was the first transgenic HSC reporter line engineered for the visualization of HSCs from aorta-gonad-mesonephros (Lin et al. 2005; Ma et al. 2011). Following this, fluorescent reporters for several genes have been generated including *runx1* and *cmyb* (Lam et al. 2010).

The existence of hemogenic endothelium was revealed by *scl β*-driven GFP line, and it also confirmed that *scl* is required for the emergence of HSC from aorta-gonad-mesonephros (Zhen et al. 2013). Other studies that visualized the emergence of HSCs from hemogenic endothelium include lineage tracing of *cmyb* and *flk1* transgenes to show budding HSCs in the vasculature (Bertrand et al. 2010) and time-lapse imaging of transgenic zebrafish embryos expressing *flk1* with GFP reporter aided in visualization of budding of HSCs from the endothelium into the vasculature (Kissa and Herbomel 2010), which is now known as endothelial-to-hematopoietic transition (EHT). These discoveries opened a new way for understanding HSC niche interactions in different major organs and also in investigating various HSC niche components.

12.4 Zebrafish as a Tool to Develop Hemostasis and Thrombosis Models

Hemostasis is defined as the physiological process that controls bleeding at the site of vascular injury, whereas thrombosis is a pathological version of hemostasis leading to blood clots in normal vessels disturbing the flow of blood through circulatory system. Since the first elucidation of clotting factors and cascade mechanisms, researchers began to develop techniques to assess the clotting factors and platelets to aid in diagnosis of clotting disorders (Kretz et al. 2015). But the genetic diversity with respect to bleeding and thrombotic disorders has not yet been completely understood resulting in variable clinical manifestations and profiling of affected patients (Ginsburg 2005). Various models have been characterized to study molecular pathways and factors contributing to this genetic variability.

One of the most common models employed for this purpose from the beginning is murine models. However, these models are not economical as well as were

time-consuming (Kretz et al. 2015). Due to its relevance to human coagulation pathways and platelet-associated reactions, zebrafish has gained importance in understanding platelet function, hemostasis, and thrombosis. There are various advantages in employing zebrafish models for studying hemostasis and have been described in the previous sections. Zebrafish has emerged as a model organism to (1) elucidate the molecular pathways involved in the generation and action of various gene products, (2) validate the genetic causes identified in human population, (3) develop genetic and chemical screens for identification of novel mediators responsible for hemostasis and thrombosis, and (4) identify genes via an unbiased approach rather than gene-by-gene approach as in mice (Orkin and Zon 1997; Santoriello and Zon 2012; Haffter et al. 1996). Zebrafish models for studying hematopoiesis are well established, and features of these model systems like high fecundity, transparent embryos, and easy accessibility of hematopoietic and circulatory system offer better features to study novel features of hemostasis in zebrafish.

12.4.1 Models for Studying Coagulation Factors

There is a high similarity between zebrafish and human genomes with the presence of about 70% of human orthologs in zebrafish. Especially the key mediators of blood clotting are highly conserved between zebrafish and other mammalian systems. Various crucial factors of vertebrate hematological system appear during the evolution of pisces, providing an evidence for the presence of a comparable system in vertebrates. Molecular and biochemical evidences for factor X, protein C, fibrinogen, and prothrombin have been explained in various fish systems (Jagadeeswaran and Sheehan 1999).

The coagulation cascade of zebrafish is comparable to mammals with similarities in thrombocytopoiesis, fibrinogen receptor GPIb expressed on thrombocytes, presence of thrombin and ADP receptors on thrombocytes of zebrafish, and high sequence homology with human coagulation and anti-coagulation factors (Meng et al. 2020). Despite certain unexpected differences in some factors, functional conservation has been established for both intrinsic and extrinsic factors as well as platelet surface receptors including F2, F7, vWF, fibrinogen, and Mpl (Weyand and Shavit 2014; Jagadeeswaran et al. 2016). Several gene editing approaches like transgenesis, mutagenesis, nucleases (zinc finger nuclease-mediated knockout), and morpholino oligonucleotides (MO)-mediated knockdown were employed for conducting loss-of-function studies in zebrafish for examination of various coagulation and thrombotic factors. MO are the most widely used gene knockdown tools in zebrafish model (Bill et al. 2009). This was followed by use of ferric chloride, phenylhydrazine treatment, or laser-mediated vascular endothelial injury on zebrafish blood vessels for understanding the coagulation cascade (Gregory et al. 2002). As the tail region is thin, thrombosis develops in caudal blood vessels of zebrafish larvae, and time to occlusion (TTO) is measured. Following laser injury, decreased TTO and time to adhesion and an increase in time of dissolution are considered the indicators of thrombus (Jagadeeswaran et al. 2016).

MO-mediated knockdown of F2 gene (codes for prothrombin) in zebrafish embryo resulted in two distinct phenotypes (Day et al. 2004). The early phenotype that stared after 1 day post fertilization (dpf) showed decreased blood flow and reduced circulating blood cells along with pericardial edema resembling F2 knock-out phenotype in mice, whereas in the late phenotype, there were no pathological changes observed during early stages except for occasional bleeding in the later stages. Occasional cerebral hemorrhage and prolonged TTO were seen in late phenotype unlike neonatal death observed in mice models. Prolonged TTO was returned to normal following the introduction of recombinant prothrombin into the circulation (Meng et al. 2020; Day et al. 2004).

A high degree of similarity was observed in between zebrafish factor VII and mammalian F7 protein (Sheehan et al. 2001). MO-mediated knockdown of F7 protein resulted in an increase in time to occlusion which explains the bleeding phenotype observed due to the delay in fibrin production due to depletion in F7 (Khandekar and Jagadeeswaran 2014). To understand the role of factor seven activating protease (FSAP), its gene (fasp) was knocked down by using antisense MO and found that there was neither alteration in thrombus production following the vascular injury nor any effect on activation of F7. Interestingly, the knockdown of hepsin, a serine protease, resulted in a reduced activation of F7 and blocked thrombus production (Khandekar and Jagadeeswaran 2014). Inconsistent results were seen for the same when performed in mice (Wu et al. 1998).

cDNA cloning of zebrafish von Willebrand factor (vWF) revealed conservation of sequences and a protein similarity of 46% (Ghosh et al. 2012). MO-mediated knockdown of this gene in zebrafish showed an inhibited thrombocyte aggregation and hemorrhage (Carrillo et al. 2010). These results provide evidence that there is a conservation of both structure and function of adhesive coagulation factor and von Willebrand factor. Fibrinogen is formed by hexamerization of three homodimer polypeptides translated from FGA, FGB, and FGG. Synthetic orthologs were identified to human fibrinogen with fgg and fgh sharing >50% similarity in amino acid sequence but not much in fga (Fish et al. 2014). To understand the function of these orthologs in zebrafish, MO knockdown studies were performed. Results like intramuscular and intracranial hemorrhage were consistent with bleeding phenotype associated with human hypo- and afibrinogenemia. GFP-incorporated Fgb was introduced into an induced thrombus, and a dysfibrinogenemia was seen (Vo et al. 2013). But with MO knockdown, complete ablation of mRNA does not occur. To overcome this problem, three frameshift mutations were introduced in fga by using zinc finger nuclease technology in order to create homozygous fga zebrafish mutants. The resultant drop in circulating fibrinogen showed bleeding phenotype and reduced survivability in zebrafish mutants. This was the first heritable coagulopathy model validated in zebrafish (Fish et al. 2014).

Along with coagulation-associated factors, certain anticoagulant factors are also conserved in zebrafish being ATIII. ZFN-mediated disruption of ATIII loci resulted in spontaneous venous thrombosis in juvenile zebrafish. The homozygous mutants that survived till adulthood succumbed to death due to intracardiac thrombosis. The bleeding phenotype and unexpected prolongation in TTO were back to normal after

the introduction of human fibrinogen. In vivo analysis of ATIII in zebrafish revealed slender functions for heparin-binding and anti-IXa/Xa activity (Liu et al. 2014). Sequence alignment revealed 55% identity and 70% similarity between zebrafish and human AT3 (Kumar et al. 2013).

Factor V mutant generated through CRIPSR-based genome editing in zebrafish revealed that there was no thrombus formation in mutant embryos in response to laser injury. Unlike in mammals, the mutant embryos survived the lethal conditions but succumbed to death in adulthood (Weyand et al. 2019). Along with these laser injury models, knockdown studies revealed the roles and importance of factor VIII, Mlck1a, protein kinase C-α, protein kinase C-β, and G6fl in hemostasis (Williams et al. 2011; Gregory et al. 2002; Hughes et al. 2012).

12.4.2 Models for Studying the Pathology of Human Blood Coagulation Disorders

Zebrafish provided a valid in vivo testing model for human genetic sequences related to coagulation disorders where the circulatory system complexity can be unveiled. Using this advantage, several coagulopathy models have been tested in zebrafish for better understanding of clinical manifestations, molecular pathway, and screening for potential therapeutic agents. MO knockdown of the zebrafish ortholog of *MASTL* revealed features like decreased thrombocyte count and downregulated *itga2b* and *mpl* expression similar to the phenotype seen in a human familial autosomal-dominant thrombocytopenia caused due to missense mutation in microtubule-associated serine/threonine-like kinase (*MASTL*) (Johnson et al. 2009). MO knockdown in zebrafish also fortified the roles of *NBEAL2* and *RBM8A* as the target genes responsible for gray platelet and thrombocytopenia with absent radii (TAR) syndromes, respectively (Albers et al. 2011, 2012).

To understand the clinical manifestations and molecular pattern of human fibrinogen deficiencies, *fga* mutants were generated by using TALEN-based gene editing. Loss of fibrinogen resulted in the absence of hemostasis and caused synthetic lethality when combined with thrombocytopenia (Hu et al. 2019). Predicted candidates to have a role in hemostasis were selected from systems biology followed by MO knockdown combined with laser injury studies which unveiled four novel genes whose roles in hemostasis were not understood earlier. Out of these four genes, two (*BAMBI* and *LRRC32*) induced thrombus formation while the remaining two (*ESAM* and *DCBLD2*) were found to have an inhibitory effect (O'Connor et al. 2009). Transcriptional profiling of human platelets and subsequent MO knockdown studies in zebrafish revealed the role of *COMMD7* and *LRRFIP1* genes in regulation of thrombus generation (Goodall et al. 2010). *Genome-wide* association studies (GWAS) and gene silencing in *Danio rerio* and *Drosophila melanogaster* revealed the role of 11 novel genes in megakaryopoiesis and platelet formation (Gieger et al. 2011).

12.5 Zebrafish as a Tool to Study Leukemia: Lymphoid and Myeloid Origin

Leukemias are the hematopoietic malignancies and can be classified into leukemia due to lymphoid origin as "lymphocytic or lymphoblastic" and leukemia due to myeloid origin as "myelogenous or myeloid." Further, these can be classified as acute and chronic types and are broadly categorized into acute lymphoblastic leukemia (ALL), chronic lymphoblastic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML) (Baeten and de Jong 2018). CLL is the most prevalent form, whereas AML has the highest incidence. ALL is the commonest form of cancer in children and adolescents (Konantz et al. 2019; Harrison et al. 2016).

A conserved hematopoietic program and unique experimental strengths make zebrafish best suited for leukemia studies (Konantz et al. 2019). Zebrafish has been used for the construction of different types of leukemia like T-ALL, B-ALL, and AML. The recent developments in genetic alteration, transplantation, and imaging techniques have significantly contributed for a better understanding of genesis, progression, and maintenance of leukemia.

12.5.1 Zebrafish Models to Study T-Cell Acute Lymphoblastic Leukemia (T-ALL)

T-ALL constitutes 25% of ALL cases in adults and 15% in children. Several mutations and rearrangements in the genes which have a role in T-ALL have been identified like histone-modifying genes, transcription factors, tumor suppressors, Hox genes, and genes regulating RAS signaling and NOTCH pathway. MYC pathway has been identified as a key regulator in the incidence and progression of T-ALL in humans. A major chunk of ALL models in zebrafish replicate T-ALL, partially because of the success of rag2 promoter in driving T-ALL. However, both B-cell and T-cell lineages express rag2 gene, and the early models induced only T-cell malignancy (Langenau et al. 2004).

The first zebrafish transgenic model of cancer was a T-cell ALL model, in which the murine *c-Myc* gene was fused to *EGFP* to promote the fusion protein expression under the lymphocyte-specific *rag2* promoter. Widespread leukemia with 52 and 32 days of mean latencies and 6% and 100% of leukemia penetrance were observed in mosaic transgene expression and stable germline transmission, respectively. The tumors grew rapidly and infiltrated the entire fish leading to death even before reaching sexual maturity. Due to this premature lethality, in vitro fertilization has been employed to maintain a stable transgenic line. This model has been used as a platform to study mutations related to c-myc-induced carcinogenesis and has paved a way for the construction of several other models linked with different types of leukemia.

Later, in order to overcome the problem of premature lethality and the use of IVF, a Cre/lox-regulated transgenic model was developed. In this system, stable

rag2-loxP-dsRED2-loxP-EGFP-mMyc transgenic zebrafish lines were developed with *EGFP-mMyc* oncogene and loxed *dsRED2* gene (Langenau et al. 2005). This conditional expression of *EGFP-mMyc* is mediated by the excision of loxed portion by introduction of Cre RNA (Langenau et al. 2005). The disease was similar to that of the previous model, but there was a significant decrease in incidence, and the latency was delayed. It was thought that such findings may be due to incomplete recombination. To overcome this problem, a novel heat shock-regulated Cre/lox system, Tg(hsp70:Cre; rag2:LDL-EMyc), was constructed (Feng et al. 2007). According to this model, the Cre inducible transgenic lines were subjected to heat shock 3 days after fertilization, and the observed mean latency, 120 dpf, and penetrance, 81%, were similar to the original model. Feng et al. studied the genetic differences between T-LBL and T-ALL and the molecular basis for the progression of T-LBL to T-ALL and constructed a Tg(hsp70:Cre; rag2:LDL-EMyc)-induced T-ALL model for overexpression of *bcl2* gene. These significantly hasten the T-LBL induction and prevented the progression into T-ALL. This phenotype of T-LBL could be due to elevated levels of S1P1 and ICAM1 which promotes homotypic cell-cell adhesion and inhibit intravasation (Feng et al. 2010). These findings proved to be useful in better understanding of novel pathways for targeted therapy of leukemia. As the AKT signaling pathway is crucial for the progression of T-ALL and autophagy suppression, the constitutive activation of akt2 gene resulted in the rapid advancement of tumors to T-ALL (Feng et al. 2010).

Another model was developed for the conditional transgenic expression of MYC using a tamoxifen inducible system (Gutierrez et al. 2011). In this system, human MYC is fused with ligand binding domain of estrogen receptor such that c-Myc oncogene is conditionally expressed in the presence of 4-hydroxytamoxifen (4-OHT) via Rag2 promoter only. Addition of 4-OHT resulted in T-ALL progression via activation of MYC; at the same time, 75% of zebrafish saw a rapid tumor cell apoptosis and T-ALL regression when 4-OHT was withdrawn. It was reported that the loss-of-function mutations in pten genes or constitutive activation of Akt2 transgene resulted in the tumor progression independent of MYC even in the absence of 4-OHT (Gutierrez et al. 2011). This model elucidated the importance of MYC-PTEN-AKT-BIM regulatory pathway in the progression of T-ALL. It was found later that the survival signaling of MKC and AKT in high-risk T-ALL is mediated by the repression of an anti-apoptotic protein, BIM (Reynolds et al. 2014). A large-scale drug screening by using the above model had identified the role of perphenazine in inducing apoptosis in T-ALL via protein phosphatase 2A (Gutierrez et al. 2014).

An alternative approach like co-injection strategy was developed to overcome the problem of maintaining stable transgenic lines. Langenau et al. integrated *rag2-EGFP* and *rag2-mMyc* transgenes for the co-expression along with GFP, such that the tumor induction is seen only in GFP+ thymocytes (Langenau et al. 2008). One of the important features of tumor cells is self-renewal which can be measured by cell transplantation, but all the then existing protocols required immune suppression of recipients prior to transplantation (He et al. 2017). To overcome this difficulty, Smith et al. in 2010 used co-injection strategy to establish a MYC- and/or

NOTCH1-induced T-ALL with clonal syngeneic strains (Smith et al. 2010). According to this model, T-ALLs that are raised from mixed genetic backgrounds can be transplanted without MHC matching or immune suppression into recipients. This system established a high-throughput screening method for rapid detection of transgenic animals and assessment by cell transplantation. This model was further put forth by Blackburn et al. in 2014 to understand the role of tumor cell heterogeneity in progression of cancer. It was reported that the continued clonal evolution leads to decrease in latency, with modulated chemotherapy resistance (Blackburn et al. 2014).

Transgenic line for expressing *rag2:ICN1-EGFP* was developed by Chen et al. in 2007, in which Notch target gene expression is driven by a GFP-tagged *Notch1* intracellular domain *(*Chen et al. 2007*)*. However, suppression of T-ALL with an overall increase in latency and incidence was seen in Tg(rag2-ICN1-EGFP) line when combined with the overexpression of bcl2. This was further investigated by Blackburn et al., where the T-ALL progression was enhanced in combination with *rag2:cMyc*. Their findings denote the primary role of Notch signaling to expand a population of pre-malignant thymocytes which require Myc or further secondary mutations or transformation (Blackburn et al. 2012). This model was also used for profiling microarray gene expression and cross-species comparisons with human and mice models to study common T-ALL gene signature and Myc-independent Notch expression pathway. These studies reported that the *Notch1* alone is not sufficient for complete induction of T-ALL but also require secondary mutations or extra oncogenes. These models can be used for further study of genes working along with Notch1 pathway in T-ALL progression.

Zebrafish is an ideal vertebrate system which can be used as a genetic screen for the identification of new potential genetic players in cancer induction and progression which can be related to human disease models. Frazer et al. employed an alkylating mutagen, N-ethyl-N-nitrosourea (ENU), to induce a random mutagenesis which can be used as a genetic screen for the discovery of potential new T-cell malignancy-prone mutants. This led to the discovery of three new mutant lines, hlk, srk, and otg, that are capable of developing transplantable T-ALL similar to oncogene mediated leukemia (Frazer et al. 2009). Ridges et al. developed a chemical screen by using the same transgenic line Tg(lck:EGFP) in order to identify potential anti-leukemia compounds. Using this model, Lenaldekar (LDK; 1H-indole-3-carbaldehyde 8-quinolinylhydrazone) was identified which is capable of eliminating both normal and Myc transformed T-ALL cells in the adult zebrafish (Ridges et al. 2012). These findings were further validated using murine xenografts and human T-ALL cell lines. By using a transgenic screen, Lobbardi et al. could identify the thymocyte selection-associated high mobility group box protein TOX. TOX was found to have an important role in T-ALL initiation and maintenance via regulation of DNA repair, growth, and genomic instability (Lobbardi et al. 2017). Further, zebrafish can be used as a model to develop patient-specific therapies for leukemia patients as shown in Fig. 12.2.

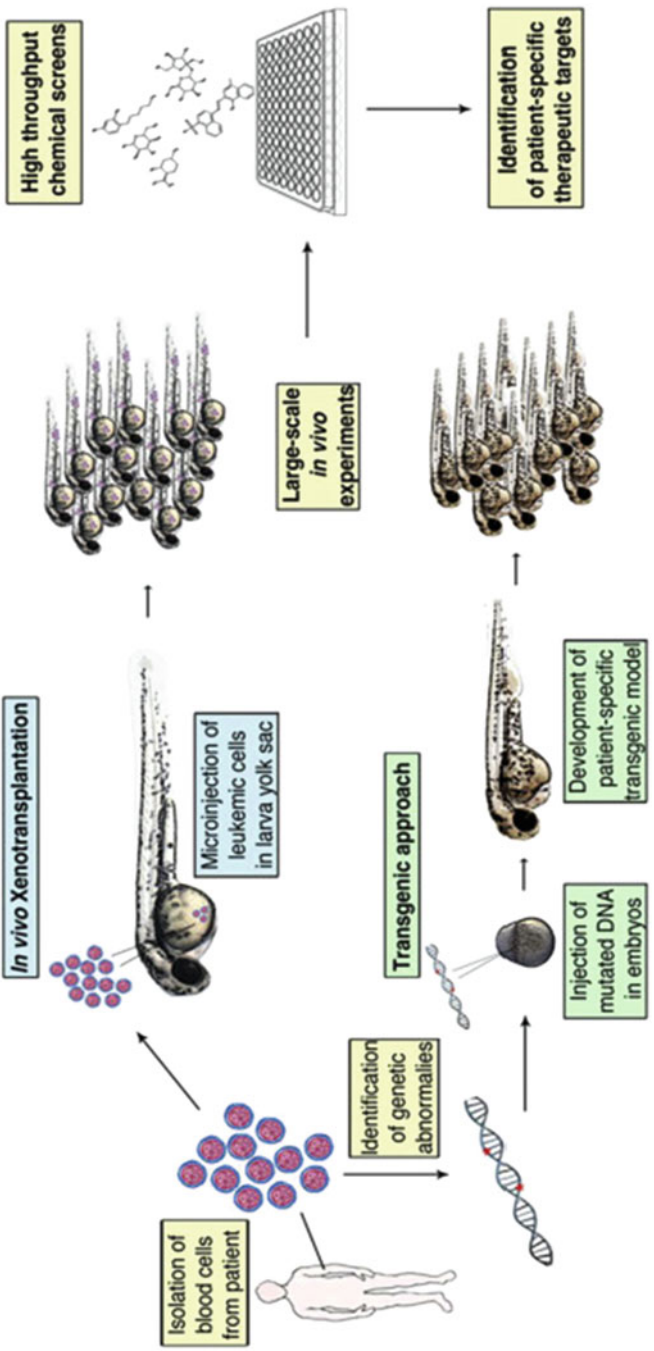

Fig. 12.2 Representation of different strategies to develop patient-specific therapies using the zebrafish. Leukemic blood cells can be isolated from patient and directly injected into the yolk sac of 2 dpf larvae (in vivo xenotransplantation). On the other side, genetic abnormalities that drive hematopoietic malignancies (red dots) can be identified and used to develop zebrafish transgenic models of human disease (transgenic approach). Given the size and the ease of manipulation, both strategies are well suited for large-scale in vivo experiments. Finally, both xenotransplanted and transgenic larvae can be used to test thousands of different chemical molecules with the aim to identify new therapeutic compounds. (The figure was reproduced with permission from reference (Zizioli et al. 2019)

12.5.2 Zebrafish Models to Study B-Cell Acute Lymphoblastic Leukemia (B-ALL)

Most common leukemia in children is B-ALL, and among all cancer-related deaths, this is the common cause of death in children. Despite its importance, designing B-ALL models in zebrafish has always been challenging because of T-cell bias of rag2 promoter. Although one and/or the other B-cell and T-cell lineages express rag2 gene and can be labeled by rag2-driven fluorescent reporters, it was observed that only T-ALL results from rag2-driven oncogene overexpression.

Until recently, the only successful B-ALL zebrafish model was constructed through the expression of global oncogene TEL-AML1 driven by the *Xenopus* elongation factor 1α promoter or the zebrafish β-actin promoter (Sabaawy et al. 2006). Low incidence (3%) and long latency (8–12 months) suggest that the additional mutations are most likely needed to induce leukemic transformation. Transgenic zebrafish which expresses the same TEL-AML1 fusion oncogene linked with *rag2* promoter failed to induce leukemia (Sabaawy et al. 2006). A recent and most promising model which was driven by human *MYC* is highly penetrant zebrafish pre-B-ALL and was constructed. This robust model was developed using a tissue-specific reporter line (Tg(lck:eGFP)), which differentially labels the B cells and T cells, and the *hMYC* fish here developed both pre B-ALL and T-ALL (Sabaawy et al. 2006). Garcia et al. in 2018 unveiled the B-ALL features in a subset of Tg(rag2:mMyc) zebrafish and constructed a zebrafish transgenic model of Myc-induced ALL and also explored B-ALL features in a subset of Tg(rag2:mMyc) (Garcia et al. 2018). These findings may help us in overcoming the T-cell bias of rag2 promoter and to find an alternate pathway for the construction of B-ALL models in zebrafish.

12.5.3 Models for Studying Acute Myeloid Leukemia (AML), Myeloproliferative Malignancy, and Myelodysplastic Syndrome (MDS)

Myeloid neoplasms are either acute or chronic clonal diseases characterized by uncontrolled proliferation in myeloid cells. Chronic myeloid malignancies like myeloproliferative neoplasms (MPNs) and myelodysplastic syndromes (MDS) have an increased potential to progress into AML. The primary genetic players in induction and progression of myeloid malignancies play a key role in hematopoietic development of zebrafish which can be exploited for elucidating the molecular mechanism behind myeloid neoplasms in humans.

12.5.3.1 Myeloproliferative Malignancies

People above 50 years of age are mostly affected with myeloproliferative neoplasms and are usually accompanied with complications like hemorrhages and thrombosis. MPN patients are presented usually with driver mutation in JAK2 or MPL or CALR or CSF3R. V617F mutation in the JAK2 gene is found to be an important genetic

event in MPN progression. Ma et al. in 2009 designed a zebrafish model equipped with ortholog of human JAK2 (V617F) through site-directed mutagenesis. Significant increase in erythropoiesis was observed in zebrafish embryos injected with jak2a (V581F) mRNA (Ma et al. 2009). A reduction in erythropoietin gene was followed by injecting TG101209, jak2a(V581F) mRNA, a JAK2 inhibitor. Overall, this model shared many common features with human polycythemia vera and can be used for better understanding of molecular details of this disease. Another common mutation noticed in MPN patients is related to CALR encoding ER residing chaperon protein called Calreticulin. Lim et al. in 2016 constructed a zebrafish model expressing mutated CALR by mRNA injection. A significantly increased hematopoietic stem or progenitor cells followed by thrombocytosis is seen resembling the essential thrombocythemia (ET) phenotype in MPN patients (Lim et al. 2016). It is understood that these findings are mediated by the upregulation of JAK-STAT signaling and the molecular machinery involved in mutant CALR tumorigenesis pathway is preserved between human and zebrafish. These two models can be employed as chemical screens for identifying potential candidates targeting JAK/STAT signaling or the treatment of MPN.

12.5.3.2 Myelodysplastic Syndrome (MDS)

About 30% MDS cases eventually progress to AML or other malignancies that are mostly resilient to conventional therapies. MDSs are usually difficult to model in animals due to their heterogeneity. In MDS patients, mutations occur most commonly in epigenetic or splicing factors. TET2 encodes for a DNA methylcytosine oxidase that catalyzes DNA demethylation within genomic CpG island; loss-of-function mutations in TET2 gene are commonly associated with AML, MPN, and MDS. Gjini et al. designed inert *tet2* zebrafish mutants using ZFN genome editing technology. Mutant zebrafish had normal embryonic and larval hematopoiesis but developed a progressive clonal myelodysplasia eventually ended in MDS by 24 months of age (Gjini et al. 2015). The same group reproduced *asxl1* haploinsufficient and null zebrafish lines to understand the role of additional sex combs-like transcriptional regulator 1 (*ASXL1*) in myeloid cell transformation. It was evident that homologous loss of *asxl1* resulted in the apoptosis of newly formed HSCs and heterozygous loss of *asxl1* along with the heterozygous loss of *tet2* led to a more penetrant MPN phenotype, whereas heterozygous loss of *asxl1* combined with complete loss of *tet2* led to AML (Gjini et al. 2019).

c-MYB transcription factor is often associated with various hematological disorders. Liu et al. in 2017 developed c-myb-gfp transgenic zebrafish expressing a hyperactivity mutant of c-myb called *c-myb*^{hyper} (Liu et al. 2017). Similar to human MDS, abnormal expansion of granulocytes began in embryonic stage and maintained throughout adulthood. Interestingly, a little portion of c-myb^{hyper} adult fish had developed AML-like or ALL-like disorders which could be due to cell cycle-related gene dysregulation or proliferation of HPCs mediated by hyperactivity of c-myb.

In another recent model, zebrafish sf3b1 mutants have shown a macrocytic anemia-like phenotype similar to MDS (De La Garza et al. 2016). sf3b1 mutation

is one of the most common mutations seen in MDS patients, and this model can be used to determine the importance of splicing in hematopoietic development and explain how the Sf3b1 dysfunction is linked to MDS progression. Furthermore, a zebrafish mutant in Prpf8 spliceosome component called *cephalophŏnus* (cph^{gl1}) was recovered via a forward genetic chemical screen (Keightley et al. 2013). Mutations at this protein resulted in Prpf8-null phenotype with defective early hematopoiesis. Craven et al. described zebrafish mutant crimsonless (crs) with ineffective hematopoiesis similar to MDS (Craven et al. 2005). It was understood that these findings are due to the oxidative stress and apoptosis of blood cells mediated by the mutations in a mitochondrial matrix protein encoded by crs. Targeting induced local lesions in genomes (TILLING) was employed by Sun et al. in 2013 to generate hypomorphic *pu.1* zebrafish allele ($pu.1^{G242D}$) with point mutation in Pu.1 protein. The downregulation of PU.1 expression resulted in a phenotype similar to that of MDS or AML in humans (Sun et al. 2013).

12.5.3.3 Acute Myeloid Leukemia (AML)

AML is one of the most usual kind of acute leukemia in the adults which can be seen in all age groups; however, it mostly affects elderly people with a mortality rate of 90% in elders and > 50% of young adult patients. Mutations in TP53, NPM1, FLT3, IDH2, chromatin, and RNA splicing regulator genes are commonly associated with AML (Papaemmanuil et al. 2016).

One of the first models of AML in zebrafish is was proposed by Zylinska et al. in 2002 for the expression of fusion oncogene, AML1 (RUNX1)-ETO (Kalev-Zylinska et al. 2002), and noticed the disruption of normal hematopoiesis, internal hemorrhages, aberrant circulation, and dysplastic circulating erythroid cells. Following this, several other AML models were designed in zebrafish for assessing the role of oncogenes and drug screening; however, premature mortality is observed in these models and could not be employed in adult animals.

A stable and first embryonic non-lethal AML model was designed for expressing MYST3/NCOA2 fusion gene under spi1 promoter. This model helped in understanding the MYST3/NCOA2 oncogenic property related to inv.(8)(p11q13) chromosomal abnormality which resulted in extensive myeloid blast cell invasion of kidneys, a common feature observed (Zhuravleva et al. 2008). Because of its early myeloid lineage specificity, spi1 promoter system for the expression of oncogene was employed in various other AML models like internal tandem duplication (ITD) of the FLT3 to understand the role of flt3 in hematopoiesis (He et al. 2014; Lu et al. 2016); tel-jak2a fusion oncoprotein (Onnebo et al. 2005, 2012); and NUP98-HOXA9 expression models. In zebrafish with transgenic lines with NUP98-HOXA9, normal hematopoiesis was restored with epigenetic therapy along with COX inhibitors or DNA (cytosine-5)-methyltransferase (DNMT) (Deveau et al. 2015).

In order to combat the embryonic lethality, Yeh et al. designed an alternative heat-shock-inducible AML1-ETO model, which on expression resulted in phenotype similar to that of AML (Yeh et al. 2008) without embryonic death. The results include identification of scl as an essential mediator in AML1-ETO-mediated effect

on hematopoietic cell line and use of trichostatin A, a histone deacetylase inhibitor restored expression of scl as well as gata1, thus ameliorating the effects of AML1-ETO. Further investigations using chemical screens revealed the role of COX2- and β-catenin-dependent pathways inside AML1-ETO, and this technique can be used for the development of potential therapeutics (Yeh et al. 2009).

Shen et al. designed a heat-shock-inducible transgenic zebrafish line depicting the murine n-myc which on induction promoted expansion of myeloblast immature cells resulting in phenotype similar to AML (Shen et al. 2013). This model provides an in vivo explanation for *MYCN*-mediated pathway in the progression of AML. Le et al. generated a heat-shock-inducible transgenic model for the expression of kRASG12D, resulting in tumors and hyperplasia closely resembling human disease (Le et al. 2007). Alghisi et al. used zebrafish model for spatial indication of the human RAS inside endothelial cells mediated by a Gal4-UAS binary system. This developed the hyperproliferation of the hematopoietic cells inside caudal hematopoietic tissue, which can be because of the downregulation of the Notch pathway.

12.6 Summary

Zebrafish is a useful tool for studying malignant as well as non-malignant hematological disorders. It provides a high-throughput screening platform for discovery of new chemical entities against hematological diseases. As the size of zebrafish is quite small along with the added advantage of transparent body, it offers multiple unique advantages for drug discovery and development compared to the rodent models. Zebrafish has been widely used for studying hematological disorders such as anemia, coagulation diseases, blood synthesis disorders, and leukemia. These studies have provided a large number of lead compounds for drug development owing to rapid live imaging and fast screening of drugs against hematological disorders. Furthermore, a great amount of transgenic mutants have been produced which have enabled to understand in-depth molecular mechanisms and signaling cascades involved in the pathology of hematological diseases. This chapter provides the summary of the advantages and limitations of zebrafish in studying hematological diseases along with the updates upon the utilization of zebrafish model for development of drugs against hematological disorders.

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Zebrafish: A Metamorphosis in Ophthalmological Research—A Literature Review

13

Tithishri Kundu

Abstract

Ophthalmological disorders causing impaired vision is a global problem affecting millions of individuals. Animal models having the same type of ocular pathology as humans and allowing high- to medium-throughput screening are needed to understand the pathophysiology of ocular disorders. High prolificity, less cost, ease of mutagenesis and similar ocular morphology render zebrafish as a better animal model compared to rodents. Zebrafish can be used as an animal model in several ocular diseases. Thus zebrafish can be useful not only to understand the pathophysiology of ophthalmological diseases but also to facilitate the development of gene-mediated and regeneration therapies for the successful management of the disease. This review discusses the advantages of zebrafish as an animal model, visual system of zebrafish with its similarities and differences from the human eye, embryology of the eye in zebrafish, visual behavioural assays, and role of zebrafish as an animal model in several ocular diseases, including aniridia, retinitis pigmentosa, Leber congenital amaurosis, diabetic retinopathy, age-related macular degeneration, corneal dystrophy, cataract, glaucoma, coloboma, microphthalmia, anophthalmia, cyclopia, etc. This review also reveals the role of zebrafish in preclinical ocular toxicity test, gene-based therapeutic management, ocular drug discovery, and the study of retinal regeneration.

Keywords

Zebrafish · Ocular disease · Visual behavioural assay · Gene therapy · Drug development · Regeneration

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13.1 Background

Impairment in vision is a global problem affecting millions of individuals (Chhetri et al. 2014). According to WHO, at least 2.2 billion people are afflicted with the loss of vision globally (WHO 2020a). Total health expenditure due to impaired vision is USD 2.3 trillion globally in 2010 and is expected to increase to USD 2.76 trillion in 2020 (Gordois et al. 2010). Cataract, glaucoma, age-related macular degeneration (Ganzen et al. 2017), diabetic retinopathy, retinitis pigmentosa (Angueyra and Kindt 2018) and coloboma are important causes of loss of vision worldwide. To combat this global problem, animal models are needed to understand the pathogenesis of ocular diseases (Richardson et al. 2017) as well as to test the ocular drugs. Zebrafish (*Danio rerio*) can be used as a suitable model for this purpose. High prolificacy, less cost, ease of mutagenesis and similar ocular morphology render zebrafish as a better animal model compared to mouse (Chhetri et al. 2014).

13.2 Zebrafish as an Animal Model

Zebrafish (*Danio rerio*) is one of the most common aquarium fish (Spence et al. 2008). It is predominantly available in the Ganges region in India (Bibliowicz et al. 2011). Recently, it is emerging as an important vertebrate model in genetics, neurophysiology, drug development and toxicity studies. Several factors can be contributed to the popularity of zebrafish as an experimental model.

Firstly, zebrafish is a cheap and sturdy vertebrate that is easier to maintain in the laboratory for a long duration. Secondly, it does not have a specific mating season. Highly prolific nature and all year round mating season account for a large number of embryos produced by zebrafish. Female fish mate every 3 to 3 days, and 250–300 eggs are generated each time. Thirdly, zebrafish is blessed with a short generation time of 3 to 4 months. Fourthly, zebrafish eggs are of 0.7 mm in diameter which is larger compared to other aquatic fishes. Furthermore, the eggs are optically transparent, which makes them suitable for experimentation. Fifthly, fertilisation and development of zebrafish occur in vitro in the laboratory. The embryos are smaller in size, less than 1 mm in diameter (Kari et al. 2007). Owing to the small size, embryos can be kept in 96-well micro-titre plates (Mathias et al. 2012). Sixthly, zebrafish larva is also transparent (Chakraborty et al. 2009) in nature leading to the visibility of all organs. This above-mentioned characteristic is responsible for the suitability of zebrafish larva for experimentation (Fishman 1999; Eisen 1996). Seventhly, easy permeability of embryos to smaller molecules and drugs made them suitable for the administration of drugs and dye-staining (Kari et al. 2007). Eighthly, embryos of zebrafish can be utilised for screening of compounds in 50 microliter volumes. Ninthly, medium-throughput screening (1000 compounds daily) of compounds is possible with zebrafish assay leading to its usefulness in preclinical drug and toxicity studies. Tenthly, high degree of similarity (approximately 75%) exists between genomes of humans and zebrafish. Eleventhly, digestive, cardiovascular and nervous system of zebrafish are analogous to mammals. Twelfthly, several genetic

techniques can be utilised in zebrafish. In the 'knockdown' technique, antisense oligodeoxynucleotide can be used to suppress specific functions of proteins. On the other hand, mRNA or plasmid can be injected into zebrafish to express specific proteins, known as 'knock-in' technique. This 'knockout/knock-in' technique is a widely popular technique used in mice for experimental purposes (Kari et al. 2007).

Zebrafish Information Network (ZFIN) has a central online database consisting of information on full sequence of zebrafish, pattern of gene expression, transgenic model, etc. (www.zfin.org). Genomic sequence of zebrafish can also be accessed in UCSC (ftp.//hgdownload.cse.ucsc.edu/goldenpath/danRer4/).

Zebrafish model has its own share of disadvantages. Firstly, though vertebrate, zebrafish is considered as a lower animal and placed far from humans in the evolutionary tree. The closer is the animal to human, that animal model has a better predictive value to measure the outcome of human diseases. Secondly, some of the organs and tissues of zebrafish (lung, skin, prostate, etc.) are dissimilar to that of the animals. The above-mentioned features suggest that though zebrafish cannot replace the mammalian models, it can be used as an adjunct to the mammalian model.

13.3 Visual Systems of Zebrafish

13.3.1 Various Parts of the Eye in Zebrafish

As both zebrafish and humans belong to the vertebrate family, several similarities can be observed in their visual system. Both human and zebrafish have binocular vision, lens and five layered retina, enabling them to have a high acuity vision.

On the other hand, several dissimilarities are noted in the visual system of humans and zebrafish. While the human has frontally located eyes with extensive binocular overlap, zebrafish has laterally placed eyes with smaller binocular overlap (Maurer et al. 2011). Extensive binocular overlap in human enables us to have superior depth perception, i.e. to locate how far the object is from us. Lens in human is ellipsoidal, whereas zebrafish lens is spherical (Chhetri et al. 2014). While humans lack a UV-sensitive colour vision, UV-sensitive cone cells present in the retina of zebrafish allow them to detect food and predators. It also facilitates them to communicate among themselves (Cronin and Bok 2016). Humans have cone-rich fovea in the retina, whereas zebrafish does not have fovea in their eye. Last but not the least, anatomy and myelination of the optic nerve are also different in human and zebrafish. All the fibres of optic nerve cross over to the opposite side at optic chiasma in zebrafish. But only medial half of optic nerve fibres cross over to the opposite side in human. Lateral half of optic nerve fibres project to the same side of the brain. In zebrafish, whole optic nerve is myelinated, whereas some parts of the optic nerve are devoid of myelination in human. Table 13.1 depicts the similarities and differences between the eye of human and zebrafish.

Table 13.1 Similarities and differences between the eye of human and zebrafish

	Human eye	Zebrafish eye
Similarities		
High acuity vision	Yes	Yes
Binocular vision	Yes	Yes
Presence of lens	Yes	Yes
Presence of five layered retina	Yes	Yes
Differences		
Position of eye	Frontally placed	Laterally placed
Binocular overlap	Extensive	Less
Depth perception	Better	Less
Shape of lens	Ellipsoidal	Spherical
Presence of UV-sensitive cone cell	No	Yes (have UV-sensitive colour vision)
Presence of fovea	Yes	No
Crossover of optic nerve fibre	Medial half of optic nerve fibres cross over to the opposite side at optic chiasma	All the nerve fibres cross over to the opposite side at optic chiasma
Myelination of optic nerve	Some parts of optic nerve is non-myelinated	Whole nerve is myelinated

13.3.2 Structure of the Retina

Like all vertebrates, both human and zebrafish have retina which consists of five layers. These layers are (external to internal) outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL) (Villegas 1961). Photoreceptors rods and cone cells are situated in the ONL. Zebrafish has a tetrachromatic vision. Four types of cone cells are present in the retina of the zebrafish—SSC (short single cone), LSC (long single cone), UV cone (ultraviolet-sensitive cone) or blue-sensitive cone and DC (double cone consisting of red- and green-sensitive cones). On the other hand, human do not possess the UV cone. Four types of cone photopigments are present in the zebrafish, i.e. UV wavelength-sensitive photopigment (λ-362 nm), short wavelength-sensitive photopigment (λ-415 nm), middle wavelength-sensitive photopigment (λ-480 nm) and long wavelength-sensitive photopigment (λ-570 nm) (Bilotta and Saszik 2001). On the contrary, zebrafish is in the possession of single type of rod cell and single type of rod photopigment named rhodopsin. First rod and cone cells develop at 5 to 6 day post fertilisation (dpf). But till 15–21 dpf, only cone cell functions are present in zebrafish (Chhetri et al. 2014; Saszik et al. 1999).

INL contains horizontal cell, amacrine cell and bipolar cell. Horizontal cell causes contrast enhancement, whereas amacrine cells are responsible for the detection of change in illumination and processing of movement (Chhetri et al. 2014; Lagnado 1998). Zebrafish horizontal cells are of two types: Type A cells have numerous processes and small body, whereas Type B cells have few processes and larger cell body. Both horizontal and amacrine cells provide inhibitory signal in the retinal pathway (neurotransmission from photoreceptor to ganglion cell through bipolar cell). Horizontal and amacrine cell send inhibitory inputs through the inhibitory neurotransmitters GABA and glycine, respectively (Bilotta and Saszik 2001; Lagnado 1998). Transmission of neuronal impulses are mediated through the bipolar cell from photoreceptors in ONL to ganglion cells in GCL of the retina. 13 types of bipolar cells are observed in zebrafish.

GCL contains ganglion cells. Ganglion cells are responsible for the fine-tuning of retinotectal projection. OPL and IPL contain synapses. Synapses between photoreceptors and bipolar cells, horizontal cells are present in OPL. On the contrary, synapses between bipolar cells and ganglion cells, amacrine cells exist in IPL.

13.4 Embryology of the Zebrafish Eye

Like all vertebrates and human, the eye of the zebrafish is developed from three types of cells (Richardson et al. 2017; Fadool and Dowling 2008). Origin of different parts of the eye in zebrafish is depicted in Table 13.2.

Embryology of the eye of the zebrafish starts with the formation of the optic vesicle from the forebrain on 11-h post fertilisation (hpf) in zebrafish. The optic vesicle is a flat structure that is attached to the forebrain by optic stalk. Optic vesicle later deepens and gives rise to the optic cup. Both neural retina and RPE are developed from optic cup on 15 hpf.

Surface ectodermal cells overlying the optic cup can lead to the formation of lens in zebrafish. In zebrafish, surface ectodermal cells give rise to lens placode around 16 hpf. Central cells of the lens placode elongate and move posteriorly to form primary lens fibre cell (Richardson et al. 2017). On the contrary, peripheral cells of the lens placode migrate anteriorly and form anterior lens epithelium. Delamination of the cells in lens placode from surface epithelium can result in the formation of lens around 24–26 hpf. At 30 hpf, corneal epithelium is formed from the surface ectoderm.

Vessels in the retina are developed from the central retinal artery around 24–29 hpf. Photoreceptors in the retina start to appear around 4 to 5 dpf. Different types of cone cells, i.e. SSC, LSC and DC, appear on 4 to 5, 7 and 10 dpf,

Table 13.2 Origin of different parts of the eye in zebrafish

Neuroectoderm	Retina, retinal pigment epithelium (RPE), ciliary body, iris (muscles)
Surface ectoderm	Lens, conjunctival epithelium, corneal epithelium
Mesenchymal cell	Cornea (endothelium, stroma), iris (stroma), ciliary muscle, sclera

respectively (Chhetri et al. 2014). On the contrary, rod cells appear around 15–40 dpf. Ganglion cells form at 28 hpf in the retina. They reach tectum on 2 dpf (Bilotta and Saszik 2001; Easter Jr and Nicola 1996). Horizontal and amacrine cells are formed approximately at 50 hpf. Bipolar cells are developed much later around 60 hpf. Optic fissure closes around 48 hpf in the eye of the zebrafish denoting the end of the morphogenesis of the eye.

13.5 Visual Behavioural Assays in Zebrafish

Zebrafish uses vision to hunt for food and also to escape predators. As a teleost animal, zebrafish has a high acuity vision. Several visual behavioural assays can be conducted in zebrafish to screen ocular genetic defects. Among the visual behavioural assays, the most popular are optokinetic response (OKR) and optomotor response (OMR).

13.5.1 Optokinetic Response (OKR)

Optokinetic response (OKR) is a tracking movement observed in the eye in response to a moving stimulus (Fleisch and Neuhauss 2006). This is a type gaze stabilising movement (Maurer et al. 2011). This movement is used to keep the motion of the image in the retina to a minimum so that the image is fixed at the retina. The above-mentioned phenomenon is responsible for the high-acuity vision in zebrafish. OKR develops at 3 dpf in zebrafish.

Measurement of OKR is associated with three steps, i.e. immobilisation of zebrafish larva, providing the optokinetic stimulation and recording the OKR (Huang and Neuhauss 2008). Zebrafish larvae are usually immobilised using 6% methylcellulose solution (Brockerhoff 2006). Optokinetic stimulation can be provided easily using an OKR apparatus which is a revolving optokinetic drum with vertical black and white stripes. Stimulation can also be delivered using computer-generated signals. Different property of stimulus, i.e. contrast, frequency, colour and velocity, can be provided with variation using a computer. Recording of the OKR can be carried out by visual inspection using microscope. Recording and measurement of OKR can also be done using electrooculography (Distler et al. 1999; Blakemore and Donaghy 1980; Young and Sheena 1975; Hoffmann et al. 1998), sclera search coil technique (Robinson 1963; Fuchs and Robinson 1966; Van Alphen et al. 2001) or infrared devices (Hermann and Constantine 1971).

OKR method can be utilised to screen several ocular genetic defects, i.e. defective development of lens (bumper mutant), defective melanin in RPE (sandy mutant), defective optic nerve growth (grumpy, sleepy, pinscher mutant), misrouting of axon (belladonna mutant), defect in retinal ganglion cell (lakritz mutant), defective INL or OPL (dropje, steifftier mutant) and defective retinotectal impulse transmission (macho, blumenkohl mutant) (Chhetri et al. 2014; Neuhauss et al. 1999).

OKR method is advantageous as it is cheap and easy to implement. The small size of zebrafish larvae constitutes the flaw in this method (Huang and Neuhauss 2008).

13.5.2 Optomotor Response (OMR)

Optomotor response (OMR) is the ability of zebrafish to move towards the perceived motion (Fleisch and Neuhauss 2006). Non-responsiveness towards the direction of perceived motion denotes a visual defect in zebrafish. OMR develops around 7 dpf in zebrafish.

Moving stripes of the alternate black and white screen are displayed to the zebrafish larvae. Larvae follow the direction of perceived motion (Muto et al. 2005). As this is a computer-generated stimulus, varied strength of stimulus can be applied. In adult zebrafish, a rotating drum consisting of alternate white and black stripe is used to elicit OKR.

OMR method can be utilised to screen several ocular genetic defects, i.e. defective development of lens (bumper mutant), defective melanin in RPE (sandy mutant), defective optic nerve growth (pinscher mutant), misrouting of axon (belladonna mutant), defect in retinal ganglion cell (lakritz mutant), defective INL or OPL (dropje, noir mutant) and defective retinotectal impulse transmission (macho, blumenkohl mutant) (Chhetri et al. 2014; Neuhauss et al. 1999).

Large-scale screening can be possible with OMR which is one of the paramount advantages of this test. On the contrary, this test cannot be implemented before seventh day of the larva (Fleisch and Neuhauss 2006). Though a school of larva can be tested together in OMR, a single fish has to be examined at a time in this test. Thus OMR is time-consuming during the testing of visual behaviour in adult fish. Ethanol exposure and abnormal lighting environment during eye development elicit abnormal visual function in zebrafish (Bilotta et al. 2002; Bilotta 2000).

13.5.3 Escape Response

Escape response is the ability of zebrafish to escape from a threatening stimulus (Li 2001). To test this response, a round container is used. Zebrafish normally swims freely in the container either in clockwise or anticlockwise direction. Threatening stimulus in the form of a vertical black paper is utilised to cover a part of the apparatus. Zebrafish usually avoids that area and when forced with it usually turns and swims in the opposite direction (Fleisch and Neuhauss 2006; Li 2001). This study can be elicited in adult zebrafish of 2 to 3 months. Role of circadian rhythm, dopaminergic effect on vision and course of light and dark adaptation can also be described (Li and Dowling 1997, 1998).

This response can be utilised to study retinal degeneration (nba, nbb, nbc mutant) (Li 2001; Li and Dowling 1998, 2000). Among these three mutants, nbc mutant does not possess a mutation in a retina-specific gene. It also shows extra-retinal defects (Li and Dowling 2000).

This method is reliable and robust. High-throughput screening can be possible using this method. The main disadvantage is that it can only be carried out in adult fish, not in larvae.

13.6 Startle Response (SR) and Visual Motor Response (VMR)

Startle response is observed in zebrafish as a series of movements due to vibratory stimulation and electrical stimulation (Kimmel et al. 1974; Orger et al. 2004). This series of movement or fast start consists of a short latent period, turning away from stimulus and fast swimming in the opposite direction. In zebrafish larva, SR can be observed as a distinctive tail flip (Kimmel et al. 1974). This response can be elicited in zebrafish after 4 dpf (Chhetri et al. 2014).

To demonstrate the response, zebrafish is placed in a round apparatus containing a central pole. Fish swims in a circular manner either in clockwise or anticlockwise direction. Electrical or acoustic stimulation or visual stimulation in the form of a black tape can be delivered. Normal zebrafish shows startle response. To assess visual defect, the same test can be used under low light condition (60 $\mu W/m^2$) (Weber et al. 2008). Though the normal zebrafish elicits startle response, visually defective fishes do not. This is a modification of startle response and known as visual motor response (VMR). Utilising this test, the role of selenium in decreasing the visual developmental defect due to methyl mercury can be detected. VMR can also be utilised in nrc mutants. Using 96-well plate, large numbers of zebrafish larva are kept to test VMR. They are tested using 30-min 'light on' and 30-min 'light off' period. Normal larva increases activity immediately after 'light off' and gradually returns to their baseline activity. Though nrc mutants show similar response as normal larva due to 'light off', their loco motor response to 'light on' is sluggish compared to normal larva. Though nrc mutants show defective OKR, VMR test proves that they are not completely blind (Emran et al. 2007). chk mutant with no eyes shows no VMR (Emran et al. 2008).

This test can be used as an adjunct to OKR to screen visually defective mutants. High-throughput screening can be possible with VMR. The main disadvantage of the test is that this test cannot be demonstrated in a larva of less than 4 dpf.

13.7 Phototactic Behaviour (PTB)

Phototactic behaviour can be defined as the movement of zebrafish towards an illuminated chamber. This behaviour can be observed in a zebrafish of more than 6 dpf.

This behaviour can be demonstrated using a small box with two chambers divided by a removable partition. There are two methods by which PTB can be tested. In the first method, both the chambers are illuminated, and the partition is removed. Some fishes move from one chamber to another while others do not. After a certain period, number of fishes is counted in each chamber. In the second method, one chamber is

illuminated while the other was kept in dark. Partition is removed and fishes are allowed to move around. After a certain time period, number of fishes in both the chambers is counted. Ideally, zebrafish with normal vision should move towards the illuminated chamber showing PTB while the visually defective fish should not. In both the methods, zebrafish show PTB. But this method is not as reliable and robust as OKR (Brockerhoff et al. 1995). So this method should not be used in mutated zebrafish.

13.8 Limitations

Visual behavioural assays have their own set of limitations. Firstly, these tests are based on the behavioural response of zebrafish. Hence, it can produce a misleading result. Around 2000 mutated zebrafish have been screened using visual behavioural assays. They show abnormal OKR and OMR. But no defect is observed in retino-tectal pathway suggesting the defect beyond that (Muto et al. 2005). Secondly, visual behavioural assays can be altered due to drugs. Acute alcohol exposure diminishes fear response in zebrafish showing ambiguous result (Luca and Gerlai 2012). Thirdly, most of the assays are based on motion cues causing bias in the study (WHO 2020a). Fourthly, less number of assays are available for adult zebrafish compared to larvae. Table 13.3 describes a summary of visual behavioural assays in zebrafish.

13.9 Zebrafish Models in Human Ophthalmological Disorders

13.9.1 Aniridia

Aniridia is a condition characterised by the complete or partial hypoplasia of the iris, hypoplasia of the fovea, and decreased vision. 1:40,000 to 1:100,000 people are affected in aniridia (Hingorani et al. 2012).

Aniridia is a congenital eye disease characterised by haploinsufficiency of the PAX6 gene. In zebrafish model, a point mutation in an ultraconserved cis-element near PAX6 was observed. This mutation disturbs an autoregulatory PAX6 binding site and loss of PAX6 enhancer activity leading to aniridia (Bhatia et al. 2013). PAX6 mutation was also found in the ‘small eye’ mouse model of aniridia. Another four point mutations were also observed in human, mouse and zebrafish (Hanson et al. 1993). FOXE3 gene is associated with the development of lens and anterior segment of the eye. Deletion of the gene results in aniridia, aphakia, cataract, microphthalmia, coloboma, etc. (Krall et al. 2018)

Table 13.3 Summary of visual behavioural assays in zebrafish

Visual behavioural assays	Appropriateness of use in zebrafish embryo	Appropriateness of use in adult zebrafish	Zebrafish mutant	Human ocular disease
OKR	5–7 dpf	Yes	bumper	Defective lens development
			sandy	Defective melanin in RPE
			grumpy, sleepy, pinscher	Defective optic nerve growth
			belladonna	Misrouting of axon
			dropje, steifftier	Defect in INL or OPL layer
			macho, blumenkohl	Defect in retinotectal impulse transmission
			lakritz	Defect in GCL layer
OMR	7 dpf	Yes	bumper	Defective lens development
			sandy	Defective melanin in RPE
			pinscher	Defective optic nerve growth
			lakritz	Defect in GCL layer
			dropje, noir	Defect in INL or OPL layer
			macho, blumenkohl	Defect in retinotectal impulse transmission
ER	No	After 2 month	nba nbb nbc	Retinal degeneration
SR/VMR	After 4 dpf	No	nrc	Partial blindness
			chk	Absence of the eyes
PTB	After 6 dpf	Yes	noa	Functional defect in outer retina

13.9.2 Retinitis Pigmentosa

Retinitis pigmentosa (RP) is an ocular disorder characterised by progressive loss of vision and degeneration of rod cells. It can be autosomal dominant, autosomal recessive or X-linked recessive in nature. Several genetic loci are found to be

associated with RP. Till now 53 loci have been isolated. Among them, 18, 26 and 2 genetic loci are associated with autosomal dominant, autosomal recessive or X-linked recessive RP, respectively. Rhodopsin gene (RHO), USH2A gene and RPGR gene are most commonly related to autosomal dominant, autosomal recessive or X-linked recessive RP, respectively (Raghupathy et al. 2013a).

Autosomal dominant RP develops from mutations in PRPF3, PRPF8 and PRPF31 genes which are responsible for pre-mRNA splicing. PRPF31 and PRPF4 genes are RP related splicing factors which are part of the spliceosomal subunit tri-SnRNP. Silencing the splicing factors can result in RP model in zebrafish. Decreased expression of PRPF31 gene can result in defective photoreceptors (rod and cone cells). Photoreceptor transcripts are mainly affected in PRPF31mutant zebrafish (Linder et al. 2011). On the other hand, diminished level of PRPF4 gene causes defect in the morphology of photoreceptor and retinal gene.

X-linked RP can result from mutation in the gene RP2. In RP2 knockout zebrafish model, degeneration of rod and cone cells is noticed. RP2 ablation can lead to reduced level of GRK1 and rod transduction subunits (GNAT1, GNB1) (Liu et al. 2015). It also results in abnormal distribution of the above-mentioned proteins. Deletion of AP2 might also cause mis-trafficking of farnesylated proteins in the retina.

X-linked RP can be associated with the mutation in both the gene RPGR and RP2. Morpholino-injected knockdown of RPGR2 gene can cause a mild phenotype (MO2M) and severe phenotype (MO2S) in zebrafish (Raghupathy et al. 2013b). MO2M phenotype is associated with small eyes only, whereas MO2S phenotype has small eyes with small head. Defective retinal lamination, abnormal photoreceptors and augmented cell death in the retina are observed in both the phenotypes. Morpholino-mediated knockdown of RP2 gene is also associated with defective retinal lamination and diminished eye and brain size. They can also have other developmental defects, i.e. curved body, hydrocephalus and pericardial effusion (Raghupathy et al. 2013b). Human RP2 can rescue the small eye phenotype of zebrafish RP2 (ZFRP2) mutants partially. This result suggests ZFRP2 is the orthologue of human RP2 gene (Shu et al. 2011). Morpholino-mediated knockdown of RP2 and RPGR2 gene can cause defective ciliary function in the whole body. This proves the role of RP2 and RPGR2 gene in ciliary structure and function (Raghupathy et al. 2013b).

Mutation in RPGR2 can further affect intracellular transport of organelles specifically retrograde transport (Shu et al. 2010). RPGR2 gene has an important role in normal lamination in the retina. It also prevents apoptosis of retinal cells. Dynein-based retrograde transport mediated by RPGR2 gene helps to maintain normal function of retina.

Mutation in splicing factor, i.e. PRPF31, can result in autosomal dominant RP. Different types of mutation in PRPF31 can cause degeneration of photoreceptors. Mutation in SP117 in PRPF31 mutant can result in haploinsufficiency, whereas mutation in AD5 can cause dominant negative effect (Yin et al. 2011).

Mutation in CERKL can be linked with autosomal recessive RP. CERKL protein is present in both outer and inner segment of photoreceptors in the retina. It binds with TRX2, a mitochondrial protein, and has an important role in TRX2-mediated antioxidant pathway (Li et al. 2014). So overexpression of CERKL can protect retinal cells from oxidative stress, and vice versa.

In RP, even mild light exposure can cause ectopic phototransduction leading to the death of the rod cells. In ovl mutant, mislocalisation of visual pigment can result in ectopic phototransduction via transducer protein (Nakao et al. 2012). Forced activation of the enzyme adenylyl cyclase also plays an important role in rod cell degeneration.

In ovl mutant, ciliary transport mechanism mediated by IFT88 polypeptide is affected leading to RP. In this mutant though the cilias are generated, they are not maintained causing degeneration of the outer segment of photoreceptors (Tsujikawa and Malicki 2004).

C2ORF71 gene is predominantly present in cilia or outer segment of photoreceptors (Nishimura et al. 2010). This gene also has a key role in normal vision. Mutation in this gene can cause photoreceptor degeneration leading to RP.

13.9.3 Usher Syndrome

Usher syndrome is an autosomal recessive disorder associated with RP. It has three phenotypes, Type 1 (USH2) being the most severe. USH1 is associated with congenital sensorineural hearing loss of severe degree, vestibular areflexia and development of RP in the first decade of life. Type 2 (USH2) patients demonstrate less severe symptoms. Though vestibular function is normal, moderate sensorineural hearing loss and pre−/post-pubertal onset of RP are observed in these patients. Type 3 (USH3) patients show progressive sensorineural hearing loss and variable onset of RP.

In Usher syndrome 3A, progressive loss of vision and hearing are noticed due to the mutation in clarin 1 (CLRN1) gene. This gene is predominantly present in OPL, INL and GCL layers of the retina in zebrafish model (Phillips et al. 2013). It is also associated with photoreceptors and outer limiting membrane.

Usher syndrome 1F is associated with mutation in the protocadherin gene (PCDH15) (Ahmed et al. 2001). Though PCDH15a is responsible for normal auditory and vestibular function (Williams 2008), PCDH15b is associated with proper positioning of photoreceptors with RPE (Seiler et al. 2005; Aller et al. 2010). It is also associated with normal functioning of the retina.

PDZD7 gene mutation is observed with Usher syndrome. Earlier, Usher syndrome was thought to be of monogenic inheritance. PDZD7 and GPR98 can contribute to digenic inheritance in Usher syndrome (Ebermann et al. 2010). It also acts as a modifier of retinal disease in USH2A patients.

Harmonin (USH1C) protein is present in muller cells of the retina. This protein is also associated with maturation and normal synaptic function of photoreceptors.

Severe type of Usher syndrome (USH1C) is observed with loss of USH1C function in zebrafish models (Phillips et al. 2011).

Zebrafish MYO7A mutant shows mutation in the myosin 7A gene resulting in Usher syndrome 1B (USH1B) (Petit 2001). This mutant also reveals augmented cell death in ONL layer of the retina and diminished amplitude of a and b waves in electroretinogram (ERG) (Wasfy et al. 2014). Continuous exposure to light in this mutant zebrafish causes degeneration of photoreceptors and large hole in RPE.

Ush1a, Ush1b and Ush1c mutant zebrafish is associated with decreased visual function and augmented cell death in the retina (Williams 2008; Phillips et al. 2007). Defective swimming and balance are also observed. Ush2a mutant zebrafish is also linked with defective function of photoreceptors. USH proteins are needed for normal synaptic alignment and functions in photoreceptors.

13.9.4 Leber Congenital Amaurosis

Leber congenital amaurosis (LCA) is an inherited disorder characterised by very severe retinal degeneration with onset in the first year of life (Collin et al. 2012). 22 different genes are associated with LCA. Among them, the most important is CEP290.

In zebrafish model, mutation in CEP290 has shown diminished visual function despite the presence of fully laminated retina (Baye et al. 2011). This vision impairment can be rescued by N-terminal region of human CEP290 protein. This indicates the importance of N-terminal region of human CEP290 protein in restoring vision, and this region can be of potential importance in gene therapy.

Though crumbs gene is mainly associated with corneal dystrophy, mutation in CRB1 is also observed in LCA. Crumbs gene is of paramount importance in the formation of adherence junction. Mutation in CRB1 gene results in disruption of RPE in ‘ome’ mutant of zebrafish (Beyer et al. 2010).

RPE65 gene is associated with development of photoreceptors in zebrafish. Knockdown of this gene results in defective development of outer segments of rod cell. But cone cells remain functional. In LCA caused by mutation of RPE65, colour vision remains normal before visual impairment occurs (Schonthaler et al. 2007). This demonstrates the presence of RPE65-independent pathway for colour vision by cone cells.

Retinal-specific guanylate cyclase (Gucy2d) mutation is associated with LCA1. Knockdown of Gucy2f results in visual impairment in zebrafish. Gucy2f zebrafish model can be utilised as an inexpensive model for LCA1 caused by Gucy2d mutation in human (Stiebel-Kalish et al. 2012).

‘cct2’ mutant zebrafish is associated with LCA. CCT2 gene is responsible for regulation of cell cycle and thus plays a role in the development of the retina. CCT2 mutant zebrafish results in augmented degeneration of retinal cell and reduced vision (Minegishi et al. 2018). This defect can be rescued by human CCTβ mRNA.

Though 22 different genes are isolated as a cause of LCA, genetic basis of LCA in 30% patients is still undiscovered. CLUAP1 gene plays a major role in normal

function of cilia and photoreceptors. CLUAP1 mutant zebrafish shows degeneration of photoreceptors. This gene can be a potential candidate LCA gene (Soens et al. 2016).

Biallelic mutation in USP45 gene can be responsible for LCA. USP45 is associated with normal function of photoreceptors. Knockdown of USP45 gene in zebrafish can result in defective development of the retina and photoreceptors (Yi et al. 2019). Abnormal ERG response can also be observed.

13.10 Diabetic Retinopathy

The prevalence of diabetes mellitus was 463 million worldwide in 2019 and expected to rise to 700 million by the year 2045 (Saeedi et al. 2019). Diabetic retinopathy (DR) usually develops within 20 years of onset of the disease (Wild et al. 2004) and is responsible for 5% of the blindness in the world (WHO 2020b).

PDX1 is a zebrafish mutant for DR. It shows vascular changes in the retina, i.e. tortuous capillaries, hypersprouting and vasculopenia in arteries (Ali et al. 2020). This mutant also demonstrates characteristic degeneration in the photoreceptors.

fli-EGFP transgenic zebrafish model is a cheap and simple model to study angiogenesis of the retina in DR. This model can also be used to study hypoxia-induced neovascularisation and the role of anti-VEGF agents, i.e. sunitinib to block hypoxia-induced retinal angiogenesis (Cao et al. 2008). In normal condition, blockage of the Notch signalling pathway by DAPT results in no arterial sprouting. But in hypoxia this blockage results in vascular sprouts. This suggests targets of Notch inhibitors are shifted in hypoxia.

fli-EGFP transgenic zebrafish model shows changes in retinal vasculature in DR. MAGP1, HS6ST2, Syn2, fe, plt, mgf and plexin D1 are some of the important genes that affect development of retinal vasculature in zebrafish, whereas the genes, i.e. Sppl2, FGF8, dsl and lop, have no role (Alvarez et al. 2007).

After 30 days exposure to glucose solution, the thickness of both IPL and INL diminishes in zebrafish (Alvarez et al. 2010; Jiang et al. 2015). Degeneration of photoreceptors especially cone cells is also noticed. Zebrafish was alternately exposed to glucose solution and water for 28 days. Though the thickness of IPL and INL is similar in the control fish, thickness of both the layers is diminished in zebrafish treated with glucose. In this fish, IPL is approximately 55% of INL layer in the retina (Gleeson et al. 2007). Streptozotocin (STZ)-induced diabetic zebrafish shows changes similar to early stages of non-proliferative diabetic retinopathy (NPDR) (Olsen et al. 2010).

Short-term model of diabetic zebrafish is developed by using flk:EGFP transgenic zebrafish. Zebrafish was exposed to high level of glucose from 3 to 6 dpf (Jung et al. 2016). Changes in hyaloid retinal vessels and effects of VEGF inhibitors, i.e. ranibizumab, are also demonstrated.

In STZ-induced diabetic zebrafish, effect of nano-formulation of quercetin (NQ) was observed. Behavioural responses (e.g. OMR, OKR, etc.) and biochemical parameters (e.g. retinal lipid peroxidation, decreased glutathione, etc.) were

measured. NQ can be utilised for the management of DR, diminished lipid peroxidation and also as a free radical scavenger (Wang et al. 2020).

13.11 Age-Related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) is the third most important cause of blindness in the world. It accounts for 5% of the blindness globally. 196 million people in the world are expected to have AMD by the year 2020 (IAPB 2020a). It is a degenerative disease characterised by the deposition of a lipid material or drusen in the retina.

Light induces retinal degeneration in zebrafish which is similar to the features seen in dry AMD. N-acetyl cysteine when administered intravitreally can protect against retinal degeneration (Saito et al. 2016). N-acetyl cysteine has antioxidant property which is responsible for the above-mentioned phenomenon.

Dry/atrophic AMD is characterised by the atrophy of RPE, degeneration of photoreceptors, oxidative stress, etc. In zebrafish ATP6VOE1 model, features similar to dry AMD were introduced. Histone deacetylase 6 inhibitor Tubastatin A shows regeneration of the morphology of photoreceptors in outer segment of the retina (Sundaramurthi et al. 2020). This is because Tubastatin A shows and regulates several pathways, i.e. ubiquitin-proteasome, phototransduction, phagosome, etc., leading to the restoration of vision in zebrafish model.

Wet/neovascular AMD is associated with choroidal neovascularisation and severe visual impairment. Both in vivo and in vitro experiments were carried out in zebrafish model to establish the role of ubiquitin protein ligase E3D (UBE3D) gene in wet AMD. After knockdown of UBE3D gene, augmented angiogenesis and abnormal morphogenesis of the eye, i.e. small eyes, delayed retinal development, and retrograde transport of melanosomes was noticed (Xia et al. 2020). During in vitro experimentation, tert-butyl hydroperoxide (TBHP) was used to induce wet AMD. Proliferation of retinal cells and expression levels of UBE3D were observed. This suggests the role of UBE3D gene in the development of the retina and oxidative damage.

Breakdown of blood-retina barrier (BRB) is observed in several diseases, i.e. DR, AMD, etc. A transgenic zebrafish model Tg(l-fabp:DBP-EGFP) is developed to study the BRB. This model shows simultaneous development of BRB and blood-brain barrier (BBB) in zebrafish around 3 dpf (Xie et al. 2010). Augmented expression of claudin 5, a protein present in tight junction, is also observed.

'gnn' mutant zebrafish model shows dystrophy of cone cells especially red cones followed by degeneration of RPE. Early changes in 'gnn' mutant zebrafish resemble AMD in human, whereas late changes are similar to RP (Biehlmaier et al. 2003).

Hypoxia-induced retinopathy can be demonstrated in zebrafish similar to DR and AMD observed in humans. Zebrafish is exposed to hypoxic water following which retinal neovascularisation is noticed. Anti-angiogenic drugs, i.e. ranibizumab and pegaptanib, can also be studied using this model (Jensen et al. 2011).

13.12 Corneal Dystrophy and Cornea Plana

Corneal dystrophy (CD) is a bilateral and symmetrical disorder without any ocular inflammation. It can be classified depending on the layer of the cornea involved. Superficial CDs are Lisch, Meesmann, recurrent corneal erosion, etc. Stromal CDs include macular, granular and reticular types, whereas posterior CDs are Fuchs, endothelial, X-linked dystrophy, etc. (Weiss et al. 2008; Klintworth 2009)

Fleck CD involves the stromal layer. Mutation in the gene PIP5K3 in human results in François-Neetens mouchetée fleck corneal dystrophy. This gene in zebrafish is 70% orthologous to human and predominantly found in several layers of the retina, i.e. outer limiting membrane, INL and GCL (Boisset et al. 2008). PIP5K3 gene encodes a kinase PIKfyve which is involved in structure of cell membrane and regulates complex intracellular pathways.

'bal^{a69}' mutant zebrafish shows lens degeneration, focal corneal dystrophy and defect in hyaloids vessels. This mutation is present in chromosome 24 near laminin alpha1 (lama1) gene (Semina et al. 2006).

Cornea plana is an eye disorder characterised by flat cornea and reduced vision. It is found to be associated with keratocan mutation (Pellegata et al. 2000). Keratocan is a keratin sulphate proteoglycan which is predominantly present in the epithelium and stroma of the cornea. Keratocan gene (zKera) knockdown in zebrafish resulted in a lethal phenotype leading to severe caspase-dependent apoptosis (Yeh et al. 2008). But zKera gene knockdown leads to the development of a healthy mouse model.

13.12.1 Cataract

According to WHO, cataract is the cause of 51% blindness worldwide affecting 20 million people (WHO 2020c). Cataract is characterised by the clouding of lens and remains as one of the leading causes of preventable blindness.

In zebrafish 'cloche' mutant, defects in haematopoiesis and development in the blood vessels are observed along with cataract formation. Crystallin is a protein that is predominantly found in lens. Defect in crystallin can lead to cataract formation. The reason behind the cataract formation in 'cloche' mutant is not due to loss of γ-crystallin protein but rather its insolubility. γ-crystallin protein becomes insoluble due to misfolding of protein which is prevented by αA-crystallin (Goishi et al. 2006). Another study reveals that the cataract formation in 'cloche' mutant is not due to the loss of αA-crystallin gene but rather due to stress (Posner et al. 2019). γC-crystallin p.Gly129Cyst zebrafish mutant is associated with vacuoles and incomplete denucleation of lens similar to cataract formation in humans. This mutation causes impairment of tertiary structure of protein leading to the formation of cataract (Li et al. 2012). αA- and αB-crystallin mutants are developed in zebrafish to understand cataract formation. R49C mutant of αA-crystallin causes aggregation of γD-crystallin due to cross-linking of disulphides (Wu et al. 2018).

βγ-crystallin superfamily gene CRYBA2 is associated with congenital cataract. CRYBA2a and CRYBA2b are highly expressed during early development of lens in zebrafish which further suggests their role in congenital cataract formation (Khor et al. 2019).

Zebrafish FOXE3 model shows stages of lens development and cataract formation. It was found that dysregulation of several genes, i.e. cryba2, cryba11, mipa and hsf4, is associated with cataract (Krall et al. 2018).

CLPB mutation in zebrafish is associated with a syndrome characterised by mental retardation, brain atrophy, congenital neutropenia and formation of cataract (Wortmann et al. 2015). This may be due to the fact that the mutant peptides destroy the function of ATPase.

13.12.2 Glaucoma

Glaucoma is one of the priority eye diseases to prevent blindness worldwide (WHO 2020d). It is characterised by high intraocular pressure (IOP), optic neuropathy and impaired vision. It has been estimated that the number of person affected by glaucoma will reach 80 million by the year 2020 (IAPB 2020b).

'LrP2' mutant of zebrafish was used to develop a model for primary open angle glaucoma (POAG). This mutant shows high IOP, optic neuron damage and diminished number of retinal neurons. Myopia, age, family history and other risk factors for glaucoma can be exhibited in this model (Veth et al. 2011; Bouhenni et al. 2012).

'wdr36' mutation in zebrafish can lead to POAG by the activation of p53 stress response pathway. It depicts that the defect in p53 pathway might affect the wdr36 mutant and might influence its role in POAG (Bouhenni et al. 2012; Skarie and Link 2008).

GPATCH3 knockdown in zebrafish can lead to gonadal dysgenesis and ocular and craniofacial abnormalities. 'GPATCH3' mutant also shows features of congenital glaucoma similar to the knockdown of glaucoma-related genes, i.e. pitx2 and foxc1 (Ferre-Fernández et al. 2017). GPATCH3 gene was found in the ciliary body of human and also present in the dermis of the skin, the skeletal muscle, mesenchymal cells and the corneal endothelium of zebrafish.

PITX2 gene mutation is associated with Axenfeld-Rieger syndrome type 1 which is characterised by craniofacial, dental and umbilical defect and augmented risk of glaucoma. 'PITX2' mutant zebrafish reveals defect in the development of iris, cornea, iridocorneal angle and craniofacial abnormality. There exists a link between PITX2 and WNT pathway, and this linkage helps in the regulation of expression of collagen gene during development (Hendee et al. 2018).

FOXC1 gene mutation is also associated with Axenfeld-Rieger (AR) syndrome and congenital glaucoma. This gene is responsible for the differentiation of retinal GCL layer. FOXC1 zebrafish mutant shows decreased number of cells in GCL rather than cell death (Umali et al. 2019).

FOXC1 mutant zebrafish shows reduced expression of FOX01A gene in trabecular meshwork and augmented cell death. FOXC1 gene maintains cellular

homeostasis in the eye and confers diminished resistance to oxidative stress. This suggests the underlying pathology of AR glaucoma (Berry et al. 2008).

Zebrafish mutant 'brass' shows decreased melanin and increased IOP. This mutant also demonstrates the presence of iris hypoplasia. This finding suggests the linkage between the pigmentation pathway and the pathway related to the development of anterior segment (Link et al. 2004).

13.12.3 Coloboma

Coloboma is a rare congenital eye disorder characterised by a gap in the ocular tissue, i.e. cornea, iris, lens, choroid, etc. It is most commonly due to the failure of closure of embryonic fissure in the fifth to seventh week foetal development. Microphthalmia, anophthalmia and coloboma are a spectrum of structurally related disorder which might be present in combination. According to the recent data, coloboma is present in 2–14 persons per 1 lakh of population (AAPOS 2020).

STRA6 is a transmembrane receptor which is responsible for the uptake of vitamin A leading to the normal development of the eye. STRA6 mutation in zebrafish can be associated with colobomatous microanophthalmia (MCOPCB).

Mandibulofacial dysostosis type Guion-Almeida (MFDGA) can be present with microphthalmia, anophthalmia and coloboma (MAC). EFTUD2 mutant zebrafish shows delayed closure of embryonic fissure around 2 dpf compared to the wild type which shows closure around 30–36 hpf (Deml et al. 2015). This finding suggests the role of EFTUD2 in the normal morphogenesis of head structures including the eye.

Two ocular coloboma zebrafish mutants, i.e. 'gap' and 'noi', were developed. Earlier studies have demonstrated the role of aminoglycoside in preventing ocular coloboma. The studies have also shown abnormal apoptosis as a cause of ocular coloboma. Anti-apoptotic agents were used on 'gup' and 'noi' mutants. Though the 'gup' mutant shows improvement, 'noi' mutant does not. It suggests that the apoptosis is a pathway in some of the genotypes of ocular coloboma (Gregory-Evans et al. 2011).

IPO13 gene belongs to the importin B family protein and is associated with coloboma and microphthalmia. IPO13 mutant zebrafish shows autosomal recessive coloboma, microphthalmia and cataract (Huang et al. 2018). It suggests the role of IPO13 gene in the morphogenesis of the eye.

13.12.4 Microphthalmia/Anophthalmia

Microphthalmia and anophthalmia can be defined as the presence of small eye or no eye in the orbit, respectively. Around 14 and 3 persons per 1 lakh of population are affected by microphthalmia and anophthalmia, respectively (Verma and FitzPatrick 2007).

Zebrafish embryos were exposed to ethanol during the time retinal neurogenesis, i.e. 24 to 48 hpf resulting in microphthalmia. Retinal cell death, decreased retinal cell

differentiation, delayed development of photoreceptors and muller cells and reduced size of lens are also observed (Kashyap et al. 2007).

Zebrafish embryos were exposed to diethylaminobenzaldehyde (DEAB), an inhibitor of retinaldehyde dehydrogenase resulting in the deficiency of retinoic acid (RA) during the early phase of eye development. Embryos show microphthalmia, absent OKR or visual background adaptation (VBA) response (Le et al. 2012). In ERG, the retina did not respond to light. This fact suggests that RA deficiency in the early phase of eye development might result in microphthalmia.

GDF6 is a protein of bone morphogenetic family. Morpholino-mediated inhibition of GDF6 in zebrafish results in coloboma, microphthalmia and anophthalmia in some phenotypes of zebrafish (den Hollander et al. 2010). This fact suggests the role of GDF6 gene in eye development (Asai-Coakwell et al. 2007).

Mutation in the retinal homeobox gene (RX) results in anophthalmia or microphthalmia in human. RX3 mutation causes an anophthalmic phenotype in zebrafish (Nelson et al. 2009). This suggests the role of RX gene in eye morphogenesis.

13.12.5 Cyclopia

Cyclopia or alobar holoprosencephaly is a rare congenital disorder characterised by a single eye placed in the median position or a partially divided eye in a single orbit (Salama et al. 2015). Absence of nose and presence of proboscis can also be associated with this disease. Around 1.05 persons per 1 lakh population can be presented with cyclopia.

'AB', 'EK', 'GL' and 'TL' zebrafish mutants are exposed to ethanol during eye development. Cyclopia, delamination of the neural retina and diminished thickness of RPE were observed (Arenzana et al. 2006).Alteration in the components of visual pathway, i.e. retina and tectum, due to ethanol exposure during morphogenesis was also detected.

CYC gene encodes a protein in the TGF-β superfamily which is responsible for the growth of the mesoderm. Sonic hedgehog (Shh) has an important role for the specification of neural plate. CYC mutant zebrafish shows cyclopia and defective development of the ventral brain and floor plate. But surprisingly this mutant shows proper development of motor neurons. This suggests both CYC and Shh are responsible for the growth of the mesoderm and induction of motor neuron, respectively (Blader and Strähle 1998).

In 'cyclops' zebrafish mutant, thapsigargin or cyclopiazonic acid was used to inhibit a signal pathway which induces the development of the floor plate and ventral brain. This resulted in cyclopia in zebrafish. Thapsigargin and cyclopiazonic acid inhibit a calcium pump in endoplasmic reticulum in the signal network during early phase of the development resulting in cyclopia (Creton 2004).

Zebrafish embryos were exposed to several teratogens, i.e. ethanol, forskolin and cyclopamine, resulting in cyclopia. Suppression of Shh signal is observed in all the cases, but this is not an important cause of cyclopia. All teratogens show suppression

Table 13.4 Zebrafish models of human ophthalmological diseases

Human eye disease	Genes affected in zebrafish
Aniridia	PAX3, FOXE3
Retinitis pigmentosa (RP)	
Autosomal dominant RP	PRPF 31, PRPF4
Autosomal recessive RP	CERKL
X-linked RP	RP2, RPGR2
Usher syndrome	PDZD7
Usher syndrome 3A	CLRN1
Usher syndrome 1F	PCDH15b
Usher syndrome 1B	MYO7A
Leber congenital amaurosis	CRB1, CEP290,RPE65, Gucy 2f, CCT2, USP45
Diabetic retinopathy	PDX1, MAGP1, HS6ST2, Syn2, fe, plt, mgf, plexinD1
Age-related macular degeneration	UBE3D
Corneal dystrophy	PIP5K3, lama1
Cornea plana	zKera
Cataract	γ-Crystallin, α-crystallin, CRYBA2, FOXE3, CLPB
Glaucoma	LrP2, wdr36, GPATCH3, PITX2, FOXC1
Coloboma	STRA6, EFTUD2, IPO13
Microphthalmia	GDF6
Anophthalmia	RX3
Cyclopia	CYC

of the markers gsc and six3b in the early phase of the development (Loucks et al. 2007). Suppression of the neural crest cell marker dlx3b was also observed in the later phase of the morphogenesis. Table 13.4 demonstrates the zebrafish models of human ophthalmological diseases.

13.13 Ocular Toxicity Test in Zebrafish

Preclinical toxicity testing in several animals is an important measure in the drug development and clinical trial. Failure to do so can result in unpredictable adverse drug events leading to the failure in a clinical trial and wastage of money and resources. Zebrafish serves as an indispensable animal model for preclinical toxicity testing for the last two decades (Cassar et al. 2019). It can be used for different types of toxicity testing including neurotoxicity, haematotoxicity, cardiotoxicity, nephrotoxicity, ocular toxicity, etc.

Several ocular toxicity studies have been conducted on zebrafish. Zebrafish larvae were exposed to known oculotoxic drugs, i.e. digoxin, quinine, gentamicin, ibuprofen, etc., and controls including 0.1% DMSO (Deeti et al. 2014). Visual behavioural assays, i.e. OKR, VMR and touch response, were measured in both the study and control groups. Though gross morphological changes were not noticed in the zebrafish larva, abnormal visual behavioural response especially in OKR and

VMR was observed in the study group. Overall, VMR shows better sensitivity and specificity in ocular toxicity testing.

Zebrafish larvae were exposed to 27 compounds from 3 to 8 dpf. Visual behavioural assays (OMR, OKR and locomotor assay) were utilised to test ocular toxicity. The overall predictivity of OMR test is 70% with specificity and sensitivity of 75% and 68%, respectively. False positives were clearly identified by OMR test. This data supports the importance of OMR test to predict the adverse effect of oculomotor drugs (Richards et al. 2008).

Zebrafish larvae were exposed to random 16 compounds from 3 to 8 dpf. OMR were assessed on 8 dpf to test ocular toxicity. Aspirin, atropine, chloroquine, chlorpromazine and phenytoin show abnormal OMR which is consistent with known clinical adverse effect or in vivo model (Berghmans et al. 2008). This suggests that zebrafish can be utilised as an animal model to detect ocular toxicity of drugs.

13.14 Gene Therapy

In gene therapy, a piece of genetic material or DNA can be introduced for the treatment of genetic diseases, e.g. Alzheimer's disease, haemophilia B and cystic fibrosis. Adeno-associated virus (AAV) is the most commonly used vector or carrier in gene therapy. In several retinal disorders, i.e. Leber congenital amaurosis, retinitis pigmentosa, age-related macular degeneration, etc., and non-retinal disorders, i.e. uveitis, glaucoma, etc., gene therapy has been tried (Liu et al. 2011).

Zebrafish can be a crucial animal model for ophthalmological research and gene therapy. Transposons can be an effective gene transfer tool and an essential component in gene therapy. Invertebrate transposons have been tried in zebrafish with little success. 'Sleeping Beauty' (SB) transposon is one of the first vertebrate transposons that can be used successfully in zebrafish (Davidson et al. 2003). It utilises an abbreviated gamma-crystallin GFP cassette for tissue-specific transgenic applications.

Several genetic eye disorders, i.e. aniridia, choroideraemia and coloboma, are associated with nonsense mutation resulting in premature termination codon. Certain aminoglycosides (gentamicin, paromomycin, etc.) suppress these nonsense mutations and partially restore the functional proteins in zebrafish (Moosajee et al. 2008). This fact has been observed in 'chm^{ru848}' mutant of zebrafish in choroideraemia. This fact has also been noticed in 'noi^{tu29a}' and 'gup^{m189}' mutants of zebrafish in ocular coloboma. These models show decreased abnormal cell death and ocular defect. Bypass of nonsense mutations rep1, pax2.1 and lamb1 can lead to the treatment of several eye diseases.

Premature termination codon (PTC) read-through inducing drugs can stop the nonsense mutation and cause recovery of protein function. In zebrafish model, PTC124 drug has shown remarkable benefit in Usher syndrome 1C compared to aminoglycoside (Goldmann et al. 2011).

In zebrafish model of Usher syndrome 1C, efficacy of new generation aminoglycosides, i.e. NB30 and NB54, and new chemical compound PTC124 was observed. All the drugs show similar read-through efficacy in retinal culture. But read-through versus toxicity effect is superior in both NB54 and PTC124 (Goldmann et al. 2012). These drugs can be the potential candidates for the treatment of Usher syndrome.

13.15 Ocular Drug Discovery in Zebrafish

Zebrafish can be an ideal animal model in ocular drug discovery and research. Several small molecular drugs can be screened in zebrafish. Embryonic eye vasculature of zebrafish is used to screen small molecular drugs. Screening of 2000 small molecular drugs revealed five potential candidates that can affect retinal angiogenesis in fli1:EGFP transgenic zebrafish. These compounds are enalapril, pyrogallin, zearalenone, albendazole and mebendazole (Kitambi et al. 2009). Out of the five compounds, pyrogallin increases the vessel diameter and also decreases the number of vessels in the retina. Both enalapril maleate and zearalenone have the former action but not the latter one. All the three drugs do not cause the defect in trunk vasculature. Albendazole and mebendazole are more toxic in nature and produce defect after the early treatment.

Zebrafish can be used as an important animal model for the discovery of novel anti-angiogenic drugs for the management of retinal vascular disease. Anti-VEGFR2 (vascular endothelial growth factor receptor 2) SU5416, a tyrosine kinase inhibitor, decreases angiogenesis in both hyaloids and retinal vessels in transgenic zebrafish model Tg (fli1:EGFP) (Rezzola et al. 2014; Rezzola et al. 2016; Serbedzija et al. 1999). But this drug does not affect the trunk vasculature.

Current therapies to prevent ocular angiogenesis are anti-angiogenic drugs against VEGF or laser surgery. Screening several small molecular compounds in zebrafish, a PI3 kinase inhibitor, LY294002, was identified. It inhibits both developmental and ectopic angiogenesis of hyaloids vasculature in the eye (Rezzola et al. 2014; Alvarez et al. 2009). Furthermore, when this drug is injected intraocularly, it does not decrease visual function and have systemic adverse effects.

Hypoxia-induced retinal angiogenesis is mediated by VEGF via HIF pathway (Pugh and Ratcliffe 2003). Adult zebrafish was exposed to 10% air-saturated water resulting in hypoxia-induced angiogenesis in the retina (Cao et al. 2008; Rezzola et al. 2014). Anti-VEGF drugs sunitinib and ZM323881 block hypoxia-induced retinal neovascularisation in zebrafish.

In Von Hippel-Lindau syndrome, activation of HIF pathway and augmented VEGF is observed. Zebrafish ‘vhl’ mutant shows marked angiogenesis, macular oedema and retinal detachment. VEGF receptor tyrosine kinase inhibitor sunitinib and 676,475 suppress angiogenesis in all the tissues including the retina (van Rooijen et al. 2010).

13.16 Retinal Regeneration

Recently intense interest has been observed in the retina of teleost like zebrafish due to their regeneration capacity. Following mechanical and chemical damage or genetic lesions, regeneration of the cells in ONL, INL and photoreceptors was noticed leading to recovery of the vision (Mensinger and Powers 1999; Brockerhoff and Fadool 2011). This property has also been noticed in zebrafish fin and heart (Brockerhoff and Fadool 2011; Qin et al. 2009).

Intravitreal low-dose ouabain injection is delivered in zebrafish leading to the marked damage in GCL and INL layers of the retina. Some photoreceptor cells also show degeneration. Within 1 day post injection (dpi) of ouabain, regeneration response was observed. This fact suggests that the inner retinal damage even without affecting the photoreceptors can induce a regeneration response in zebrafish retina (Fimbel et al. 2007). This is due to the capacity of muller cells to re-enter the cell cycle to form neuronal progenitor cells that regenerate the retinal cell layers.

Constant light exposure can lead to apoptosis of rod and cone cells in albino zebrafish. INL progenitor cells migrate to the ONL layer, and regeneration of photoreceptors occurs from these cells. High expression stat3 was also observed in this regeneration process, indicating its role in signalling of muller cells to re-enter the cell cycle (Kassen et al. 2007). Another study suggests that the blockage of cell division of muller cells in zebrafish retina prevents regeneration (Thummel et al. 2008).

Two transgenic zebrafish models of rod cell death were produced using metronidazole. In the first model Tg(zop:nfsB-EGFP)$^{nt\ 19}$, all the rod cells were killed leading to muller cell division and regeneration of rod cells. In the second model Tg(zop:nfsB-EGFP)$^{nt\ 20}$, some rod cells were killed, and no regeneration was noticed. This above-mentioned phenomenon suggests that extensive amount of rod cell death in an acute manner leads to regeneration (Montgomery et al. 2010).

Muller glial cell-derived neuronal progenitor cells are responsible for regeneration. Proteins PAX6a and PAX6b present in the retina play an important role in this regeneration process. Though PAX6 has no role in the cell division process of muller cell, PAX6b and PAX6a play important role in early and late cell division of neuronal progenitor cell (NP) derived from muller cell (Thummel et al. 2010). Figure 13.1 describes the regeneration process of photoreceptors and the role of PAX6 gene in the regeneration process.

Muller cells in the retina can be reprogrammed to act like stem cell leading to retinal regeneration. This property can be utilised in trauma-induced injury and retinal degenerative disorders in human. Thus zebrafish can provide indispensable information to harness alternative therapeutic management in the treatment of human retinal degeneration.

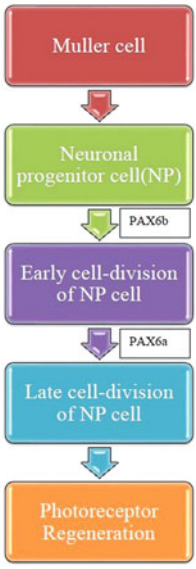

Fig. 13.1 Regeneration process of photoreceptors and the role of PAX6 gene in the regeneration process

13.17 Summary

Ophthalmological diseases causing impaired vision affect millions of people worldwide. Zebrafish can be a robust animal model in ocular diseases, i.e. cataract, glaucoma, retinitis pigmentosa, diabetic retinopathy, age-related macular degeneration, etc., due to its high prolificity, less cost, ease of mutagenesis and similar ocular morphology. Zebrafish cannot only help us understand the ocular disease process including the pathophysiology of the disease; it has also an indispensable role to help us manage ocular diseases by its role in preclinical toxicity test, ophthalmological drug discovery, gene-based therapy and the study of retinal regeneration.

Conflict of Interest The author declares no conflict of interest.

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Zebrafish: A Potential Preclinical Model for Neurological Research in Modern Biology

14

Suraiya Saleem and Rajaretinam Rajesh Kannan

Abstract

Zebrafish have recently acquired the attention of the research community as a model to study neurological pathologies and behavioural anomalies. The small size and high fecundity have made the zebrafish highly suitable for high-throughput screening. The relatively simple organisation of its nervous system and the optically transparent embryos allow real-time neurological imaging of the zebrafish embryos. Further, genetic malleability and highly varied behavioural repertoire increase its suitability as a model for neurological studies. The recent increase in ageing population and the number of patients suffering with neurodegeneration pose the pressing need for effective models for the study of neurological diseases. The current dearth of therapies for the treatment of neurological disorders is mainly due to the lack of absolute and robust in vivo models. Interestingly, zebrafish models have been found to successfully simulate Alzheimer's disease (AD) and tauopathy pathologies along with other neurodegenerative conditions. This chapter summarizes the contemporary research studies that employ zebrafish as neurological models for the development of improved translational therapeutic strategies.

Keywords

Zebrafish · Neurodegeneration · Neuroimaging · Behavioural anomalies · Alzheimer's disease · Parkinson's disease

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14.1 Introduction

Zebrafish are well established as a model system for research in genetics and development (Varga et al. 2018). The zebrafish serve as a strong model to study the neural basis of behaviour. Optical studies of neuronal circuitry and behavioural analysis are possible because of the transparency of the embryonic and larval stages. Transparency of the embryos offers the advantage of noninvasive photoablations of individual neurons in the fish (Karlsson et al. 2001). Availability of neurogenetic tools that facilitate the visualisation, comprehension and manipulation of selected individual neural circuits has made the zebrafish the choice of model to study neuronal circuitry (Kabashi et al. 2011). The combination of ease of optical interventions and genetic manipulations can make the zebrafish model a unique and robust model amongst all vertebrates (Gutiérrez-Lovera et al. 2017; Timme-Laragy et al. 2012). They also harbour several advantageous features that enable it to be a suitable model for neurological research too. Short generation time, transparent embryos and fast development external to the mother have made screening for mutant lines much easier (Hoo et al. 2016; Gioacchini et al. 2010). With the advent of recent state-of-the-art technologies, neuroimaging of the brain and spinal cord, or of individual neuron, has become noninvasive and of high quality and resolution. Imaging of neurons by means of laser ablations in intact living fish has given insights into the links between neurons and behaviour (Basnet et al. 2019). A major challenge in the field of contemporary neuroscience is to obtain a clear understanding of how social behaviour and the brain impact each other. Both have a very fine-tuning ability to regulate each other's functioning and activities. The crucial step towards unravelling this connection requires proper level of analysis to relate social and biological, behavioural and cognitive phenomena. The neural networks of both the cognitive and social processes are highly complex and difficult to comprehend (Xu and Cheng 2017). The only explanatory interface which could act as a link between the brain and behaviour is the perspective of processing information and a degree of cognitive analysis. The neuroendocrine responses of zebrafish are robust and display strong bindings on behavioural process end points. The increased sensitivity of the fish towards environmental variations which may include stress, predators, pheromones and drugs makes them highly suitable for behavioural analysis and drug toxicity studies (Bradford et al. 2017). The zebrafish show varying degrees of baseline anxiety and hence can be used to model acute and chronic drug toxicities (Spitsbergen and Kent 2003). In this chapter we focus on the various neurological models and the expanse of knowledge offered by the zebrafish for research in the field of neuroscience (Fig. 14.1).

14.2 An Introduction to Zebrafish

The zebrafish (*Danio rerio*) is a freshwater teleost fish found in shallow ponds and amongst rice paddies in the Southeast Asian region (Avdesh et al. 2012). It is found inhabiting rivers and freshwater streams in India, Nepal and Bangladesh. It is the

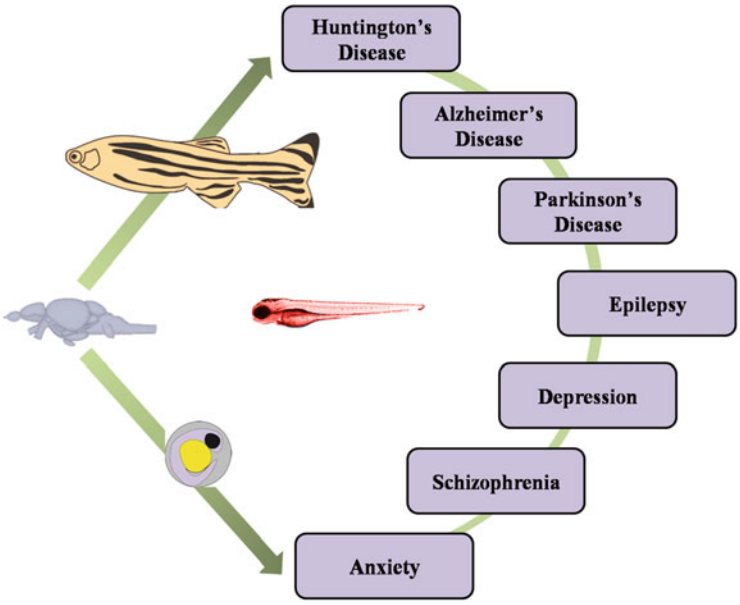

Fig. 14.1 Zebrafish neurological disease models: Utility of the zebrafish to model several neurological diseases. These include Huntington's disease, Alzheimer's disease, Parkinson's disease, depression, epilepsy, schizophrenia and anxiety. Several models have been established by means of pharmacological agents/chemicals/genetic manipulations. All life stages of zebrafish are used for neurological studies

most abundant species in the southern parts of India specially Tamil Nadu (Aoyama et al. 2015). First ever report of using zebrafish as a model organism for experimentation dates back to the 1960s (Gut et al. 2017). Streisnger et al. were the first to suggest the use of zebrafish as an animal model for basic research, after studying its embryonic and genetic characteristics (Holtzman et al. 2016). Since then, the zebrafish have emerged as a powerful tool for research and discovery (Nüssllein-Volhard 2012). Further zebrafish research got an impetus in the 1990s when two genetic mutants were produced by using the zebrafish. This research was conducted by two stalwarts in the field of science, Nobel Prize winner Christiane Nüssllein-Volhard in Tubingen, Germany, and the other by Wolfgang Driever and Mark Fishman in Boston, USA (Bradbury 2004; Brown 2017). The zebrafish possess huge similarities with human organ systems including the brain, digestive system, musculature, vascularisation and immune system (Goldsmith and Jobin 2012; Santoriello and Zon 2012). Their popularity as an experimental model in neurology is recent and is increasing leaps and bounds. Zebrafish toady have emerged as a robust model for neurological research mainly because of two reasons (Best and Alderton 2008). First, the zebrafish display a genomic organisation and neurochemistry quite similar to that observed in man (Howe et al. 2013; Woods et al. 2000). Second, most of the neurobehavioural patterns observed in man have also been

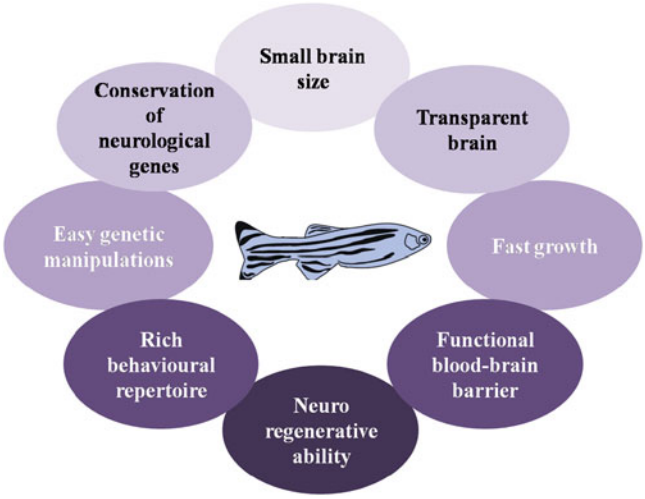

Fig. 14.2 Features which make zebrafish adept for modelling neurological diseases. These include smaller brain size facilitates whole brain imaging; transparency of brain in the embryonic and larval stages enable in vivo imaging; fast growth of the fish allows high-throughput drug screening; functional blood-brain barrier show similarity with humans; unique neuroregenerative ability, a rich repertoire of behavioural display which aids in studying complex neuronal circuitry; easy genetic manipulations by TILLING/morpholinos/knockouts/CRISPR; conservation of genes that allow genetic tractability and modelling of human diseases in the zebrafish

observed in the zebrafish larvae (Kalueff et al. 2014). These neurobehaviours include learning, cognition, sleep-wake cycle or the circadian rhythm and addiction to drugs. Further, the zebrafish harbour various other characteristic features which enable it to be used as a robust model. These additional features are included as following five points. (1) The zebrafish larvae are transparent in nature (Antinucci and Hindges 2016). This allows the researchers to be able to look within the larvae. The optical clarity thus offered by the zebrafish larvae is advantageous in studying complex metabolic processes and development in real time. (2) The small size of the larvae, 4 mm long at 7 days post fertilisation (dpf), makes it possible to undertake several assays simultaneously, thus making high-throughput screening possible (Singleman and Holtzman 2014). (3) Analysis of drugs at an early stage of development is also possible only with the zebrafish larvae (Parng et al. 2002). This enables scientists to understand the effects of drugs in vivo at crucial stages of development. (4) High fecundity rate of the fish provides the opportunity to investigate a large number of embryo/larvae/adults to accomplish high-throughput screening (Wafer et al. 2016). (5) Maintenance of the fish is also very cheap and reasonably easy (Geisler et al. 2016). These features have established the use of zebrafish as an excellent model to study human diseases, specifically neurological diseases (Fig. 14.2). The advantage of using zebrafish for small molecule screening has been extensively explained and is being put to good use by researchers worldwide.

Table 14.1 Various techniques used to establish zebrafish models for neurological research

Pharmacological agents	Genetic manipulations	Behavioural assays	Neuroimaging
Anxiolytic agents Mutagens Depressants Neuro toxic agents Carcinogens	Morpholinos Viral vectors Transposons CRISPR Zinc finger nucleases TALENS/ TILLING Morpholinos	Spatial alternation task Diving task Three-chambered assay T maze Active avoidance Light dark assay Novel object recognition assay	Calcium imaging Confocal imaging 3D reconstruction Multiphoton imaging Photoacoustic tomography PET fMRI

The overall brain structure of the fish is similar to several other vertebrates. Brain areas such as the hypothalamus and olfactory bulb are well defined even in the fish (Calvo-Ochoa and Byrd-Jacobs 2019; Braubach et al. 2012). Further, a structure similar to the mammalian hippocampus is also present (Mueller 2012; Ganz et al. 2014). It is referred to as the lateral pallium (Mueller et al. 2011). In addition, the zebrafish harbour a neurotransmitter system which is similar to the vertebrates (Horzmann and Freeman 2016). They show presence of active cholinergic, dopaminergic and noradrenergic pathways throughout the brain (Rico et al. 2011; Panula et al. 2006). Apart from this, minor ways in which the fish vary from the vertebrates are in the structure of the cerebral hemispheres and optic tectum and the layout of the forebrain. Even the blood-brain barrier (BBB) in the zebrafish is functional and active since 10 dpf. These facts suggest that zebrafish is a well-suited model for the study of neurological diseases (Table 14.1).

While the fish offers a storehouse of advantages as a model organism, a few disadvantages are also associated with the fish. The zebrafish take up drugs dissolved in the medium. Hence quantitative analysis of the actual concentration of drug taken by the fish is difficult to calculate. Further, the zebrafish display neurogenesis (Schmidt et al. 2013; Kizil et al. 2012). This phenomenon may impede its use as a model for certain neurological diseases.

14.3 Neurobiology and Genetics of Zebrafish

The zebrafish genome is well characterised and highly conserved (Ingham 1997; Dooley and Zon 2000). The zebrafish genome has been fully sequenced (Patowary et al. 2013; Vogel 2000). Additional features like external fertilisation, fast development and increased clutch size have made zebrafish a novel model organism in the field of biomedical sciences. The ability to manipulate the zebrafish genome by means of the prevalent genetic tools like forward and reverse genetics has produced several genetic models of rare genetic disorders of the central nervous system (Dong and Stuart 2004; Gao et al. 2017). The models include those for addiction, aggression, anxiety and depression. This serves as one of the most promising features for

zebrafish to be used as a model organism for study of human brain diseases. Interestingly, there occurs almost 70% similarity between the genes of zebrafish and humans (Davis et al. 2014). Approximately 70% of zebrafish genes have minimum one orthologue in the human genome, while 47% of the human genes are strikingly homologous to the zebrafish counterpart. Such a similarity is enough to foster the use of zebrafish as a model for studying human genetic diseases. Zebrafish expresses a total of 26,206 protein-coding genes more than any other vertebrate sequenced previously (Kettleborough et al. 2013). This increased number of genes might have arisen because of the fact that zebrafish has undergone genome duplication which is teleost specific (Ung et al. 2015). Redundancy which occurs as a result of gene duplication in zebrafish has led to increased novelty and variability to the genome. As a result of increased variability, studying gene functions for similarity, divergence or redundancy is possible. This can be achieved by analysis of the functional aspect of the duplicated genes, specifically those associated with neurobehavioural phenotypes. The main architectural organisation of the zebrafish brain is similar to that of humans. G protein-coupled receptors play an important role in neurodegenerative diseases (Martin et al. 2005). Though zebrafish harbour a fairly large number of G protein-coupled receptors, the expression patterns and signalling properties of several of these proteins are similar to those of mammals (Nakano et al. 2017). Despite the presence of a few differences between the mammalian and the teleost central nervous system, a number of reports suggest functional homology in key brain areas between humans and teleosts. Zebrafish also display remarkable memory and learning skill as well as the ability for complex decision-making (Santana et al. 2012). Similar to the rodent models, zebrafish display extraordinary responses to fear, stimuli and sensitivity to drugs and chemical or pharmacological manipulations (Cheng et al. 2014). The area of the zebrafish brain which functions similar to the human hippocampus is the lateral pallium of the telencephalic region; both are responsible for memory and learning (Furlan et al. 2017). While the brain area similar to the amygdala is the habenula (Okamoto et al. 2012; Amo et al. 2010), both are being associated with responses to fear and emotions (Turner et al. 2016). Zebrafish possess several genes which are considered to be orthologues of those found in the humans. The psen1 and psen2 genes, psenen, ncstn, aph1b, bace1, bace2, mapta, maptb, apoea and apoeb are considered to be orthologues of human PSEN1 and PSEN2, PSENEN, NCTN, APH1b, BACE1, BACE2, MAPT and APOE genes, respectively (Jiang et al. 2018; van Bebber et al. 2013; Wilson and Lardelli 2013). The ability to regenerate neurons has provided the zebrafish model an additional advantage (Gemberling et al. 2013). It is a very important characteristic feature observed in the zebrafish central nervous system which enables researchers to explore this facet for developing new therapeutics in humans (Beffagna 2019). Exploring the regenerative ability of the zebrafish may open up newer areas of neuroprotective studies, neurogenesis, signalling mechanism and functional integration of the neuronal cells. The zebrafish model system has been recently extensively used to study several neurological behaviour patterns, namely, cognition, memory, learning and discriminatory behaviour. Sophisticated automated behaviour analysis and video recording by high-resolution video tracking devices have authenticated

the behavioural study in the zebrafish. Comprehensive classification and characterisation of behaviour in zebrafish open up the avenues of drug analysis and high-throughput screening of pharmacological modulators. Studies related to psychiatric and neurological disorders also have a promising future when studied in zebrafish models. The zebrafish therefore has emerged as a potential model system for elucidating the roles of conserved genes and studying various disorders of the central nervous system. The characterisation and proper validation of zebrafish models of neurological disorders also offer the platform for studies related to effective pharmacological therapeutics.

14.4 The Emerging Utility of Zebrafish in Neuroscience Research

Zebrafish models for central nervous system research include studying embryos, larvae as well as adult fish (Khan et al. 2017). All the life stages of the zebrafish have been found to be powerful sources of information for various brain disorders (Becker and Becker 2008). There still exists a wide scope of further research using the zebrafish model system, with regard to the functional and evolutionary relevance of various genes involved in diseases of the brain (Meshalkina et al. 2017). The quantitity of neuroscience research is 10.8% of the total 17,151 records available on zebrafish research at the Zebrafish Information Network, the Zebrafish Model Organism Database and Web of Science (Kinth et al. 2013). The earliest reported work on zebrafish dates back to 1951. Up to the 1980s, there was a lag phase in the world of zebrafish research. The impetus returned only in the 1990s with a robust 226 publications in 1996 which grew to a huge 1929 in the year 2012 (Kinth et al. 2013). Neuroscience research in zebrafish occupies fourth position only after research in development, biochemistry and cell biology (Wilson et al. 2002). The USA occupies the first position while India ranks on the twenty-first position in zebrafish research. Zebrafish being native to the Asian subcontinent demands greater attention from the research fraternity in Asia, specifically India, to augment more extensive research on this promising model organism. Interestingly, neuroscience research has seen a sudden increase in interest and research output in the last few years. Taken together, marked amount of evidence suggests that the zebrafish is rapidly growing to become a leading model system for translational neuroscience research (Guo 2009). It has the ability to simulate almost all brain disorders occurring in the human system.

14.5 Zebrafish Models of Neurological Disorders

14.5.1 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder (Nopoulos 2016). It is associated with motor disabilities and cognitive and psychological problems (Roos 2010). The gene responsible for the pathogenesis of HD was identified as early as 1993 (Finkbeiner 2011). However, till today researchers have not been successful in identifying a cure for this debilitating disease. The disease is caused by a mutation in the Huntingtin (HTT) gene (Myers 2004). HTT is a protein of 350 kDa molecular weight which is ubiquitously expressed by the brain. The pathological hallmarks of HD striking the neuropathological changes occur in the striatum including the loss of medium spiny neurons, gamma-aminobutyric acid (GABA)-utilising neurons of the striatum and neurons in the deeper layers of the cerebral cortex (Neueder et al. 2017). HD affects almost 5–10 people per 100,000 people worldwide.

The endeavour towards the first model of HD using zebrafish was made in 1998 by Karlovich et al. (Karlovich et al. 1998). They isolated the HD cDNA in zebrafish. The protein product of this cDNA was found to share 70% identity to human HTT. Expression of polyQ-expanded HTT in the zebrafish embryos caused accumulation of large aggregates which were insoluble in SDS. This is a key feature in HD pathology (Karlovich et al. 1998). The model thus generated allowed monitoring the HTT aggregates in real time in vivo in the fish. This opened up new insights into the field of HD research. This zebrafish model of HD helped identify two anti-prion compounds of the *N*′-benzylidene-benzohydrazide class as novel inhibitors of polyQ aggregation (Schiffer et al. 2007). This model also highlighted the fundamental question whether inclusion bodies of polyQ-expanded HTT were actually pivotal to the disease. The zebrafish orthologue of the HTT gene encodes 4 glutamines in comparison to the 35 encoded by humans. The HTT protein in the zebrafish is responsible for energy metabolism, iron signalling, lipid, cholesterol homeostasis and development of the brain (Henshall et al. 2009). Another model explored the deficiency of HTT by using antisense morpholino oligonucleotides (Lumsden et al. 2007). These zebrafish displayed cellular iron deficiency, decreased haemoglobin and iron stores in the yolk. This model helped in enunciating the role of HTT in helping release iron from the endocytic compartments into the cytosol. Zebrafish models of HD are made by stably expressing the GFP-fusion protein with transcriptional factors (Wu et al. 2016). This protein is under the control of the rhodopsin promoter. The phenotype of this plasmid is the increased aggregation of the fusion protein and loss of rhodopsin expression from the retina. Compounds like verapamil, calpastatin, clonidine and 2′,5′-dideoxyadenosine helped destroy the aggregates of HTT in the zebrafish model. These compounds had been screened for their activity in the cell culture model thus authenticating the cell culture model system. This acts as a proof of principle for therapeutic relevance of the zebrafish models. In this model the morpholino causes knockdown of the gene responsible for making the HTT (protein). Zebrafish lacking HTT display massive apoptosis of neuronal cells,

abnormal development of the eyes and head and brain ventricles enlarged in size. Older fish having huntingtin knockdown developed lower jaw abnormalities with absence of most branchial arches. They displayed decreased expression of brain-derived neurotrophic factor (BDNF), impaired neural tube formation, reduced cartilage biogenesis and increased activity of metalloproteinases ($ADAM_{10}$ and N-cadherin) (Wiatr et al. 2018). This model brought to light the fact that neurulation defects in the embryos depend on damage to N-cadherin-mediated cell adhesion (Jimenez-Sanchez et al. 2017). In another study, quinolinic acid (QA) was introduced into the telencephalon of the adult zebrafish to study the effects of HD on neuron death (Skaggs et al. 2014). The QA induced neuronal cell death and migration of microglia into the central nervous system indicating neuroinflammation. On the other hand, it induced cell repair and neurogenesis in the fish. Another genetic model of HD was prepared by deletion of N17 coupled with 97Q expansion (mHTT-ΔN17-exon1). This resulted in rapidly progressing movement anomaly. The N17 was intact and 97Q expansion (mHTT-exon1) displayed slower and progressive movement deficit. Zebrafish models to study HD are proving to be essential in unravelling important concepts and notions involved in HD pathogenesis (Das and Rajanikant 2014). Responsiveness to pharmacotherapies related to HD remain to be explored in the zebrafish model system.

14.5.2 Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of dementia (Schachter and Davis 2000). The number of people suffering with AD is predicted to increase to 66 million by 2030 AD. The main pathological hallmark of AD is accumulation of amyloid beta plaques and neurofibrillary tangles (Bondi et al. 2017). Memory loss is the earliest symptom of the disease. Progressive loss of motor ability, onset of hallucinations, delusions and aggressive behaviour are also observed (Weller and Budson 2018). Examination of brain sections from AD patients has revealed immense neuronal loss and neurodegeneration from the early stages of AD (Neugroschl and Wang 2011). There are two types of AD. AD pathologies are occurring below 60 years by genetic causes are termed as familial AD (FAD), while the sporadic AD (SAD) is the general age-related AD having the late symptoms above 65 years. (Apostolova 2016). The mutations responsible for AD have been extensively studied from FAD cases. The genes responsible for AD are PRESENILIN 1 (PSEN1), PRESENILIN 2 (PSEN2), apolipoprotein e (ApoE) and amyloid-beta precursor protein (APP) (Bekris et al. 2010). Apart from the genetic mutations, several other factors like hypertension, high cholesterol and obesity may also play a role in developing AD (Pegueroles et al. 2018; Alford et al. 2018).

Several genetic manipulations have been performed in the zebrafish to study AD. Zebrafish embryos have been injected with psen1 morpholinos (Newman et al. 2014). These embryos are viable. These embryos have revealed that psen1 is responsible for histaminergic neuronal development. Injection of morpholinos against psen2 has revealed that psen2 regulates Notch signalling while the embryos

continue to remain viable (Nery et al. 2017). Other models of AD in zebrafish have been made by introducing morpholinos against Psenen and Aph1b genes (Leimer et al. 1999; Groth et al. 2002). The resultant phenotypic aberrations include defective somatogenesis and impaired neuron formation in the fish. Amyloid beta plaques are the main pathological hallmarks of AD. Several models have been made focussing on amyloid beta. Morpholinos have been employed to block Appa and Appb proteins (Song and Pimplikar 2012; Abramsson et al. 2013). Inhibition of Appa had showed very little effect on the embryo, while inhibition of Appb resulted in reduced length of the body with impaired neural development and cellular movements. In an interesting study, researchers made a double deficient embryo. Morpholino targeting of App showed amyloid beta deficient, while those overexpressing APP showed an abundance of amyloid beta (Zhang et al. 2013). Deficiency of amyloid beta was achieved by blocking beta secretase inhibitor (Timme-Laragy et al. 2012). These larvae displayed major cerebrovascular defects. However it was observed that treatment with amyloid beta $_{1–42}$ peptide could rescue the cerebrovascular function in the embryo to a certain extent. This suggested that amyloid beta $_{1–42}$ plays a constructive role in regulating normal cerebrovascular functions (Liu et al. 2007; Cunvong et al. 2013). This is an interesting finding of amyloid beta which has been revealed by studies in the zebrafish model of AD. Zinc finger (ZNFs) motifs have also been utilised to manipulate the zebrafish genome to prepare AD models (Swarthout et al. 2011). bace1 and bace2 mutations have been introduced in the zebrafish genome by using ZFNs (van Bebber et al. 2013). bace1 knockout fish showed decreased myelination and an enhanced number of mechanosensory neuromasts. bace2 mutants displayed a unique migration of melanocytes which was not observed in the bace1 mutants. This model shed light on the fact that bace1 and bace2 genes are non-redundant in the zebrafish (van Bebber et al. 2013). Several pharmacological models of AD have been made using zebrafish. Neurotoxins like scopolamine, carbachol, pilocarpine and physostigmine have been used on larvae and adults to obstruct the cholinergic neurotransmitter mechanism in the zebrafish (Kim et al. 2010; Hsieh and Liao 2002; Bailey et al. 2013; Eddins et al. 2010). Similarly, pentylenetetrazole, picrotoxin, phenylpyrazole and valproate have been used to inhibit GABAergic neurotransmitter system (Baraban et al. 2005; Yang et al. 2017; Connaughton et al. 2008; Stehr et al. 2006; Baronio et al. 2018). Further neurotoxins have also been used to study AD in zebrafish (Santana et al. 2012). Pentylenetetrazole is a neurotoxin which blocks the GABAergic system and has been used; it resulted in seizure-like state and convulsions in the zebrafish. Similarly, okadaic acid, a potent inhibitor of phosphatases 1 and 2, have been introduced in the zebrafish to create an AD model (Koehler and Williams 2018). Apart from these, several zebrafish transgenic lines have been constructed to study AD. A group of researchers developed a zebrafish AD transgenic line by expressing the mutant human TAU protein fused with the GFP (Paquet et al. 2009). This construct was under the control of neural-specific GATA2 promoter. Another group developed a stable transgenic zebrafish line expressing the human Tau protein in 4-repeat isoform, under the control of the eno2 promoter (Bai et al. 2007). It displayed ubiquitous expression of Tau protein in

the brain. Another zebrafish line expressing the mutated human Tau protein TAU-P301L was developed (Bai and Burton 2011). This model displays the hyperphosphorylation of Tau and can be used to study the early stages of pathology development and also the conformational changes in the Tau phosphorylation. Recent endeavours by a research group have established amyloid toxicity like model in zebrafish by injecting synthetic derivatives of the amyloid peptide into the zebrafish brain (Bhattarai et al. 2017a, b, 2020). This model has been used to study the amyloid pathology as well as the regenerative response. These models of AD in the Zebrafish hold immense promise to open up newer avenues in the field of AD therapeutics (Newman et al. 2014; Xia 2010; Saleem and Kannan 2018).

14.5.3 Parkinson's Disease

Parkinson's disease or parkinsonism (PD) refers to the hypokinetic neurodegenerative disease (DeMaagd and Philip 2015). The symptoms include akinesia (voluntary movements are impaired), bradykinesia (voluntary movements are slow), impairment of gait and balance, freezing phenomenon and rigidity. PD is the most prevalent movement disorder in the world (Emamzadeh and Surguchov 2018). Dopaminergic cell loss in the substantia nigra pars compacta and α-synuclein protein body inclusions known as Lewy bodies are the main clinical hallmarks of the disease (Rizek et al. 2016). Familial cases of PD are associated with mutations in alpha-synuclein (snca), leucine-rich repeat kinase 2 (lrrk2), vps35, PTEN-induced putative kinase 1 (pink1), parkinsonism-associated deglycase (dj-1) and parkin RBR E3 ubiquitin protein ligase (PARK2) genes. Zebrafish have been used extensively to study Parkinson's disease (PD) (Klein and Westenberger 2012; Inamdar et al. 2007). The use of zebrafish to study PD has been facilitated by the fact that they harbour genes which are highly conserved in PD (Makhija and Jagtap 2014). Mutations in ATP13A2 gene have been associated with PD. Zebrafish genome possesses the orthologue for ATP13A2 which displays very high homology with its human counterpart (Lopes da Fonseca et al. 2013). A zebrafish model possessing the ATP13A2 knockdown was created. Complete knockdown of ATP13A2 resulted in to increased mortality of the embryo, while a partial knockdown resulted in behavioural anomalies when compared to control zebrafish larvae. All stages of zebrafish (embryos, larvae and adults) exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been used to study PD (Sarath Babu et al. 2016; McKinley et al. 2005). It has been observed that MPTP causes specific loss of dopaminergic neurons and a decrease of the neurotransmitters dopamine, norepinephrine and serotonin levels and impairs motility in zebrafish larvae and adults (Vaz et al. 2018; Lam et al. 2005; Sallinen et al. 2010). Another strong model of PD has been made in the zebrafish by use of 6-hydroxydopamine (6-OHDA) (Feng et al. 2014; Vijayanathan et al. 2017; Wang et al. 2011). 6-OHDA when injected intramuscularly into the adult zebrafish leads to a decrease in the levels of dopamine and norepinephrine (Matsui and Sugie 2017). However, in the zebrafish models in which 6-OHDA was administered into the ventral diencephalon, immense loss of

dopaminergic neurons in the posterior tuberculum and two other dopaminergic areas was observed resulting in bradykinesia (Vijayanathan et al. 2017; Zhang et al. 2015a). Exposure to 6-OHDA to the zebrafish larvae causes decreased levels of tyrosine hydroxylase and reduced locomotor activity (Li et al. 2018). The use of herbicide paraquat has successfully helped in creating PD models in zebrafish (Zhang et al. 2016; Nellore and Nandita 2015). Administration of paraquat in adult zebrafish leads to locomotor anomalies, impaired memory, anxiolytic and aggressive behaviour, decrease in mitochondrial viability and an increase in antioxidant enzymes. Administration of rotenone also leads to the development of a zebrafish PD model (Ramkumar et al. 2018; Ünal et al. 2019). Fish exposed to rotenone reported lower levels of dopamine and tyrosine hydroxylase, impaired motor function, anxiety and olfactory anomalies. Other models of PD using zebrafish have been made by genetic manipulations. One such model is Tg(dat: CFP-NTR) zebrafish line which expresses the cyan fluorescent protein and the nitroreductase enzyme, both under the control of the dat promoter (Godoy et al. 2015). When these fish were exposed to metronidazole, it was metabolised by the nitroreductase to release a cytotoxic product which led to apoptotic death of the dopaminergic neurons. Recently, researchers have used nanotechnology to induce parkinsonism to the fish. Exposure of zebrafish to titanium dioxide nanoparticles caused parkinsonian-like pathogenesis in the zebrafish larvae. The nanoparticles caused premature hatching of embryos followed by abnormal development. The nanoparticles accumulated in the brain of the larvae, causing loss of dopaminergic neurons, increased production of reactive oxygen species and impaired locomotor activities. Besides it also led to an increase in the expression of PD-related genes pink1, parkin and uchl1 (Hu et al. 2017). Knockdown of the β- and γ-synucleins in the zebrafish results in complete absence of synucleins (Chandra et al. 2004; Greten-Harrison et al. 2010; Burré et al. 2010). Overexpression of γ1-synuclein leads to protein aggregates in the brain, while overexpression of α-synuclein could have been a promising model for PD but fails to be one today because the extent of lethality caused is huge (Lulla et al. 2016). Further research into this aspect to restrict the effects of overexpressed α-synuclein will lead to promising results in the field of PD research (Prabhudesai et al. 2012). Morpholinos have been used to knock down PINK1, Parkin, DJ-1, leucine-rich repeat kinase 2 (LRRK2) and Fbxo7 in the zebrafish. PINK1 knockdown leads to mitochondrial dysfunction which leads to apoptotic cell death and to increase in reactive oxygen species and abnormal neuronal morphology (Anichtchik et al. 2008). Knocking down of Parkin causes mitochondrial dysfunction and dopaminergic loss of neurons (Flinn et al. 2009). Loss of DJ-1 led to increase in the expression of the apoptotic genes p53 and Bax (Bretaud et al. 2007). Absence of leucine-rich repeat kinase 2 (LRRK2) showed embryonic lethality and caused immense developmental defects and retardation in the zebrafish (Prabhudesai et al. 2016; Xiong et al. 2017; Ren et al. 2011). Morpholino knockdown of the zebrafish Fbxo7 orthologue resulted in abnormal patterning of dopaminergic neurons and motor impairments while polymorphic Fbxo7 orthologues prove to be protective (Chen et al. 2014).

14.5.4 Epilepsy

To study epilepsy and seizures in zebrafish, hyperthermia-induced models have been studied. Hyperthermia-induced models are generated by exposing the zebrafish to varied ranges of temperatures. So far, only one hyperthermia-induced model has been characterised. Zebrafish larvae were exposed to increased bath temperatures. The epileptic discharges thus released from the forebrain of the larvae were measured. Some epilepsy models were created by genetic manipulations. Morpholino based knock down of bomb mutant are lgi1a morphant, scn1lab, and the scn1lab morphant (Teng et al. 2010; Cowell 2014). Several chemicals have been used to create models of epilepsy using zebrafish. The chemicals used in establishing epilepsy are PTZ, domoic acid, 4-aminopyridine, maprotiline, bemegride, bicuculline, physostigmine, amoxapine, semicarbazide, aminophylline, acetylsalicylic acid, picrotoxin, strychnine, caffeine, pilocarpine, kainate, ginkgotoxin, RDX, linopirdine, XE991 and allylglycine (Kundap et al. 2017; Tiedeken and Ramsdell 2007; Liu and Baraban 2019; Hortopan et al. 2010). All the chemicals used have been able to induce seizures in the zebrafish making them pertinent models for epilepsy.

Genetic manipulations have been employed to generate epileptic models in the zebrafish. Morpholinos against genes like mind bomb, lgi1a, lgi1b, kcnq3, tnt, ocrl1, pk1a, scn1lab, kcnj10a, chd2, stx1b, cnnm2a and cnnm2b, scn1lab, cntnap2ab and stxbp1b have been used to generate seizures in the fish (Chege et al. 2012; Suls et al. 2013; Zdebik et al. 2013; Schubert et al. 2014; Zhang et al. 2015b; Baraban et al. 2013). Knockdown of kcnj10a not only induced seizures but also showed ataxia and loss of posture. Ocrl1 mutants are expressed by occasional twitching, while chd2 mutants showed whole body twitching and whirlpool-like movements. Further overexpression of mutant fhf1b1 Tol2 has also been reported to qualify as a successful model of PD.

14.5.5 Schizophrenia

Schizophrenia is a heterogeneous disorder; thus modelling it poses a significant problem. However, pharmacological and genetic manipulations that lead to schizophrenia-like symptoms have been attempted to create zebrafish models of schizophrenia. MK-801, a pharmacological chemical agent, was introduced into the zebrafish system, and it was observed that it affected locomotion in varied ways in the zebrafish larvae and adults (Seibt et al. 2011). Anomalies in circling behaviour, aggression, social withdrawal and memory deficits were observed. Another chemical called ketamine has also been used to induce schizophrenia-like behaviour in the zebrafish (Zakhary et al. 2011). It has been observed to affect memory and locomotion in the fish. Interestingly, low doses of ketamine have been observed to increase aggressive behaviour in the fish while high doses of it decreases such behavioural displays. Further, inhibitory shoaling cohesion has also been observed in the zebrafish treated with ketamine. Phencyclidine when treated to the zebrafish results

in accelerated circling behaviour in the fish while its social shoaling behaviour remained intact (Bruni et al. 2016; Kyzar et al. 2012). Amphetamine increased the locomotion of fishes by ceasing the freeze moments in the fish (Khan et al. 2017), while treatment with lysergic acid diethylamide (LSD) decreased the social proximity by increasing inter-fish distances. Mescaline caused no effect on the locomotory behaviour of the fish; however, it has decreased social proximity of the fish (Neelkantan et al. 2013). Proline on the other hand increased locomotion and distance travelled by the fish while decreased shoaling behaviour in the fish (Savio et al. 2012). Methionine had a significant impact on the memory of the fish impairing learning and cognition in them (Wang et al. 2016).

Genetic manipulations by means of morpholinos have been used to generate models of schizophrenia in the zebrafish. Disrupted-in-Schizophrenia 1 (DISC1) is a gene well-known as a risk factor for mental illnesses including schizophrenia. It is a scaffold protein involved with the dopamine system. Knocking down of disc1 in the zebrafish causes abnormalities in the brain ventricles, forebrain and axonal extensions. Though the fish showed normal shoaling behaviour, there was decreased perception of its environment as observed in lack of bottom-dwelling tendency and preference for dark areas. On the molecular level, an impaired hedgehog signalling was observed. Knockdown of th1/2 (TH) and kif17 (KIF17) led to minor impairments in locomotory behaviour, and dose-dependent morphological imperfections along with stunted growth in the larvae were observed, respectively. Knockdown of kctd13 (KCTD13) had led to increased apoptotic cell death and reduced expression of neuronal markers by introducing human mRNA against it. while shank3a/b (SHANK3) knockdown led to reduction in the size of the brain and trunk size and eye deformities.

14.5.6 Anxiety and Depression

Although anxiety-related disorders are estimated to be one of the most prevalent neuropsychiatric conditions, research has still not been able to decipher most of their pathological mechanisms. Stress and anxiety have been studied by modelling several vertebrates and invertebrates for the study. Recently zebrafish have emerged as the choice of organism for anxiety research. Modelling anxiety in zebrafish involves novelty-based paradigms, pharmacological exposures and genetic manipulations. The use of recent technologies like 3D reconstructions and bioinformatic interventions is also being used for studies in anxiety in zebrafish. Several anxiogenic or anxiolytic agents have been used to study their effects zebrafish models. The anxiolytic compounds that have been currently used are tranylcypromine (TCP), lysergic acid diethylamide (LSD), monoamine oxidase inhibitors (MAOIs) and dizocilpine (MK-801). Tranylcypromine, an antidepressant drug, blocks degradation of serotonin and causes an increase in cell proliferation in the cerebellum (Stewart et al. 2012). LSD, a potent hallucinogen, regulates serotonin receptors. It causes anxiolytic-like actions in the fish in both types of treatment, acute and repeated. MAOIs, widely used as treatment for anxiety disorders, reduce

anxiety-like behaviour in the fish. MK-801 treatment results in hyperlocomotion in the fish. Further it also affects perception and preference for place, increases exploratory behaviour and reduces aversion to predators. Several behavioural paradigms have also been used to assess anxiety-related states. The novel tank diving assay is being currently used to test various high-throughput drug testing on the basis of psychotropic analysis. The novel tank diving test assessed the anxiety level in the fish. When the fish are released into the novel environment, they first dive to the bottom. After spending sometime at the bottom, they then get acclimatised to the environment. They then diverge to explore the top. The various parameters which can be used in this test to assess anxiety levels are exploration, speed and frequency of escape-like erratic behaviours. In case the fish displays inhibition in exploration, reduced speed and more erratic behaviour, these are indicators of high levels of anxiety in the fish. The presence of predators are the universal stressor for the zebrafish. Visual contact with the predator has been seen to increase cortisol levels in the fish. Further, genetic manipulations have also been adopted to create anxiety-like phenotype in the zebrafish. Mutation in the gene, nevermind (nev), causes disruption in the optic nerve while muscular morphology is normal. However it impacts their axon projections on both lateral and ventral sides of the tectum, causing corkscrew swimming, a typically abnormal locomotion pattern displayed by the fish. In this swimming pattern, the fish display rotator movement along their longitudinal body axis. Sphingosylphosphorylcholine knockout fish have displayed erratic movements and escape responses even in the absence of stress or stimuli. Another transgenic zebrafish line harbours mutation in the Lhx2, which is a transcription factor involved in the retinotectal axonal growth. Several other mutations may lead to anxiety-related behaviour in the zebrafish. Studies in this field are still in its infancy with a lot still remaining to be explored.

14.6 Assays for Assessing Cognition in Zebrafish

Zebrafish are emerging as an attractive model organism to study cognitive neuroscience and bias. Several assays have been developed to study cognition in the zebrafish. The three-chambered task typically analyses learning to avoid and the perception of spatial and nonspatial escape and discrimination. In the conditioned place preference (CPP), there has been a focus on the role of motivational learning and positive response and the influence of fear and anxiety upon negative reinforces. The active avoidance assay analyses learning and memory capabilities in the fish. The fish are made to learn to actively avoid certain areas by the help of negative reinforcement on them. T maze assay assesses the memory of the fish. In this, it tests their spatial analysis ability and memory of positive reinforcement. Inter- and intratrial habituation behaviour is another behavioural analysis which assesses short-term and long-term learning and memory in the fish. Latency to initiate the ability to explore assesses changes in cognitive functions which arise due to age. Novel object recognition assay revolves around the capability of the zebrafish to explore, inspect, recognise and remember features of objects exposed to them.

Homebase formation explores spatial memory and space recognition in the zebrafish. Pre-pulse inhibition program processes the modulations in the startle response and makes it readily available as paradigms to assess neurological disorders. Several other pharmacological chemical agents used as stressors to create AD, PD and schizophrenia models can also be used to study cognitive disorders. This is simply because they affect that part of the brain which is involved with learning and memory in the fish.

14.7 Zebrafish Neuroimaging Models

The zebrafish have been recently used to image neurons and the brain. Presence of a relatively simple nervous system facilitates this process. The fact that the zebrafish embryos and larvae are transparent acts as an added advantage to the neuroimaging process. It also enables researchers to not just visualise neuronal proteins in the neurons but also enables them to comprehend real-time neurological processes. The ability to study neuronal processes in real time has a major role in creating an understanding towards neurodegenerative diseases and brain disorders. Several zebrafish models have been made to facilitate neuroimaging for various diseases. Calcium imaging technique has been adopted to study development in the zebrafish model. It has been used to study reticulospinal neurons in the larval zebrafish and Mauthner cell circuit in the living fish. Aequorins, bioluminescent photoproteins, have also been used to study in vivo neuron death in the zebrafish. A stable transgenic zebrafish line has been created to allow detection of tau conformational changes and hyperphosphorylation as early as second day of embryonic development. The zebrafish are able to process and display the pathological features much earlier as compared to the time taken by other rodent models; this aids in neuroimaging in the zebrafish. A transgenic zebrafish line harbouring GFP in the microglia helped view the morphological changes in the fish post treatment in AD. It also provided an insight into microglial phagocytosis and clearance of amyloid beta in the diseased brain. In another transgenic zebrafish model which GFP targeted in the mitochondria, in vivo fragmentation of mitochondria and real-time imaging of it were possible. These studies shed new insights into these phenomena to enable understanding of the complex biological mechanisms. Confocal imaging of mitochondrial oxidation was also studied by another group. Zebrafish models have also facilitated real-time imaging to expand our knowledge on cell division cycle and related events. Recent state-of-the-art techniques like laser axotomy and time-lapse imaging have also been applied to zebrafish. Interestingly these have led to unravelling of valuable information pertaining to neuroregeneration and degeneration in the zebrafish. Of great interest is the fact that zebrafish have been found suitable to study three-dimensional reconstruction of images to produce a 3D volumetric representation of the specific object. Immense endeavour is being vested in this aspect whereby fluorescent probes are being developed and two-photon and multiphoton microscopy techniques are being developed using zebrafish models. The most recent development in this field is the multi-usage chamber called

UniverSlide adapted for 3D live observation of bioimaging of the zebrafish larvae. Neuroimaging in zebrafish has therefore unearthed a huge amount of information but still holds promise to bring about breakthrough research in the field of neuroimaging. It therefore stands as a most promising and robust model system in this field.

14.8 Neural Circuits in Zebrafish

Some of the features of the zebrafish which make it a suitable model to study neuronal circuits are that their brain remains small in size at both larval and adult stages of life. Being small in size, studies related to neuronal activity pattern and their measurements are highly reasonable. Zebrafish brains also facilitate reconstruction of neuronal circuitry diagrams by electron microscopy. All these repertoire of information gained from studies in zebrafish aid in enhancing knowledge on neuronal computations and complex brain functions. Calcium-sensitive dye has been used to image activity patterns of the sensory axons evoked due to odour. Activity patterns can be read by electrophysiological recordings and multiphoton calcium imaging. Imaging neural activity in the zebrafish larvae is achieved by the use of differential interference contrast (DIC) optics or fluorescent labels. Calcium indicators are advantageous for monitoring neuronal activity because the signals generated from them are significantly large and they also pose less dye phototoxicity problems. Calcium has several advantages in terms of neuroimaging. It can flow into neuronal cells and measure the electrical activities. The larval zebrafish were injected with calcium green dextran into their muscle to image neuronal activity. Confocal microscopy then revealed high-quality, high-resolution images of neurons. This application can help in studying not only the dendritic morphology but also the axonal structures along with the synaptic terminals of neurons. Another group explored the activity of escape behaviour in fish by eliciting avoidance response as a result of tapping one side of fish head. This stimulus results in a very rapid escape response that causes the fish to bend and turn away from the end that was tapped. Imaging of the neuronal circuitry of the fish revealed that there was a huge outburst of activation of axial motor neurons on the part of the head that was tapped. Detailed imaging revealed that this activity involved not just the large motor neurons but also the smaller secondary motor neurons. Another work which focussed on imaging neuronal circuitry in the zebrafish brain was focussed on the reticulospinal neurons. The regulatory mechanism of the reticulospinal neurons over the hindbrain neurons was studied. Imaging data revealed that touch on the head resulted in activation of a large set of hindbrain neurons, whereas touch on the tail activates only the Mauthner cell group. The larger conclusion that could be drawn from the observed facts was that the directionality of response depended on the pattern of activation of hindbrain neurons. The study of hindbrain neurons has elaborated the possibility of imaging neuronal populations in the vertebrate brain to explore cell functionality that were difficult to unearth with conventional electrophysiological approaches. Researchers captured the neuronal activity generated by tail beats of fish while in swimming phase. It reveals the active status of the spinal interneurons during this activity. To

overcome the problem of electrode usage, a novel labelling approach was developed. In this, calcium indicator is injected into the blastomeres in the zebrafish embryo. The fish are then allowed to continue development. As a result of injecting the indicator at an early stage of development, a huge population of neurons display the indicator making imaging easy and highly effective. This method has been used to study motor neurons and neurons in the auditory system of zebrafish. The main advantage of the blastomere method is that the neurons are filled with the calcium indicator without destroying their integrity and connections. However there are two problems linked to this method. First is the increase in dilution of the indicator. The concentration of the injected indicator gradually decreases with the developing fish as a result of which at later stages only a very small quantity is left in the cells. This makes identifying the cell type post imaging difficult. Secondly, since many cells are possessing the indicator, the problem of absorption of the illuminating light arises. The solution to this problem is achieved by shifting the injection of the dye to a later stage of development. Another solution is the use of transgenic lines of zebrafish which express high levels of GFP in some neuronal populations. Presence of GFP makes neurons easily identifiable. Another possibility is the generation of transgenic lines with a calcium-sensitive GFP. The advantage of using zebrafish for imaging neuronal circuitry also lies in the fact that it harbours relatively small number of neurons in its central nervous system. This allows imaging of the neurons in the truest sense of functionality and structure. Further, the data obtained can be extrapolated to higher vertebrates and help solve the complexities of neuronal circuitry.

14.9 Conclusion

The zebrafish is becoming quite popular as a model for studying structure and function of neuronal cells. Small brains of the zebrafish are particularly useful for qualitative, quantitative and exhaustive studies of neuronal integrity, activity and connectivity patterns.

Zebrafish serves as the most apt genetic model organism. It possesses easy genetic malleability and tractability. The findings from the zebrafish can be extrapolated to higher vertebrates and humans. Recent research into the zebrafish genome promises to yield novel genes, which can create a deeper insight into the complex organisation of the central nervous system. Specific mutant and transgenic lines of fish can provide powerful tools to perform imaging and further research. Zebrafish have the potential to achieve breakthrough discoveries in the field of neuroscience. Ongoing endeavours to establish better zebrafish models for various neurological, psychiatric and other diseases promise to establish zebrafish as a powerful and robust model system for studies in neurobiology. Zebrafish therefore holds potential to take the studies on neurobiology into translational therapeutic research.

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Zebrafish Screening for Skin Diseases

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Abstract

Kidneys play a vital role in maintaining body homeostasis, and its disorders are a global health problem, affecting more than 750 million people. Recently, zebrafish has become popular to model various renal disorders in comparison to traditional models utilising mice and rats. The models developed include modelling of acute kidney disorders using nephrotoxic agents (gentamicin, cisplatin, acetaminophen, citrinin, patulin and anticancer antibiotic adriamycin), precision cellular laser ablation, renal fibrosis and mechanical obstruction of pronephros tubules. The other popular models include genetic models to assess tubular disorders, glomerular disorders, renal ciliopathies and congenital anomalies. Although, in demand, there are various limitations in modelling zebrafish to assess renal disorders, which needs to be addressed.

Keywords

Zebrafish · Renal disorders · Acute kidney injury · Genetic models

15.1 Introduction

The largest organ of the body—skin—acts as a first barrier protective against various environmental injury and pathogens in all animals. Over years various animal models have been developed to probe pathophysiology of various dermatological disorders that also facilitate the testing of newer treatments (Zomer and Trentin 2018; Eisenhoffer et al. 2017). The rodents (mice and rats) play a key role in modelling for studying various aspects of skin physiology and biochemistry due to

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their ease of handling, rapid reproducibility and economic accessibility. But rodents differ in skin microanatomy in comparison to humans as they lack a multilayered epidermis, apocrine and eccrine glands (Table 15.1). In a study systemically comparing transcriptomes of human and mouse skin reported that only 30.2% of skin-associated genes are identical, thereby illustrating probable explanation for translational gaps in extrapolation of murine studies to humans. To overcome this limitation, various genetic models including 'knockout' models or transgenic mutants have been developed but are limited by their cost, size of litters and long generation time (Zomer and Trentin 2018; Eisenhoffer et al. 2017; Cline and Feldman 2016; Candi et al. 2014; Gerber et al. 2014; Agarwal et al. 2020). However, studies in larger animals like pigs have increased translational ability owing to its physiological closeness to humans but are expensive and genetically heterogeneous. Hence the need of alternate models and the zebrafish (*Danio rerio*) has been explored to bridge this gap (Zomer and Trentin 2018).

Zebrafish has demonstrated large similarity in development and genetics of the skin to humans. The zebrafish skin possesses three compartments (epidermis, dermis and hypodermis) similar to humans. The various advantages offered include transparency of embryos for several days and recognition of the epidermis and dermis by 24 h post fertilisation (hpf), basement membrane by 32 hpf and lamina lucida and densa by 48 hpf. Keratinocytes with distinct cell-cell borders are demonstrated by 1 day post fertilisation (dpf); almost 400 melanocytes develop by 60 hpf and differ from mammalian melanocytes in that they retain melanin even after transferring melanosomes to keratinocytes. The epidermal cells can proliferate and are replaced only upon injury/death unlike mammalian epidermis, and the non-differentiated epithelial cells in intermediate stratum provide a potential reservoir of stem cells for keratinocytes. A comparison of the morphology of the human, mice and zebrafish skin has been tabulated (Table 15.1) (Zomer and Trentin 2018; Eisenhoffer et al. 2017; Cline and Feldman 2016; Candi et al. 2014; Gerber et al. 2014; Agarwal et al. 2020; Li et al. 2019).

15.2 Zebrafish Model for Hereditary Pigmentary Disorders

Pigmentary disorders comprise a group of hereditary/acquired disorders characterised by varied degree of hyperpigmentation and/or hypopigmentation, related to defects in melanin synthesis/development of melanocytes. Due to genetic similarities and transparency of the larval skin, zebrafish possessing stripes over its body and fins has been used to study development and disease associated with melanocytes.

Morpholino oligos (MOs): These are one of the powerful tools widely used to manipulate genes and analyse embryos of zebrafish. They have become standard knockdown tools to study developmental biology in zebrafish. MOs are p-chiral nucleic acid analogues wherein methylenemorpholine rings substitute for ribose/deoxyribose moieties and non-ionic phosphoramide linkages can replace ionic phosphates in comparison to natural DNA and RNA that can pair with

Table 15.1 A comparison of morphology of human, mice and zebrafish skin[a–d]

Characteristics	Human skin	Mouse skin	Zebrafish skin
Epidermis	Thicker (>100 μm) and multilayered (5–10 layers)	Much thinner (<25 μm) and less layers (2–3) in comparison to human epidermis → reduced barrier function & ↑percutaneous absorption (affects drug delivery and absorption)	Bilayered epithelium
	Keratinized epithelium Stratum granulosum Stratum spinosum Stratum basale Rete ridges	Cornified layer Granular layer Spinous layer Basal layer Contains γδ dendritic epidermal T cells (DETCs)	Cuticle Micro ridges Superficial stratum Intermediate stratum Basal stratum
Basement membrane	Epidermis is adherent to basal tissue Contains lamina lucida (contains integrin and laminin) Lamina densa (contains collagen)	Non-adherent and loose	Hemidesmosomes attach basal stratum to underlying dermis. Lamina lucida (contains integrin and laminin) Lamina densa (contains collagen)
Dermis	Contains melanocytes, blood vessels, hair. Also rich in eccrine sweat glands and neutrophil defensins	Rich in hair follicles (in comparison to humans) Absent eccrine sweat glands	Contains melanocytes, scales (source of calcium), and blood vessels
Hypodermis (subcutaneous tissue)	Fat cells	Along with fat cells contains panniculus carnosus (muscle layer)	Adipocytes
Melanocytes development and migration	Originate from neural crest cells (multipotent) near dorsal aspect of neural tube and melanoblasts → Activation by microphthalmia-associated transcription factor (MITF) → Activation of genes (tyrosinase, dopachrome tautomerase) → growth, differentiation and survival of melanocytes		
	Most steps in differentiation and specification are similar to zebrafish	Most steps are similar to zebrafish, except melanoblasts, migrate along lateral pathway only	Melanoblasts reach final destination and form pigment pattern by migrating along ventral and lateral pathways

[a]Cline and Feldman (2016)
[b]Candi et al. (2014)
[c]Zomer and Trentin (2018)
[d]Eisenhoffer et al. (2017)

complementary target mRNA without being recognised by enzymes including DNase and RNase. MOs target 5′ untranslated region (UTR), prevent the reading of translation initiation complex and hence reduce target RNA expression or target critical splicing events of pre-mRNA in nucleus (Li et al. 2011, 2019). The various models based on this include the following:

1. Oculocutaneous albinism (OCA): It is an autosomal recessive disorder characterised by mutation in multiple genes. Mutation in OCA2 gene (P-gene) causes hypopigmentation in the hair, skin and eyes and also associated visual anomalies (brown OCA/OCA type II). Bier et al. have identified zebrafish with oca2 mutant with similar phenotype (Li et al. 2019). Several other mutations contributing to autosomal recessive albinism C10orf11 (differentiation of melanocytes), Slc45a2 (increases pH of melanosomes), TYRP1 (related to OCA3) and TYR (related to OCA1) could be modelled in zebrafish using MO knockdown.
2. Hermansky-Pudlak syndrome (HPS): It is characterised by OCA associated with pulmonary fibrosis, bleeding diathesis and congenital nystagmus. The zebrafish mutants with snow white (snw)/HPS gene 5 (hps5) mutations have helped in better understanding of disease.
3. Vitiligo: It is a depigmentation disorder with multifactorial inheritance, characterised by progressive epidermal melanocytes disappearance. Various causes attributable include oxidative stress, autoimmune response, genetic susceptibility and metabolic abnormalities. Zhou et al. treated zebrafish with IL-17 and observed dyspigmentation and pigment cell death by autophagy (Li et al. 2019). This model could be used to assess newer treatment option for vitiligo.
4. Dowling-Degos disease (DDD): It is a rare autosomal dominant dermatosis characterised by reticular hyperpigmentation in flexural areas (groin, neck and axilla). Li et al. knocked down suspected protein O-fucosyltransferase 1 (POFUT1) gene using pofut1-MO. The resulting embryos at 48 hpf displayed hypopigmentation and abnormal distribution of melanin at 72 hpf with significant decrease in melanin and tyrosinase activity (Li et al. 2019).
5. Menkes disease: It is an X-linked recessive hereditary disorder due to mutations in ATP7A gene characterised by copper deficiency manifesting as hypopigmentation, seizures and neurodegeneration. A unique model of combined genetic alteration and nutrient deficiency in zebrafish has been developed. The calamity mutant with viable ATP7A allele in zebrafish identified by chemical screen when exposed to mild deprivation of copper leads to hypopigmentation.
6. Dyschromatosis universalis hereditaria (DUH): It is a rare disorder characterised by both hypo- and hyperpigmentation involving all parts of the body. ABCB6 mutation has been identified as a causative agent and has been confirmed by Liu et al. after knocking out abcb6 using MOs (Li et al. 2019).

15.3 Melanoma

It is a malignant tumour arising from melanocytes that can be highly aggressive. Approximately 5–12% of melanomas are hereditary due to mutations in CDKN2A that leads to defect in proteins $p14^{ARF}$ and $p16^{INK4A}$ that are tumour suppressors regulating G1 checkpoint and stabilise expression of p53. The mutations in nucleotide excision repair (NER) pathways fail to repair bulky DNA lesions created by ultraviolet (UV) radiation. The other mutations include signalling pathways BRAF, NRAS, NF1 (regulating proliferation), PTEN and KIT (regulating growth and metabolism), TP53 (leads to resistance to apoptosis), TERT (replicative life span) and ARID2 (involved in cell identity) (Li et al. 2011). Various drugs have been developed targeting melanoma-specific mutations but are often rendered inactive as melanoma cells alter their sensitiveness towards treatment. Hence there is need of novel treatment options. Zebrafish offer unique opportunities due to its transparent larval skin and highly conserved function of melanocyte genes to mammalian melanocyte genes (Davis et al. 2019). Various transgenic models designed include the following:

1. BRAF gene mutation: It leads to a successful formation of nevi, and the crossing of these transgenic mutants with p53-deficient mutants results in highly invasive melanomas. Lister et al. combined BRAF gene mutants with temperature-sensitive mutants with mutation in melanocyte-inducing transcription factor alpha (mitfa) (Li et al. 2019). They demonstrated that changes in mitfa levels associated with melanoma progression/regression (Cline and Feldman 2016; Cooper 2017).
2. Mutants expressing human GNA11, $GNA11^{R183C}$ and $GNA11^{Q209L}$ hyperactive versions tend to develop large lesions. These models can greatly contribute to understanding aetiology and biology of melanoma (Cooper 2017).

15.4 Psoriasis

It is a chronic immune-mediated disease characterised by systemic and severe cutaneous manifestations, affecting an estimated 3% of global population. Its pathogenesis involves a complex interaction between genetic and environmental factors leading to abnormal proliferation of keratinocytes and infiltration of immune cells across different skin layers. It leads to release of various inflammatory mediators leading to symptomatology of psoriasis. But exact pathogenesis is unclear; hence, to elucidate exact mechanism, various in vivo and in vitro preclinical models have been designed. Zebrafish with its close resemblance with humans genetically offer an attractive model (Martinez-Navarro et al. 2019).

1. Mutant models:
   (a) Penner/lethal giant larva 2 (pen/lgl2): It was the first mutant model generated with a defect leading to overgrowth of the epidermis and failure in forming

hemidesmosomes in the basal membrane. Hence the lack of pen/lgl2 disrupts the basal keratinocytes localisation, and loss of elements regulating the same allows for the study of associations in psoriasis (Cline and Feldman 2016; Martinez-Navarro et al. 2019).

(b) Hepatocyte growth factor activator inhibitor 1a (Hai1a)/serine peptidase inhibitor, Kunitz type 1a (Spint1a) and Clathrin interactor 1a (clint1a): They are characterised by proliferation of the epidermis and keratinocytes (mesenchymal characteristic). The loss of spint1a in both models leads to infiltration of neutrophils and loss of keratinocytes contact leading to their abnormal mobility and prone to apoptosis. It also leads to antagonist role between Hai1a and its target matriptase 1a (St1a). This model plays a crucial role to uncover crucial players on defects caused by loss of Hai1 and St1a. Hai1a models in zebrafish at 24 hpf enable study and visualisation of real-time immune response under high resolution (Cline and Feldman 2016; Martinez-Navarro et al. 2019).

(c) Psoriasis/m14: Phenotype due to the loss of functional mutation of atp1b1a (encoding beta subunit of sodium potassium ATPase). The zebrafish with this mutation exhibits overproliferation of the epidermis and keratinocytes differentiation defect closely resembling psoriasis. This model allows the study of regulation of growth of the epidermis (Cline and Feldman 2016; Martinez-Navarro et al. 2019).

2. Morphant model/knock-down models using MOs technology [tumour necrosis factor alpha (TNFα)-TNF receptor 2 (TNFR2)]: Knockdown of tnfr1 using MOs demonstrated little effect on mobilisation of neutrophils in developmental larvae at 3 dpf, but knockdown of tnfa and tnfr2 had stronger effects. This model can be further enhanced by using transgenic zebrafish [Tg (mpx: GFP)] which possess green fluorescence expressing neutrophils that greatly help in determining the location of neutrophils. In addition the morphants of tnfa and tnfr2 through NF-kB pathway triggered production of proinflammatory molecules interleukin1b (il1b), prostaglandin-endoperoxide synthase 2b (ptgs2b) and others resembling mutant spint1a and clint1a phenotype. It was also observed using H2O2-detecting probe that keratinocytes of tnfr2-deficient larvae activate the enzyme dual oxidase 1 (DUOX1) creating gradients of H2O2 that can be sensed by neutrophils with the help of tyrosine kinase Lyn. This model thus provides not only evidence of immune pathology in psoriasis but also a robust platform to assess management of these inflammations due to oxidative stress (Cline and Feldman 2016; Martinez-Navarro et al. 2019).
3. Environmental inducible model: Apart from genetic influence, environmental factors are suspected to affect psoriasis onset and development. Exploring these environmental insults and their mechanisms remains a challenge. The various zebrafish models to elucidate the same include the following:

(a) The germ-free zebrafish model: The gut microbiota has been associated with not only the pathophysiology of various chronic inflammatory disorders but also the efficacy of therapeutic agents on these disorders. Various resident microorganisms are loosely organised in the skin, and studies suggest

profound changes in DNA and RNA due to these microbes in individuals with psoriasis. This model of zebrafish based on exclusion of commensal microbiome has provided new tool to assess constituents of microbes triggering inflammatory mediators (Martinez-Navarro et al. 2019).

(b) TRPV4 (transient receptor potential vanilloid 4): The triggering events for psoriasis are often mechanical in nature which leads to enhanced proliferation and altered differentiation of keratinocytes in the epidermis. Evidence suggests that polymodal transient receptor potential (TRP) channels especially TRPV4 play a critical role. Hence drugs targeting TRPV4 or similar channels could be tested using zebrafish models (Martinez-Navarro et al. 2019).

15.5 Model for Selective Pruritus

Pruritus is an unpleasant sensation eliciting scratching behaviour mediated through pruritic receptors (G protein-coupled receptors) on the somatosensory neurons. These pruritic receptors on activation open downstream transient receptor potential (TRP) channels, which in turn facilitates neuronal activation. Zebrafish serves as a valuable tool for studying nociception as the orthologues of genes Trpa1 and trpa1b involved in mammalian itching are preserved in zebrafish. The somatosensory neuronal response to pruritus-inducing agents was evaluated in transgenic zebrafish (elavl3:CaMPARI) expressing neuronal activity indicator CaMPARI, which is a fluorescent protein which in presence of 405 nm light and calcium gets converted from green to red (Esancy et al. 2018).

Using this approach the activity of trigeminal nerves was visualised in 3 dpf larvae of zebrafish post application of pruritogen. Allyl isothiocyanate (AITC) 50 μM was used as a positive control, and other groups were exposed to imiquimod (100 μM), histamine (100 μM), serotonin (500 μM), SLIGRI-NH2 (100 μM), deoxycholic acid (100 μM) and loxoribine (100 μM) solutions that were injected cutaneously into the upper lip 5 dpf zebrafish. The counts of photo-converted cells, behaviour pattern (swimming pattern/locomotor activity) and lip rubbing activity were evaluated. Only imiquimod elicited statistical significant dose-dependent baseline locomotor activity along with activation of neurons. Pruritogenic activity demonstrated as frequent rubbing of lips against the walls of its tanks. AITC-injected zebrafish exhibits nocifensive behaviour (Esancy et al. 2018).

15.6 Model for Wound Healing Research

The wound healing in mammals is a multistep process involving clotting of blood, inflammation, re-epithelialisation, vascularisation and formation of granulation tissue. Although mammals could be used to study the process of healing, they are costlier, time-consuming and challenging technically. Hence use of lower organisms

like zebrafish is gaining momentum. It has been observed that steps in wound closure in zebrafish are similar to humans. A model of wound healing has been studied by inducing 2 mm wound on to the flanks of adult zebrafish (6–12 months) using a dermatomal laser. Rapid re-establishment of the barrier at 12 h post wounding (hpw) was demonstrated using methylene blue dye that gets absorbed by damaged tissue but not by undamaged/regenerating tissues. The inflammatory response could also be analysed by using transgenic lines expressing green fluorescent protein (GFP) Tg(mpx:GFP) that labels neutrophils or Tg(lyz:DsRED2/GFP) that labels neutrophils and macrophages (Richardson et al. 2013).

15.7 Drug Discovery: Evaluation of Percutaneous Absorption of Drug and Drug Delivery Systems Using Zebrafish

Transdermal route of drug delivery for systemic drug administration is gaining attention because it provides uniform plasma concentration with minimal inter-individual variation and side effects. Being non-invasive, it is preferred by many patients, but the development of such formulation faces many challenges like the drug molecule must be of low molecular weight (< 500 Da) and possess balanced lipophilicity for passive diffusion across the skin. The assessment of absorbability of a medicine through the skin is determined by measuring plasma concentration achieved. Various in vitro and in vivo models have been developed to assess quality and efficacy of topical and transdermal formulations including excised human skin, rabbit, pig, rat, mouse and guinea pig. Many disadvantages of using guinea pig as a model for evaluating transdermal formulations include dilution and diffusion into surrounding water, and exposure to direct chemical solution results in dermal absorption as well as absorption through gills (Morikane et al. 2020; Kagotani et al. 2020).

1. Leakage test after transdermal administration of felbinac: 4–6-month-old AB strains of male zebrafish maintained at 28 °C were anesthetised using solution of Tricaine (0.168 mg/ml) for 15–30 s in a tank. Depending on the size of the head, the tip of 1.5 ml microcentrifuge tube was cut using fine scissor/cutter to 5–10 mm. Anaesthetised fish was lifted using skimmer spoon and slip gently into centrifuge headfirst; breathing was ensured by leaving gills out of the hole. Then the zebrafish was fixed by pouring agarose solution (prepared by mixing in 1:1 ratio of agarose and low-melting agarose in water to produce 3% aqueous solution) until base of tail fin. After agarose hardens, monitor vitals by observing mouth movements and gills. The head of the zebrafish fixed was immersed into anaesthetic solution followed by the following:
   (a) 100 µl felbinac-red dye solution (21.2 µg felbinac + 75 µg red dye) applied to tube followed by sampling of anaesthetic (Tricaine) solution at 0, 1 and 2 h. The concentration of felbinac was quantified using high-performance liquid chromatography (HPLC).

(b) 100 µl of 1 mM 4-Di-2ASP (fluorescent dye) was administered to tail fin for 2 h, and a filter paper with 39 µg 4-Di-2ASP dissolved in 5 µl of ethanol was fixed onto tail fin directly for 2 h. Then wash zebrafish thrice with water and capture fluorescent signal using microscope with GFP filter (Morikane et al. 2020).

2. Dose response study after transdermal administration of felbinac: As explained above, dose ranges of 5.3, 10.6, 21.2 and 42.4 µg of felbinac in 5 µl solutions were applied to filter paper and affixed to tail fin for 1 h. After washing with water thrice, blood samples were collected, and felbinac concentrations were measured using HPLC.
3. Evaluation of lecithin-based drug delivery system: The embryos of wild AB strain of zebrafish were dechlorinated using pronase solution (2 mg/ml). Following which, 12 embryos were transferred to 6-well plates containing 4 ml of 0.3x Danieau's solution with or without lecithin-ceramide and incubated at 28 °C for 96 h. The images were captured at 72 hpf, and larvae were exposed to emulsions for 5 days to evaluate toxicity. The Danieau's solution was refreshed every day, and survivals were recorded every day. To visualise the dermal delivery of phosphatidylcholine 70 (PC70) ceramide, the 1% PC70 Nile red emulsions were prepared (200 µg/ml of PC70 and 1 ng/ml of Nile red), or 1 ng/ml of NR solution was added to 72 hpf zebrafish larva followed by incubation at 2 and 20 h. Then the fish were washed with breeding water for 18 h followed by capture of fluorescent image of larva using BZ-X710 microscope (Kagotani et al. 2020).

15.8 Conclusion

The various advantages offered by zebrafish present a unique opportunity to obtain insight into pathogenesis and also test potential therapies in the field of dermatology. Zebrafish potentially allowing use of various molecular tools, genetic manipulation and therapeutic testing platform could be a potential replacement for the existing animal models to study various cutaneous disease/disorders in humans.

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Growing Importance of Zebrafish in Translational Neuroscience

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Abstract

Zebrafish was introduced as a vertebrate model organism with enormous application in neuroscience to improve our understanding of brain development, homeostasis, dysfunctions, and genetic and behavioural phenotypes. The neuroscience research community is showing increasing interest in zebrafish as a complementary vertebrate model because it shares physiological and morphological characteristics with mammals and has a diverse repertoire of behaviours. Zebrafish has proven its potential as a model organism in recapitulating the genotypes and phenotypes of a wide range of human neurological disorders. It is a suitable preclinical model organism for studying a multitude of neurological conditions since adult zebrafish, larvae, and even embryos are acquiescent to pharmacological, experimental, and genetic manipulation. The utility of zebrafish as a model organism to examine the neurophysiological and pathological processes underlying major neurological disorders is discussed in depth.

Keywords

Zebrafish · Neurogenesis · Neurodegenerative diseases · Multiple sclerosis · Epilepsy · Spinal cord injury · Glioma

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16.1 Introduction

Over the last four decades, zebrafish (*Danio rerio*) has gained prominence as a versatile vertebrate model organism in behavioural, psychiatric, and translational neuroscience areas due to several unique advantages (Table 16.1). Zebrafish is inexpensive and easy to maintain and breed, its embryos develop externally, and the transparency of the eggs enables in vivo imaging, real-time tracking, and manipulation of neural circuits. Because of their high anatomical, physiological, metabolic, and genetic similarity to humans, both larvae and adult zebrafish have become increasingly useful in neuroscience. Zebrafish has already established itself as a preferred model organism in neuroscience due to its versatility in genetic, pharmacological, and experimental manipulations, as well as the availability of a large number of transgenic lines (Fig. 16.1). Additional advantages of using zebrafish as a model organism in neuroscience research include a well-characterized behavioural repertoire and the ability to compare its patterns with physiological outcomes. It follows the concepts of 3Rs (replacement, reduction, and refinement), required by a multitude of national and international animal welfare regulatory agencies. George Streisinger introduced the scientific world to zebrafish as a model organism to explore the genetic basis of vertebrate nervous system development (Streisinger et al. 1981). As a dependable model organism, zebrafish provides an excellent opportunity to conduct steadfast scientific research in developmental neuroscience and gain understanding of the underlying mechanisms of various neurological disorders from a translational standpoint (Kalueff et al. 2014; Fontana et al. 2018). This chapter highlights the growing importance of zebrafish in fundamental and translational neuroscience research, recognizing its potential as a model organism (Fig. 16.2). Furthermore, we have provided a broad overview of the utility of zebrafish as a versatile and complementary model organism for studying neuronal regeneration, major neurological disorders, and basic principles for the integration of known mechanisms for central nervous system (CNS) repair.

16.2 Zebrafish Brain Organization

In zebrafish, the CNS framework typically begins at the onset of gastrulation (≈ 6 h post-fertilization [hpf]). At 24 hpf, the visually discernible morphogenetic boundary of the forebrain, midbrain, and hindbrain frameworks forms the fully grown brain structures such as the pallium, subpallium, thalamus, and cerebellum (Fig. 16.3). The forebrain develops into vital functional units of the brain such as the retina, hypothalamus, diencephalon, and telencephalon, which are in charge of sensory input acquisition and processing as well as behavioural guidance. The telencephalon consisting of the pallium, subpallium and olfactory bulb is associated with the regulation of social behaviour, memory, and emotion. The diencephalon is composed of the thalamus, pineal body, and habenula. Diencephalon regulates attention, alertness, and circadian behaviour. The pallium in zebrafish has functional similarities to the hippocampus and amygdala in mammals, while the subpallium

Table 16.1 Characteristics of zebrafish that make it a suitable model organism for studies in translational neuroscience

• Small in size, short generation time, high fecundity, and fertilization and embryonic development are ex utero
• Optical transparency of the embryo allows the direct, real-time monitoring of the developing nervous system and in vivo imaging, including calcium imaging can be conveniently carried out
• Practical and inexpensive model system
• The statistical power of a large number of zebrafish larvae analysed simultaneously by automated systems makes it a very promising model organism
• The aqueous environment facilitates drug treatment
• Systemic drug administration is possible via the intraperitoneal or oral route, reducing the amount of drug used, improving dosage checks and allowing direct dose comparisons with rodents
• The effectiveness of the drug, bioavailability and toxicity can be determined readily.
• Zebrafish hold genomic homology to humans
• Share >70% genetic homology with humans, including the conservation of many biological pathways
• Share >80% homology in disease-causing genes with humans
• The zebrafish genome is sequenced and available for annotation
• Since zebrafish is a vertebrate, a relatively high degree of physiological and anatomical homology with mammals is apparent in most organs, including the nervous system (forebrain, midbrain, hindbrain, diencephalon, telencephalon and cerebellum)
• Reduced complexity of the zebrafish nervous system enables to perform large-scale activity in the whole brain and is ideal for high-throughput in vivo screening studies
• The lateral pallium is assumed to act similarly to the human hippocampus
• Evolutionary retention of the basic anatomical structure, cell populations and chemical properties of zebrafish and the human nervous system
• The blood–brain barrier (BBB) is structurally and functionally similar to that of higher vertebrates and developed by 3 days post-fertilization (dpf)
• Small molecule absorption and distribution via BBB in zebrafish is comparable to mammals
• Shared cortical neurotransmitters including acetylcholine, dopamine, γ-aminobutyric acid (GABA), glutamate, histamine, norepinephrine and serotonin
• No glial scar is observed
• The remarkable regenerative capacity of the central nervous system facilitates cross-comparative studies with non-regenerative species
• Multiple relevant features of the central nervous system appear within 1 day of development, allowing for direct observation of brain development during embryogenesis
• Potential for scale-up and automate high-throughput analysis of a wide range of human homology neurobehavioral catalogue
• Possess all of the classical sense modalities (vision, olfaction, touch, balance, pain, etc.) and share an overall homology with humans
• Genetic manipulation easy and transgenic zebrafish lines develop rapidly, unlike the time-consuming development of rodent models
• Heritable mutations can be easily introduced by zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and CRISPR/Cas9
• Vast repertoire of transgenic lines
• Automated interaction behaviour analysis and video tracking can be performed under natural conditions

(continued)

Table 16.1 (continued)

• Gene duplication may also lead to a higher degree of tuning of programs, promoting successful endogenous regeneration
• In some instances, sub-functionalized gene duplicates provide a rare opportunity for the study of pleiotropic phenotypes
• Contributes to the 3Rs (replacement, refinement, reduction) by replacing specific mammalian experiments and reducing the numbers of mammals required
• The abundance of molecular and research information available on the zebrafish model system provides unique opportunities (Table 16.4)

is similar to mammalian basal ganglia. The midbrain is necessary for hearing and vision, and its two major structures are the tectum and the tegmentum. The tectum and its supporting neurons form the zebrafish's startle and reflex response centre. The hindbrain produces neurons and motor neurons which innervate the branchial arches and regulate jaw, eyes, head, and whole-body movement, respectively. The cerebellum is correlated with functions such as motor control, sensory stimuli, and learning. Regardless of anatomical differences, the general structural organization is conserved across taxa, ensuring that its core functionality is maintained (Vaz et al. 2019). In adult zebrafish, stem cells from the brain and spinal cord ventricular zone are responsible for extensive neurogenesis throughout the brain and spinal cord rostrocaudal axis (Fig. 16.3). It may be noted that pluripotent stem cells are renewable cell and tissue substitutes for the treatment of a variety of neurological disorders such as Alzheimer's and Parkinson's diseases, cerebrovascular diseases, spinal cord injury, amyotrophic lateral sclerosis, and cerebral palsy, to name a few.

16.3 Modelling Neural Development.

Neuroregeneration involves the restoration of lost connections through and neurogenesis, which is critical in the treatment of specific neurological disorders such as neurodegenerative and cerebrovascular diseases, as well as traumatic brain injury. Given the remarkable neuroregenerative capacity of zebrafish, a potential approach would be to compare them with non-regenerative organisms to identify cellular and molecular differences that could eventually support neuroregeneration in humans (Fig. 16.3, Table 16.2). The presence of multiple neurogenic niches in zebrafish makes it possible to study neurogenesis in different brain areas within the same model. The transparent zebrafish embryo enables the structural development of the central nervous system to be observed at a single-cell resolution. Besides, the zebrafish larva is an appropriate model for longitudinal neuronal development studies. The availability of a wide variety of transgenic lines that express fluorescent reporters in post-mitotic neurons (huc/elavl3+), GABAergic interneurons (dlx5/6+), glutamatergic neurons (vglut2a+), or oligodendrocytes (olig2+) makes it an appealing and versatile model system to implement (Park et al. 2000; Zerucha et al. 2000; Shin et al. 2003; Kimura et al. 2006).

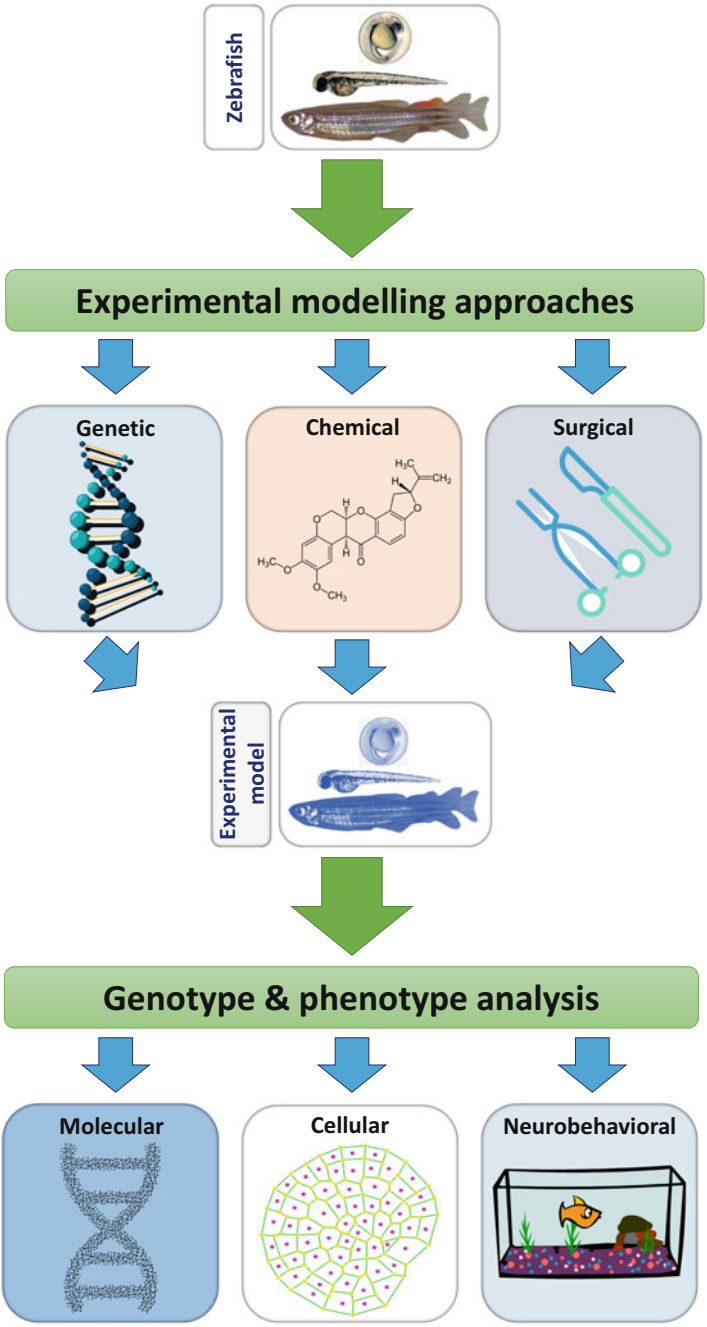

Fig. 16.1 Pictorial representation of the development of the zebrafish model system for experimental research. The embryos, larva, and adult zebrafish may be subjected to genetic, chemical, or surgical manipulations to create specific experimental models. Thus developed zebrafish model can be characterized/analysed by molecular, cellular, and neurobehavioural approaches to prove the hypothesis

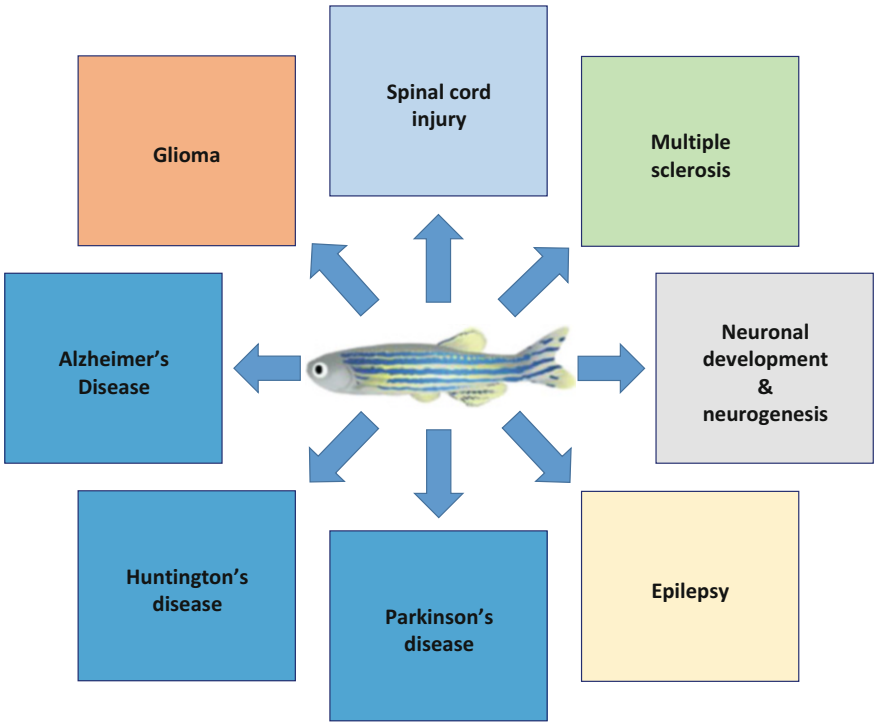

Fig. 16.2 Zebrafish as a versatile model organism provides numerous research opportunities in basic and translational neuroscience, some of which have been discussed in this chapter

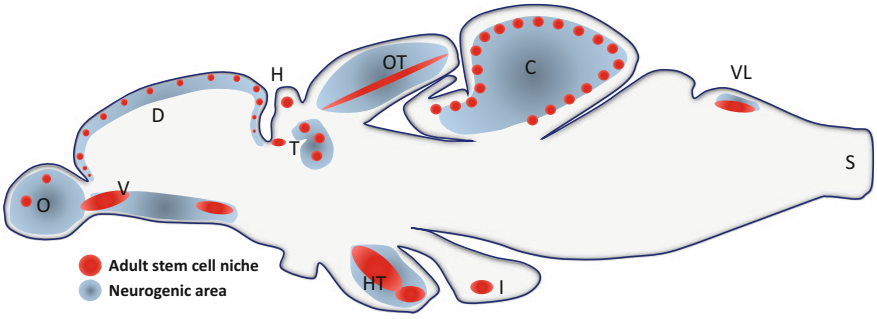

Fig. 16.3 Simplified sagittal view of the adult zebrafish brain and main domains along with the zone of proliferation and neurogenesis. *O* olfactory bulb, *D* dorsal telencephalon, *V* ventral telencephalon, *H* habenula, *T* thalamus, *HT* hypothalamus, *I* inferior lobe, *OT* optic tectum, *C* cerebellum, *VL* vagal lobe, *S* spinal cord

Cerebroventricular microinjection of amyloid β42 derivative into the adult zebrafish brain induces Alzheimer's disease-like phenotype, activates ependymoglia (a cell that transports hormones from neurosecretory cells), and boosts neurogenesis

Table 16.2 Essential biomolecules and cellular markers expressed during neuroregeneration in zebrafish

Markers	Cell type	Biomolecules	Biological process
GFAP Nestin Vimentin S100B	*Neural progenitor cell*	FGF Notch Shh CXCR5 LTC4	Cell proliferation
PSA-NCAM	*Neuroblast*	FGF Notch GATA3 BDNF IGF GDNF CXCR5 GAP-43 L1-CAM miR-133b	Neurogenesis
HuC/D NeuN NeuroD Prox1 MAP2 ChAT	*Neuron*	IGF miR-133b	Neuronal survival
		GDNF L1-CAM CRP Contactin-2 Tenascin-C miR-133b	Axonal regrowth

by upregulating interleukin-4 (IL-4) in microglia and neurons. Subsequently, IL-4 functions as a signal transducer, activating the phosphorylation of signal transducer and activator of transcription 6 (Stat6) through its receptors on neural stem cells (NSCs) (Bhattarai et al. 2016). Furthermore, studies have revealed the involvement of numerous cellular and molecular processes such as apoptosis, cell adhesion, cell cycle, and inflammatory and immune response in the zebrafish traumatic brain injury model (Barbosa et al. 2015). While paraquat-induced neurotoxicity caused redox imbalance and mitochondrial dysfunction, partially mimicking Parkinson's disease phenotypes, quinolinic acid promoted ependymoglial cell proliferation and telencephalon neurogenic programme in adult zebrafish. Ependymoglial cells rely on various cellular and molecular mechanisms to produce neurons, which contribute to both constitutive and restorative neurogenesis. For example, if induction of the Wnt signalling pathway regulated the neuron differentiation of the ependymoglial cells in response to injury, the modulation of the Sonic hedgehog and Notch activities restricted the differentiation process. Numerous injury paradigms targeting the adult zebrafish brain in different regions collectively showed that multiple cellular and intrinsic molecular factors (Table 16.2) eventually regulate neuroregenerative processes in particular areas of the brain (Barbosa et al. 2015). Furthermore, the zebrafish model of brain injury has contributed to the revision of the long-held belief

that inflammation is deleterious to neuroregeneration. Acute inflammation, in fact, is a potent inducer of neuroregeneration in zebrafish (Kyritsis et al. 2012).

NSCs are multipotent progenitor cells responsible for the production of neurons and glia and are predominantly quiescent in mammals. On the other hand, quiescent NSCs in zebrafish proliferate and differentiate after brain injury, ensuring efficient functional recovery. Radial glia and neuroepithelial cells, two proliferative yet differentially responsive NSC populations found in the adult zebrafish brains, remind us of the limited and heterogeneous neuroregenerative potential of mammals. Zebrafish offers rare and valuable understanding into the proliferation and differentiation abilities of NSCs post-injury, which are difficult to obtain by using rodent models alone. Zebrafish models of neuronal pathology have demonstrated that NSCs have distinct molecular signatures that support a specific type of regeneration response that differs from their developmental and homeostatic programmes. However, a better understanding of quiescent NSC regulation and heterogeneity is a prerequisite for enabling them to contribute therapeutically to human brain repair and recovery. Despite teleost-specific neurulation processes, zebrafish is seen as an invaluable model for studying neural crest, as its developmental mechanisms are well-conserved across vertebrates (Rocha et al. 2020). For example, initial zebrafish genetic screens have identified more than 400 mutations, many of which affect the development of NCCs (Rocha et al. 2020). According to a recent zebrafish study, teratogens cause neurocristopathy and craniofacial anomalies by altering the gene expressions of cranial neural crest (NC) cells (Liu et al. 2020).

Developing brain is vulnerable and sensitive to environmental contaminants and chemicals. Exposure to such neurotoxins is linked to several neurological pathologies, such as autism, schizophrenia, and Parkinson's and Alzheimer's diseases. Zebrafish is particularly suitable as a complementary model organism for developmental neurotoxicity studies since it can accelerate the risk assessments and the observed neurotoxic effects can be extrapolated to humans. Several endpoints for developmental neurotoxicity such as gene expression patterns, neural morphogenesis, and neurobehavioural profiling are used to study the impact of environmental and chemical toxins. Lincomycin (an antibiotic) increased acetylcholinesterase and ATPase activity, oxidative stress, and cell death in zebrafish larvae, according to a recent study (Cheng et al. 2020). Lincomycin reduced the expression of genes involved in zebrafish larvae neurotransmitter and neurodevelopmental systems as well as swimming activity, in a dose-dependent manner, contributing to a change in their motor behaviour (Cheng et al. 2020). In zebrafish embryos, bisphenol F (BPF), a chemical used in the production of epoxy resins and plastics, caused significant peripheral motor neuron developmental toxicity, resulting in locomotion suppression and a reduction in distance travelled (Yuan et al. 2019). BPF significantly activated microglia and astrocytes, implying that the neuroinflammatory response was over-activated. BPF exposure was found to have a significant effect on apoptotic and neurodevelopmental processes, correlating with the phenotypic findings. Although silica nanoparticles (SiNPs) are among the most productive nano-powders widely used in various fields, their neurotoxic effects remain at the exploration stage. The alteration in zebrafish neurobehavioural phenotypic pattern revealed an

observable effect of SiNPs on disrupting light-dark preference, reducing exploratory behaviour, and impairing memory (Li et al. 2020). Furthermore, SiNPs increased the expression of genes involved in Parkinson's disease progression and autophagy.

16.4 Modelling Major Neurological Disorders

As assessed by disability-adjusted life year (DALY), the prevalence, incidence, deaths, and strain of neurological disorders continue to rise globally (GBD 2016 Neurology Collaborators 2019). Neurological diseases have a wide range of aetiologies, including but not limited to genetic or congenital abnormalities, lifestyle, infections, environmental factors, and direct injury to the brain. An important step forward in the development of effective treatments is exploratory research into the pathogenesis of neurological disorders, the translational potential of which depends on the use of aetiologically relevant model systems that recapitulate some of the clinical features and related complexity of human disease of interest. Complementing rodent models, zebrafish enable the multilevel study of a disorder, from molecules to behaviour, to provide a thorough understanding of the underlying mechanisms of the disease, thereby supporting novel approaches to treatment (Figs. 16.1 and 16.4). In addition, zebrafish exhibit a wide variety of complex neurobehavioural responses to stimuli, real-time monitoring, and analysis of the same through automated video tracking systems which contribute to drug screening and behavioural phenomics (Oliveira 2013).

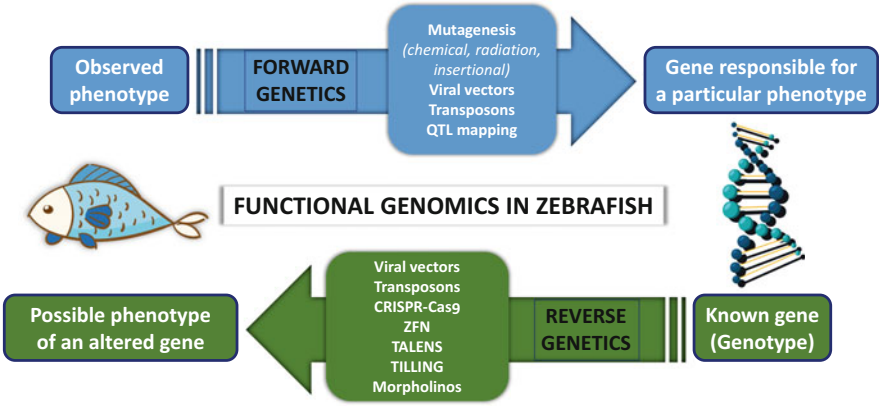

Fig. 16.4 Functional genomic approaches followed in zebrafish research. *QTL* quantitative trait locus, *CRISPR* clustered regularly interspaced short palindromic repeats, *ZFN* zinc finger nuclease, *TALEN* transcription activator-like effector nucleases, *TILLING* targeting induced local lesions in genomes

16.5 Alzheimer's Disease (AD)

AD is the most prevalent type of dementia and is a progressive and irreversible neurodegenerative disorder that impairs memory, thinking, communication, and behavioural skills. While approximately 10–15% of the cases are genetic, many are sporadic and are due to several risk factors. There is currently no cure or disease-modifying therapy for AD. AD is characterized by a poorly understood loss of the cholinergic neuronal system caused by amyloid beta (Aβ) deposition and intracellular neurofibrillary tangles comprised of hyperphosphorylated tau proteins.

Zebrafish possess human orthologous genes that play important roles in AD and emerged as an ideal vertebrate organism to study AD pathology and to validate neurotherapeutics. AD is associated with mutations in amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-1 (PSEN2) genes, orthologous to the zebrafish appa, appb, psen1, and psen2 genes (Newman et al. 2011). The knockdown of APP and/or PRNP (appa, appb, prp1, and prp2) in zebrafish decreased cell adhesion and neuronal apoptosis, suggesting a unique and conserved genetic interaction between APP and PRP (Kaiser et al. 2012). This interaction is unusual since prp1 has been found to interact genetically with appa, but not with appb, and prp2 does not interact with appa. Interestingly, the capacity of appa and appb to inhibit neuronal death depends differentially on prp1 abundance. Similarly, the contribution of microtubule-associated tau protein to AD was demonstrated by studying the redistribution of tau protein from neuronal axons to neuronal soma, forming pathogenic neurofibrillary tangles in stable transgenic zebrafish expressing human tau with a mutation (TAU-P301L) (Tomasiewicz et al. 2002). Besides, embryos expressing tau protein exhibit neurobehavioural deficits in escape response following a mechanical stimulus (Xi et al. 2011).

Zebrafish not only exhibit memory and cognitive deficits similar to humans but can also display complications with locomotion, as evidenced by startle response, avoidance tasks, and movement tracking (Best et al. 2008; Nery et al. 2014). For example, studies on the startle response of zebrafish larva to acoustic stimuli have revealed that donepezil exposure increases startle response while decreasing habituation, a response similar to that observed in rodents (Best et al. 2008). Babin et al. (1997) have shown that apoA-I and apoE genes involved in AD are abundantly expressed in zebrafish and can be used to explore the influence of apolipoproteins in embryonic and larval nutrition, as well as the role of apoE in neuroregeneration and morphogenesis. Transgenic zebrafish expressing fluorescently labelled TAU swiftly recapitulated key pathological characteristics of tauopathies, including human TAU protein conformational changes and phosphorylation, tangle formation, and neuronal and biobehavioural disturbances, and apoptosis and was used in both time-lapse microscopy imaging and drug development against AD. AR-534, a recently developed GSK3β inhibitor, reduced TAU phosphorylation and TAU-induced neuronal cell death in transgenic zebrafish (Paquet et al. 2009). Further, zebrafish model of inhibitory avoidance memory deficits induced by muscarinic antagonist, scopolamine, demonstrated that quercetin and rutin pre-treatments prevented the amnesia (Richetti et al. 2011). Scopolamine impairs the memory of zebrafish without

inducing locomotor defects or anxiety-like behaviour. Non-insulin-dependent diabetes mellitus (NIDDM) and Alzheimer's disease are the two most common amyloid diseases, both of which are attributed to the presence of amyloid plaques composed of toxic islet amyloid polypeptide (IAPP) and Aβ. By producing bacterial endotoxins such as lipopolysaccharide (LPS), the gut microbiome plays critical roles in the pathological development of neurological disorders. Zero-dimensional carbon quantum dots (CQDs) mitigated the catalytic effects of LPS on Aβ and IAPP amyloidosis (Koppel et al. 2020). Furthermore, CQDs rescued the impaired zebrafish embryonic hatching and suppressed free radical generation in vivo, implying its potential as a treatment for NIDDM and AD. Barbereau et al. (2020) used the TAU-P301L transgenic zebrafish line to demonstrate that Tau neurotoxicity altered brain-derived neurotrophic factor (BDNF) system, which could be targeted to develop an effective therapeutic strategy.

16.6 Parkinson's Disease (PD)

PD is a common progressive neurodegenerative hypokinetic disease caused predominantly by dopamine neuron loss in the substantia nigra. Although the cause of PD is obscure, akinesia, bradykinesia, gait and balance disturbances, rigidity, and freezing phenomenon are the manifestations of this disease. The standard clinical treatment for PD is primarily symptomatic since disease aetiopathophysiology is not thoroughly elucidated. The existence of Lewy bodies (LB), Lewy neurites (LN), and fibrillary aggregated α-synuclein combined with degeneration of dopaminergic nigrostriatal neurons are cardinal neuropathological lesions identified for clinical PD. While α-synuclein gene mutation is responsible for the β-pleated sheath aggregates found in LBs and LNs, protein conformational changes of which cause neurotoxicity. Genetic mutations in lrrk2, dj-1, parkin, and pink1 genes also result in PD.

Zebrafish brain encompasses the forebrain, midbrain, and hindbrain areas, enabling the region-specific study of neurochemicals and structures involved in PD-related motor and non-motor behaviours. Additionally, zebrafish have a well-characterized dopaminergic system. Evolutionarily conserved molecular markers of zebrafish and their protein products (uch-l1, dj-1, lrrk2, pink1, snca, and park2) are expressed in the ventral diencephalic dopaminergic neurons (Son et al. 2003; Bai et al. 2006; Flinn et al. 2008; Pienaar et al. 2010). Further, zebrafish is widely used for the study of PD pathogenesis due to the rapid life cycle, genetic similarity to humans, and altered locomotor activity following exposure to neurotoxins. The pathophysiology and neurobehavioural characteristics of the disease are investigated using transgenic and knockout models with the help of PD-related protein homologues, since zebrafish is readily available for a variety of genetic manipulations (Fig. 16.4). For example, PINK1 downregulation is the second leading cause of autosomal recessive Parkinson's disease. Pink1 deficiency reduced the number of dopaminergic neurons, hindered mitochondrial function and response to tactile stimuli, and decreased swimming behaviour in zebrafish (Anichtchik et al.

2008; Xi et al. 2011). Knockdown of β- or γ1-synucleins (functionally nearest to human α-synuclein) elicits motor deficits in zebrafish, which is far more drastic when both are abolished (Milanese et al. 2012).

Neurotoxins such as rotenone, paraquat, maneb, 6-hydroxydopamine (6-OHDA), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are often used to elicit Parkinson's disease-like complications in zebrafish. While MPTP caused specific dopaminergic neuron loss; decreased dopamine, serotonin, and norepinephrine levels; and impaired motility in zebrafish larvae, an adult zebrafish showed a significant reduction in swimming velocity (bradykinesia), an inconsistent swimming pattern, and an increment in freezing episodes (dyskinesia) (Lam et al. 2005; Sallinen et al. 2009; Sarath Babu et al. 2016). MPTP-injured zebrafish brain proteomic analysis showed disturbed transcriptional regulation of pink1, park2, dj-1, and lrrk2 (Sarath Babu et al. 2016). Paraquat and rotenone treatment also altered mitochondrial physiology, decreased dopamine, and modulated PD-related genes, caused behaviours similar to PD (Bortolotto et al. 2014; Wang et al. 2017).

16.7 Huntington's Disease (HD)

HD is an incurable, autosomal-dominant, inherited, polyglutamine disorder, triggered by an expansion of a recurring CAG triplet series in the huntingtin gene on chromosome 4. The huntingtin (HTT) protein's expanded polyglutamine sequence is harmful to brain cells, resulting in a progressive neurodegenerative HD characterized clinically by the gradual development of a triad of motor [involuntary muscle movement (chorea)], cognitive, and psychiatric symptoms.

Length-dependent misfolding, oligomerization, abnormal morphology, development, and toxicity of HTT polyQ fragments akin to those observed in rodent models and patients have been documented in zebrafish (Schiffer et al. 2007; Das and Rajanikant 2014). Hsp40 and Hsp70 chaperones or the ubiquitin ligase C-terminal Hsp70-interacting protein reduced the aggregation and adverse effects of mutant fragments. Strikingly, transgenic zebrafish expressing the mutated polyQ fragment lacking the 17 amino acids of the HTT N-terminal tail (mHTT-ΔN17-97Q) exhibited severe neuron-specific toxicity, replicating one of the pathological features of HD for the first time (Veldman et al. 2015).

Interestingly, quinolinic acid (QA), which induces HD-like brain injury in rodents, not only causes microglial infiltration and cell death in zebrafish but also promotes neurogenesis, leading to a rapid recovery (Skaggs et al. 2014).

16.8 Epilepsy

Epilepsy is the fourth commonly diagnosed chronic neurological disease, characterized by repeated unprovoked seizures caused by a rapid spike in electrical activity in the brain. While standard anti-epileptic drugs (AEDs) decrease seizures,

nearly one-third of epileptic patients develop resistance to treatment, emphasizing the importance of continuing therapeutic invention.

Since zebrafish do not have a cortex, qualitative homology can be drawn for functional characterization between zebrafish and human epilepsy, relying on complex model systems to study specific subtypes of seizure. Zebrafish larvae exhibit epileptiform-like interictal and ictal states, emphasizing the existence of common epileptic pathophysiology and phenotype (Meyer et al. 2016). Unbiased quantitative phenotypic readings such as the Racine scale can report unrelated epileptiform activity (neuromuscular effects) (Baraban et al. 2005). Since real-time changes in metabolism, particularly mitochondrial dysfunction, which is one of the underlying causes of epilepsy, have been well established in zebrafish, using it as a model organism provides a starting point for leveraging bioenergetics for drug screening (Kumar et al. 2016). Further, electrophysiological events in zebrafish can be captured in real time using high-throughput strategies (Ghannad-Rezaie et al. 2019). For instance, a high-throughput screening (HTS) method in zebrafish has identified neurotherapeutics for epilepsy and Dravet syndrome (Baraban et al. 2005; Sourbron et al. 2019). The pharmacological (pentylenetetrazole, kainite, picrotoxin, etc.) and transgenic (scn1lab, gabrg2, mecp2 etc.) zebrafish models of epilepsy have been developed and tested using a variety of physiological and neurobehavioural endpoints (Cunliffe 2016). Typical behaviours for epilepsy-like states in adult zebrafish are hyperactivity, erratic swimming, loss of body posture, spasm-like corkscrew swimming, and CNS electrical discharges (Stewart et al. 2012; Zdebik et al. 2013). With distinct AEDs, zebrafish brain electroencephalograms and neurobehavioural analyses have identical profiles to those found in rodents (Orellana-Paucar et al. 2013).

16.9 Multiple Sclerosis (MS)

MS is a debilitating, chronic, autoimmune disorder that affects the CNS. A concerted attack on myelinated axons by the immune system contributes to demyelination and neuronal loss that clinically manifest as impaired sensory, motor, visual, and autonomic systems. Genetic and environmental factors have been proposed as causes of MS, although actual aetiology is not fully understood. Efforts are being made to elucidate the underlying pathological mechanisms in the hope that they can be targeted for therapeutic intervention.

In zebrafish, the deposition of myelin sheath as tight concentric wraps around the axons by oligodendrocytes is a highly regulated process. The mRNA for myelin basic protein (mbp) is detectable 2 days post-fertilization, and mbp is observable by 3 days post-fertilization in zebrafish (Buckley et al. 2010). While the majority of myelin and oligodendrocytes genes in zebrafish are conserved, there are a few variances. The major myelin protein is P0 in zebrafish, as opposed to mammalian myelin proteolipid protein (PLP or lipophilin) (Jeserich et al. 2008). Using multiple zebrafish mutants and transgenic reporter lines, researchers discovered how neuronal subtypes and axonal length are related to myelination and how oligodendrocytes can

develop in the absence of axons (Czopka 2016; Osorio-Querejeta et al. 2017). The majority of studies to date have focussed on elucidating the mechanism of remyelination and oligodendrocyte progenitor cell (OPC) differentiation in zebrafish using toxin-induced demyelination and genetic cell ablation models. For instance, exposure of adult zebrafish optic nerve to lysophosphatidylcholine (LPC) triggered microglial influx, reduction of $Olig^{2+}$ cells, and focal demyelination within 8 days (Münzel et al. 2014). Remyelination, settling of microglial response, and OPCs re-entry are all observed at 28 days after exposure without any reduction in axonal number, emphasizing oligodendrocytes and myelin-specific toxicity and the speed of remyelination in zebrafish. Surprisingly, recovery from injury took much longer in older zebrafish (>15 months) with a reduced microglial response, and myelin was not fully recovered.

To study remyelination mechanisms in vivo, a transgenic zebrafish model of demyelination was created using the bacterial nitroreductase enzyme (NTR) expressed in oligodendrocyte lineage cells and controlled by the mbp and sox10 promoters, which converts the prodrug metronidazole (Mtz) into a cytotoxic DNA cross-linking agent (Chung et al. 2013). Mtz exposure caused a rapid decrease in myelination and oligodendrocyte number within 48 h. Mtz withdrawal, on the other hand, resulted in effective remyelination of the demyelinated CNS within 7 days. In addition, de- and remyelination processes could be easily monitored in live animals by mCherry, suggesting that transgenic zebrafish may be a useful animal model for studying remyelination in vivo and high-throughput screening of pro-myelinating therapeutics. For example, using the NTR/MTZ-induced transgenic zebrafish demyelination model, it has been shown that sulfasalazine (an anti-inflammatory and immunomodulatory drug) temporarily modulates the immune response to promote myelin repair and remyelination and thus resolves MS pathology (Kim et al. 2015).

Experimental autoimmune encephalomyelitis (EAE) is a widely recognized model of demyelinating diseases, such as multiple sclerosis and acute disseminated encephalomyelitis (ADEM). Recently, the zebrafish model of EAE was developed through the subcutaneous injection of myelin oligodendrocyte glycoprotein 35–55 (MOG), and body weight changes, clinical signs, and survival were monitored (Kulkarni et al. 2017). The model was further validated by known modulators of rodent EAE such as fingolimod, dexamethasone, dimethyl fumarate, and SR-1001. The benefit of the zebrafish EAE model is the rapid development of the observable disease phenotype by 3 days post-immunization and a significant impairment by 7 days post-immunization. However, additional work is needed to corroborate and appreciate what drives autoimmunity in this model, as MOG is not presently recognized in the zebrafish genome.

16.10 Spinal Cord Injury (SCI)

SCI is a damage to the spinal cord that causes a loss of function, such as sensation and/or movement. The glial scar, which is composed of reactive astrocytes, inflammatory immune cells, and fibroblasts, is a major physical barrier for the regeneration

of damaged spinal cord in mammals. Because of the lack of effective neurorestorative therapies, the majority of affected patients remain paralysed for the rest of their lives.

Due to the existence of two effective neural regeneration programs, namely, neurite outgrowth and remyelination, zebrafish is used as a promising model of SCI. Fish are paralysed caudal to the injury site, reflecting the level of injury but achieving functional restoration within 4–6 weeks (Becker et al. 2004). The transection or crushing of adult zebrafish spinal cord elicits the proliferation and differentiation of local neural progenitor cells into new neurons that integrate into existing circuitry (Becker et al. 1997; Hui et al. 2010). A high-density microarray profiling of the temporal transcriptome dynamics post-SCI in zebrafish revealed the expression of genes unique to cell migration and proliferation, neural patterning, axonal regrowth, neurogenesis, inflammation, and apoptosis (Hui et al. 2014). In addition, growth-associated proteins (tubulin), cell adhesion molecules, flotillins, microtubule-interacting zRICH proteins, and Reggie 1 and 2 have been reported following SCI or optic nerve injuries in zebrafish (Rasmussen and Sagasti 2017).

Glial cells respond differently to SCI in zebrafish than in mammals. According to the model proposed by Goldshmit et al. (2012), SCI triggers proliferation and migration glial cells to the lesion site, extending along the anteroposterior axis and acquiring a bipolar morphology (bridging glia). At 2–3 weeks' post-injury, the elongated glial cells form a link between the severed spinal cord ends and function as a support for regenerating axons. The signalling through Fgf receptors influences the proliferation, migration, and glial cell differentiation. Inhibition of Fgf signalling either by induced expression of a dominant-negative Fgf receptor or by a small molecule inhibitor (SU5402) affects the formation of glial bridges, blocking axonal regeneration. Alternatively, the spatiotemporal activation and distribution of microglia and macrophages along with blood-borne macrophage depletion were observed after 10 days post-SCI in zebrafish (Hui et al. 2010). A functionally diverse subset of macrophages exists in zebrafish with early expression of M2 type macrophage genes following SCI (Hui et al. 2014; Bollaerts et al. 2017). Evidence indicates that the initial short pro-inflammatory state appears to be followed by a persistent M2 polarized macrophage-induced anti-inflammatory reaction primarily in zebrafish SCI.

Despite significant progress over the last decade, renewal of the spinal cord after injury is still understudied in zebrafish. Ependymo-radial glial cells appear to contribute to the regenerative process.

16.11 Glioma

Glioma is a type of most common malignant primary brain tumour that originates from glial cells. It is an umbrella term for various types of glial tumours such as oligodendroglioma, astrocytoma, and glioblastoma. The malignant and invasive nature of gliomas prevents a complete resection of the tumour, resulting in poor prognosis and significant neurological morbidity and mortality.

A zebrafish glioma model is beneficial for monitoring tumour invasion and helps with the prognosis of the disease. Stable germline mutations in nf1a and nf1b and orthologues of neurofibromin 1 (NF1) were created to explore the roles of NF1 in brain tumorigenesis (Shin et al. 2012). The loss of nf1 promoted tumorigenesis, as evidenced by the earlier onset high-grade glioma and malignant peripheral nerve sheath tumours as well as their increased penetrance in adult nf1a(+/−); nf1b(−/−); and p53(e7/e7) zebrafish, supporting the potent tumour suppressor role of nf1a and nf1b. In another study, the zebrafish model revealed the major role of Akt and Rac1 signalling in glioma genesis and tumour progression when dominant-active (DA) human Akt1 or Rac1(G12V) (DARac1) was overexpressed in the ptf1a domain (Jung et al. 2013). DARac1 co-expression with DAAkt1 promoted glioma genesis by increasing survival, proliferation, and epithelial-to-mesenchymal cell transition, as indicated by higher tumour incidence, invasiveness, advanced histologic grade, and reduced survival. Further, zebrafish glioma model that ectopically expressed a constitutively active zebrafish Smoothened (Smoa1) in neural progenitor cells delineated the tumorigenic potential and mechanism of activated Shh signalling (Ju et al. 2014). Further, zebrafish as a brain tumour model was validated by overexpressing a human version of oncogenic KRAS [KRAS(G12V)] (Ju et al. 2015). Temporary expression of transgenic KRAS(G12V) in progenitor and/or putative neural stem cells induced brain tumorigenesis. Its expression controlled by the krt5 gene promoter promoted brain tumours in ventricular zones (VZ) at low incidence. The majority of the other tumours were made up of spindle and epithelioid cells, similar to malignant peripheral nerve sheath tumours (MPNSTs). When expressed under the control of the gfap gene promoter, KRAS(G12V) induced brain tumours in both VZs and brain parenchyma at a high incidence. Immunohistochemical findings confirmed that the canonical RAS-RAF-ERK pathway was strongly activated. The findings suggest that zebrafish can be used to study the cellular origins and molecular mechanisms of brain tumorigenesis, as well as a high-throughput screening platform for the discovery of oncogenic RAS inhibitors. Gamble et al. (2018) demonstrated that an embryo-larval zebrafish xenograft model can be successfully used to quantitate glioblastoma proliferation, micro-tumour formation, blood vessel association, and individual cell invasion by evaluating the role of laminin alpha 5 (lama5) on U251MG cell progression. Using a lama5 morpholino, they revealed that lama5 decreased U251MG spreading by 23% and doubles the development of blood vessel-dependent micro-tumours. Individual cell analysis revealed that lama5 significantly reduced invasion of mobile U251MG cells.

Although clinically relevant zebrafish model recapitulates the pathology of human malignancies, currently available glioblastoma orthotopic models do not facilitate HTS for drugs discovery. To address this issue, Pudelko et al. (2018) developed a reliable, automated assay for establishing orthotopic glioblastoma tumours in zebrafish that does not necessitate technically difficult intracranial transplantation of single embryos. They transplanted both glioblastoma cell lines and patient-derived cells into zebrafish blastulas and used real-time in vivo light sheet microscopy and time-lapse microscopy to monitor the transplant behaviour. Transplanted glioblastoma cells migrated quickly to the developing nervous system,

forming an orthotopic intracranial tumour within 24 h. Not only did the assay system show that the model developed could mimic human brain tumours, but it could also be used for in vivo drug screening.

16.12 Conclusion

As nervous system diseases continue to affect the world's growing population, it is critical to develop, validate, and refine non-mammalian and mammalian model systems in translational neuroscience to gain a better understanding of disease aetiology, pathophysiology, and treatment modalities. Zebrafish is a powerful animal model for a better understanding of the central nervous system and its diseases. It possesses biologically conserved CNS traits as well as a diverse set of complex neurobehavioural paradigms, providing countless opportunities for phenotype-based drug discovery and translational neuroscience (Fig. 16.1). However, like all animal model systems, zebrafish has also some critical limitations that need to be considered for translational purposes (Table 16.3). For instance, a limited number of validated zebrafish behavioural paradigms exist as opposed to human-based studies or rodent models. Moreover, methodological inconsistencies may also affect the reproducibility of the data during behavioural tests. Zebrafish have a polygenic sex determination instead of heteromorphic sex chromosomes; therefore, colonial sex ratios may vary. They may not exhibit aberrant sexual dimorphism as compared to mammals, limiting their ability to access sex-dependent differences. Differences between zebrafish and mammalian anatomy, metabolism, and physiology may also limit the translation of results from zebrafish to humans. Genome duplication is another phenomenon that significantly raises the overall complexity of genetic analyses. It should be noted, however, that genome duplication does not render zebrafish unsuitable as a model organism. Rather, studying genes and their functional significance can help us understand molecular interactions better. Non-availability of inbred lines in which all individuals are identical and homozygous limits the use of zebrafish as an effective substitute model organism. However, zebrafish, as a flexible model organism, offers many other critical advantages (Table 16.1). Moreover, the zebrafish model is not expected to recapitulate the complex human phenomenology entirely. As one of the most thoroughly studied teleosts, with a wealth of information available, zebrafish is now being considered as a resourceful model organism for both basic and translational neuroscience research (Table 16.4). In the coming days, the in-depth characterization and empirical assessment of zebrafish using molecular tools and sophisticated automated neurobehavioural tracking systems would undoubtedly improve the validity of this promising model system. Moving forward, zebrafish will continue to grow in popularity as a versatile complementary vertebrate model organism for providing valuable insights into the central nervous system, its disorders, and experimental therapeutics.

Table 16.3 Significant limitations of the zebrafish model system

• Non-mammalian model organism. Modelling of chronic injuries and neurodegenerative diseases partially recapitulates the phenotypes observed in the human brain and are generally characterized in embryos or juvenile animals
• The difference in the cellular composition of the brain
• Genomic duplication with redundant functions causes phenotypic buffering and complicates disease pattern generation
• Since zebrafish embryos are developing externally, it is not possible to fully control the chemical dose absorbed
• Empirical drug dose extrapolation: The lack of general guidelines for calculating mammalian equivalent doses of zebrafish
• Factors influencing the pharmacodynamic and pharmacokinetic properties of drugs are not well understood
• Metabolism and excretion of drugs are difficult to predict
• Underlying brain regenerative mechanisms are poorly understood and yet unknown
• Using zebrafish larvae for small molecule studies is unreliable as using adult zebrafish, where the BBB is fully functional and mimics the physiology of mammals better
• Difficulty in administering water insoluble chemicals
• Zebrafish are surrounded by a protective membrane in the early stages of life that may limit the diffusion of certain chemicals
• A difference exists in the size ratio of the cortex to the brain stem between humans and zebrafish
• Zebrafish neural tube is characterized by eversion during early neurulation, whereas the mammalian neural tube undergoes evagination
• Non-availability of inbred lines in which all individuals are identical and homozygous
• Some of the physiological, cellular and molecular aspects of astroglia may differ between zebrafish and humans
• Zebrafish do not have the 'heteromorphic sex chromosomes' (the X and Y chromosomes), but a polygenic sex determination
• Colonial sex ratios may vary and may not exhibit aberrant sexual dimorphism compared to mammals, limiting the ability to access sex-dependent differences
• Exothermic—physiology is not identical to humans
• Methodological inconsistencies in behavioural tests may affect the reproducibility of data
• The existence of relatively small number of validated zebrafish behavioural paradigms
• Fish are not approved by the US Food and Drug Administration (FDA) for preclinical screening of drugs

Table 16.4 Diverse online resources for the zebrafish community

• The Zebrafish Information Network (ZFIN): A database of genetic and genomic data for the zebrafish (*Danio rerio*) as a model organism *http://zfin.org/*
• Zebrafish International Resource Center (ZIRC): Provides a central repository for wild-type and mutant strains of zebrafish (*Danio rerio*) and materials and information about zebrafish research *http://zebrafish.org/home/guide.php*
• European Zebrafish Resource Center: Zebrafish stock collection, containing approximately 30,000 mutations from the laboratory of Christiane Nüsslein-Volhard (Tübingen I, Tübingen 2000 and Tübingen EU screen) and the Sanger Mutagenesis Project as well as transgenic and wildtype lines from diverse sources *https://www.ezrc.kit.edu/*
• National BioResource Project—Zebrafish *https://shigen.nig.ac.jp/zebra/index_en.html*
• China Zebrafish Resource Center (CZRC) *http://en.zfish.cn/*
• EUFishBiomed: Promotes the exchange of information within the fish community *https://www.eufishbiomed.eu/en-gb/home*
• Zebrafish: Peer-reviewed journal to focus on the zebrafish model *https://www.liebertpub.com/loi/zeb*
• Zebrafish Genome Project: The Sanger Institute started the zebrafish genome sequencing project in 2001 and has generated several genome assemblies of the Tuebingen strain *https://www.sanger.ac.uk/data/zebrafish-genome-project//*
• Zebrafish Genome (Santa Cruz) *http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=352094793&clade=vertebrate&org=Zebrafish&db=danRer7&redirect=manual&source=genome.ucsc.edu*
• Zebrafish Genome (Ensembl) *http://asia.ensembl.org/Danio_rerio/Info/Index*
• The Zebrafish Gene Collection (ZGC): NIH initiative that supports the production of cDNA libraries, clones and sequences to provide a complete set of full-length (open reading frame) sequences and cDNA clones of expressed genes for zebrafish *https://genecollections.nci.nih.gov/ZGC/*
• Z Brain Atlas *https://engertlab.fas.harvard.edu/Z-Brain/home/*
• ZebraFish Anatomy Portal (ZFAP): An anatomical resource for the zebrafish *Danio rerio* containing 3D models of zebrafish covering the range of development 24 h until adulthood *http://www.fishnet.org.au/*
• International Zebrafish Society (IZFS) *https://www.izfs.org/*
• ZF-HEALTH—Zebrafish Regulomics for Human Health: Large-scale integrating project funded by the European Commission *http://zf-health.org/*
• Morpholino Database *http://www.morpholinodatabase.org/*
• The Zebrafish Book: A guide for the laboratory use of zebrafish *Danio* (Brachydanio) rerio* *https://zfin.org/zf_info/zfbook/zfbk.html*
• ZFIN Protocol Wiki: Zebrafish experimental protocols and tips *https://wiki.zfin.org/display/prot/ZFIN+Protocol+Wiki*
• Life Science Teaching Community Resource Community (LifeSciTRC.org) *https://www.lifescitrc.org/collection.cfm?collectionID=2663*

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Supporting the Next Generation of Risk Assessment in Toxicology: The Design of AOPs Based on the Alternative Model Zebrafish

17

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Abstract

The adverse outcome pathway (AOP) is a risk assessment approach that emerged as part of a paradigm shift proposal for toxicity testing in the twenty-first century, in an effort to lower costs and increase throughput in risk assessment and management. From the knowledge available in the literature, an AOP describes intrinsic relationships between a molecular initiating event (MIE), a toxicity trigger, and an adverse outcome (AO), or an apical toxic effect. These events are linked by key events (KEs) which in turn are linked together by key event relationships (KERs). AOs can be observed at different levels of biological organization (e.g., tissue, individual, population), leading to an apical toxic endpoint of toxicological relevance (Ankley et al., Environ Toxicol Chem 29: 730–741, 2010). In the case of endpoints frequently used for ecotoxicological risk assessment, it is interesting that they are demographically relevant (i.e., reproductive dysfunction, developmental vascular toxicity, early stage mortality, pigmentation pattern, heritable mutations in offspring). The development of AOPs does not present restrictions on the type of information (e.g., in silico, in vitro, and in vivo data) that supports it; it is clear that public toxicological data from numerous model organisms, such as *Danio rerio* (zebrafish), used over the past few years to assess the safety of chemicals to the environment, can be a great source of knowledge to be used to develop these models. In this chapter, we discuss importance of developing AOPs based on zebrafish data and the relevance of this to risk assessment. Second, we provide an overview of the AOP strategies and examples developed using zebrafish data and some of the AOPs based on this

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species. Finally, we present some perspectives and conclusions about the discussion covered in the chapter.

Keywords

Danio rerio · Adverse outcome pathway · Ecotoxicology · Toxicity testing in the twenty-first century · Alternative methods

Abbreviations

AChE	Acetylcholinesterase
AFSA	Animal-Free Safety Assessment
AhR	Aryl hydrocarbon receptor
AO	Adverse outcome
AOP	Adverse outcome pathway
AOP-KB	AOP knowledge base
ER	Estrogen receptor
ESL	Early stage mortality
KE	Key event
KER	Key event relationship
MIE	Molecular initiating event
NRC	National Research Council
QSARs	(Quantitative) structure-activity relationships
SETAC	Society of Environmental Toxicology and Chemistry

17.1 AOP: Concept, Importance, and Current Framework for Development

The adverse outcome pathway (AOP) is a risk assessment approach that was first conceptualized at the Pellston Workshop, organized by the Society of Environmental Toxicology and Chemistry (SETAC) in Oregon, USA, in April 2009 (Ankley et al. 2010; Jeong and Choi 2017; Vinken 2018). The AOP model emerged as part of a paradigm shift proposal for toxicity testing in the twenty-first century (Ankley et al. 2010; Bradbury et al. 2004; Gibb 2008), in an effort to lower costs and increase throughput in risk assessment and management (Krewski et al. 2010).

From the knowledge available in the literature, an AOP describes intrinsic relationships between a molecular initiating event (MIE), a toxicity trigger, and an adverse outcome (AO), or an apical toxic effect. These events are linked by key events (KEs) which in turn are linked together by key event relationships (KERs) (Ankley et al. 2010; Leist et al. 2017; Vinken et al. 2017). An AOP must obey a sequential progression of events, departing from the MIE to the in vivo AO of interest, both considered as specialized KEs (OECD 2017).

A general framework for an AOP is depicted in Fig. 17.1a. The AOP is triggered when a chemical interacts with a macromolecule (e.g., protein or DNA), causing a series of responses at the cellular level. Together, this interaction and its cellular responses make up a pathway of toxicity. The concepts of AOP and toxicity pathway are completely aligned and can often be derived from the same scientific data. However, in AOPs it is possible to observe biological events (e.g., triggered by a MIE) down to the population level, whereas toxicity pathways happen only at the cellular level (Ankley et al. 2010). According to the National Research Council (NRC), this is defined as a common cellular pathway that, if sufficiently disturbed by a stressor, will cause an adverse health result (Gibb 2008).

Adverse outcomes can be observed at different levels of biological organization (e.g., tissue, individual, population), leading to an apical toxic endpoint of toxicological relevance (Ankley et al. 2010). In the case of endpoints frequently used for ecotoxicological risk assessment, it is interesting that they are demographically relevant, so that risk assessors can predict or deduce adverse results in populations. Reproductive dysfunction, developmental vascular toxicity, early stage mortality, pigmentation pattern, and heritable mutations in offspring are some great examples of relevant endpoints within this context and will be discussed in some AOPs covered in this chapter.

Practically demonstrating, the inhibition of AChE by organophosphates and carbamates (MIE and KE1) (Fig. 17.1b), caused by the binding of these chemicals to specific enzyme sites, is a typical case of a biological event that occurs at the molecular level. After this inhibition, the synapses of the cells start to accumulate acetylcholine (KE2). The accumulation of this neurotransmitter, in turn, leads to an excitatory response in the muscles and the brain (KE3). Such events culminate in the appearance of symptoms of neurotoxicity, which lead to the death of individuals (AO and KE4). Considering biological plausibility, such outcome could easily cause a decline in animal populations exposed to environmentally relevant concentrations of any of these chemicals (Russom et al. 2014). By design, an AOP contains only one MIE and a single AO but several KEs and KERs (OECD 2017). This is due to the fact that MIE and AO are the two anchors that support an AOP, while KEs and KERs are the response matrix (OECD 2017; Vinken 2018). Therefore, to design an AOP, a researcher must clearly define the beginning (MIE) and end (AO) of the proposed path. As demonstrated in the previous example, different chemicals that act by the same MIE and toxicity pathway can cause the same AO in an individual and its population or in a diversity of species, as in the specified example. In the case of the AOP in Fig. 17.1b, the relationship between AChE inhibition (MIE) in the brain and an increase in the mortality rate (AO) was observed in insect species, crustaceans, fish, amphibians, birds, and mammals, among others (Russom et al. 2014), giving this AOP great taxonomic applicability. The latter refers to the variety of species to which an AOP can be applied and is directly related to the degree of phylogenetic similarity of the toxic responses observed across taxa.

In addition to becoming an interesting approach in the medical sciences with respect to understanding the mechanisms of action of cancers (Helm and Rudel 2020; Proctor et al. 2018), the development of AOPs has gained importance in

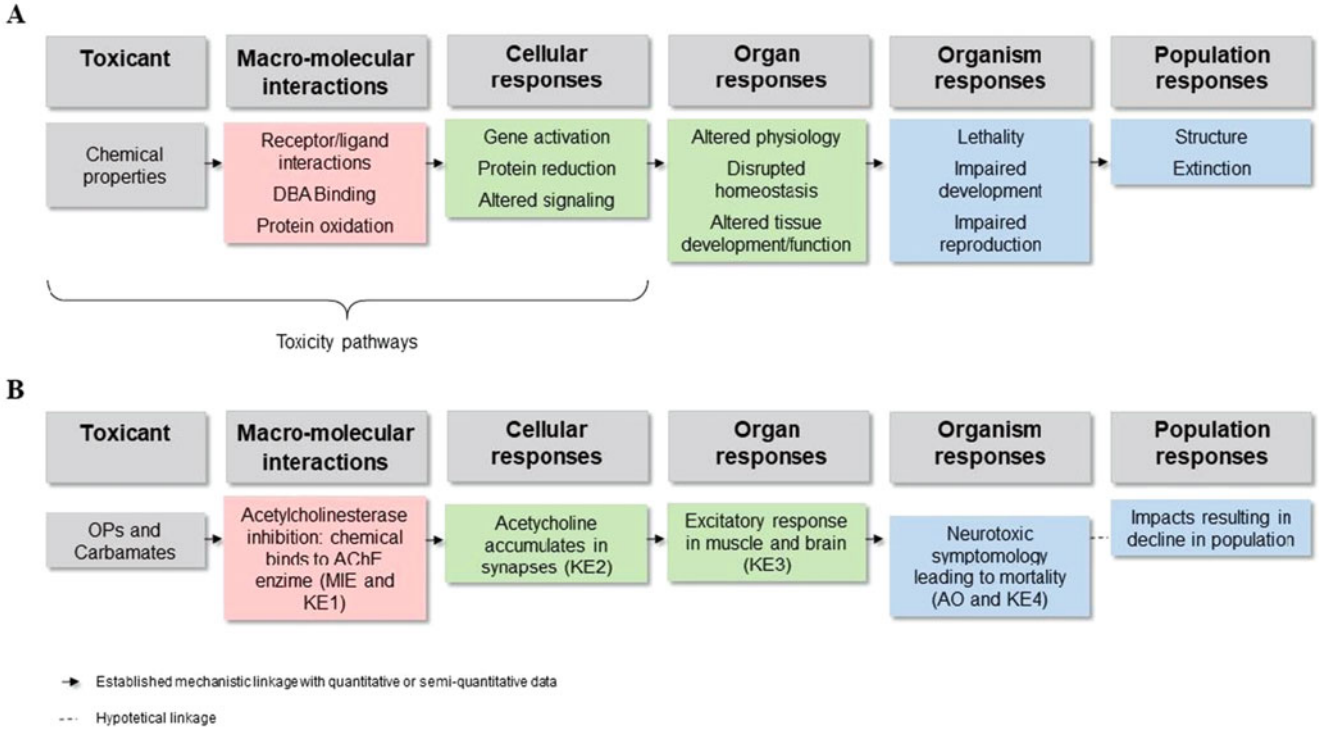

Fig. 17.1 (**a**) The framework of an adverse outcome pathway (AOP). The pathway begins with the molecular initiating event (MIE), which triggers a succession of key events (KEs), leading to the adverse outcomes (AOs) of interest. (**b**) AOP of acetylcholinesterase inhibition leading to acute mortality. Pink boxes correspond to MIE, while green and blue boxes are KEs and AO, respectively. *OPs* organophosphates. (Adapted from Ankley et al. (2010) and Russom et al. (2014))

several areas of toxicological sciences (Vinken et al. 2017). In fact, this model was initially introduced in ecotoxicology (Ankley et al. 2010). The implementation of the AOP concept facilitates the toxicity assessment of an entire group of chemicals that operate through the same MIE/KEs in the AOP (Jeong and Choi 2017; Perkins et al. 2019). In addition, multiple types of information can be used to build an AOP, including in silico, in vitro, ex vivo, and in vivo clinical and epidemiological data (Delrue et al. 2016; Edwards et al. 2016; Vinken 2019; Vinken et al. 2017).

According to the OECD, the complete development of an AOP follows three consecutive steps: identification of information blocks (MIE, KEs, AO), graphical representation, and assessment of the AOP (OECD 2017). The blocks of information from an AOP can be identified through a thorough review of the specialized scientific literature or through in vitro and in vivo tests (when there is not enough data available to support AOP). The graphic representation of an AOP, or sum of data, is done through the graphic representation of the MIE, the main events of intermediate stages, and the AO in a linear flow diagram (Ankley et al. 2010; Vinken 2018). This graphic version of AOP allows the visualization of the sequence of events at different biological levels of organization (OECD 2017). Even the uncertainties and gaps about certain KEs and their relationships can be conceived within an AOP.

Regarding the assessment of the AOP, it takes place in two stages, which include the implementation of the Bradford Hill criteria for assessing the weight of evidence and the discussion of a series of key questions (Table 17.1) determined by the OECD to test the confidence (Becker et al. 2015; OECD 2017). These graphical models can be classified as qualitative AOPs, just a graphical representation connecting a MIE to an AO, or quantitative AOPs (qAOPs), graphical models that establish quantitative relationships between key events within the AOP and a particular stressor. This specific type of AOP will be covered in Sect. 17.2.2 of this chapter.

Table 17.1 Assessment of an AOP

Step 1	Step 2
1. Concordance of dose–response relationships	1. How well characterized is the AOP?
2. Temporal concordance among the key events and adverse outcome	2. How well are the initiating and other key events causally linked to the outcome?
3. Strength, consistency and specificity of association of the adverse outcome and the molecular initiating event	3. What are the limitations in the evidence in support of the AOP?
4. Biological plausibility, coherence and consistency of the experimental evidence	4. Is the AOP specific to certain tissues, life stages or age classes?
5. Alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP	5. Are the initiating and key events expected to be conserved across taxa?
6. Uncertainties, inconsistencies and data gaps	

Step 1: Bradford Hill criteria for assessing the weight of AOP evidence. Step 2: key questions used to test AOP's confidence. Adapted from OECD (2017)

AOP adverse outcome pathway

In partnership with several other institutions, the OECD has created a database to record and store information about AOPs, the AOP knowledge base (AOP-KB) (aopkb.oecd.org/index.html). According to its own definition, AOP-KB is a fusion of individually developed platforms, such as Effectopedia (www.effectopedia.org/) and AOP-Wiki (www.aopwiki.org/), synchronized and organized in a way that users have the possibility to capture, review, browse, and comment on AOPs shared by the AOP stakeholder community. This database allows all stakeholders to create AOPs by entering and linking information about MIEs, KEs, AOs, and chemical initiators. The AOP-KB serves as a central repository for storing all AOPs developed under the OECD guidelines.

Effectopedia is a system used to capture quantitative information and establish new models, as well as to provide a standard visual representation of AOPs and associated test methods. The AOP-Wiki is an open-source and collaborative collection of AOPs, through which developers can work together to build and refine these models. This library contains all AOPs in development or already developed, so far, together with the scientific evidence that supports each of them.

AOP Wiki has almost 350 AOPs (September 2020) for a multitude of toxicological effects, which are relevant to human toxicology and ecotoxicology. In this electronic library, these AOPs can be classified according to their identification number (ID), title, point of contact (AOP author), and author status. Table 17.2 contains a list of author status and their respective definitions (https://aopwiki.org/aops), which describes the different stages of development for an AOP.

Table 17.2 Phases of development of AOP based on the author status defined by their respective points of contacts

Development stage	Definition
Under development: not open or comment; Do not cite	This is the default status assigned when a new AOP page is created in the AOP-Wiki. It is used to indicate that the project team is actively developing the pages and that the author(s) have new content they expect to add such that commenting on or citing the existing content is premature.
Open for comment; do not cite	This status is used to indicate that the authors have added the primary content they wish to include and they invite the community to comment on that content via the Discussion pages. However, this designation indicates that the authors do not feel the AOP should be cited in its current form.
Open for citation and comment	This status is used to indicate that the author(s) have added the content they wish to include on their AOP page (and the associated KE and KER pages) and they invite the community to comment on that content via the Discussion pages and cite the AOP in its current form, if desired. This designation usually indicates that the authors stand behind their contribution and take responsibility for the scientific content.
Open for adoption	This refers to "adoption" in the sense of new authors taking over responsibility for further development of the AOP. It should not be confused with an AOP that should be considered for endorsement or use. This status is used to indicate that the primary author(s) of the AOP are no longer actively working on the page, but would like to invite others from the community to take-over development of the AOP. An open for adoption status also signals the curators of the AOP-Wiki that the authors feel the content provided warrants further development. AOPs that are open for adoption will not be deleted from the AOP-KB without first consulting the current Point of Contact.
Not under active development	This status indicates the primary author(s) of the AOP are no longer actively working on the page. Others may still contact the authors about taking-over development of the pages if desired. However, the content provided may or may not warrant further development. AOPs with this status designation are subject to deletion at the discretion of the curators of the AOP-KB.

Adapted from OECD (2016)

Furthermore, since the development of AOPs does not present restrictions on the type of information that supports it and due to its versatility in relation to its taxonomic applicability (Russom et al. 2014; Jeong and Choi 2017), it is clear that public toxicological data from numerous model organisms, such as the fishes *Pimephales promelas* (fathead minnow), *Oncorhynchus mykiss* (rainbow trout), *Cyprinus carpio* (common carp), and *Danio rerio* (zebrafish), used over the past few years to assess the safety of chemicals to the environment (Dubińska-Magiera et al. 2016; Ensenbach et al. 1996; Štěpanova et al. 2012; Tillitt et al. 2010), can be a great source of knowledge to be used to develop these models.

Fish species are widely used as models to assess the toxicity of aquatic contaminants to vertebrates and to determine quality parameters of aquatic ecosystems (OECD 1992, 2000, 2013, 2016, 2018). The zebrafish is the most common species in testing trials to assess the safety of chemicals (Hartung 2017) and one of the most used in animal testing around the world, being surpassed only by mouse and rat (Hickman et al. 2017).

In this chapter, we discuss importance of developing AOPs based on zebrafish data and the relevance of this to risk assessment. Second, we provide an overview of the AOP strategies and examples developed using zebrafish data and some of the AOPs based on this species. Finally, we present some perspectives and conclusions about the discussion covered in the chapter.

For this purpose, all AOPs deposited in the AOP-KB and listed on the AOP-Wiki were analyzed, and we discuss AOPs whose MIE and AO are well established and applicable to zebrafish (i.e., supported by data from that organism). In addition, only "open for citation and comments" AOPs were included in the discussion in this chapter. This status is used by the AOPs author(s) to indicate that they have added the content they wish to include on their AOP page (and the associated KE and KER pages) and they invite the community to comment on that content via the discussion pages and cite the AOP in its current form, if desired (OECD 2017).

17.2 AOPs Developed for Non-mammalian Species

17.2.1 Relevance of Non-mammalian-Based AOPs

Amphibians, reptiles, fishes, and birds are non-mammalian vertebrates. Commonly, these organisms are used as an alternative to replace mammals in animal testing owing it to its low maintenance costs and ethical concerns in animal experimentation (Doke and Dhawale 2015). Fathead minnow, rainbow trout, common carp, and zebrafish are among the most commonly used models. Despite the somewhat distant evolutionary kinship to mammals, the zebrafish, for example, shares about 70% of protein coding genes with humans, which leads to certain degree of similar biological responses when exposed to a stressor (Howe et al. 2013; Segner and Baumann 2016). The last 40 years of studies with this animal have generated an immeasurable amount of toxicological data (e.g., omics, histological, cellular,

population), which can now be used in the construction of AOPs, providing mechanistic information to support risk assessment.

However, a brief survey conducted in the AOP-KB database reveals that, despite the amount of information about this model accumulated in recent decades, only 9 of the 180 AOPs developed to date are supported by data from these animals. In addition, the AOP-Wiki has only 24 AOPs being supported by data from this model among the 349 that are registered on the platform.

The AOP "Aryl hydrocarbon receptor (AhR) activation leading to early life stage mortality, via increased COX-2" (AOP 21), for instance, has taxonomic applicability to zebrafish but also to *Oryzias latipes* (medaka or Japanese rice fish) and *Gallus gallus* (chicken). With that AOP it is possible to predict that any chemical substance capable of activating an aryl hydrocarbon receptor, via COX-2, will potentially increase the mortality rate of bird and fish species in early developmental stages (Fig. 17.2a) (aopwiki.org/aops/ 21). Thus, this AOP is very relevant to understand mechanisms of adverse effects of any chemical with the ability to activate AhR in these animals.

In addition to this, there is also the AOP of "AHR activation to ELS mortality" (AOP 150) that connects AO to AhR activation. It associates the activation of this receptor with an increase in the mortality rate of the early stages of birds and fish, via deregulation of the vascular endothelial growth factor (Fig. 17.2b) (aopwiki.org/aops/150). The importance of this sharing of biological events (MIEs, KEs, and AOs) between AOPs will be discussed in the following sessions.

Another interesting AOP described in recent years, based on data from zebrafish and other non-mammalian models, is that of "Developmental vascular toxicity" (AOP 43) focused on vasculogenesis-angiogenesis during embryonic development (Fig. 17.2c) (aopwiki.org/aops/43). This AOP, as described by the authors, is important in the toxicological context in order to predict adverse effects that certain stressors may cause during embryonic development. Through this route, the authors suggest that it will be possible to identify useful information (e.g., minimum concentration of a stressor responsible for a specific KE) with the purpose of establishing relevant AOs for risk assessment and optimizing resources through the creation of predictive methods relating the toxicity of the development with vascular disruptions. In addition, computational models based on this AOP may emerge as new screening techniques to predict apical endpoints caused by exposure of embryos to chemicals in different dose-over-time scenarios.

Another valuable, recently established AOP is the "Inhibition of tyrosinase leads to decreased population in fish" (AOP 292). This path was designed to assess demographic changes in fish populations caused by the inhibition of the enzyme tyrosinase (Fig. 17.2d) (aopwiki.org/aops/292). Such AOP is fundamentally interesting to establish apical pigmentation effects related to photosensitivity, as well as to evaluate skin toxicity and teratogenic effects, since the reduction of the activity of this enzyme leads to a drop-in melanin levels and changes in the pigmentation pattern of these animals, which is intrinsically related to these three endpoints.

Such AOPs show very well how the risk assessment of chemicals may become simpler and cheaper in the coming decades. However, the construction of these

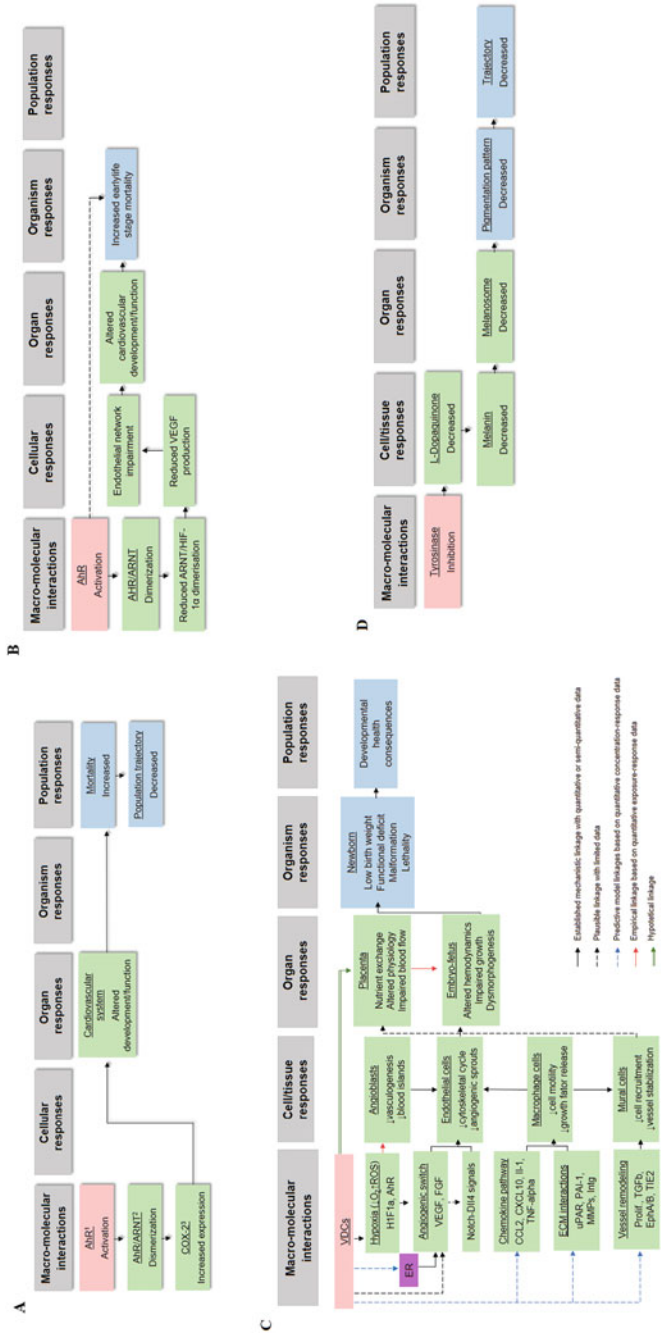

Fig. 17.2 Adverse outcome pathways (AOPs) based on non-mammalian models. (**a**) AHR activation to ELS mortality. (**b**) AhR-mediated mortality, via COX-2. (**c**) Developmental vascular toxicity. (**d**) Inhibition of tyrosinase in fish. Pink boxes correspond to molecular initiating event (MIE), while green and blue boxes are key events (KEs) and adverse outcome (AO), respectively. *ER* estrogen receptor

biological maps will make it possible to identify numerous AOs (at individual and/or population level) of ecotoxicological relevance, resulting from interactions between biological macromolecules and a specific chemical, using public toxicological data from model animals such as those mentioned above.

17.2.2 Use in Risk Assessment

AOPs enable toxicity assessment of chemical groups acting through the same MIE (Jeong and Choi 2017). Therefore, these models can be used to develop strategies in order to maximize the amount of useful information about these substances. For example, through the construction of quantitative AOPs (qAOPs) for certain compounds, it is possible to identify the minimum concentration by which a specific chemical trigger adverse effects on taxons. qAOPs allow pinpointing concentrations of a chemical related to key events within an adverse effect pathway (Muller et al. 2015; Perkins et al. 2019). This, in turn, enables the development of essential in vitro tests with the purpose of establishing chemical categories and their structure-activity relationships with minimal experiments.

AOPs including KEs conserved in different species can be applied at a wide range of taxa (vertebrates and invertebrates). These KEs common in different genres, families, classes, and even phyla are due to genetic and physiological similarities between animals. For example, the inhibition of acetylcholinesterase caused in many animals by exposure to organophosphates and carbamates (different chemical classes) is a perfect case of a key event (Fig. 17.1b) that can happen basically in almost all organisms that have this enzyme (Russom et al. 2014). Since these KEs can be measured by in vitro assays, it proves the feasibility of their use in risk assessment.

In a practical way, the AOPs accurately described provide mechanistic information with different applicability (Jeong and Choi 2017; Leist et al. 2017; OECD 2017). The AOP "Alkylation of DNA leading to heritable mutations" (AOP 15) (Fig. 17.3a), for example, supported mainly by data from *Drosophila melanogaster* (fruit fly) and *O. latipes*, makes it clear that a chemical substance capable of causing alkylation of germ cell DNA will certainly cause an increase in the rate of mutations in the offspring of males of these species (aopwiki.org/aops/15). Thus, this adverse effect pathway can be used by regulators in the risk management of this type of substance, such as, for example, the establishment of maximum concentrations of specific chemicals that cause DNA alkylation legally permitted in aquatic environments. Furthermore, because the AOP in question is aimed at any substance capable of alkylating germ cell DNA, it can be taken as a basis to allow or veto the release of new alkylating agents produced by the pharmaceutical and chemical industries.

AOPs have great relevance and different applicability for risk assessment; the inclusion of quantitative information can turn an AOP into a qAOP; it would be possible to do robust risk analysis by using it, besides the possibility of being used in the establishment of (quantitative) structure-activity relationships (QSARs), as the MIE and AO of an AOP have numerous chemical-specific interactions with

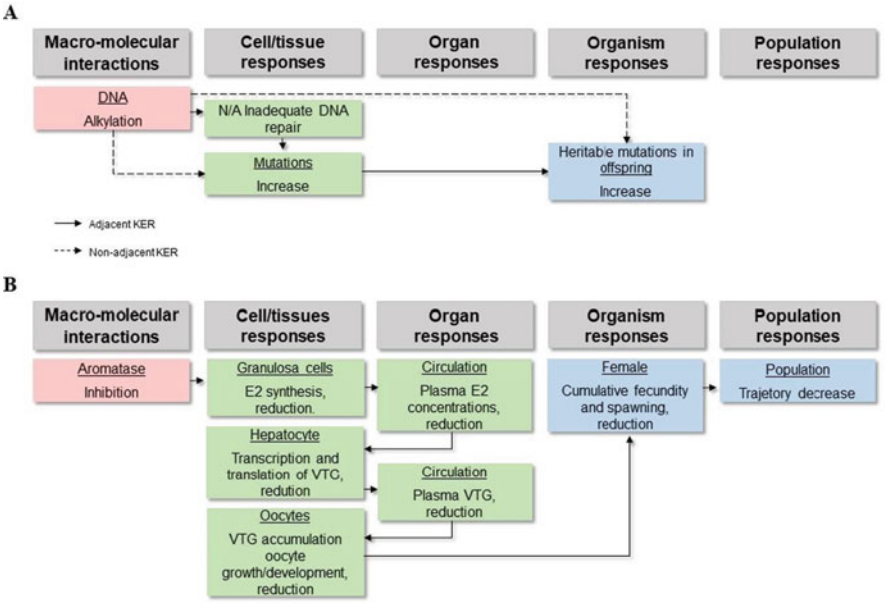

Fig. 17.3 Examples of some adverse outcome pathways (AOPs) relevant to risk assessment. (**a**) Alkylation of DNA leading to heritable mutations. (**b**) Aromatase inhibition leading to reproductive dysfunction. Pink boxes correspond to molecular initiating events (MIE), while green and blue boxes are key events (KEs) and adverse outcome (AO), respectively. *N/A* N-alkyl

biological systems, and this can be used as a basis in the construction of these mathematical models. QSAR models, in the first place, establish the supposed relationship between the structure of a chemical and its biological activity, and, second, they make it possible to predict the activity of new chemicals (Schultz et al. 2003; Vinken 2013).

In addition, AOPs (1) encourage the creation of new in vitro methods and (2) enable the development of prioritization strategies, making risk assessment process faster and less costly (Vinken 2013; Perkins et al. 2019). In the first case, this happens when an AOP when describing specific KEs can be scientifically ascertained, such as a biological activity. For example, the AOP "Aromatase inhibition leading to reproductive dysfunction" (AOP 25) (Fig. 17.3b)—which establishes the relationship between the inhibition of aromatase in fish gonads and the alteration of the population structure of these animals—not only encourages the development of other quantitative assays but also paves the way for the development of in silico models to predict aromatase's inhibition by other chemicals (aopwiki.org/aops/25) (Wittwehr et al. 2017). This AOP (which will be described in detail in later sessions) was initially designed for only females of the Osteichthyes class; however, it is currently known that it is applicable to other classes of fish, as well as to species of birds, reptiles, and amphibians, because they are oviparous species.

In the second case, which concerns which chemical should be prioritized to be subjected to more complex and expensive risk assessment tests, AOPs are great points of reference. The information established by an AOP in relation to a chemical and its activities in biological systems can increase confidence and/or validate in vitro tests, which essentially can be used in the screening of chemical risk. This allows the establishment of a specified risk for defined chemicals; thus, priority is given to animal tests only for chemists whose risks to be established cannot be verified through alternative models.

17.3 Current Zebrafish-Based AOPs

17.3.1 Description of Specific AOPs

There are currently nine AOPs fully described for zebrafish (but not exclusive to the species) on the AOP-Wiki platform. However, this same AOP bank records 19 adverse effects pathways with taxonomic applicability to this model. This means that new AOPs are being developed based on data from this model, but that includes data from other organisms, to help understand how certain stressors trigger biological events that lead to an adverse outcome in an individual and/or their population.

Because they are supported by data from various animal models, these AOPs (with intermediate events conserved between taxa) can be extrapolated to multiple species in order to predict ecotoxicological effects. In this way, risk assessors will have more information about the endpoints caused by a specific chemist or a class of them for proper assessment and management of the risks that these stressors offer to the environment (Bradbury et al. 2004). The following is the description of AOPs based mainly on zebrafish data, some already mentioned in this chapter, which are certainly of value for risk management.

One of the first AOPs developed for the purpose of predicting toxic effects was the "Estrogen receptor antagonism leading to reproductive dysfunction" (AOP 30) (aopwiki.org/30) (Fig. 17.4). In general, this AOP describes the relationship between estrogen receptor antagonism (ERs) and the cumulative fertility reduction endpoint in female fish that reproduce continuously. The disruption of these receptors, in general, is detrimental to human health, and, therefore, this AOP is particularly interesting because with the inclusion of quantitative data in this model, it can be used to characterize the risk of chemists regarding their disrupting endocrine abilities. In addition, it is known that when ERs interact with antagonist ligands, they undergo a change in their conformation and consequently in their function. Because of this, the interaction between an ER and an antagonist causes a reduction in the synthesis of vitellogenins (Vtgs) in female hepatocytes during the reproduction period. Inevitably, this reduces the levels of Vtgs available in the plasma, and, therefore, the oocytes do not accumulate these calf proteins in sufficient quantity to guarantee their normal growth, which is essential for an embryo to reach the larval

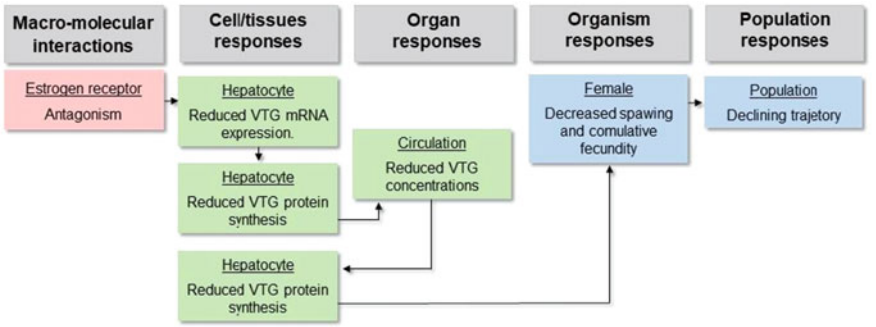

Fig. 17.4 Estrogen receptor antagonism leading to reproductive dysfunction. Pink boxes correspond to molecular initiating event (MIE), while green and blue boxes are (key events) KEs and adverse outcome (AO), respectively

stage. This is the case of butachlor, for example, which negatively affects the fertility and homeostasis of adult sex hormones of zebrafish, as well as promoting an increase in the levels of VTG II transcripts (Chang et al. 2012).

Another interesting AOP, mentioned earlier, is AOP 25 (Fig. 17.3b). In this model, the inhibition of the aromatase enzyme in female fish reduces the synthesis of 17beta-estradiol (E2), leading to a drop in its concentration in plasma. These two events lead to a reduction in Vtgs in the circulation of these animals, and this, as already mentioned, leads to a reduction in the cumulative fertilization of these animals. Up until this moment, some computational models were developed for this AOP comprising different sections (Wittwehr et al. 2017); for instance, the linkage of aromatase inhibition to vitellogenin levels in fathead minnows proposed by Cheng et al. (2016) and the population model suggested by Miller et al. (2015) could be used to predict potential impacts in population dynamics.

Like AOP 30 (Fig. 17.4), this aromatase inhibition pathway is also strictly related to reproductive dysfunction, which is one of the most important endpoints in OECD test 229 (Fish Short Term Reproduction Assay) (OECD 2012). This type of apical effect caused by an ER antagonist chemical or an aromatase inhibitor usually indicates a decline in the fish population. In view of this, the importance of these two AOPs is evident, first, for the development of alternative in silico methods in order to predict the abilities that a new synthetic has (a) to be an antagonist of ERs or (b) to inhibit the aromatase enzyme and, second, to support and encourage the creation of in vitro methods that quantify ER antagonism or inhibition of the aromatase enzyme.

Similarly, two other graphic models that are certainly excellent starting points in the development of these alternative methods are AOPs 21 and 150 (Fig. 17.2a, b), referred to in Sect. 17.2.1. Both AOPs have both MIE, AhR activation, and AO, early life stage mortality, in common, but they have different key events that occur in different cell pathways. In the case of AOP 21, the activation of AhR by a stressor

causes an increase in the expression of COX-2. The dysregulation of this enzyme, somehow not elucidated in AOP, is directly related to changes in cardiovascular tissue, which leads to the observed AO. Regarding the AOP 150, the same MIE is responsible for the reduction of the vascular growth factor, generating damage to the endothelial tissue, which alters the development and function of the cardiovascular tissue, resulting in the AO. Interestingly, even though these two AOPs differ on KEs at the cellular level, they have the same KE at organ level. This intermediate event sharing between AOPs is quite frequent and is an interesting thing to be addressed.

17.3.2 Common Events Across AOPs

In the topic just above, we have several key events being shared between AOPs 25 and 30 (Figs. 17.3b and 17.4) that trigger the same AO but are triggered by different MIEs, and we also have AOPs 21 and 150, which have identical MIE and AO and only one KE in common. To talk about the importance of this sharing of key events, let's take the first two AOPs as examples. In the case of these two graphic models, most KEs are common to each other and culminate in reproductive dysfunction, which can lead to a change in the structure of the species populations to which these AOPs apply. Giving importance to the complexity of reproductive dysfunction, together with their respective KEs sequences, the two MIEs proposed by these AOPs are just two of the several cascades of possible biological events, which are capable of causing changes in the structure of species populations.

By the sharing of KEs, in general, it is possible to build networks of AOPs (Perkins et al. 2019; Villeneuve et al. 2014). In relation to the endpoints discussed above, the creation of networks could be done with all AOPs important in understanding changes in the population trajectories of fish species, connecting them to each other from these intermediate events. The relevance of networks like these, built through collaborations between toxicologists and other scientists, lies in the fact that they can contribute to the documentation of the complexity of an adverse outcome (e.g., extinction) that can sometimes be caused by multiple MIEs and have different toxicity pathways.

In addition, AOP networks can also serve as a reference point for the development of alternative in vitro methods for testing strategies, supported by key AOP events. This favors, for example, the possibility of effective and large-scale testing of a large amount of chemicals for their ability to generate changes in fish populations.

Furthermore, according to the AOP-Wiki database, when two or more AOPs are being developed by different teams and they share KEs or the same AO, it is important that the leaders of each team try to cooperate with each other to develop only a single AOP, if possible. The database system works best if AOPs do not have duplicate entries.

17.4 Conclusions and Perspectives

The lack of understanding about the mechanisms of toxicity, in the field of (eco) toxicology, makes it impossible to define new sublethal apical endpoints (e.g., hepatotoxicity), as well as limiting the usefulness of information originated from methods and tests with model organisms. In the field of toxicological risk assessment, the ideal would be that all data obtained in one species and with a chemical product could be extrapolated to other species, as well as to other chemicals. Apart from that, classic toxicological models (based on animal experimentation) can be expensive and immersed in ethical controversies (Groh et al. 2015). Thus, the proposal of AOPs came with the objective of optimizing all types of data (old/new/in silico/in vitro/in vivo) produced with model species in toxicological studies and to extrapolate this knowledge to other taxa. This new paradigm will certainly take toxicology and risk assessment sciences into a new era, when it will be possible to carry out the risk assessment of chemicals using methods in silico, but not exclusively.

Furthermore, considering that it is possible that not all AOPs under development and/or developed to date are registered in the OECD databases, as is the case with the AOP of reproductive toxicity mediated by silver nanoparticles proposed by Ma et al. (2018), it is important to alert all researchers working on the construction of these maps about the minimum requirements needed (e.g., weight of scientific evidence, confidence assessment, only one AO) so that they can serve as a risk assessment tool. In general, any AOP built to be applied in the environmental risk assessment process must be in line with OECD requirements. Each criterion necessary to make a consolidated and useful AOP that can be applied in risk management is described in the OECD's AOP development guide, last updated in 2017 (OECD 2017).

Currently, anyone interested in the field of AOPs can have access to a historical perspective on this new paradigm, available online and free on the AOP-Wiki (https://aopwiki.org/training/aops/), as well as virtual training for the construction of these models. These courses are now hosted on the Animal-Free Safety Assessment (AFSA) Collaboration website.

Although still very recent, the potential of this new toxicology paradigm and its applicability is significantly great. For example, AOPs can easily be adopted by the medical sciences, although initially they were assigned to the field of ecotoxicology. However, they must be constantly refined and updated (Vinken 2013).

In the coming years, through the continuous refinement of these maps, the inclusion of quantitative descriptions, as well as toxicodynamic relationships between key events and the toxicokinetic characterization that leads to the emergence of an AO, will become more and more frequent within the process of building AOPs. Consequently, this will make it possible, for example, to carry out a risk assessment based solely on AOPs. To reach that, however, the scientific community must make use of AOPs already available (qualitative and quantitative) as tools in the risk assessment process; they can be used, for example, to establish classes of chemicals that should be prioritized in an ecological risk assessment (Groh et al. 2015).

At the moment, among the AOPs mentioned in this chapter, the one that brings toxicologists closer to a future in which risk assessment using only AOPs will be possible would be AOP 25 (aromatase inhibition). This is because there is an enormous amount of quantitative information from scientific evidence that supports the KEs and KERs of this model. In fact, this AOP has proven to be useful for the development of alternative methods used by regulatory bodies, as we already mentioned. Trials involving the MIE and some KEs of this AOP have already been carried out for use in screening and risk assessment programs (Hecker et al. 2011).

While promising, there are still some challenges for the future of AOPs. For example, an AOP does not represent the complexity of biological processes that take place within a pathway, hence the need to build networks of AOPs, connected by shared key events. Because through this collaborative approach it will be possible to better understand how the KEs of the AOPs are connected to each other and how they and their relationships (KERs) contribute to the manifestation of an AO in an individual or in their population (Vinken 2013). In addition, the inclusion of quantitative data and dose-response relationships in AOPs is still very fickle in the process of making these maps; however, this should become a basic criterion for any AOP.

In addition, another obstacle imposed on AOPs is the lack of scientific evidence to support these models. Anyone can suggest an AOP, but a minimal amount of information, including exposure data and toxicokinetics, is required. This leads to the need to establish a collaborative, transparent, and objective system for the construction of relevant AOPs, which can be validated as official methods used in risk assessment.

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Zebrafish: A Novel Model in Psychopharmacological Research

18

Prasan Ramchandra Bhandari

Abstract

The zebrafish (*Danio rerio*) has recently become a powerful animal model for research purposes and drug discovery due to its ease of maintenance, genetic manipulability and ability for high-throughput screening. It has emerged as a model species for translational research in various neuroscience areas, including pharmacogenetics and neuropharmacology. Due to their physiological (neuroanatomical, neuroendocrine, neurochemical) and genetic homology to mammals, robust phenotypes, and value in high-throughput genetic and chemical genetic screens, zebrafish are ideal for developing valid experimental models of major neuropsychiatric disorders and discovering novel therapeutics. Both larval and adult zebrafish are presently used to enhance our understanding of brain function, dysfunction and their genetic and pharmacological modulation. This article provides a review of the developing utility of zebrafish in the analysis of complex brain disorders (including, e.g. depression, autism, psychoses, drug abuse and cognitive deficits) and also covers zebrafish applications towards the goal of modelling major human neuropsychiatric and drug-induced syndromes.

Keywords

Zebrafish · Model · Neuropsychopharmacology

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18.1 Introduction

Psychiatric disorders are a varied set of diseases that influence all features of mental function including social interaction, thinking, feeling and mood. Though psychiatric disorders put a huge economic burden on society, the drugs offered to treat them are often palliative with inconsistent efficacy and unbearable side effects. The development of new drugs has been slowed down by a lack of knowledge about the aetiology of these diseases. Hence it is imperative to further explore psychiatric disorders using a combination of human molecular genetics, in vitro pharmacological and biochemistry experiments, animal models and investigation of the non-biological basis of these diseases, such as environmental effects (Norton 2013). Furthermore, as the population ages, there is an increasing need for efficacious and safe therapies for the treatment of neurological diseases. A restricted number of treatment options are presently available to recover the cognitive dysfunction, and research is limited by the need for in vivo models.

Animal models of these disorders are widely used in behavioural neuroscience to explore brain abnormalities, screen drugs and establish behavioural phenotypes of gene-targeted or transgenic animals. Noting a deficit of fresh ideas and especially new paradigms for animal models, there exist a challenge and a pressing need for important directions for further research in this field. Current models in biological psychiatry focus on a handful of model species, and the majority of work relies on data generated in rodents. However, the inclusion of other species and an adoption of comparative viewpoints in behavioural neuroscience could also lead to increases in knowledge relevant to biological psychiatry. To achieve this goal, the current focus on mammalian species must be expanded to include other species, including non-mammalian taxa (Maximino et al. 2015). This review will look at the utility of the zebrafish in the study of neuropsychiatric diseases and neuropsychopharmacological research.

18.1.1 Rationale for the Use of Zebrafish as a Novel Model in Psychopharmacological Research.

In the above-mentioned scenario, a small aquatic vertebrate, the zebrafish (*Danio rerio*) (Fig. 18.1), is rapidly becoming a new accepted model organism in biomedical research.

Major universities and research centres worldwide have established zebrafish facilities, and the US National Institutes of Health have recently constructed the world's biggest zebrafish centre, with the ability to house up to 19,000 tanks and 100,000 fish. The usefulness of both adult and larval zebrafish in neuroscience has developed strikingly in the past decades since it is a vertebrate species with high physiological and genetic homology to humans, and also due to the ease of genetic manipulation and comparable central nervous system (CNS) morphology. Because of its both rapid development (Fig. 18.2) and a relatively long life span, zebrafish are currently used to model various human brain disorders. Additionally, the close

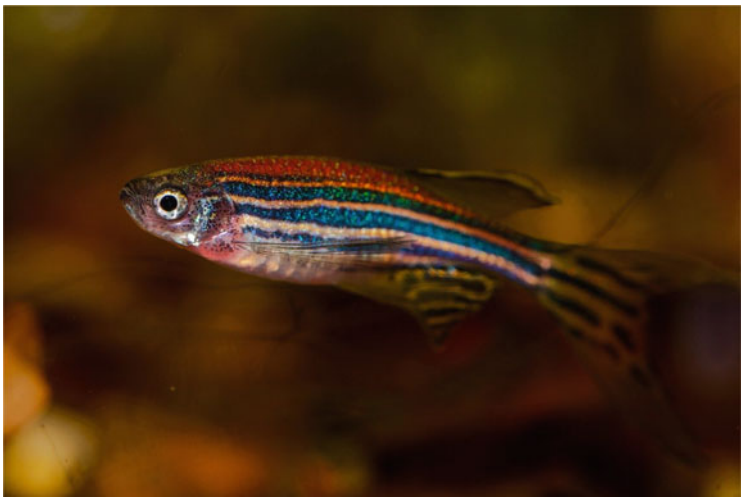

Fig. 18.1 Zebrafish. (Image source: Pixabay)

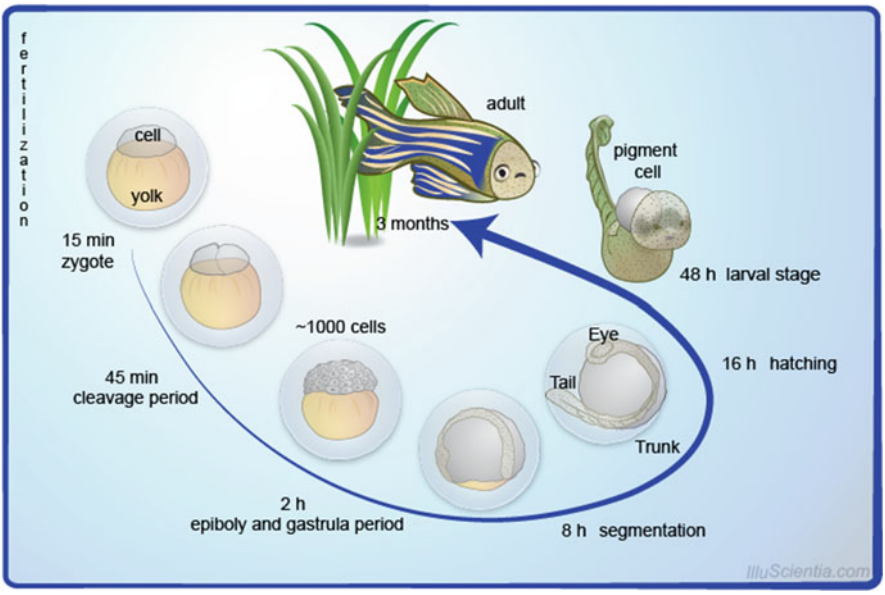

Fig. 18.2 Zebrafish life cycle. (Image source: Wikimedia Commons)

parallels between mammalian and zebrafish behavioural paradigms hint towards the evolutionarily conserved nature of many behaviours (and deficits of their control) across species, suggesting face and construct validity of zebrafish models. Zebrafish are especially cost-efficient and easy to breed and can be housed in large numbers in a relatively small space. Thus, zebrafish is rapidly becoming an accepted model

organism in pharmacogenetics and neuropharmacology. Both larval and adult zebrafish are currently used to increase our understanding of brain function, dysfunction and their genetic and pharmacological modulation.

There is a striking similarity between zebrafish and mammalian (rodent) models, including both general macro-organization of the brain and cellular morphology. The knowledge gained from these studies is very relevant to human brain functioning. In addition, zebrafish possess all main neuromediator systems, including transmitters, their receptors, transporters and enzymes of synthesis and metabolism, similar to those observed in humans and rodents (Kalueff et al. 2014).

Modulatory neurotransmitters which signal through G protein-coupled receptors control brain functions which deteriorate in degenerative brain diseases. In the past decade, several of these systems have been mapped in the zebrafish brain. The main structural designs of the systems in zebrafish brain bear a resemblance to that of the mammals, notwithstanding differences in the development of the telencephalon and mesodiencephalon. Modulatory neurotransmitter systems which degenerate in human diseases include dopamine, noradrenaline, serotonin, histamine, acetylcholine and orexin/hypocretin. While the amount of G protein-coupled receptors in zebrafish is evidently larger than in mammals, numerous receptors have similar expression patterns, binding and signalling properties as in mammals. Zebrafish are sensitive to neurotoxins including MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and exposure to this neurotoxin induces a decline in dopamine content and number of detectable tyrosine hydroxylase immune reactive neurons in distinct nuclei. Sensitivity to important neurotoxins, many available genetic methods, rapid development and large-scale quantitative behavioural methods in addition to advanced quantitative anatomical methods render zebrafish an optimal organism for studies on disease mechanisms (Panula et al. 2010).

Furthermore, zebrafish are sensitive to all major classes of neurotropic drugs, including antipsychotics, mood stabilizers, anxiolytics, antidepressants, ethanol, sedatives/hypnotics, stimulants, hallucinogens, antiepileptics, anaesthetic/analgesics and cognitive enhancers (Kalueff et al. 2014). Because of their relatively small size of zebrafish larvae, assays can be undertaken in a 96-well plate, and as the larvae can live in as little as 200 mul of fluid, only a few milligrams of compound are needed for screening. Thus in vivo analysis of the effects of compounds can be undertaken at much earlier stages in the drug discovery process (Ali et al. 2011). It is perceived that zebrafish models of complex brain disorders and drug-induced conditions are a rapidly emerging critical field in translational neuroscience and pharmacology research.

18.1.2 Anxiety and Anxiolytics

Zebrafish is becoming progressively more accepted in neurobehavioral research. By using the novel tank test (Fig. 18.3) as a sensitive and efficient behavioural assay, zebrafish anxiety-like behaviour is bidirectionally altered by drugs affecting the gamma-aminobutyric acid, monoaminergic, cholinergic, glutamatergic and

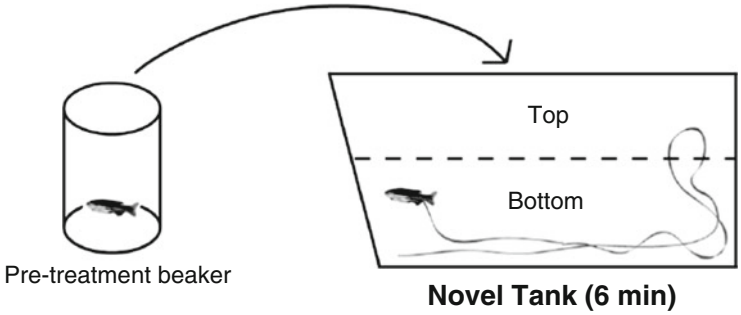

Fig. 18.3 Novel tank test for anxiety (Cachat et al. 2011)

opioidergic systems. Complementing human and rodent data, zebrafish drug-evoked phenotypes obtained in this test support this species as a useful model for neurobehavioral and psychopharmacological research (Stewart et al. 2011a).

Cachat et al. have devised a protocol and described a battery of assays to characterize anxiety-related behavioural and endocrine phenotypes in adult zebrafish. They have demonstrated how to use the 'novel tank' test to assess behavioural indices of anxiety (including reduced exploration, increased freezing behaviour and erratic movement), which are quantifiable using manual registration and computer-aided video tracking analyses. Furthermore, authors have described how to analyse whole-body zebrafish cortisol concentrations that correspond to their behaviour in the novel tank test. This protocol is an easy, economical and effective alternative to other methods of quantifying stress responses in zebrafish, thus facilitating the swift acquisition and exploration of large amounts of data. Fish anxiety-like behaviour can be either reduced or exaggerated depending on stress or drug exposure, with cortisol levels generally expected to parallel anxiety behaviours. This protocol can be accomplished over the course of 2 days, with an adaptable testing duration depending on the number of fish used (Cachat et al. 2010a).

Bencan et al. have established a technique to evaluate novel environment diving behaviour of zebrafish as a model of stress response and anxiolytic drug effects. In a novel tank, zebrafish dwell in the bottom of the tank initially and then increase their swimming exploration to higher levels over time. It was earlier established that nicotine, which has anxiolytic effects in rodents and humans, considerably reduces the novel tank diving response in zebrafish. The specificity of the diving effect was corroborated with a novel vs. non-novel test tank. The novel tank diving response of zebrafish was tried when given three anxiolytic drugs from two different chemical and pharmacological classes: buspirone, chlordiazepoxide and diazepam. Buspirone, a serotonergic (5HT(1A) receptor agonist) anxiolytic drug with some D(2) dopaminergic effect, had a distinct anxiolytic-like effect in the zebrafish diving model at doses that did not have sedative effects. In distinction, chlordiazepoxide, a benzodiazepine anxiolytic drug, which is an effective agonist at GABA-A receptors, did not produce signs of anxiolysis in zebrafish over a broad dose range up to those that caused sedation. Diazepam another benzodiazepine anxiolytic drug did produce

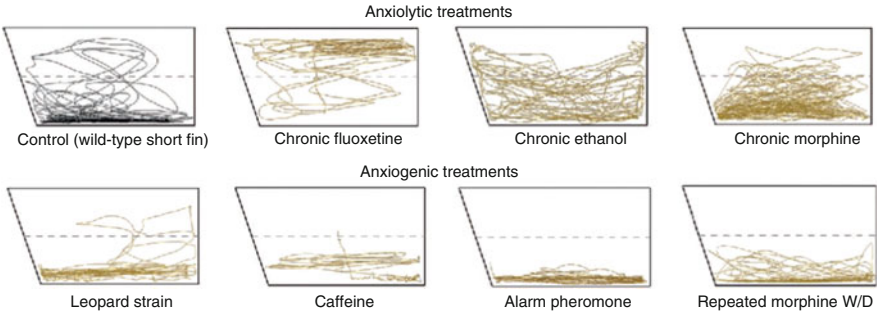

Fig. 18.4 Zebrafish anxiety/anxiolytic responses (Cachat et al. 2010a)

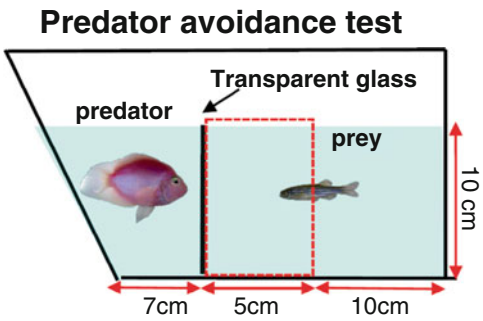

Fig. 18.5 Predator avoidance test (Audira et al. 2018)

an anxiolytic effect at doses that did not cause sedation. The zebrafish novel tank diving task can be useful in discerning anxiolytic drugs of several classes (serotonergic, benzodiazepines and nicotinic) (Bencan et al. 2009).

Current data has established behavioural responses of adult zebrafish to a varied gamut of putative anxiolytic and anxiogenic agents. Experimental manipulations encompassed exposure to alarm pheromone, chronic exposure to fluoxetine, acute exposure to caffeine as well as acute and chronic exposure to ethanol. Acute (but not chronic) alarm pheromone and acute caffeine produced significant anxiogenic effects, including reduced exploration, augmented erratic movements and freezing behaviour in zebrafish tested in the novel tank diving test. However, ethanol and fluoxetine had robust anxiolytic effects, comprising higher exploration and decreased erratic movements (Fig. 18.4). Additionally, investigators have established a simple and effective method of assessing zebrafish physiological stress responses (based on a human salivary cortisol assay) and presented that alterations in whole-body cortisol levels in zebrafish parallel behavioural indices of anxiety (Egan et al. 2009).

The chronic unpredictable stress (CUS) paradigm is a commonly used model of experimental stress, wherein rodents are exposed to a series of chronic stressors, like restraint, crowding, isolation, novelty, temperature change, light, noise and/or predator exposure (Fig. 18.5). Current studies have effectively applied CUS in zebrafish, which affects shoaling, exploration and anxiety behaviours, besides modifying brain

proteome profiles and neurogenesis (the hallmark of affective disorders in rodent models); they also demonstrate chronic stress-induced memory deficits and raised cortisol levels, similar to depression-like states in humans and rodents (Chakravarty et al. 2013).

Piato et al. determined the effects of UCS (unpredictable chronic stress) protocol during 7 or 14 days on behavioural and physiological parameters. The effects of stress were assessed in relation to anxiety and exploratory behaviour, memory, expression of corticotrophin-releasing factor (CRF) and glucocorticoid receptor (GR) and cortisol levels. UCS protocol enhanced the anxiety levels, diminished cognitive function and raised CRF whereas decreased GR expression. Furthermore, zebrafish subjected to 7 or 14 days of UCS protocol presented increased cortisol levels. This is a complementary model for learning the neurobiology and the effects of chronic stress in behavioural and physiological parameters. Additionally, this protocol is less time-consuming than regular rodent models usually used to study chronic stress. These outcomes endorse UCS in zebrafish as an adequate model to preclinical studies of stress, although further studies are warranted to determine its predictive validity (Piato et al. 2011).

Anxiolytic effects of nicotine have been accepted in studies with rodents and humans. Understanding the neural basis of nicotine-induced anxiolysis can benefit both in developing better aids for smoking cessation besides the potential development of novel nicotinic ligands for treating anxiety. Levin et al. assessed whether a zebrafish model of anxiety would be sensitive to nicotine. When zebrafish are positioned in a novel environment, they dive to the bottom of the tank. In the wild, diving could benefit to escape predation. The investigators verified the anxiolytic effect of nicotine on the novelty-elicited diving response and subsequent habituation. Zebrafish placed in a novel tank spent the bulk of time at the bottom third of the tank during the first minute of a 5-min session and later show a gradual diminution in time spent at the tank bottom. Nicotine treatment at 100 mg/L for 3 min by immersion before testing produced a noteworthy reduction in diving throughout the session, while 50 mg/L was effective during the first minute when the greatest bottom dwelling was seen in controls. Nicotine effects were reversed by the nicotinic antagonist mecamylamine given along with nicotine, but not when administered shortly before the test session after prior nicotine dosing. This suggests that the effect of nicotine on diving was because of the net stimulation at nicotinic receptors, an effect that is blocked by mecamylamine, and that once invoked, this effect is no longer dependent on continuing activation of nicotinic receptors. Nicotine-induced anxiolytic effects can be modelled in the zebrafish. This preparation will help in the investigation of the molecular bases of this effect (Levin et al. 2007). Collectively, results confirm zebrafish as a valid, reliable and model of stress and affective disorders.

18.1.3 Depression and Antidepressants

Supplementing genetic and experimental manipulations, pharmacological models are also extensively used in brain research. For example, depression-like behaviours in humans and rodents can be induced by reserpine, which diminishes brain monoamines by irrevocably blocking the vesicular monoamine transporter. The drug evokes strong pro-depressant effects in humans, besides causing hypoactivity, motor stereotypies, lethargy and anhedonia in rodent (Kyzar et al. 2001). Reserpine treatment and related neurochemical and behavioural deficits are frequently used as a model of depression in rodents but can likewise induce depression-like behaviour in zebrafish (including hypolocomotion and disrupted shoaling, resembling motor retardation and social withdrawal symptoms observed in clinical depression).

However, d-amphetamine enhances brain monoamines' levels and leads to hyperactivity and anxiety, though the effects of these agents on behaviour and in relation to monoamine levels continue to be poorly understood, compelling further experimental studies to comprehend their psychotropic action. Kyzar et al. studied the acute and long-term effects of reserpine and d-amphetamine on zebrafish behaviour in the novel tank test. Largely, d-amphetamine (5 and 10 mg/L) evokes anxiogenic-like effects in zebrafish acutely, but not 7 days later. In distinction, reserpine (20 and 40 mg/L) did not arouse obvious acute behavioural effects but evidently reduced activity 7 days later, approximating motor retardation witnessed in depression and/or Parkinson's disease. Results validate that zebrafish are exceedingly sensitive to drugs bidirectionally modifying brain monoamines, usually paralleling rodent and clinical findings. Together, this highlights the prospect of zebrafish tests to model complex brain disorders associated with monoamine dysregulation (Kyzar et al. 2001).

Interestingly, antidepressants (such as selective serotonin reuptake inhibitors, SSRIs) regulate some of the mutant phenotypes, paralleling recognized effects of these drugs in altering glucocorticoid signalling and relieving stress disorders in human patients, which also endorses the translational significance of serotonergic modulation of zebrafish stress responses (Ziv et al. 2013).

Subchronic exposure to dosages of 100 μg/L fluoxetine and 20 mg/L of ketamine reduced anxiety/depression-like behaviours, leading to upregulation of serotonin synthesis and elevated whole-body cortisol levels. These results demonstrate the possible efficacy of fluoxetine and ketamine coadministration (Pittman and Hylton 2015).

18.1.4 Psychoses and Antipsychotics

Modelling neurological diseases have demonstrated to be extraordinarily difficult because of the phenotypic complexity of each disorder. The zebrafish has become a convenient model system to study abnormal neurological and behavioural activity and holds promise as a model of human disease. The growing use of adult zebrafish in behavioural studies has generated the need for new and improved protocols.

Blazina et al. pursued to evaluate the swimming behaviour of zebrafish against a water current using the newly developed spinning task. Zebrafish were separately located in a beaker comprising a spinning magnetic stirrer, and their latency to be swept into the whirlpool was documented. The investigators categorized that larger fish (>4 cm) and lower rpm reduced the swimming time in the spinning task. There was also a dose-related decrease in swimming after acute treatment with haloperidol, valproic acid, clonazepam and ethanol, which modify coordination. Essentially, at doses that reduced swimming time in the spinning task, these drugs influenced absolute turn angle (ethanol increased and the other drugs decreased) but had no effect of distance travelled in a regular water tank. These results recommend that the spinning task is a useful protocol to complement evidence to the assessment of zebrafish motor behaviour (Blazina et al. 2013).

While most of the disease modelling using zebrafish has made use of adults, larvae too have remarkable potential for the high-throughput screening of prospective therapeutics. The additional development of larval disease models will reinforce their ability to add to the drug screening process. Ellis et al. have utilized zebrafish larvae to model the symptoms of bipolar disorder by treating larvae with sub-convulsive concentrations of the GABA antagonist pentylenetetrazole (PTZ). A variety of therapeutics that act on diverse targets, besides those that have been used to treat bipolar disorder, were tried against this model to evaluate its predictive value. Carbamazepine, valproic acid and baclofen were found to oppose several aspects of the PTZ-induced variations in activity. Lidocaine and haloperidol aggravated the PTZ-induced activity changes, and sulpiride had no effect. By equating the degree of phenotypic rescue with the mechanism of action of each therapeutic, the investigators have presented that the low concentration PTZ model can produce a number of intermediate phenotypes that model symptoms of bipolar disorder, may be worthwhile in modelling other disease states and will help predict the efficacy of novel therapeutics (Ellis and Soanes 2012).

Since psychotropic drugs affect behaviour, alterations in behaviour can be used to discover psychotropic drugs. The original exemplars of most neuroactive medicines were revealed in humans, rodents and other model organisms. Most of these findings were made by chance, but the development of behaviour-based drug discovery can be made more methodical and efficient. Fully automated platforms for investigating the behaviour of embryonic zebrafish capture digital video recordings of animals in each individual well of a 96-well plate before, during and after a series of stimuli. By merging the in vivo relevance of behaviour-based phenotyping with the scale and automation of modern drug screening technologies, systematic behavioural barcoding characterizes a means of discovering psychotropic drugs and provides an authoritative, organized methodology for unscrambling the complexities of vertebrate behaviour (Kokel and Peterson 2011).

Schizophrenia is a severe mental illness characterized by positive and negative symptoms and cognitive deficits. Decrease in glutamatergic neurotransmission by NMDA receptor antagonists simulates symptoms of schizophrenia. Modelling social interaction and cognitive impairment in animals can be of immense help in the effort to develop innovative treatments for negative and cognitive symptoms of

schizophrenia. Studies have established that these behavioural variations are, in some cases, sensitive to remediation by antipsychotic drugs. The zebrafish has been suggested as a candidate to evaluate the in vivo effects of numerous drugs and to discover new pharmacological targets. Seibt KJ et al. investigated the capability of antipsychotic drugs to reverse schizophrenia-like symptoms induced by the NMDA receptor antagonist MK-801(dizocilpine). Results exhibited that MK-801 (5 μM) administered pre-training delayed memory formation while both atypical antipsychotics sulpiride (250 μM) and olanzapine (50 μM) improved MK-801-induced amnesia. An identical change was detected in the social interaction task, where atypical antipsychotics reversed the MK-801-induced social interaction deficit; however, the typical antipsychotic haloperidol (9 μM) was ineffective to reverse those behavioural deficits. Hence, MK-801-treated zebrafish showed some behavioural features witnessed in schizophrenia, such as cognitive and social interaction deficits, which were reverted by current available atypical drugs (Seibt et al. 2011).

Additionally, these same investigators categorized the behavioural effects of MK-801 and investigated the effect of typical and atypical antipsychotic treatments on locomotors activity as well on the hyper locomotion induced by MK-801 in zebrafish. MK-801 (20 μM) increased the locomotors behaviour as measured by the amount of line crossings, distance travelled and the mean speed in the tank test after 15, 30 and 60 min of exposure. All tested antipsychotics counteracted MK-801-induced hyperactivity on all parameters analysed and at doses that, given alone, had no effect on spontaneous locomotors activity. The results recommend a similar profile between typical and atypical antipsychotics in the reversal of locomotor disorders induced by MK-801. Likewise, an anxiolytic effect was verified at 30 and 60 min of MK-801 exposure that was not reversed by antipsychotics tested in this work. Furthermore, olanzapine, which alone caused an anxiolytic response, when given with MK-801 potentiated the latter's effect on anxiety. Accordingly it validated the value of the zebrafish, a simple to use animal model, in evolving some behavioural features observed in schizophrenia, which may indicate a new approach for drug screening (Seibt et al. 2010).

18.1.5 Mood Stabilizers/Lithium

After more than 60 years from its therapeutic discovery, lithium still remains the exemplary treatment for bipolar disorder and has been extensively utilized as a mood stabilizer because of its capability to diminish manic and depressive episodes, efficacy in long-term mood stabilization and usefulness in decreasing patient suicide risks. There has been a great effort to describe lithium cellular and system actions, directing to improve treatment effectiveness and diminish side effects. In order to entirely evaluate the therapeutic and toxicological effects of lithium, diverse experimental models have been proposed.

In spite of the growing interest about the effects of antidepressants and anxiolytics on behavioural responses in zebrafish, insufficient studies have been implemented

assessing the actions of lithium on this experimental model. Studies have shown that drugs used for treatment of mood disorders, such as lithium, are able to modulate circadian rhythm. Lithium chloride (LiCl) promoted a 0.7 h lengthening of the circadian period in a specific zebrafish line, able to detect maturing and developmental activity of the circadian clock. LiCl and LiCl plus forskolin treatments promoted a significant increase in the pigmentation in zebrafish embryos, due to synthesis of melanin in neural crest-derived melanophores.

So far only an inadequate number of studies evaluated the toxicological impact of lithium ion zebrafish development and demonstrated morphological, physiological and behavioural effects that may be informative regarding human findings. Additional studies devoted to characterize and assess the underlying mechanisms of the toxic effects and the potential impact of exposure on developing and adult individuals are necessary to establish safe clinical management guidelines for women with bipolar disorder of childbearing age and safety disposal guidelines for pharmaceutical neuroactive compounds (Siebel et al. 2014).

18.1.6 Alcohol Research

Zebrafish are at the forefront of neurobiological research and have been gaining acceptance as a feasible and effective behavioural model in a range of research applications. This model turns out to be even more attractive bearing in mind the behavioural changes that follow exposure to compounds that are water soluble.

Alcohol abuse and dependence are a rapidly growing problem with scarce treatment options available. The zebrafish has become a popular animal model for behavioural neuroscience. This species may be appropriate for investigating the effects of alcohol on the vertebrate brain.

As such, numerous studies have associated both acute and chronic ethanol exposure in the modulation of zebrafish behaviour. Within this field there seems to be a common drift across multiple studies. As with many drugs, ethanol appears to influence behaviour in a dose-dependent manner (Echevarria et al. 2011).

Alcohol tolerance is often observed following chronic exposure to low concentrations of alcohol. Alcohol sensitization also called reverse tolerance (a progressive increase in the effect of alcohol over time) is frequently detected ensuing recurrent separate exposures to higher concentrations of alcohol. These two phenomena might underlie the development and maintenance of alcohol addiction. The phenotypical characterization of these responses in zebrafish may be the first important steps in establishing this species as a tool for the analysis of the molecular and neurobiological mechanisms underlying human alcohol addiction (Tran and Gerlai 2014).

Considering these efforts, the activity of adult zebrafish is normally computed using indirect activity measures that are either scored manually or identified automatically from the fish trajectory. The exploration of such activity measures has produced vital understanding into the effect of acute ethanol exposure on individual and social behaviour of this vertebrate species. Bartolini et al. have recently

established a tracking algorithm that reconstructs fish body shape to explore the effect of acute ethanol administration on zebrafish tail-beat motion in terms of amplitude and frequency. Their research demonstrate a significant effect of ethanol on the tail-beat amplitude as well as the tail-beat frequency, both of which were found to strongly decrease for high ethanol concentrations. Such a direct measurement of zebrafish motor functions is in agreement with evidence based on indirect activity measures, proposing a corresponding perspective in behavioural screening (Bartolini et al. 2015).

Luchiari et al. analysed the effects of alcohol on performance of zebrafish in a recently developed latent learning paradigm. It was found that acute exposure to 1.00% alcohols after chronic freshwater disrupted learning performance, so did exposure to freshwater after chronic alcohol treatment (withdrawal). It was also found that exposure to chronic alcohol diminished the effect of subsequent acute alcohol suggesting development of tolerance. These results demonstrate that analysis of learning performance of zebrafish allows detection of alcohol-induced functional changes. The simplicity and scalability of the employed task also imply the utility of the zebrafish in high-throughput drug screens (Luchiari et al. 2015).

Even Mathur et al. measured the effects of both acute and chronic ethanol exposure on anxiety-like behaviours in zebrafish, using two behavioural paradigms, the novel tank diving test and the light-dark choice assay. Acute ethanol exposure exerted significant dose-dependent anxiolytic effects. Nevertheless, withdrawal from repetitive intermittent ethanol exposure disabled recovery from heightened anxiety. These results validate that zebrafish display diverse anxiety-like behavioural responses to acute and chronic ethanol exposure, which are remarkably similar to these effects of alcohol in humans. Due to the accessibility of zebrafish to high-throughput screening, results advocate that genes and small molecules identified in zebrafish will be of relevance to understand how acute versus chronic alcohol exposure have opposing effects on the state of anxiety in humans (Mathur and Guo 2011).

18.1.7 Nootropics/Cognitive Enhancers

Piracetam, a derivative of γ-aminobutyric acid, exerts memory-enhancing and mild anxiolytic effects in human and rodent studies. To scrutinize the drug's behavioural profile further, Grossman et al. measured its effects on behavioural and endocrine (cortisol) responses of adult zebrafish. Generally, acute piracetam did not affect zebrafish novel tank and light-dark box behaviour at mild doses (25–400 mg/L) but produced nonspecific behavioural inhibition at 700 mg/L. No effects on cortisol levels or inter-/intra-session habituation in the novel tank test were observed for acute or chronic mild non-sedative dose of 200 mg/L. However, fish exposed to chronic piracetam at this dose performed significantly better in the cued learning plus-maze test. This observation parallels clinical and rodent literature on the behavioural profile of piracetam, supporting the utility of zebrafish paradigms for testing nootropic agents (Grossman et al. 2011).

18.1.8 Epilepsy and Antiepileptics

Rodent seizure models have considerably added to our basic understanding of epilepsy. Yet, medically intractable forms of epilepsy continue, and the essential mechanisms underlying this disease remain unclear. Baraban et al. have established that seizures can be produced in a simple vertebrate system, e.g. zebrafish larvae. Exposure to a common convulsant agent (pentylenetetrazole, PTZ) induced a stereotyped and concentration-dependent sequence of behavioural changes ending in clonus-like convulsions. Extracellular recordings from fish optic tectum revealed ictal and interictal-like electrographic discharges subsequent to the application of PTZ, which could be blocked by tetrodotoxin or glutamate receptor antagonists. Epileptiform discharges were inhibited by generally used antiepileptic drugs, valproate and diazepam, in a concentration-dependent manner. Upregulation of c-fos expression was also observed in CNS structures of zebrafish exposed to PTZ. Taken together, these results exhibit that chemically induced seizures in zebrafish demonstrate behavioural, electrographic and molecular changes that would be expected from a rodent seizure model. Consequently, zebrafish larvae signify a powerful new system to study the underlying basis of seizure generation, epilepsy and epileptogenesis (Baraban et al. 2005).

Mussulini et al. performed a detailed temporal behaviour profile characterization of PTZ-induced seizure in adult zebrafish. The behavioural profile during 20 min of PTZ immersion (5, 7.5, 10 and 15 μM) was characterized by stages defined as scores: (0) short swim, (1) increased swimming activity and high frequency of opercular movement, (2) erratic movements, (3) circular movements, (4) clonic seizure-like behaviour, (5) fall to the bottom of the tank and tonic seizure-like behaviour and (6) death. Animals exposed to distinct PTZ concentrations presented different seizure profiles, intensities and latencies to reach all scores. Pre-treatment with diazepam significantly attenuated seizure severity. Finally, the brain PTZ levels in adult zebrafish immersed into the chemoconvulsant solution at 5 and 10 μM were comparable to those described for the rodent model, with a peak after a 20 min of exposure. The PTZ brain levels observed after 2.5-min PTZ exposure and after 60-min removal from exposure were similar. Altogether, these results showed a detailed temporal behavioural characterization of a PTZ epileptic seizure model in adult zebrafish. These behavioural analyses and the simple method for PTZ quantification could be considered as important tools for future investigations and translational research (Mussulini et al. 2013).

Additionally, Gupta et al. investigated the effect of established anticonvulsants, such as valproic acid, carbamazepine, gabapentin, diazepam, lacosamide and pregabalin, against pentylenetetrazole (6 μM) seizures in adult zebrafish. Different phases of seizures (increase swim activity, rapid whirlpool-like circling swim behaviour and brief clonus-like seizures leading to loss of posture) were elicited in zebrafish on exposure for 15 min to 6 μM pentylenetetrazole. The exposure of zebrafish to an increasing concentration of the anticonvulsants alongside 6 μM pentylenetetrazole showed concentration-dependent elevation of seizure latency against pentylenetetrazole-induced seizures except for pregabalin, which failed to

produce any anticonvulsant activity in zebrafish. Additionally the proconvulsant activity of caffeine was also estimated using suboptimal concentration (4 μM) of pentylenetetrazole in adult zebrafish. Reduction in seizure latency of different phases of seizures was detected with increasing concentration of caffeine compared with its respective control group. In view of the above findings, the results of the study suggested that adult zebrafish produce the expected anticonvulsive and proconvulsive effects and could potentially be used as a screen in future epilepsy research (Gupta et al. 2014).

18.1.9 Autism Spectrum Disorder (ASD)

Autism spectrum disorder characterizes additional collection of serious behavioural deficits, affecting ~1–2% of the general population. While the prevalence of ASD is lower than depression or anxiety (which affect >10–15% of the adult population worldwide), autism causes a huge amount of human suffering, which (if expressed in patient-years, i.e. the number of patients multiplied by the length of time for which the patient suffers from the disease) signifies a pressing unmet medical need. In addition to severe behavioural and cognitive impairments, ASD is characterized by high (~90%) heritability, representing one of the most heritable brain disorders in humans (Zafeiriou et al. 2013).

Animal (rodent) models of ASD-like behaviour are widely used to study genetics, circuitry and molecular mechanisms of ASD. The evolutionarily preserved nature of social behaviour and its molecular pathways proposes that alternative experimental models can be established to supplement and boost the existing rodent ASD paradigms. The zebrafish is rapidly becoming a popular model organism in neuroscience and biological psychiatry to study brain function, model human brain disorders and explore their genetic or pharmacological modulation. Representing highly social animals, zebrafish emerge as a strong potential model organism to study normal and pathological social phenotypes, as well as several other ASD-like symptoms (Stewart et al. 2014).

Thus, as a result of over four decades of developmental biology research with zebrafish, this species has become one of the most powerful vertebrate tools and may therefore offer uniquely efficient answers to the conundrum of the developmental abnormalities associated with ASD (Cachat et al. 2010b).

18.1.10 Drug Abuse and Withdrawal

Drug-induced disorders are a significant area of biomedical research. One of the main examples is addiction, a prevalent disorder frequently associated with drug abuse. Supplementing traditional (rodent) models, zebrafish are effective translational models to study reward and drug abuse. Both larval and adult zebrafish show high sensitivity to various drugs of abuse, as well as tolerance, clear preference

(reward stimuli) for these agents and withdrawal symptoms (Cachat et al. 2010b; Stewart et al. 2011b).

Rising evidence involves the zebrafish as a promising model species for reward and addiction research. Modelling drug abuse-related behaviour in both adult and larval zebrafish produced a wealth of clinically translatable data, also demonstrating their sensitivity to numerous drugs of abuse and the ability to develop tolerance. Quite a few studies have also applied withdrawal paradigms to model the adverse effects of drug abuse in zebrafish (Collier and Echevarria 2013).

For instance, the effects of alcohol in zebrafish have been studied for more than a dozen years, revealing numerous behavioural changes in fish that resemble those seen in rodents and humans (Tran and Gerlai 2013; Cachat et al. 2013; Kyzar et al. 2012; Grossman et al. 2010). Acute alcohol reduces zebrafish fear/anxiety at lower doses, whereas higher doses induce lethargy and sedation. Additionally, chronic alcohol exposure also demonstrates parallels between zebrafish and mammals. Similarly, withdrawal from chronic alcohol exposure leads to several behavioural and physiological abnormalities that resemble anxiogenic withdrawal symptoms in humans or rodents.

Though considered as 'dangerous' drugs of abuse, hallucinogenic agents frequently have low to mild addictive properties and are of importance because of their strong psychotropic effects and potential for treating brain disorders, including depression, anxiety and post-traumatic stress (Nutt et al. 2013; Stewart and Kalueff 2013).

Strongly affecting human and animal CNS, several hallucinogenic drugs (such as serotonergic psychedelic, glutamatergic dissociative and cholinergic deliriant agents) have lately been screened in zebrafish (Neelkantan et al. 2013). These studies not only showed noticeable behavioural and physiological responses to these psychoactive drugs (similar to those in humans) but also established remarkable parallels amongst the pharmacological profiles of drugs in zebrafish, humans and rodents.

Cachat et al. have studied the effects of ethanol, diazepam and morphine and caffeine withdrawal on zebrafish behaviour. Generally, discontinuation of ethanol, diazepam and morphine produced anxiogenic-like behavioural or endocrine responses, demonstrating the utility of zebrafish in translational research of withdrawal syndrome (Cachat et al. 2010b).

Ketamine is a non-competitive glutamatergic antagonist used to induce sedation and analgesia. In sub-anaesthetic doses, it induces hyperlocomotion, impairs memory and evokes stereotypic circling in rodents. Zebrafish has emerged as a promising new animal model to screen the effects of psychotropic compounds. Reihl et al. have investigated the effects of sub-anaesthetic doses of ketamine on anxiety, locomotion, habituation and social behaviour of adult zebrafish. Acute 20 min exposure to 20 and 40 mg/L of ketamine reduced anxiety, impaired intra-session habituation, evoked circular swimming and disrupted zebrafish shoaling. Additionally, ketamine reduced whole-body cortisol levels and elevated brain c-fos expression in zebrafish. These findings demonstrate the sensitivity of zebrafish to behavioural and physiological effects of sub-anaesthetic doses of ketamine, further supporting the utility of this

species as a model for neuropharmacological research, including testing ketamine and related drugs (Riehl et al. 2011).

An indole alkaloid, ibogaine, is the principal psychoactive component of the iboga plant, used by indigenous peoples in West Africa for centuries. Modulating multiple neurotransmitter systems, the drug is a potent hallucinogen in humans, although its psychotropic effects remain poorly understood. Cachat et al. exposed adult zebrafish to 10 and 20 mg/L of ibogaine, testing them in the novel tank, light-dark box, open field, mirror stimulation, social preference and shoaling tests. In the novel tank test, the zebrafish natural diving response (geotaxis) was reversed by ibogaine, inducing initial top swimming followed by bottom dwelling. Ibogaine also attenuated the innate preference for dark environments (scototaxis) in the light-dark box test. These results support the high sensitivity in zebrafish models, dose-dependently affecting multiple behavioural domains and strongly support the developing utility of aquatic models in hallucinogenic drug research (Cachat et al. 2013).

Mescaline and phencyclidine (PCP) are potent hallucinogenic agents affecting human and animal behaviour. Kyzar et al. scrutinized the effects of mescaline and PCP in numerous zebrafish paradigms, comprising the novel tank, open field and shoaling tests. Mescaline and PCP dose-dependently augmented top activity in the novel tank test, also decreasing immobility and disturbing the patterning of zebrafish swimming. Largely, these studies indicate high sensitivity of zebrafish models to hallucinogenic compounds with complex behavioural and physiological effects (Kyzar et al. 2012).

Lysergic acid diethylamide (LSD) is a powerful hallucinogenic drug that intensely affects animal and human behaviour. Quite a few behavioural paradigms (the novel tank, observation cylinder, light-dark box, open field, T-maze, social preference and shoaling tests), as well as modern video tracking tools and whole-body cortisol assay were utilized to describe the effects of acute LSD in zebrafish. On the whole, findings in these studies show sensitivity of zebrafish to LSD action and support the use of zebrafish models to study hallucinogenic drugs of abuse (Grossman et al. 2010).

Serotonin syndrome (SS) is a severe fatal disorder linked with raised brain serotonergic function. With the growing use of serotonergic drugs, SS distresses a large portion of general population, becoming a foremost biomedical concern. SS-like behaviours have also been described in animals subsequent to the administration of serotonergic drugs. While clinical and rodent studies have provided substantial understanding into the aetiology of SS, its precise mechanisms and risk factors remain poorly understood. The necessity to develop more effective psychotropic drugs also entails extensive high-throughput screening of novel compounds using sensitive in vivo tests. The use of zebrafish in neuroscience research is speedily escalating due to their homology to humans, robust behavioural and physiological responses, genetic tractability and low costs. Overall, zebrafish exposed to serotonergic agents and their combinations exhibit a characteristic top dwelling (surfacing behaviour) and hypolocomotion which may represent potential markers of SS-like states in zebrafish. This behaviour in zebrafish models positively correlates with brain concentrations of serotonin, suggesting the developing utility of zebrafish for

studying SS. Forthcoming research is anticipated to foster high-throughput screening of drug interactions and pharmacogenetics studies identifying zebrafish mutations implicated in pathological SS-like states (Stewart et al. 2013).

18.1.11 Pain/Analgesics

Acute and chronic pain conditions are frequently incapacitating, causing severe physiological, emotional distress and economic burden and disturb a great percentage of the global population. Nevertheless, the development of therapeutic analgesic agents based primarily on targeted drug development has been essentially unsuccessful. An alternative methodology to analgesic development would be to develop low-cost, high-throughput, untargeted animal-based behavioural screens that model multifaceted nociceptive behaviours to screen for analgesic compounds. Curtright et al. have described the development of a behavioural-based assay in zebrafish larvae that is effective in identifying small molecule compounds with analgesic properties. Modelling thermal hyperalgesia, the addition of the noxious inflammatory compound and TRPA1 agonist allyl isothiocyanate, sensitized heat aversion and reversed cool aversion leading larvae to avoid rearing temperature in favour of otherwise acutely aversive cooler temperatures. The investigators have demonstrated that small molecules with known analgesic properties are able to inhibit acute and/or sensitized temperature aversion (Curtright et al. 2015).

Nociception is the sensory mechanism used to perceive indications that can harm an organism. The understanding of the neural networks and molecular controls of the reception of pain remains a continuing challenge. Mice and rat animal models have been comprehensively used for nociception studies. However, the study of pain and nociception in these organisms can be rather laborious, costly and time-consuming. Malafloglia et al. have charted the explanations why zebrafish presents a novel and attractive model for studying pain reception and responses and the most interesting findings in the study of nociception that have been obtained using the zebrafish model (Malafoglia et al. 2013).

Gonzalez-Nunez et al. have described the zebrafish opioid system, a unique experimental method to unravel the molecular mechanisms that underlie opioid activity. They have established zebrafish opioid receptors and peptides. Their expression patterns during development and in the adult organism have been identified. Additionally, their pharmacological profiles and biochemical properties have been determined. Moreover, developmental studies in the zebrafish have generated valuable information about the developmental roles of the opioid receptors. Building on these findings, it has been demonstrated that the zebrafish opioid receptors and peptides present molecular, pharmacological and biochemical profiles that are primarily comparable to those of their mammalian counterparts and from which results can hence be extrapolated to higher vertebrates. Accordingly the zebrafish represents a straightforward model to study opioid activity and can be very beneficial not only for the investigation of the complex endogenous systems that

regulate the action of opioid agents but also for in vivo tests of novel analgesic drugs (Gonzalez-Nunez and Rodríguez 2009).

18.2 Conclusion

Though similar to any model organism, the zebrafish also has its limitations. For example, a documented limitation in zebrafish genetics is the dearth of well-characterized inbred strains. Hence, forthcoming efforts may be desirable to increase the number of existing zebrafish strains and advance our understanding of the strain variances in fish neural phenotypes.

In general, no experimental animal model can target the entireness of any complex brain disorder detected clinically in human patients. Several questions remain to be addressed in future zebrafish studies.

Several significant neuropsychiatric domains were not discussed in-depth here because of their coverage by current comprehensive literature elsewhere. Similarly, aging-related psychiatric disorders and cognitive decline are getting increasing acknowledgement in clinical psychiatry and have recently been successfully modelled in zebrafish.

In summary, zebrafish models are becoming a significant tool anticipated to progress neuroscience and neurogenetics. Zebrafish sensitivity to all major neurotropic drugs and the capability to respond to them in an analogous manner as humans support their usefulness for pharmacological research.

Thus, zebrafish prove to be progressively worthwhile in translational brain research and are well matched to meet the fast developing challenges of this field.

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Pharmacological Modeling of Gastrointestinal Disorders in Zebrafish for Drug Discovery and Development

19

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Abstract

Zebrafish as a pharmacological model is gaining popularity due to its similarity in both transcriptional and developmental physiology with humans. In this chapter, zebrafish as a relevant model for gastrointestinal tract (GIT) diseases including inflammatory bowel disease (IBD) and GIT cancers is outlined. Information on the relevance of zebrafish models in cancer metastasis, host-microbe interactions, and screening of new drugs is also included in this chapter. In IBD, zebrafish as a tool is used to understand both the pathogenesis and treatment outcomes such as immune suppression or disease regression through drug screening. TgBAC, a transgenic zebrafish line, has revealed two major factors which results into IBD including depletion of epigenetic repression as well as excessive production of tumor necrosis factor. The features of human IBD disease pathology are observed in chemically induced zebrafish models, which are well established and commonly used to test the new chemical entities (NCEs). In addition, zebrafish models are also used to understand IBD immunology including expression of chemokine responses which are difficult to investigate in murine models and to understand intestinal development and GIT transit. Factors such as high fertility, low maintenance, and physiological and developmental similarities in the GIT with humans make zebrafish an efficient model to explore GIT cancers. In addition, comparative genomics to humans, transparent body, and rapidly developing embryos are prominent factors for the successful use of zebrafish models in GIT cancers.

Keywords

Zebrafish · Inflammatory bowel disease · Intestinal cancer · Host-microbe interactions · Pharmacological model

19.1 Introduction of Zebrafish as a Model for Gastrointestinal Tract Disorders: Similarities and Differences with Humans

The application of zebrafish (*Danio rerio*) being a whole animal model has been widely increased since the last decade in various research fields, starting with developmental biology as it possesses high fecundity, translucency in embryonic

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and larval stages, short generation gap, small size, ease to oversee, and genetic versatility (Flores et al. 2020). As a vertebrate it shares more similarities with humans than invertebrate research models. Further, it shares approximately 70% of genomic orthologues of transcriptional and developmental physiology of the intestinal tract with humans (Howe et al. 2013), which makes it a more interesting animal model in studies of vital operations underlying intestinal inflammation and injury (Yang et al. 2014).

The zebrafish gastrointestinal tract (GIT) exhibits biological homology with mammalians in its development, function, and organization. The exocrine and endocrine pancreas, intestine, and gallbladder along with the liver are all existing, and cellular constitution of GIT is relatable to the mammals as well. Although zebrafish GIT shares similarities in its evolution, organization, and outcome with mammals, it lacks the glandular stomach (Carten and Farber 2009). The mammalian intestine is composed of five distinct parts each with a unique function comprising of (a) the glandular stomach, which performs mechanical digestion of food; (b) duodenum, which carries out chemical digestion of food; (c) jejunum which is involved in the transport of nutrients across the lumen into the circulation (absorption); (d) ileum which carries out the absorption of bile salts into the systemic circulation; and (e) colon which transports water and salts into the systemic circulation. On the other side, the intestine of zebrafish is categorized into three histologically distinct sectors: (a) an anterior intestinal bulb which functions similar to the mammalian ileum and absorbs the bile salts (Lickwar et al. 2017; Brugman 2016; Ng et al. 2005); (b) middle intestine which plays role in the digestion of proteins and fats; and (c) posterior intestine which is functionally similar with the colon and aids in the absorption of ions and water (Wallace et al. 2005). The mammalian stomach possesses a strong acidic pH of up to 1.4 (Dressman et al. 1990), which inhibits the growth and multiplication of various microbes; in zebrafish similar function is played by various digestive enzymes including renin, nothepsin, cathepsin, and lipase (Wallace et al. 2005; Wang et al. 2010).

The development of GIT in zebrafish starts at ~18 somite stage, while in mammals it starts at ~1–2 somite stage. At mid to late (~18) somite stage, the lumen of the gut tube develops from the primitive endoderm and extends anterioposteriorly with multiplication and differentiation of endodermal cells (Wallace et al. 2005). The size of the gut lumen decreases towards the posterior which is similar to mammalians GIT development. In both systems cranial gut develops first which is then followed by posterior as well as the midgut. At 76 h post fertilization (hpf), enteroendocrine cells are present in the anterior gut epithelium which stimulates enzyme secretion from the surrounding tissue and modifies the muscular contractions and leads to the development of intestinal folds in the rostral and middle intestinal regions and initiation of peristalsis movement in the GIT (Pack et al. 1996). These intestinal folds serve the purpose of intestinal crypts present in the mammalian gut epithelium as zebrafish intestinal epithelium lacks it (Brugman 2016; Wallace et al. 2005; Wang et al. 2010).

In both mammals and zebrafish, GIT is covered with protective layer of mucus, and it is produced by goblet (G) cells (Jevtov et al. 2014; Hossain et al. 2018); the

difference lies in the location and distribution of G cells; in zebrafish these cells are distributed throughout the mucosa as it lacks the intestinal crypts, while the absorptive enterocytes occupy anterio-mid intestinal epithelium which is similar to mammals (Gebert and Jepson 1996; Rombout et al. 1985). The crypts of Lieberkühn are the embryonic stem cells which are absent in zebrafish GIT, leading to the apoptosis of epithelial cells of GIT, when the differentiation, growth, and migration of epithelial cells start at the base of folds and take 5–7 days to reach the apical region in the rostral epithelium whereas 7–9 days in the middle intestinal region (Brugman 2016). Paneth cells are absent in zebrafish, while histologically a distinct type of cells (i.e., M-like cells) is found in the ventral region of the mid intestine having vacuoles large in size serving in storage of luminal content. Histologically zebrafish GIT is composed of three layers, namely, mucosa, muscularis externa, and serosa, while in mammals it contains submucosal layer in addition to the above three layers that connect the mucosa with muscularis externa. The submucosa contains network of capillaries, lymphatic vessels, and nerve plexus (Flores et al. 2020). The enteric nervous system is simple and more easy to understand in zebrafish than in mammals, consisting of only one myenteric plexus without any clear demarcation in the type of ganglia (Wallace et al. 2005). On the fifth day of the larval stage, the GIT is completely matured and consists of the mouth, pharynx, esophagus, anterior intestinal bulb, and posterior intestine, along with the anal aperture (Ng et al. 2005; Wallace et al. 2005). Figure 19.1 shows the comparison between zebrafish and mammalian intestines. These all histological and functional similarities between the GIT of zebrafish and mammals make it a suitable model of choice for studying the GIT diseases of humans and higher vertebrates. In this chapter, we have covered the details of zebrafish being a pertinent pharmacological model to study diseases like inflammatory bowel disease, GIT cancers, cancer metastasis, screening of new drugs, and host-microbe interactions.

19.2 Zebrafish as a Tool for IBD Disease Understanding

IBD is associated with several persistent inflammatory states of the small intestine as well as colon due to an imbalance in gut microbiota and immune system. Zebrafish models have been successfully utilized to understand the IBD as the morphology, anatomy, and architecture of the human gastrointestinal system are similar to zebrafish (Ng et al. 2005). Zebrafish model has been employed to analyze the microbiota of the intestine and therapeutic approaches, along with the genes involved. Moreover, it has been revealed based on evolutionary history that zebrafish and humans share 70% of the genome (Wallace et al. 2005). Furthermore, due to their mutational and transgenic susceptibility, they are considered as a potential model to study IBD pathogenesis. The zebrafish models can be utilized to study genetics, pathology, toxicology, and screening of drugs in gastrointestinal tract-associated diseases including IBD (Hanyang et al. 2017; Ryan et al. 2013). Currently, transgenic studies are preferred to generate models for investigating a disease condition and to understand pathogenesis, and zebrafish models are used

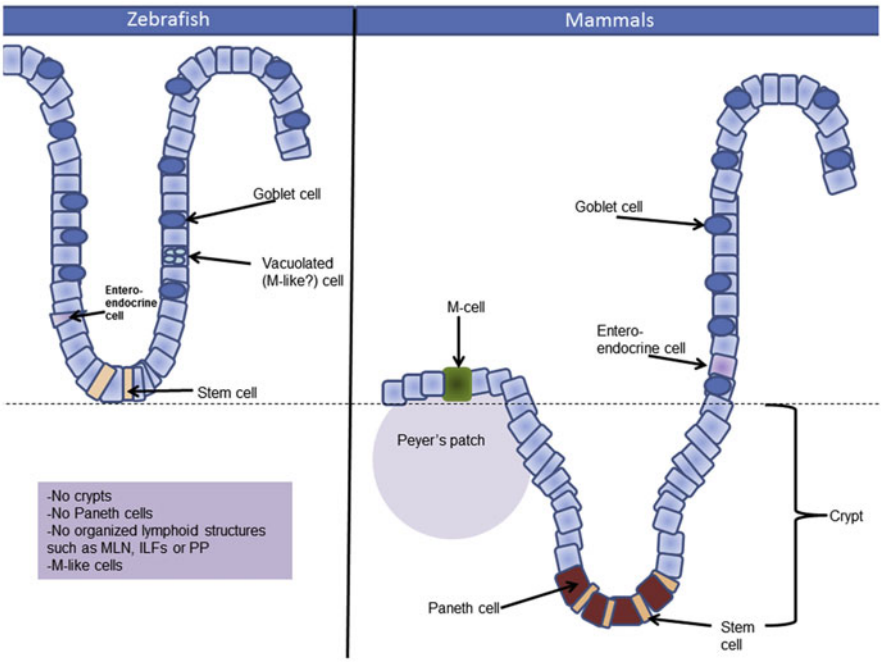

Fig. 19.1 Difference in cell types and structures between the zebrafish and mammalian (small) intestines. Zebrafish do not have Paneth cells, crypts, and organized lymphoid structures such as mesenteric lymph nodes (MLN), isolated lymphoid follicles (ILFs), or Peyer's patches (PP). (The figure was reproduced from reference (Brugman 2016) under the Creative Commons Attribution License (CC BY))

successfully to study epigenetic changes, forward and reverse genetics by exploiting high-throughput sequencing, and genome-wide association studies (Lieschke and Currie 2007; Haramis et al. 2006). The zebrafish model for screening of drug is also a leading topic because drugs can provoke highly reproducible tissue alterations (Oehlers et al. 2011a). Therefore, zebrafish is a promising model to understand IBD pathogenesis and its treatment. The main aim of using the zebrafish model is to understand and explore the IBD pathogenesis and halt the progression of IBD through screening of drugs or by suppressing the immune system.

19.2.1 Zebrafish as a Tool to Study IBD Genetic Susceptibility

To understand the mechanism of intestinal pathogenesis, zebrafish is considered an excellent model because some of the gut genes such as nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 are conserved among mammals and zebrafish (Oehlers et al. 2011b). The genomic study has detected 163 IBD susceptible regions through single nucleotide polymorphism (SNPs) in

humans. The expressed proteins in this region revealed the occurrence of injured intestinal epithelium and defense, involving epigenetic alterations, an association among host immune system and microbes and endothelium stress (Van Limbergen et al. 2014).

The study of a transgenic zebrafish line, namely, TgBAC, revealed that the loss of epigenetic repression and excessive production of tumor necrosis factor-α (TNF-α) leads to IBD (Marjoram et al. 2015). It includes a mutation in aa51.3^{pd1092}, which resulted in injured epithelium cells of the intestine. Additionally, a mutation in a conserved ubiquitin-like protein, responsible for methylation, is also marked. This mutant promotes excessive methylation at the promoter site of TNF-α leading to enhanced expression of TNF-α by unblocking the promoter site from transcriptional inhibition (Thakur et al. 2014). The increasingly expressed TNF-α induced characteristic features of microbe-associated inflammation, including apoptosis and occurrence of shredded cells of the epithelium, barrier dysfunctioning, and arousal of immune cells (Pack 2015). Hence, this study recognized a susceptible gene for IBD and influence researchers to utilize genome-wide studies in zebrafish to recognize other IBD susceptible genes.

The genome-wide study recognizes a cdipt^{hi559} mutant, which is incapable of synthesizing phosphatidylinositol (PI). Interference in PI signaling is associated with inflammation and gastrointestinal ailments. The mutant cdipt^{hi559} possess characteristics of IBD such as decreases abnormal mucus secretion, excessive microbial growth, goblet cell apoptosis, and increased infiltration of leukocytes. Additionally, upregulation of endoplasmic reticulum (ER) stress markers and acute phase genes was induced. This study also revealed an association between ER stress-mediated gastrointestinal inflammation and PI signaling (van Dieren et al. 2011). Genome-wide-associated analysis showed that SNPs close to macrophage-stimulating 1 (MST1) as well as MST1R encoding the macrophage-stimulating protein and also macrophage-stimulating protein receptor Ron are susceptible to Crohn's disease (Goyette et al. 2008). The zebrafish exhibits eosinophilia in the intestinal region and increased expression of MMP9 marker showing goblet cells dysfunctioning and inflammation.

It is known that NOD (NOD1/2) genes are conserved in the genomes of zebrafish and humans. They are responsible for coding intracellular sensory proteins of microbes that induce innate immunity. These NOD genes are highly demonstrated in neutrophils as well as epithelial cells of intestine. Moreover, the mutation in NOD1 and NOD 2 genes are strongly correlated with Crohn's disease along with ulcerative colitis respectively (Hugot et al. 2001). Moreover, to examine the effect of NOD genes functioning, morpholino oligomer (MO) knockdown models are being used. It is shown that MO knockdown of NOD genes leads to enhanced susceptibility towards microbial infections and impairing of dual oxidase gene expression which resulted in the generation of reactive oxygen species (ROS) in bacteria (Ogura et al. 2001).

19.2.2 Zebrafish as a Tool to Study IBD Immunology

Zebrafish is utilized to study IBD as it possesses several reporter genes of immune cell together with the larvae bear optical transparency. Additionally, the live imaging of zebrafish helps in studying the functioning of immune cells, pro- or anti-inflammatory cytokine generation, and association among immune cells and microbes. Various chemically and genetically induced zebrafish models were studied to investigate the pathogenicity of IBD. The chemical induction is done via trinitrobenzene sulfonic acid (TNBS), dextran sodium sulfate (DSS), or oxazolone and examined through varied morphological alterations, overexpression of cytokines, and increased white blood cell count (Oehlers et al. 2011a). The intrarectal administration of oxazolone to adult zebrafish model is associated with epithelial and goblet cell damage, infiltration of neutrophils and eosinophils, and increased impact of IL-1β, IL-10, and TNF-α (Moos et al. 2010). Similarly, intrarectal administration of TNBS in adult zebrafish model resulted in dose-dependent survival of zebrafish with no alteration in goblet cells, thickening and shedding of villi, damaged epithelial integrity, and enhanced expression of the IL-10, IL-1β, and IL-8, whereas TNBS in larvae zebrafish model can also induce dose-dependent survival; enlargement of the intestinal lumen; shedding of villi; enhancement in goblet cell; overexpression of IL-1β, TNF-α, IL-8, and MMP9; enhanced expression of TNF-α in the intestinal lumen; infiltration of myeloid cells; and modulation of lipid metabolism (Oehlers et al. 2012; Fleming et al. 2010). Figure 19.2 shows the histological features of TNBS-induced enterocolitis in zebrafish (Geiger et al. 2013).

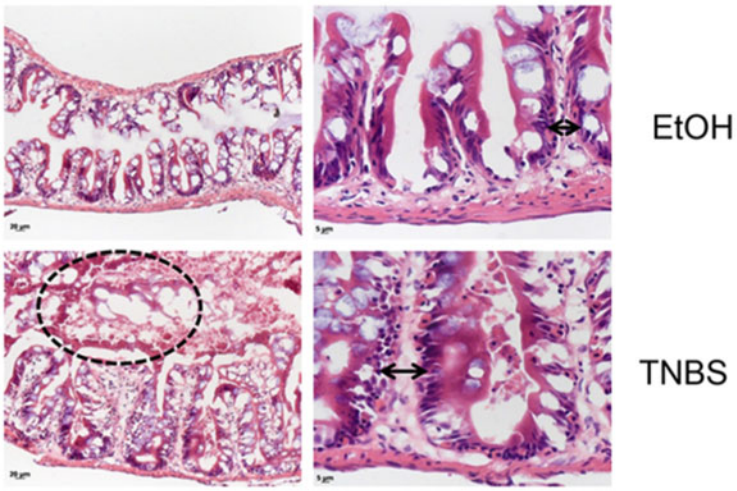

Fig. 19.2 Histological features of TNBS-induced enterocolitis in adult zebrafish. Representative H&E stained sections from TNBS- or vehicle (30% ethanol)-treated animals, at two different magnifications. *Arrowheads* indicate villi thickness in TNBS- and vehicle-treated intestinal sections. A region showing luminal sloughing of cellular debris in the TNBS-treated intestine is marked by a dotted line. (The figure was reproduced from reference (Geiger et al. 2013) under the Creative Commons Attribution License (CC BY))

Furthermore, the chemical induction of zebrafish larvae with DSS showed microbiota-associated infiltration of neutrophils; increased expression of CCL20, IL-1β, IL-23, IL-8, MMP9, and decreased PCNA; suggesting a more mucosecretory phenotype; and increased concentration of proliferating cells, while glafenine induction leads to apoptosis and ER-mediated stress (Mizoguchi 2012).

The pro-inflammatory together with anti-inflammatory cytokines are known to manage a significant balance of immune responses, but in IBD the cytokines are unbalanced. The MO knockdown is utilized to explore the IBD disease pathogenesis based on cytokine actions. Genomic studies revealed that IBD patients contain a mutant IL-23 allele. The IL-23 cytokine is responsible for innate immunity and developing Th17 cells. Also, there is a constitutive expression of IL-23 in zebrafish, and it can be induced with LPSs which in turn aids in studying innate immunity involved in IBD. Likewise, IL-10 is also conserved and is known as an anti-inflammatory cytokine whose mutation or impairment can cause IBD. The upregulation of IL-10 can be induced by LPS in zebrafish models and helps in studying inflammation in the pathogenesis of IBD (Grayfer and Belosevic 2009). In the case of Crohn's disease, increased expression of IL-22 is observed, which bears immunomodulatory functions and stimulates secretion of mucous and antibacterial protein production and promotes regeneration of the epithelium in mice. It serves a crucial role in inflammation as well as innate immunity. The loss of function in IL-22 can lead to enhanced expression of IL-1β and TNF-α in zebrafish. The knockdown studies of IL-22 can help in understanding intestinal inflammation and adaptive immunity in zebrafish (Costa et al. 2013). Apart from cytokines, several signaling pathways and inflammatory factors are responsible for causing IBD. Some of the chemokine responses are hard to investigate in murine models but can be studied in zebrafish models such as the expression of CXCL8 that promotes IBD pathogenesis (Zlotnik et al. 2006).

The heat shock proteins also have crucial roles in inflammation and immune responses of the intestine, as well as they are known to reduce the IBD pathogenesis. The expression of heat shock proteins is increased in chemically induced zebrafish models (Crawford et al. 2011). Additionally, the expression of melanin-concentrating hormone (MCH) is associated with IBD pathogenesis and is conserved in humans, fishes, and mice. The upregulation of these hormones in the intestine has been observed in zebrafish models which could help in examining the pathogenesis of IBD (Geiger et al. 2013). Furthermore, the expression of several transcription factors and associated adapter proteins responsible for IBD can be studied in the zebrafish model, which in turn helps in gaining a better understanding and examination of disease (van der Vaart et al. 2013).

19.2.3 Zebrafish as a Tool to Study Intestinal Development and GIT Transit

Coordinated contractions in the gut are a prerequisite to blend as well as propel the food via the GIT to maintain normal gastrointestinal function. Alteration or

abnormalities in these contractions deregulate the pace of substance that transits through GIT, with probably adverse consequences. In preclinical studies assessing the intestinal transit of different pharmacological agents, zebrafish-based models have been developed that offers an easy and noninvasive examination of luminal content because it passes through the gut. Prototypically, this was demonstrated in a study conducted by Field and colleagues. They mixed the larval feed with a non-digestible yellow-green fluorescent tracer which followed the bowel movement for 24 h. Together with this they also probed the role of Ret protein in gastrointestinal contraction by using a knockdown model of MO gene by using its antisense oligonucleotide. They observed a decrease in the transit speed in the antisense-treated zebrafish group in contrast to the untreated one. Similarly, Holmberg and colleagues demonstrated the effect of tetrodotoxin in zebrafish to explore its role in gut motility.

To explore the neuronal development in correlation to intestinal motility in zebrafish, Holmberg et al., using the digital motion analysis, examined the intestinal activity before starting exogenous feeding. After 3 days post fertilization (dpf), inside the gut they noticed erratic and spontaneous contraction waves, which later (at 4–7 dpf) acquired more different patterns of contraction. They observed as well as quantified the retrograde along with anterograde waves of contraction which were projecting orally and anally with the intestine, respectively. They also distinguished the local rectal contraction waves. The contractions rate of both the anterograde intestinal and local rectal significantly enhanced at the time of first few days of the growth phase. They also examined the occurrence of developing and growing neurons in the zebrafish gut by performing immunohistochemistry of HuC/HuD and HNK-1 along with acetylated tubulin proteins. Similarly several studies were demonstrated to study the GIT transit in zebrafish. To advance our knowledge and for screening new pharmacological agents for IBD and other gastrointestinal diseases treatment, zebrafish presents a high potential and valuable preclinical model. Owing to its transparency, accessibility, and ease of screening, it is a versatile and most prominently used model to study various other diseases as well.

19.2.4 Zebrafish as a Model for Pharmacological Screening of Anti-IBD New Chemical Entities: Chemical Models

Preclinically, various screening models based on the zebrafish have been developed to test the new chemical entity and to study disease pathologies. To induce the IBD in the zebrafish, oxazolone, DSS, and TNBS are commonly used. As discussed, these models cause IBD by upregulating the expression of cytokines, changing the gut morphology, and inducing leukocytosis. For the first time, the Brugman group induced the IBD model in adult wild type along with myeloperoxidase reporter transgenic zebrafish by injecting hapten oxazolone intrarectally. They successfully established the enterocolitis model as confirmed from molecular and histological findings. They observed the stiffening of bowel wall, loss of the intestinal fold architecture, and reduced goblet cell and immune cell infiltration. Moreover, in the

intestine oxazolone provoked the release of several genes which are encoding for the colitis-associated pro-inflammatory cytokines IL-1β, anti-inflammatory cytokine IL-10, and TNF-α.

Another model based on chemical induction in zebrafish was developed by submerging the zebrafish in the pre-defined media having TNBS. Moreover, TNBS induces gut inflammation as well as damages the gut normal activities in zebrafish larvae. Fleming et al. exposed zebrafish to 75 μg/mL TNBS and found changes in intestinal morphological features and altered goblet cell numbers. Similarly other group used the same dose of TNBS to induce IBD, and they noticed increased neutrophil recruitment combined with an abridged intestinal segment-ll length. This however was not associated with damage of intestinal morphology. They confirmed the development of inflammation in the intestine by estimating the inflammatory cytokines IL-1β, TNF-α, as well as MMP-9 and the extent of leukocytosis. Moreover, besides the inflammation, they also observed the peristalsis movement loss and manipulated lipid metabolism.

DSS-produced IBD is the most widely used model to study IBD pathogenesis in rodents. Similarly, to study IBD pathogenesis in the zebrafish, here DSS immersion was also used to develop an enterocolitis model. A dose of 0.5% (w/v) DSS is generally used to induce enterocolitis and intestinal mucus accumulation in the zebrafish model. After exposing the zebrafish to DSS, Oehler et al. reported the neutrophil infiltration in the intestine, reduced global cell proliferation rate, and enhanced pro-inflammatory genes including CCL20, IL-1β, IL-8, MMP-9, and TNF-α. In contrast to TNBS models, acidic mucins were evident to be aggregated mainly in the intestinal bulb, but the relative goblet cell remained unaffected. However, the redundancy of mucins plays a protecting character inside the gut epithelium during intestinal-associated inflammation and damage. The different mucin phenotype of DSS-induced IBD in the zebrafish model allowed the ability to reveal the regulation of mucins by various pharmacological agents. For instance, in the zebrafish model, retinoic acid can restrain both intestinal and basal mucus secretion, thereby aggravating the experimental enterocolitis.

19.3 Zebrafish as a Tool to Develop GIT Cancer Models

Zebrafish is considered an attractive cancer model due to its long history in the past as a cancer model. Combined with various efficient genetic tools and genomics knowledge, it is now a very suitable model for investigation of GIT cancers (Field et al. 2003). From the last few years, the literature focusing on the similarity among mammalian and zebrafish GI tract has been grown considerably. In particular, the characterization of transcriptional profiles and cell type comparison revealed that the physiology and development of mammalian and zebrafish GIT bear various likeness (Etchin et al. 2011). The transcriptional regulators controlling the intestinal development, physiology, and around 70% of human genes have been highly conserved in zebrafish and mammals. In addition, gene expression patterns, transcription regulation, and several metabolic features are highly conserved in intestinal epithelial cells

of the zebrafish as well as mammals. The GIT of both are layered by a protective mucous sheath, namely, mucin 2 (Muc2), which is a structural gel-forming component (Hason and Bartůněk 2019). The gut portion of adult zebrafish has regions that are similar to the mammalian small intestine as well as colon such as the posterior region of zebrafish gut performs water and ion absorption similar to the mammalian colon. It has various digestive enzymes that are characteristically similar to gastric markers of mammals (Taylor and Zon 2009). Additionally, various established markers of mammalian intestinal and colon like villin and fabp2 are expressed varyingly along the intestinal lining of zebrafish. These similarities among GI tract of mammals and zebrafish made them very suitable model to study cancer (Howe et al. 2013).

The zebrafish shows high susceptibility towards a wide range of chemical carcinogens and gives rise to various types of neoplasm in several tissues which are histopathologically similar to mammalian and human cancers (Field et al. 2003). Cancer modeling in zebrafish is extremely versatile and can be accessed through various approaches such as by mutating the gene and stable transgenes or by inducing transient downregulation or overexpression of certain genes. They are considered superior because to develop a cancer model in zebrafish various approaches can be utilized such as transgenics, carcinogenic treatment, and transplanting mammalian tumor cells. They are great traceable animal models for identifying an upregulating or suppressing factor in metastasis. Tumors can easily be produced in various GIT organs of the zebrafish such as the liver, intestine, and pancreas by mutating a particular gene or by stimulating signaling pathways by using chemicals (White et al. 2008). Additionally, transgenic strategy allows the production of particular kinds of tumors by overexpressing certain types of oncogenes. Zebrafish offers a novel way to understand the interaction among host vasculature and transplanted tumor cells by xenotransplantation of human or mammalian tumors into the zebrafish model. Moreover, they are also utilized for studying angiogenesis, which is crucial for the progression of tumor as well as a successful target for the antitumor treatments (Langenau et al. 2003). The vascular architecture of zebrafish shows a high similarity with the humans and swiftly generates a single blood circulatory loop at 25 hpf. The vascular endothelial cells of zebrafish may be easily marked by the fluorescent protein to observe neovascularization at the early stage in the surrounding tumor cell. Zebrafish models have also been utilized for studying tumor metastasis (Mathias et al. 2009). The fluorescently stained tumor cells are clearly visible in zebrafish which aids in examining the process of metastasis and can also be accurately traced at the cellular level. The double pigment mutant of zebrafish line, namely, *casper* zebrafish line, bears a fully transparent body in the adulthood, which is extremely beneficial in noninvasive tracking the fluorescently labeled tumor cells. Of note, the cancer stem cells consist of a very little portion of the tumor cells which are very few for feasible transplantation in a mammalian model to study metastasis (White et al. 2013). However, owing to the very small size of zebrafish, very few cancer stem cells are necessary for transplantation. Moreover, low maintenance and high fertility in zebrafish make them a great model for understanding and studying GIT cancers.

19.3.1 Zebrafish Model for Tumor Metastasis

Metastasis is a complex as well as multistep procedure where the tumor cells enter into the vascular system that spread throughout the parenchymatous tissues. Moreover, zebrafish models are successfully employed to analyze the process of metastasis as well as angiogenesis. The comparison between human and genomic sequences depicts that several genes of cell cycle, oncogenes, and tumor suppressor genes are evolutionarily conserved. Apart from comparative genomics, there are several other benefits also to model zebrafish for studying metastasis like large-scale forward genetics can be applied to these conserved pathways of cancer. The acquired immunity in zebrafish generally matures after 14 dpf, which makes suitable conditions for the endurance of transplanted tumor cells as well as metastasis (Letrado et al. 2018). Moreover, the process of metastasis can be easily examined via the transparent zebrafish body. For a better understanding of metastasis, the fluorescent labeling of transplanted tumor cells can be done, and within 48 hrs of stained transplanted tumor cells, angiogenesis and metastasis can be observed (Hason and Bartůněk 2019). In zebrafish, mainly the mutagenic screens such as cell proliferation, genomic instability, and cell differentiation are observed in transparent and rapidly developing embryos for studying cell cycle phenotypes (Payne and Look 2009). The zebrafish model offers a straightforward way to check whether a mutation generating embryonic phenotype also develops cancer in adults. Zebrafish models are highly advanced to study cancer as the growth of the tumor can be established in cell culture as well as allografts or xenografts (Baeten and de Jong 2018). The versatile benefits of the zebrafish model make it suitable for studying and understanding gastrointestinal (GI) tract tumor metastasis.

Liver cancer is leading the second place in terms of cancer-associated deaths globally. In addition, hepatocellular carcinoma counts for nearly 90% of the cases of liver cancer. Several zebrafish models have been utilized by exploiting various expression systems as zebrafish liver tumors bear molecular hallmarks similar to mammalian liver cancers (Astone et al. 2017). A liver-specific transgenic system was developed by mifepristone-mediated expression of the oncogenic $kras^{V12}$ through permanent genetic recombination via Cre-loxP system. This model will help in studying liver tumors developed from a small number of cells or single cell via clonal expansion. Overall, 20–40% of liver cancers are due to mutation in the gene coding for β-catenin (Wrighton et al. 2019). A transgenic zebrafish was developed which expresses activated β-catenin specifically in hepatocytes. This zebrafish model is utilized to screen the druggable procedures which mediate β-catenin-induced liver development which recognized two antidepressants and two c-Jun N-terminal kinase inhibitors as potent therapeutic agents (Lam et al. 2006).

Similarly, pancreatic cancer is also a fatal genetic ailment showing maximum of 5-year survival rate (Nguyen et al. 2011). The mutations in the zebrafish model to study liver cancer are presented in Table 19.1. The pancreatic cancers are majorly pancreatic ductal adenocarcinomas, and most of them are having point mutations in the KRAS gene. The KRAS-induced pancreatic cancer in the zebrafish model is generated depending on the Gal4/UAS transgenic system, in which the Gal4

Table 19.1 Zebrafish animal models of hepatocellular carcinoma

S. No	Transgene name	Expression system	Liver condition	Reference
1.	*edn1* (zebrafish)	Constitutive	Hepatocellular carcinoma	Pai et al. (2013)
2.	*Kras-G12V* (zebrafish)	Induced by mifepristone	Hepatocellular carcinoma	Nguyen et al. (2012)
3.	*Kras-G12V* (zebrafish)	Constitutive	Hepatocellular adenoma	Nguyen et al. (2012)
4.	*Kras-G12V* (zebrafish)	Inducible	Hepatocellular adenoma and carcinoma	Chew et al. (2014)
5.	*Kras-G12V* + $p53^{M214}$ (zebrafish)	Constitutive	Hepatocellular adenoma	Nguyen et al. (2012)
6.	*Kras-G12V* + *RhoA* (zebrafish)	Inducible	Hepatocellular adenoma and carcinoma	Chew et al. (2014)
7.	*Kras-G12V* + *RhoAG14V* (zebrafish)	Inducible	Hepatocellular adenoma and carcinoma	Chew et al. (2014)
8.	*Kras-G12V* + *RhoAT19N* (zebrafish)	Inducible	Hepatocellular carcinoma	Nguyen et al. (2012), Chew et al. (2014)
9.	*Myca* (zebrafish)	Induced by mifepristone	Ascites of liver tumor	Sun et al. (2015)
10.	*Myca* + *p53M214* (zebrafish)	Induced by mifepristone	Ascites of liver tumor	Sun et al. (2015)
11.	*Mycb* (zebrafish)	Induced by mifepristone	Ascites of liver tumor	Sun et al. (2015)
12.	*Src* (zebrafish)	Constitutive	Chronic inflammation and hepatocellular carcinoma	Lu et al. (2013)
13.	*Src* + *p53M214* (zebrafish)	Constitutive	Hepatocellular carcinoma	Lu et al. (2013)

transcriptional activator leads to the effects of multiple transgenes within the control of upstream activating sequence (UAS) regulatory elements (Astone et al. 2017). Zebrafish models allow the examination of various KRAS mutations and observe other involved proteins modulating KRAS expression, which can further lead to recognize mew oncologic targets. The involvement of several other signaling pathways such as Notch, TGF-β, Hedgehog, and Wnt has also been utilized based on Gal4/UAS system to study pancreatic cancers in zebrafish models (Guo et al. 2015a).

Moreover, zebrafish models were used to analyze the colorectal cancer. The zebrafish colorectal xenografts are utilized to test Guanidine alkaloids, clinically tested combinational and pineapple extract bromelain. Although gastric cancers are one of the leading cancers worldwide with regard to death rates, there are very few zebrafish models to study gastric cancers (Chang et al. 2019). Wu et al. examined

chemotherapeutic therapies of gastric cancer patients in zebrafish models. Another study was conducted for gastric carcinoma using zebrafish where a herbal formulation was proven to inhibit the growth of metastasis and xenografted cells by preventing the phosphorylation of ERK, Akt, and EGFR signaling pathway proteins (Tsering and Hu 2018). The effect of sandensolide extract was also investigated in zebrafish xenograft model in case of human oral squamous cell cancer (Roel et al. 2016). These studies favor the successful utilization of zebrafish models in studying cancer cell metastasis.

19.3.2 Zebrafish Model for Drug Screening

The zebrafish model has become a crucial model for studying drug effects. It can be well adapted for development, embryology, genetics, and cell biology studies. The zebrafish model bears important features such as low maintenance costs, easy drug administration, transparency which allows continuous examination of developing organs and cells, and a short reproductive cycle. These characteristics made the zebrafish model more suitable for drug screening and bio-assaying such as for investigating toxicity, metastasis, angiogenesis, and apoptosis (Parng et al. 2002). The choice of a suitable model is crucial to ensure the accuracy and validity of a therapeutic agent. Zebrafish can be used successfully for the screening of drugs, known bioactive compounds, or FDA-approved drugs to aid in the development of the drug discovery process and repurposing efforts for various diseases including cancers (Stoletov and Klemke 2008). Drug discovery progress is first analyzed via in vitro assays which include cytotoxicity, cell proliferation, cell activation, activation of several pathways, motility, as well as altered morphology. In a second step, in vivo screening of drug is performed in which endpoints like enhanced survival are examined (Konantz et al. 2012). The zebrafish model is advantageous as it can combine both the procedures in a single model because it is simultaneously an in vivo as well as high-throughput animal model; hence, the zebrafish model may accelerate the success rate in drug development along with reducing the cost and time (Lu et al. 2013). Zebrafish models have been utilized to evaluate metastasis, angiogenesis, and invasion. As discussed earlier, one of the most important features of zebrafish is its transparency allowing investigation of labeled tumor cells and examining the response of a particular drug molecule in vivo in a high-throughput manner. For instance, tumor cells marked with fluorescent-labeled dye, namely, CM-Dil, were injected in embryos which allowed fast examination of tumor progression, metastasis, and angiogenesis effect of any cancer drug molecule (Xiang et al. 2009). Transgenic zebrafish model, namely, *vegfr2*:grcfp, in which the expression of the green fluorescent protein is restricted only to blood vessels was utilized to screen a library of compounds having antiangiogenic activity (Lee et al. 2009). By this approach, one lead molecule, namely, rosuvastatin, was identified that can prevent the progress of intersegmental vessels in the zebrafish model (Tran et al. 2007). Two novel antiangiogenic compounds were discovered, namely, EM011 (9-bromonoscapine) and indirubin-3′-monoxime (IRO) which were not described

earlier (Karna et al. 2012). Additionally, the zebrafish tumor xenograft presents a unique tool for assessing and exploring the field of drug discovery as well as gene targeting in metastasis and tumor angiogenesis. Moreover, hepatocellular carcinoma (HCC), mainly hepatic cancer, is responsible for around 90–95% cases of liver cancer. In zebrafish models of HCC, *kras-V12* transgenic larvae induced by mifepristone when cured with MEK1/2 inhibitor, namely, PD98059, showed inhibition of liver growth in almost 49% cases (Liu et al. 2012). Similarly, rapamycin-mediated inhibition of PI3K-AKT-Mtor signaling procedure resulted in the restoration of normal liver phenotype and more potent antitumor effects in *kras-V12* transgenic larvae. Currently, liver tumors induction in doxycycline-regulated *xmrk* showed activation of downstream targets of signal transducer and activator of transcription 5 (STAT5) and mitogen-activated protein kinase 1/2 (MEK1/2) which resulted in increased tumor growth and increased apoptosis in tumor regression (Zhan et al. 2010). Exposure of zebrafish to PD98059 and nicotine hydrazide which can inhibit MEK1/2 and STAT5 showed decreased tumor progression (Liu et al. 2012). Therefore, it has been concluded that zebrafish is a versatile platform which is having a bright future in the field of drug screening.

19.3.3 Zebrafish Model for Intestinal Cancer

As discussed earlier, zebrafish is a great animal model which can be utilized for analysis of forward as well as reverse genetics of tumors, toxicology, and metastasis. Various zebrafish transgenic lines and mutants have also justified the successful application of zebrafish models to study several tumors and human diseases (Amatruda et al. 2002). Several experiments have been conducted that show maturity of tumors which can take place in wild inside all organs of zebrafish. Moreover, the histopathological results of intestinal neoplasia in humans are relatable to that of zebrafish (Dooley and Zon 2000). It has been shown that mutation in the *APC* gene leads to the human familial adenomatous polyposis (FAP) syndrome and development of colorectal cancer followed by somatic inactivation of other alleles by carriers of germline mutation. Zebrafish apc mutants have a stop codon that mimics the mutation found in FAP patients. In addition, homozygous apc mutation in zebrafish showed aberrantly developed liver, gut, and pancreas and died in 96 hpf. They also showed disorganized villi, pseudostratification of stroma, and loss of goblet cells. Moreover, the heterozygous *apc*-gene loss in the zebrafish is similar to cancer phenotype in mammals (Hurlstone et al. 2003). Similarly, a zebrafish with *k-ras* mutation is used to study tumorigenesis as expression of $kras^{G12D}$ in the posterior intestine leads into the production of intestinal tumors, and it is the same as that of human *H-RAS*, *N-RAS*, and *K-RAS* mutation-related malignancies (Nguyen et al. 2011; Bos et al. 1987). Further, tp53 zebrafish mutations are used to study intestinal tumors as homozygous dominant negative *tp53 is orthologous to cancer mutation of human TP53 cells* and combinational effects of the target gene and tp53 mutation lead to the formation of these tumors (Berghmans et al. 2005). Some of the zebrafish animal models of intestinal tumors are listed in Table 19.2.

Table 19.2 Zebrafish animal models of intestinal tumors

S. No	Gene	Mutation	Phenotype	Stage	References
1.	*mpc*1	Knockdown of mutation cluster region 1	Intestinal differentiation failed, tumorigenesis	96 hpf	Sandoval et al. (2017)
2.	*Apc*	Stop codon in the mutation cluster region	Intestinal and liver tumors	15 months	Hurlstone et al. (2003)
3.	*Apc* + *DMBA*	Stop codon in the mutation cluster region	Adenomas of the intestine	14 months	Haramis et al. (2006)
4.	$tp53^{M214}$	Point mutations in the DNA-binding domain	Adenocarcinoma and hyperplasia of the intestine	12 months	Berghmans et al. (2005)
5.	$K\text{-}RAS^{G12D}$	Heat shock protein-inducible Cre/lox	Intestinal tumors and hyperplasia	0.8–3.4 months	Le et al. (2007)
6.	*cagA*	Constitutive expression of *B-actin*	Intestinal carcinoma, hyperplasia, dysplasia, and mucosal fold fusion	12 months	Le et al. (2007)
7.	$cagA^{EPISA}$	Constitutive expression of *B-actin*	Not specific	12 months	Neal et al. (2013)
8.	*cagA*	Constitutive expression of *FABP2*	Intestinal carcinoma, hyperplasia, dysplasia, and mucosal fold fusion	12 months	Neal et al. (2013)
9.	*cagA* + $tp53^{M214}$	Constitutive expression of *FABP2*	Intestine-associated adenocarcinoma	12 months	Neal et al. (2013)

19.4 Zebrafish as a Tool to Study the Intestinal Microbiome and Host-Microbe Interactions

19.4.1 Intestinal Microbiome

Intestinal or gut microbiota is the total number of microorganisms including bacteria, archaebacteria, and fungi that are an inhabitant of host GIT. These microbiomes have various functions in mediating growth and differentiation of intestinal epithelium,

immunity development, and homeostasis of hosts' health (Semova et al. 2012; Umesaki et al. 1999; Thaiss et al. 2016). The intestinal microbiome helps in consuming more energy from food by degrading the indigestible compounds of food into simpler degrading products, thereby increasing the availability of nutrients and production of vitamins which are essential for the proliferation of gut epithelium. Bates et al. had studied and documented the part of the intestinal microbiome in the maturity as well as regulation of GIT. He studied the germ-free (GF) larvae of zebrafish which had suffered from loss of gastrointestinal motility as there is no brush border intestinal alkaline phosphatases activity, reduced number of secretory cells, and immature glycan expression, while these deficiencies and functional loss could be corrected by providing microbiota from a healthy microflora donor (Bates et al. 2006).

Host and environmental factors decide which species of the microbiome will reside in the intestinal tract (Rawls et al. 2006; Roeselers et al. 2011). Host selection pressure dominates in selecting the species of the intestinal microbiome by exerting immune response against pathogenic and not against commensals microorganisms. As zebrafish is an aquatic vertebrate, the genus of *Propionibacterium* is the dominant microbiome, whereas in mice and humans, *Bacteroides* and *Firmicutes* are the dominant microbiome. However, there are six common microbial phyla in humans and zebrafish (Bates et al. 2006; Eckburg et al. 2005; Rawls et al. 2004). The usefulness of zebrafish being a device to study human microbiome interaction along with various intestinal diseases is possible as various researchers had studied and demonstrated the colonization of zebrafish larval gut with the common human anaerobic and aerobic gut microflora including *Eubacterium limosum*, *Akkermansia muciniphila*, and *Lactobacillus paracasei*, respectively (Arias-Jayo et al. 2018; Toh et al. 2013). In recent years, demand for *Lactobacillus plantarum* as a probiotic supplement to improve mental and physical health has increased drastically and invited research studies in this sector with the use of zebrafish. Davis and colleagues had analyzed the impact of supplementation of *L. plantarum* in zebrafish larvae and observed its beneficial effect on physical and mental health through enhancing expression of gamma-aminobutyric acid (GABA) and inhibitory neurotransmitter and through facilitating the availability of serotonin in the central nervous system (CNS) (Davis et al. 2016).

19.4.2 Immune Surveillance of Zebrafish

Optical transparency in the early stages of life makes the zebrafish an interesting animal model in studies of developmental immunology. Information about immune development in zebrafish and its similarities with the mammalian immune system paves the way in the understanding of host-microbiome interaction and pathogenesis of diseases of the intestinal tract (Brugman 2016). On the third dpf, zebrafish larvae open their mouth, and at seventh dpf, the complete digestive system is functional and they start feeding on the paramecium; at this stage colonization of GIT epithelium with microbiome starts (Holmberg et al. 2007).

19.4.2.1 Innate Immunity

The system of innate immune regulates homeostasis of the microbiome. Intestinal macrophages which are phagocytic active and having a regulatory function of complement pathways have a role in maintaining the equilibrium of gut microflora (Ellett et al. 2011). It has been shown that failure of homeostasis of the intestinal microbiome in the adult zebrafish can result from *irf8* mutants as a result of intestinal macrophage damage. Also, there is a role of the microbiome in maintenance and development of immune system (Earley et al. 2018). Bates et al. had shown that the alkaline phosphatase, an enzyme involved in physiological barrier function, is secreted by brush border of the healthy gut epithelium. It denatures bacterial lipopolysaccharides and toxins secreted by various pathogens and regulates the number of infiltration of neutrophils in the intestine, while stress-induced dysbiosis leads to a more severe form of inflammation characterized by pronounced infiltration of neutrophils in the gut (Bates et al. 2007). In the early stages of life, i.e., in the larval stage, the innate immune system protects the larvae from various pathogens, whereas the adaptive immune system develops at the 4 wpf and acts predominantly in adult stage of life. All mucosal surfaces of aquatic and vertebrate organisms are colonized with diverse group microflora. Interaction of microflora with gut epithelium releases various antimicrobial peptide substances (AMPs) (Reddy et al. 2004), which are conserved between zebrafish and humans including cathelicidins, β-defensins, hepcidin, and histone-derived peptides (Fraenkel et al. 2009). Hepcidin, an iron-regulating enzyme that serves as a crucial part in the transportation of iron into red blood cells (RBCs), binds with transferrin iron transporter and inhibits the growth of *E. coli*, *Vibrio anguillarum*, *Bacillus subtilis*, and *Staphylococcus aureus* as iron is the limiting factor in their growth and multiplication (Fraenkel et al. 2009). Neutrophils of zebrafish larvae share common morphology and function with that of mammalian neutrophils which serve as the primary line of defense to the infective and inflammatory injuries. Oxidative burst and secretion of reactive oxygen species (ROS) are the microbicidal properties of neutrophils which are also noticed in teleost species (Lieschke et al. 2001; Harvie and Huttenlocher 2015). Appearance and participation of increased number of eosinophils in the gut in presence of parasites share the similarity with eosinophils of mammals (Balla et al. 2010). Also, zebrafish shares similarities in the class of toll-like receptors (TLRs); unlike mice and humans, TLR3 activation and myeloid differentiation primary response (MyD88) pathways lead to pathogenesis of various inflammatory diseases (Bates et al. 2007; Yang et al. 2017). Traver et al. demonstrated the presence of dendritic cells (DCs) along with natural killer (NK) cells that resemble precursors of mammalian DCs and NK cells and are component of and actively participating in the innate immune system.

19.4.2.2 Adaptive Immunity

Adaptive immunity in the zebrafish larvae is formulated by T cells, B cells, and the major histocompatibility complex (MHC)-1 and MHC-2 genes (Rauta et al. 2012), which protect the organism by mounting the specific immune response against every foreign antigen which becomes functional by 4–5 weeks postfertilization (wpf) (Lam et al. 2004; Langenau and Zon 2005). Primary lymphoid tissues including lymph

nodes and payer's patches are absent in zebrafish, while the enterocytes of the mid intestine are highly phagocytic and perform the function of antigen-presenting cells (APCs). Hence, we can appreciate that the lymphatic system of zebrafish shares many correspondences with that of the higher vertebrate immune system (Oehlers et al. 2011c). Up to date not much information is present on the maturity of B cells. However, Page et al. had concluded parities exist between the zebrafish and mammalian immune system as various developmental stages of pro-B, pre-B, and immature or mature B cells occur at different locations in early and late stages of life (Page et al. 2013). In adult zebrafish, intestinal immunity is mostly overlooked by B cells. Further, B cells function as APCs, which correlates between the innate and adaptive immunity (Lewis et al. 2014; Zhu et al. 2014). After oral administration of *Mycobacterium marinum* which is similar to *M. tuberculosis* in mammals, the enterocytes of the later mid intestine engulf this bacterium into the vacuoles. In parallel to the human system, most bacteria colonize the intestinal epithelium at the base, which is the same site for localization of the leucocytes (Løvmo et al. 2017). IgM and IgD of zebrafish are equivalent to the immunoglobulins of mammals. Based on all these, developmental and functional similarities of immune system with mammalian immune system make it easy to evaluate the pathogenesis of inflammatory conditions commonly observed in humans by using zebrafish as a whole animal model.

19.4.3 Zebrafish as a Model of Infectious Diseases of GIT

During early stages of life, zebrafish larvae survive by consuming *Paramecium caudatum*. Several researchers had used this *P. caudatum* as a vehicle to deliver the microorganisms causing food-borne infections in humans (Flores et al. 2019; Stones et al. 2017; Fan et al. 2019). *P. caudatum* internalizes the infective microorganisms in the storage vacuoles which are acidic. Hence, the microbes pass through a similar acidic environment of a human stomach. After the breakdown of *P. caudatum* in the anterior intestinal bulb of zebrafish larvae releasing infective organisms into the mid intestine which follows the same pathogenesis of food born infection in humans by consumption of contaminated food.

19.4.3.1 Bacterial Commensals and Pathogens

Edwardsiella and *Aeromonas* spp.

Edwardsiella and *Aeromonas* spp. are the major opportunistic pathogens of mammals (Lee and Wendy 2017). The characteristic result of food poisoning in adult humans is mild to moderate gastroenteritis, while in old and immunosuppressed persons, it leads to severe forms of diarrhea (Clarridge et al. 1980; Gracey et al. 1982). Pressley et al. found that infection of zebrafish with *E. tarda* resembles edwardsiellosis in humans. Both end with drastic mortality and development of hemorrhagic septicemia as a result of the release of pro-inflammatory mediators such as IL-β and TNF-α (Pressley et al. 2005).

Researchers are employing zebrafish model to evaluate the novel therapeutic approaches against edwardsiellosis and to know the mechanism underlying it; Guo et al. studied the vaccine while Udayangani et al. studied the efficacy of nanoparticles against antibiotic-resistant spp. of bacteria causing edwardsiellosis (Udayangani et al. 2017; Guo et al. 2015b). *Aeromonas hydrophila*, *A. caviae*, and *A. veronii* are the common etiological agents infecting humans (Clarridge et al. 1980). Saraceni et al. studied by immersing the zebrafish larvae or wounded adult zebrafish in to the medium of *A. hydrophila* and concluded that there is release of the pro-inflammatory mediators IL-β as well as TNF-α, on the localizations of intestinal epithelium by the infective bacteria. Also increased infiltration of neutrophils and dysbiosis of a healthy microbiota and increased pathogenic bacterial load further aggravate the condition (Yang et al. 2017).

Vibriosis

Vibrio cholera mainly causes gastroenteritis in humans, while *V. parahaemolyticus* and *V. vulnificus* also cause intestinal pathologies, soft tissue injuries, and bacteremia in humans. Uncooked or infected seafood is the major source of vibriosis in humans which is mainly characterized by nausea, vomiting, and abdominal cramps (Johnston et al. 1986). The application of zebrafish being a model to analyze pathogenesis as well as therapeutic approach provides valuable information as vibrios are the commensals of zebrafish (Runft et al. 2014). In their study, they developed vibriosis in adult zebrafish by immersion method and observed the development of cholera toxin-independent diarrhea with notably increased production and excretion of mucin.

19.5 Summary

This chapter outlines the use of zebrafish models for IBD, GIT cancer, and host-microbe interactions. The existing similarities among zebrafish as well as humans with regard to both genetic structure and immunological features make them superior models as compared to other intestinal inflammatory models. Transgenic zebrafish models offer distinct features, including specific tumor induction by overexpressing certain types of oncogenes. Pathogenesis of various inflammatory diseases can be studied using zebrafish owing to its similarities with mammals in having diverse microflora, common neutrophil morphology, similar eosinophils appearance and participation in presence of parasites, and similar toll-like receptors (TLRs). The low maintenance costs are one of the important features of zebrafish models, which make these experimental tools highly interesting for researchers. However, there is the need for skilled personnel, and intensive caring of respective models is challenging.

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Zebrafish Models for Liver Diseases

20

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Abstract

Zebrafish (*Danio rerio*) emerged as a leading model in designing diverse mammalian diseases. It shares high resemblance in genotypic modalities with human. Despite difference in structural aspects, functionality of human liver is considerably consistent with that of zebrafish. It elicits comparable signalling cascade post chemical insult to liver and is highly conserved in genetic makeup with human. External fertilization, transparent embryo-larva and rapid ontogenic process make it suitable to study developmental defects in liver of zebrafish and its various regulatory pathways. Zebrafish model is now gaining popularity over rodent models in drug screening and basic research due to the advantage of smaller size, ease of handling, low cost of maintenance, relatively less ethical issues and increasing understanding of its genome. This chapter discusses about the history of zebrafish research in liver diseases, a comparative account of structural similarity and differences of human and zebrafish liver and possibilities of designing diverse liver pathological conditions in zebrafish. The liver pathogenesis due to

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various chemicals, polycystic conditions, genetic mutations, gene transfer and cancers have been discussed. The advantages, limitations and future prospects of zebrafish in liver disease modelling are also discussed.

Keywords

Chemical-induced liver damage · Model organism · Genetically engineered model · Hepatocellular carcinoma model · Zebrafish model

20.1 Introduction

The liver is the largest visceral organ and secretory gland, exocrine as well as endocrine, of the human body. It performs metabolic, synthetic, storage and secretory functions. It plays central role in metabolism of endogenous biochemicals like hormones, enzymes, etc. and exogenous molecules like nutrients, drugs and toxins (detoxify). It metabolizes molecules and transforms into water-soluble form, thus facilitating their excretion via the kidney. It synthesizes vital molecules including clotting factors, plasma proteins, transport proteins, cholesterol, phospholipids, glycogen and hormones and activates vitamin D. It stores glycogen, vitamins and minerals. It secretes bile and some hormones like insulin-like growth factor and thrombopoietin. It plays role in RBC synthesis and breakdowns as well as serves as site for blood storage. It is a complex organ and is considered to play more than 500 different functions in the human body. Due to central function in multiple processes, the liver is highly liable to get diverse challenges that lead to various diseases and disorders caused by aberrant development and metabolism, infection, immune mediated and neoplasm. As substituting all these functions is not yet possible by an artificial liver and there are a number of limitations in the availability of the liver for transplantation, finding novel therapeutic approaches becomes of prime importance.

In search of precise mechanism of liver disease, a number of in vitro, ex vivo and in vivo models have been designed. Based on the data through these models, in silico predictive software were also developed. The rodent model has considerable advantages due to highly conserved genetic makeup with human. However, high-throughput screening is difficult with mammalian models due to longer duration of time required to generate large population, difficulty in manipulation in embryo as it develops in the uterus, strain-specific diverse physiology and cost of housing and management (Wilkins and Pack 2013).

The research on development of zebrafish liver disease model was founded early in 1937. A detailed timeline of development of zebrafish liver disease model is given in Fig. 20.1.

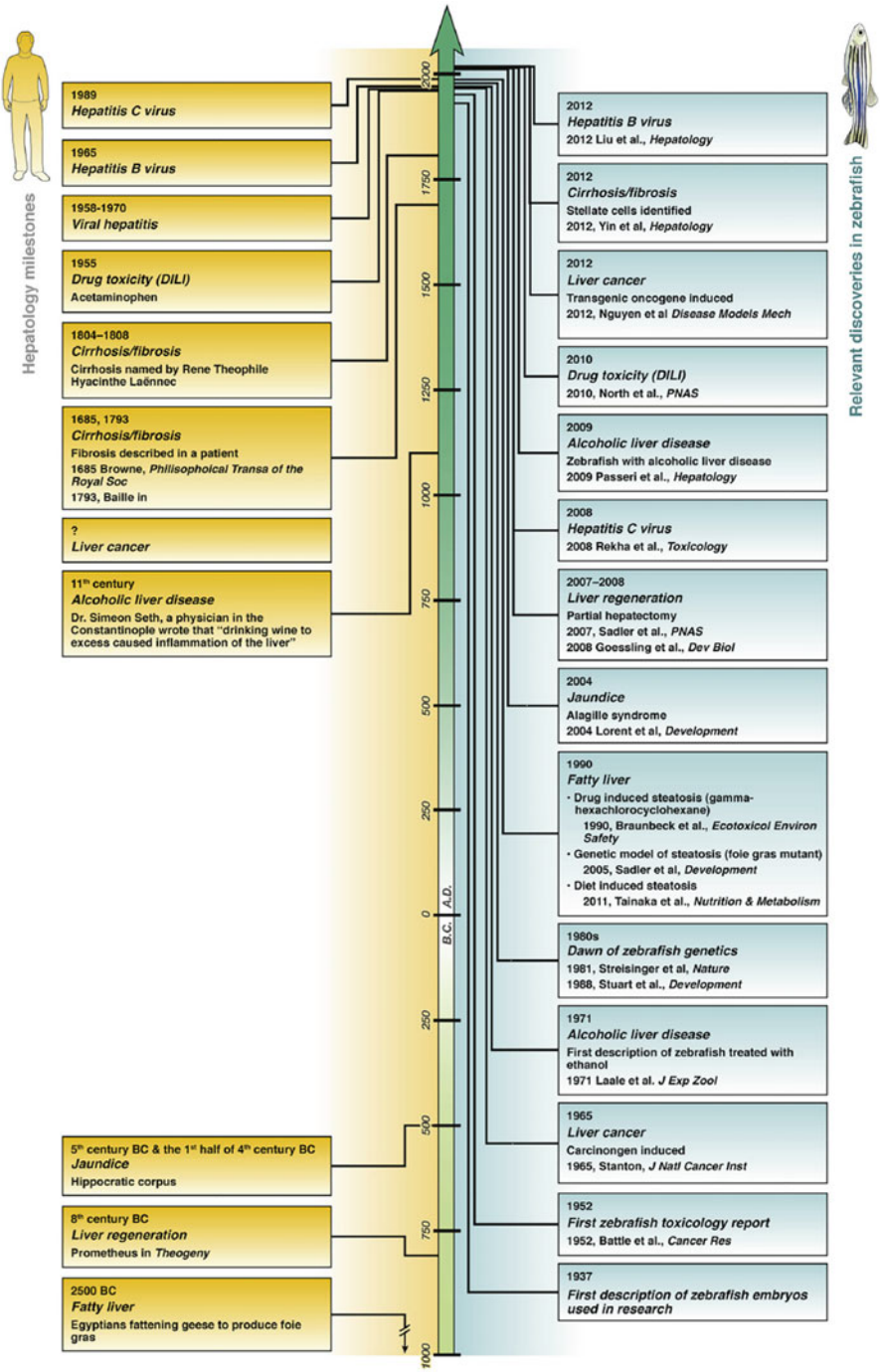

Fig. 20.1 Timeline of hepatology advances in zebrafish research. Major milestones or historic events in human liver pathology are indicated in yellow, and corresponding milestones in zebrafish research are indicated in green. The timeline indicates years B.C. and A.D (Goessling and Sadler 2015)

20.1.1 Comparative Anatomy and Physiology of Zebrafish Liver

The zebrafish liver performs similar functions as of human. It has high conserved genetic pattern as of mammals. The gene expression profile after challenging with hepatotoxic material have similar pattern in in vitro and in vivo model of human, rodent and zebrafish (Cassar et al. 2020). Despite its unique anatomical arrangement of the liver, zebrafish has similar physiological processes as of human and other mammals. Anatomically, zebrafish liver has highly conserved cell types with mammals except the hepatic stationary macrophages, i.e. Kupffer cells which is absent in it. The zebrafish liver is architected in three lobes (one ventral and two laterals) without pedicle, which separate lobes in the mammalian liver. Instead of the portal triad structure of the human liver, in zebrafish liver, the hepatocytes are arranged in tubular structure separated with intercalated bile ducts and blood vessels. There is no specific pattern of distribution of blood vessels in it including central hepatic and portal vein. These vessels are lined by endothelial cells (Goessling and Sadler 2015). The bile ducts consist of two types of cells, first, small pre-ductal biliary epithelial cells which are present in the intracellular lumen and second, intrahepatic biliary canal-forming cells called large columnar cholangiocytes (Katoch and Patial 2021). The bile ductules are located centripetally with hepatocytes forming tight junctions with hepatocytes to prevent leakage of bile. It further merges to form intrahepatic bile duct and cystic duct which finally leave the liver from the hilum to pour the secretion into the gallbladder. The common bile duct pours it into the intestine. The sinusoids are present at the basal side of the hepatocytes at the periphery of the tubules. Hepatic stellate cells store lipid droplets and interspersed between endothelial cells and hepatocytes (Pham et al. 2017). It is described as myofibroblasts which get activated on hepatic injury, and to initiate repair process, extracellular matrix is secreted (Goessling and Sadler 2015) (Fig. 20.2).

20.1.2 Liver Development and Maturation in Zebrafish

The origin of the zebrafish liver cells is from the foregut endoderm same as that of mammals. The development of zebrafish liver takes place in three phases: (1) specification, (2) differentiation and (3) growth.

The first phase of zebrafish liver cells development starts after 22 h post-fertilization. The progenitor cells develop initially that further develops hepatoblasts, the precursor of hepatocytes. At the same time, from the anterior lateral part of the foregut endoderm, the expression of transcription factors starts.

It expresses as follows:

1. Haematopoietically expressed homeobox (hhex) which contributes in regulation of specification and growth of the hepatopancreatic ductal system (Villasenor et al. 2020), along with some other genes that govern morphogenesis of the hepatopancreatic duct (HPD) system and digestive tract systems (Gao et al.

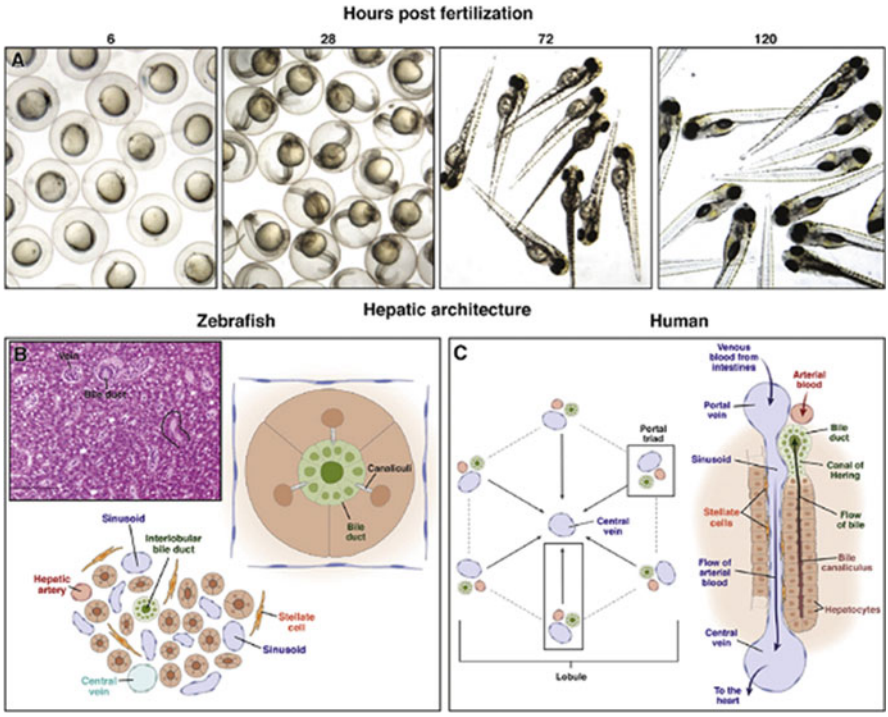

Fig. 20.2 Comparative anatomy of human and zebrafish livers. (**a**) Live zebrafish at 6, 28, 72 and 120 h post-fertilization show that a large number of synchronously developing larvae can be easily cultured. Cellular anatomy and architecture of the liver in (**b**) zebrafish and (**c**) humans. (**b**, inset) A histological section of adult zebrafish liver stained with H&E (Goessling and Sadler 2015)

2019)**,** regulate signalling of VEGFC/FLT4/PROX1 to control blood and lymphatic vessel formation (Gauvrit et al. 2018) and regulate thyroid growth and differentiation (Elsalini et al. 2003).

2. Prospero homeobox 1 (prox1) regulates hypothalamic catecholaminergic neurons development; the sex-determining region Y (SRY)-box 32/17 (SOX 32/17), GATA 4–6 and forkhead box A (FOXA) regulate maintenance and specifications of endoderm (Shin 2005; Tiso et al. 2002).

The differentiation phase starts at 24 hpf and lasts up to 50 hpf. It starts with left side bending of the foregut. At 32 hpf, the earliest cells of the liver express ceruloplasmin. It proceeds with differentiation of hepatoblasts into hepatocytes, i.e. hepatic and intrahepatic biliary cells which further form biliary ducts. At 48 hpf, liver fatty acid-binding protein (L-FABP) and transferrin were expressed (Her et al. 2003). Between 3 and 5 dpf, the growing liver completes the development of the network of bile excretion by hepatocytes growth and differentiation. The keratin 18, a type of cytokeratin, is expressed at the stage of biliary cell maturation.

The growth phase starts at 50 hpf and lasts till juvenile stage. It is characterized by proliferation and polarization of hepatocytes, hepatic vasculature growth and development and expansion of biliary system. From 55 to 72 hpf, endothelial cells are essential for the polarization of adjacent hepatocytes and the promotion of development of biliary system (Field et al. 2003).

20.2 Liver Disease Models of Zebrafish

The liver anatomy and physiology can be altered by various methods in zebrafish. The liver disease models are very similar to rodent models, and similar test compounds can be dissolved in water of aquarium or injected directly in zebrafish. This can adversely affect the hepatocytes of zebrafish by altering its external physical parts and mitochondrial and endoplasmic damage ultimately leading to oxidative stress in cellular organelles (Katoch and Patial 2021).

20.2.1 Chemical-Induced Liver Cirrhosis and Necrosis Models

20.2.1.1 Fatty Liver Disease Models

Alcohol Induced

Excess consumption of alcohol leads to alcoholic liver disease by increase lipid accumulation in hepatocytes, and chronic exposure may contribute in the development of hepatocellular carcinoma (Passeri et al. 2009). Exposure of 2% ethanol to 4 dpf zebrafish larva promotes development of abnormal enlargement of the liver, overexpression of genes involved in hepatic metabolism and alteration of metabolic state leading to accumulation of excess fat droplets. Activation of the transcription factor family sterol regulatory element-binding proteins (SREBPs) found to play a key role in development of hepatic steatosis post alcohol exposure in zebrafish larvae. The SREBP is reported to modulate biogenesis of lipids specifically cholesterol, triglycerides and fatty acids. The inactive SREBP is taken from ER to Golgi complex by Scap (escort protein), where it is cleaved by MBTPSs protease (membrane-bound transcription factor site 1 and membrane-bound transcription factor site 2). Inactivation or knocking out of any of these leads to failure of development of alcohol-induced hepatic steatosis. In addition to it, many genes related to lipogenesis and lipid intake were found to be markedly overexpressed (Passeri et al. 2009).

Non-alcoholic Fatty Liver Disease (NAFLD)

The fatty liver caused by alcohol consumption is related to high-fat diet, resistance to insulin and altered metabolism. This causes excess accumulation of fats in hepatocytes, liver cirrhosis and fibrosis that may progress to hepatocellular carcinoma (Willebrords et al. 2015).

Overfeeding-Induced NAFLD

Fatty liver is observed in a 5 dpf zebrafish larva on feeding 180 mg/day for 10 days. The larvae were found to develop hepatic necrosis and formation of lipid vacuoles in hepatocytes with steatosis of vasculature and high glucose, cholesterol and triglycerides level. The study was supported by modulation of expression of various genes related to glucose metabolism, fat metabolism, inflammation, lipid peroxidation, oxidative stress and ER stress. The genes related to lipid and glucose metabolism were suppressed and suggested inhibition of endogenous lipid formation on overfeeding (Ma et al. 2019).

High Fructose Diet-Induced NAFLD

On exposure to 4% sucrose from 5 to 7 dpf, zebrafish larvae were observed with high inflammation and oxidative stress in ER and develop high lipid accumulation (Sapp et al. 2014). Similar results were obtained by exposing adult zebrafish to 6% fructose (Ferrari et al. 2018). High fructose diet leads to overexpression SREBP-1c that stimulates ACC level which increases production of free fatty acids along with FAS to induce enhanced production of triglycerides. The process contributes in the development of steatosis. The chronic exposure leads to induce inflammatory cytokines including TNF alpha, IL-6, IL-1 beta and TGF beta that activate stellate cells and promote liver fibrosis (Katoch and Patial 2021).

High-Fat Diet-Induced NAFLD

The development of lipid globules in hepatocytes can be induced in a 5 dpf larva by feeding on diet with high fat content (30 mg/day) for 7 or 10 days. The overexpression of the genes related to lipolysis, ER stress and inflammation was observed. The severity of hepatic damage is dose dependent (Dai et al. 2015).

High Cholesterol-Induced NAFLD

Zebrafish larva of 8 dpf when fed on diet with 5% cholesterol developed high mortality rate. It was observed with steatosis in hepatic cells and macrovasculature, enhanced generation of reactive oxygen species and oxidative stress with altered level of SOD and MDA. Genes responsible for lipid production, inflammation, oxidative stress and fibrosis were found to be overexpressed while genes related to lowering of lipid were underexpressed (Ma et al. 2019).

20.2.1.2 Drug-Induced Liver Injury Model

As one of the human vitals, the liver is an important organ to be studied in drug discovery and development process. Damage to the liver is a leading cause of rejection of molecules to proceed for further stages of drug discovery process. The adverse event with liver can be assessed based on the pathological presentations in histopathological studies and/or liver enzyme levels which are comparable to mammalian system. This also provides information of interaction of drug with the liver at different stages of development (Vliegenthart et al. 2014).

Paracetamol

Administration of paracetamol beyond its therapeutic dose may lead to production of high level of its toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). Higher plasma level of this metabolite depletes glutathione in hepatocytes and leads to oxidative stress which resulted in hepatocytes damage (Vliegenthart et al. 2014).

The 3 dpf larva of zebrafish was utilized for this study. Various amounts of paracetamol were dissolved in aquarium to attain concentration 1000, 2000 and 5000 μM. The larva was allowed to expose in the stipulated concentration till the stage of 6 dpf. Severe necrosis of hepatocytes was observed after dose of 5000 μM (Pandya et al. 2015).

In adult zebrafish, liver toxicity is presented with hepatic necrosis, haemorrhages and sinusoids widening at dose level of 10 mM for 24 h (Swarnalatha 2017).

Valproic Acid

As a side effect at its therapeutic dose for epilepsy, valproic acid causes hepatocyte damage. It leads to severe fatty liver and vacuolization of hepatocytes after administration of dose 67, 200 and 600 μM in 3 dpf zebrafish embryo and adult zebrafish for 48 h (Driessen et al. 2013).

20.2.1.3 Antibiotic-Induced Liver Injury Model.

Mitoxantrone

It is used in therapeutics for its anticancer effect. For the study, the embryos were exposed to 100 μg/L. Post 48 h exposure, the hepatotoxicity biomarkers were unregulated, viz. gclc, gsr and nqo1, though fab10a downregulated. Post 72 h of exposure, it induces formation of lipid vacuoles, chromatin condensation, swelling and irregularity in arrangement of hepatocytes. The presence of histopathological abnormalities and altered gene expression related to oxidative stress and detoxification after exposure to mitoxantrone further confirmed its hepatotoxic activity (Liu et al. 2018).

Tetracycline Hydrochloride

Tetracycline hydrochloride is one of the earliest well-known antibiotics. At dose higher than 583.8 μM, it is lethal to zebrafish. Post 72–120 hpf exposure to zebrafish embryo at lethal concentration resulted in non-transparent dark-coloured embryo. It inhibits the liver and decreases its size along with delay in yolk absorption. It degenerates the liver at the rate of 13.5–37.5% (He et al. 2013).

Erythromycin

Erythromycin is a very popular antibiotic for the treatment of infections especially of respiratory tract, nose, ear, throat, skin and lungs. It is non-lethal at its solubility level to zebrafish. At dose of 5000 μM, erythromycin has similar toxic effects as of tetracycline. The liver degeneration rate of erythromycin is 11.3–46.3% (He et al. 2013).

20.2.1.4 Preservative-Induced Liver Injury Model

Bisphenol A (BPA)

BPA is classified as an endocrine disruptor which also damages hepatocytes in zebrafish. It is utilized in the synthesis of plastics used to manufacture containers for preservation of food items. It can leach in food items and leads to toxicity after consumption of such food material.

Post 60 days exposure of 100 μg/L BPA, zebrafish hepatocytes were observed with accumulation of lipid and loss of glycogen. The body mass index was found to be increased. The *ppar*-gama and CCAAT/enhancer-binding protein alpha found to be modulated post BPA administration which are involved in regulating lipid metabolism (Ngo et al. 2017).

Bisphenol S (BPS)

It is used as a substitute to bisphenol A in manufacturing of container for preservation of food. In male zebrafish, it has been observed to inhibit the transcription of SREBP1 and ppar-alpha, the lipid metabolizing genes, when exposed to BPS at dose of 1000 μg/L for 120 days. This resembles the non-alcoholic-type chronic exposure-induced fatty liver disease. On exposure to females, the offspring from them were observed with higher amount of yolk lipid accumulation (Wang et al. 2019).

20.2.1.5 Pesticide-Induced Liver Injury Models

Tricylazole

It is an agricultural fungicide. The embryo of the transgenic strain of zebrafish Tg (fabp10a:DsRed) was exposed to 25 mg/L tricylazole. The liver is adversely affected by it and found to be of smaller size with unevenly developed hepatic regions, and absorption of yolk sac was inhibited. Anti-apoptotic genes (bcl2) and cholangiocyte (marker sox9b) differentiation were inhibited. Induction of pro-apoptotic gene (bax, capspase-3, tnf alpha, P53) was noted (Qiu et al. 2019).

The adult transgenic strain of zebrafish Tg (fabp10a:DsRed) was exposed to 10 mg/L tricylazole. The resulted effect observed was complete depletion of reserved glycogen, high blood glucose level, low triglycerides and high cholesterol. Along with the fatty acid-transporting genes, genes responsible for the production of enzymes for carbohydrates metabolism are found to be downregulated, and insulin, fatty acid and prolactin production genes are found to be upregulated (Qiu et al. 2019).

20.2.1.6 Carcinogen-Induced Liver Injury Models

Diethylnitrosamine (DEN)

It is a carcinogen of 2A class. The larva of zebrafish 72 hpf is exposed to 100 μg/mL and 22 h post exposure ApoFlamma H 675 (fluorescent dye) for 2 h and examined. The increase in intensity indicated hepatocellular damage and death due to necrosis which was further confirmed by histopathological studies (Kang et al. 2013).

Thioacetamide (TA)

It is a carcinogen of 2B class. On exposure to TAA for 72 h to 3 dpf, larvae induced hepatocytic accumulation of fat globules affecting nucleus. The adipogenesis was upregulated along with genes related to fibrosis. It inhibits anti-apoptotic genes and induces the apoptosis-inducing genes (Rekha et al. 2008).

Thioacetamide induces hepatic steatosis in zebrafish larvae. Thioacetamide 0.025% exposure from 3 to 5 dpf leads to enhanced accumulation of lipid globules and apoptotic gene upregulation (jnk-1, Caspase-3 and 8, bad, p-38a, bax) resulting in liver damage (Goldsmith and Jobin 2012).

20.2.1.7 Heavy Metal-Induced Liver Injury Models

Arsenic

The arsenic is a carcinogen of A class. It mainly causes damage in DNA and proteins of hepatocytes. On exposure of arsenic for 96 h to a concentration of 192 μM, liver fragility increases with time. It resulted in depletion of intracellular glycogen, cellular swelling, abnormality in cell shapes, enlargement of cell nucleus, breakage of chromatin, nuclear fragmentation and dissolution (Lam et al. 2006).

20.2.1.8 Mycotoxin-Induced Liver Injury Models

Aflatoxin

A family of toxins called aflatoxin is produced by the fungi *Aspergillus parasiticus* and *Aspergillus flavus* which mostly grow on agricultural crops like cotton seed, maize, peanuts, etc. It is a known carcinogen and hepatotoxic agent (Zuberi et al. 2019). The aflatoxin (AFB1) 0.15 μg/μL (in 10 μL volume) was injected intraperitoneally to 2.75 mpf transgenic zebrafish Tg(1-fabp: HBx-Cherry) and Tg(1-fabp: GFP-mCherry). Post 2.25–3 months of injection, various factors related to cell division and lipid production were observed to be enhanced. Post exposure at age of 3–5.75 mpf, fish were found to be swollen due to membrane damage and influx of fluid in cells leading to cytoskeleton damage by extracellular membrane fibrosis. There was infiltration of liver cells with lipid that leads to alteration in metabolism and enhanced glycogen accumulation ultimately leading to apoptosis altering expression of various genes related to lipogenesis and cell cycle (Lu et al. 2013).

Ochratoxin

Various *Aspergillus* fungi chiefly *Aspergillus carbonarius* and *Aspergillus ochraceus* and some *Penicillium* fungus chiefly *Penicillium verrucosum* produce a group of mycotoxins called ochratoxin. It naturally contaminates agricultural products like grains, dried foods and cereals. It is a known teratogenic chemical affecting the liver of the offspring.

At 0.5 μM concentration, embryo of zebrafish exposed till 96 hpf twofold inhibited the coagulation cascade-related gene expression indicating alteration in liver synthetic function. It limits the expression of marker genes of liver characteristic with shrunken cytoplasm, vacuolization and peripheral nucleus (Wu et al. 2018).

20.2.2 Polycystic Liver Disease Model

The biliary epithelial is smooth in a normal liver, but in polycystic liver disease, the liver epithelial is having fluid-filled sacs, which progressively increase in size, throughout the liver. It increases the size of the liver and pushes the surrounding organs which lead to compromise of their functions. The pathological presentations include the bile duct system inflammation, fibrosis, cirrhosis and rarely cancers. The origin of this disease is in genetic mutations in SEC63 and PRKCSH genes which encode for glycoprotein translocator protein of ER. By antisense morpholino oligonucleotides, SEC63 and PRKCSH gene knockdown hepatic cyst models have been demonstrated (Tietz Bogert et al. 2013).

20.2.3 Genetically Engineered Model of Liver Disease

20.2.3.1 Transgenic Models

Transgenic model of zebrafish is a genetically engineered zebrafish to observe the specific gene or its protein product functionally in disease condition. It allows to study the genetic origin of certain pathological conditions as well as provides cellular-level understanding of pathological processes and its modification by drug administration. It gives a flexibility to study the action of a drug for specific pathological condition and study the specific molecular mechanism (Wrighton et al. 2019).

To screen the possible drug molecule for treatment of liver disease, various genes can be inserted in genome of zebrafish, and specific pathological conditions can be imparted. At various levels of liver development and maturation, various genes play regulating role. These genes can be modulated to achieve specific pathological state of liver cells (Chu and Sadler 2009).

The oncoprotein Gankyrin is having a regulatory capacity of cell cycle by interacting with CDK4. Gankyrin, found to be highly expressed in hepatocellular carcinoma, is structurally an alpha helical series of ankyrin repeat (33-amino acid). The overexpression of it in a transgenic zebrafish unregulated the genes related to fatty acid translocation-membrane transporters, fatty acid-binding protein and lipid metabolism. The adipogenic gene upregulation by engineering zebrafish through hepatitis B virus X protein (HBx) resulted in fatty liver (Her et al. 2011).

Through the mutation in fibroblast growth factor (FGF) receptor, regulation of liver homeostasis can be dysregulated. The resulted transgenic zebrafish develop severe hepatic steatosis (Zaret and Grompe 2008; Jung 1999).

Treatment with mifepristone for 4 weeks leads to constitutive expression of kras (V12) in a transgene zebrafish which develops liver tumour (Nguyen et al. 2012).

Various zebrafish models engineered with transgene were developed for various liver diseases, for example, hepatic steatosis was induced in Tg (lfabp:dnfgfr1-egfp) by dominant-negative fibroblast growth factor (dnfgfr1) (Tsai et al. 2013), in Tg (hand2:EGFP) by hepatic stellate cells (Yin et al. 2012), in Tg(−2.8fabp10a:gfp-YY1) by ying yang 1 (yy1) (Her et al. 2013), etc.

20.2.3.2 Mutant Models

Mutation is change in nucleotide sequence of genome. A mutant is an organism harbouring that altered characteristic. By altering the genetic makeup, zebrafish can be induced with liver pathologies. Mutation in various proteins/genes like stk11 (van der Velden et al. 2011), slc16a6a (Hugo et al. 2012) and guanosine monophosphate synthetase (Nussbaum et al. 2013) may lead to hepatic steatosis. One of the initial mutant models reported was gene-encoding Fgr protein mutation that leads to hepatic steatosis. Cannabinoid receptor mutation (Liu et al. 2016), vacuole membrane protein 1 mutation (Kim et al. 2015) and electron transfer flavoprotein mutation (Kim et al. 2015) also may lead to hepatic steatosis.

A mutation of frame shift type and stop codon generation was observed due to insertion of a trap cassette of 172 bp gene between intron of 11 and 12 exons foie gras (foigr) gene. This resulted in accumulation of large fatty globules and apoptosis in hepatocytes in 5 dpf larvae. The apoptosis was due to ER stress, and the UPR gene, atf6, regulated it up to some extent (Goldsmith and Jobin 2012).

These models avail specific advantage of altering any specific gene to understand its role in any physiological and pathological process.

20.2.4 Hepatocellular Carcinoma Model

The modelling of hepatocellular carcinoma can be done by modulating genes related to primarily Wnt signalling pathway and Ras/MAPK signalling pathway. By alteration in beta-catenin (CTNNB1) signalling, axin (Villanueva and Llovet 2011), xmrk, kras (V12), human RAF1, mutation in UHRF1 (Mudbhary et al. 2014), telomerase reverse transcriptase (TERT) (Lee 2015), tumour protein 53 (Farazi and DePinho 2006), hepatitis B x antigen (HBx) (Levrero and Zucman-Rossi 2016), HCV core protein (HCP) (Rekha et al. 2008), NS3 and NS5A hepatocellular carcinoma can be induced. Some hormones like 17 beta estradiol can increase the size of the liver, and nutritional deficiencies like of selenium in cirrhotic cases may lead to HCC (Wilkins and Pack 2013; Wrighton et al. 2019).

Easy observation of proliferation of liver cell and angiogenesis and tumour implant observation in transparent embryo and larva are the added advantages of zebrafish HCC model.

20.3 Limitation and Advantages of Zebrafish

20.3.1 Advantages

Zebrafish offers many advantages over other animal models. The genome of zebrafish is fully sequenced thus having good feasibility to easily manipulate it genetically (Shin 2005). It has the ability to fertilize externally, produce abundance of offspring (clutch size, 100–200 eggs/spawning) and have transparent embryo-larva to easily observe developmental stages and corresponding cellular activity;

embryo-larva grow rapidly into various developmental biological stages with overall short life span. The housing and maintenance of zebrafish are inexpensive and with fewer ethical and legal restrictions (Goessling and Sadler 2015).

20.3.2 Limitations

Zebrafish are having several duplicate genes; thus generation of knockout strains is complicated. Administering drug is generally through solubilizing in water of aquarium which has several limitations like exposure to whole body that may lead to undesirable effects on other than target organ, individual variation in dosing and effect on the food intake; excretion is difficult to assess, and water-insoluble drugs are challenging to administer. The study is difficult with low sample availability and multiple time points for blood sampling; smaller amount of tissue is available for transcriptomics (Wrighton et al. 2019). The cell culture has not developed extensively and poorly developed stem cell technology. The physiological parameters are considerably diverse than mammals. The cellular architecture is not similar to human/mammals like liver anatomy, and framework is considerably different. The in-bread strains available are not very well characterized (Wrighton et al. 2019; Engeszer et al. 2007).

20.4 Conclusion and Future Prospects of Zebrafish in Liver Disease Modelling

The zebrafish is sharing a considerable homology and conserved genetic patterns as of mammals. The presence of most of the organ system, specific cells, enzyme function and metabolic system makes many physiological process of zebrafish very close to mammals. Despite having difference in structural architect, zebrafish successfully mimicked human physiology in such a small in vivo system. Having a transparent embryo and larva availed opportunity for scientists to observe the cellular changes clearly with developmental and manipulation in developmental process easily. The large clutch size, easy to reproduce, small life span, faster growth-life stages and relatively less ethical-legal restrictions render zebrafish as an ideal system for in vivo studies and drug screening. For liver diseases, studies with zebrafish are having upper hand compared to other animal models.

Further, studies in utilization of more advanced tools in genetic manipulation and mutant formation may lead this organism as an organism of study choice. The use of CRISPR/Cas9 for mutation and drug target identification may reveal more information about the suitability of zebrafish as a model of preclinical testing in drug discovery research. Its application in toxicological studies has been accepted by the FDA.

Moreover, studies with various dyes to elucidate genotyping and phenotyping characteristics are needed for this organism. Availability of such data may attract

scientist to use it for studying variety of conserved biological processes that may contribute in disease pathologies.

It has wider prospective in discovery of orphan drug and individualization of therapy. The insertion of gene, study of the respective gene product and modulating its expression are relatively convenient in zebrafish.

Overall, with the application of advanced technological input, generation of more data about tools to perform various types of studies and genotypic and phenotypic predictions, factors affecting it may develop this organism as a vital preclinical study model.

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Zebrafish Model for Neurotoxic Drug Screening: Methodologies and Protocols

21

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Abstract

Zebrafish is one of the emerging animal models for drug screening. A neuronal system similar to humans makes zebrafish a potential candidate as an animal model for neurotoxicity and neurogenic diseases. Additionally, due to its transparent larvae, it provides mechanistic insights of drug actions in real time. Behavioural, proteomics and metabolomics studies of zebrafish help to understand the various changes that occur in the brain function and pathways due to the neurotoxic effects. However, to successfully carry out the experiments, efficient, easy and cost-effective protocols should be followed. This chapter provides detailed protocols of experiments for a comprehensive neurotoxicity study using the zebrafish model. Zebrafish behaviour is robust. The behavioural study is a non-invasive and quick method to assess neurotoxicity. Novel tank test, colour preference test, social behaviour and cognitive behaviour analysis of zebrafish are well documented in the literature, and numerous researches have been reported. The recent development of omics techniques such as metabolomics and proteomics, along with bioinformatics, provides an excellent opportunity to study alteration of protein expression and neurochemicals due to induction of any neurotoxic drug. The focus of this chapter is the systematic designing of experiments for neurotoxic study.

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Keywords

Behavior tracking · Proteomics · Metabolemics · Neurotoxicity · Protocols · Bioaccumulation

21.1 Introduction

No animal model is ideal for simulating humans. However, several models have emerged as an excellent animal model; the zebrafish model is one among them. It has recently gained a lot of attention due to several advantages such as transparent embryo/larvae, numbers of offspring, short life span, genetic similarities with humans and low maintenance cost. In the last decade, zebrafish has become a potential model organism for neurotoxicological research (Egan et al. 2009; Lawrence and Lawrence 2014). Recent studies demonstrated it as a very effective animal model to mimic human neurological diseases. The genetic and neuroanatomic similarity of zebrafish with humans makes it ideal for translational research (Kalueff et al. 2016). Furthermore, the zebrafish behaviour model is well established and can be helpful for neurotoxicological drug screening. Due to the transparent embryo/larvae, it is possible to visualize neurological changes in real time, which is one of the unique advantages in the zebrafish model.

Neurological diseases are a new concern for world health. Recent studies found that neurological disorders such as Alzheimer's disease, epilepsy, Parkinson's disease and stroke affect up to one billion people globally (Siuly and Zhang 2016). Approximately 6.8 million individuals die every year because of neurological disorders (Siuly and Zhang 2016). Furthermore, the increasing number of patients at lower age groups is a warning sign for the human society. The proper cause of these neurological diseases is still unknown. However, the ecotoxicological factors cannot be ignored, as several reports have speculated a strong correlation between environmental toxicants and neurological disorders (Cannon and Greenamyre 2011). With the growing number of new industrial compounds, the risk factors are increasing too. In view of this, screening of a large number of environmental contaminants is required. The zebrafish model is one of the most potential candidates for fast and efficient drug screening. The effects of neurotoxicity can sometimes emerge long after a drug exposure, at the later stages of the life cycle involving several neurological dysfunctions. Due to the rapid development of zebrafish, the time required for executing developmental neurotoxicological study is less when compared to higher animal models (mammalian models). Moreover, the use of mammalian models for drug screening engages higher cost and regulatory restrictions.

Therefore, zebrafish could be considered as a comprehensive animal model organism for neurotoxic drug screening. However, profound experimental strategies are required to ensure consistent and accurate research output. With the aid of sophisticated tools such as behaviour tracking systems, liquid chromatography and high-quality fluorescent imaging techniques, an in-depth toxicological research is possible. Hence, this chapter focuses on detailed experimental methodologies and

protocols for neurotoxicological research. In this chapter, the experimental protocols of the following topics will be discussed.

1. Behavioural model.
2. Biochemical assays related to neurotoxicity (oxidative stress, AChE and cortisol).
3. Metabolomics of brain samples.
4. Proteomics of brain samples.
5. Immunohistochemistry.
6. Accumulation of drug.

21.2 Limitation of the Zebrafish Model

Zebrafish model possesses numerous advantages, although several deficiencies are also obtained when compared to mammalian models, such as translatability of toxic potencies (Cassar et al. 2020). In most of the zebrafish studies, the drug administration route is in-water dosing, which may yield unique exposures compared to the typical mammalian administration routes. In this administration route, as the fish are immersed in the treatment solution, it could impact the translatability of the results.

21.3 Neurotoxicity Assessment of Drugs Using Zebrafish Model

Brain and nervous system are the most complex systems in humans. The nervous system consists of the central and peripheral nervous system and has numerous components and functions which are yet to be fully understood. It is not surprising that more than 600 human disorders afflict the nervous system (Siuly and Zhang 2016), and several of them could have been caused by ecotoxicological factors. The zebrafish model can be used to examine numerous chemicals for their potential neurotoxicity. On the other hand, the neuroprotective effect of various chemicals can also be examined using zebrafish. In the literature, a number of drugs have already been screened using the zebrafish model. Table 21.1 shows various chemicals and their effects on the zebrafish model.

21.4 Experimental Protocols

The success of any animal model research depends on the design of the experiments and the protocols followed, keeping in mind the limitation of resources in the existing facility. Accuracy in the results, fast sample processing, repeatability and cost-effectiveness are those factors that should be optimized in laboratory practices. Several protocols have been reported in the literature for any specific analysis that leads to confusion in the mind of a researcher, especially newcomers. In this chapter, we will discuss those protocols that work most effectively in our experiments. Additionally, these protocols are cost-effective and time-saving.

Table 21.1 Some examples of drugs that have been studied in zebrafish model for neurotoxicity assessment

Drugs	Time of drug exposure	Experiment done	Remarks	Reference
Minocycline, rasagiline	Embryo: 2 dpf	Locomotor activity, immunohistochemistry and morphological assessment	Rasagiline shows neuroprotective effect against	Cronin and Grealy (2017)
Rotenone	Embryo: 3 dpf	Locomotor activity, immunohistochemistry, morphological assessment and gene expression analyses	A loss of DAnergic neurons	Kalyn et al. (2019)
$HgCl_2$	Embryo: 5 hpf	Swimming activity, Western blot and metabolomics	Impaired neuromotor functions	Abu Bakar et al. (2017)
Atrazine	Embryo: 2 hpf	Activity of acetylcholinesterase (AChE), morphology	Upregulation of AChE activity	Wang et al. (2015)
Tralopyril	Embryo: 2 hpf	Gene expression	Affected amino acids, energy and lipids metabolism	Chen et al. (2020)
Chlorpropham	Embryo: 6 hpf	Heartbeats and viability	Induces apoptosis, generates ROS	Lee et al. (2020)
Trichloroethylene	Embryos: Immediately after fertilization	Morphological assessment, heartbeats, photomotor response and visual-motor response	Altered hatching, morphology, behaviour and heart rate	Horzmann et al. (2020b)
Acrylamide	Adult zebrafish	Behaviour, proteomics and metabolomics	Proteomic alteration	Faria et al. (2018)
Atrazine	Adult zebrafish	Behaviour, microarray evaluation and histopathology	Genes related to cell function, cancer and reproductive and nervous systems	Horzmann et al. (2020a)
Sodium selenite	Adult zebrafish	Behaviour and biochemical analysis	Prevents paraquat-induced neurotoxicity	Müller et al. (2017)

In the first section of the experimental protocol, we will discuss the common routes of drug exposure in zebrafish. The behavioural study of zebrafish with the aid of an automated tracking system has been described. The protocols of biochemical analyses such as reactive oxidative species (ROS), MDA, AChE, catalase and cortisol have been given. A detailed methodology of proteomics and metabolomics using LC-MS/MS techniques is one of the most important aspects discussed here. Lastly, the immunohistochemistry and accumulation of drug have also been discussed.

21.4.1 Mode of Drug Exposure to Zebrafish

Drugs are exposed to adult zebrafish mainly by mixing them in water (in-water solution). Oral and parenteral doses of several drugs are also possible for adult zebrafish study. Drugs can also be provided by mixing them in the feed or by oral gavage (Collymore et al. 2013).

For zebrafish larvae, microgavage using microinjection manipulators and stereomicroscope has been successfully delivered by Cocchiaro and Rawls (2013). However, in-solution exposure to neurotoxic drugs is the most common route of exposure for embryo/larvae. Microinjection to the embryo is also a possible method for gene editing, but it requires very minute control. A highly accurate microinjection system is required to control the typical injection volumes of 500 pL or 1 nL (Rosen et al. 2009).

21.4.2 Behavioural Model

Zebrafish have a similar brain structure like humans, which includes the olfactory bulb, cerebellum and spinal cord. The neurochemistry of zebrafish and humans are also identical. The zebrafish brain comprises all the major components essential for neurotransmission such as neurotransmitter receptors, transporters and enzymes of synthesis and metabolism. All the important neurotransmitter systems such as dopamine, GABA, serotonin, noradrenaline, histamine and acetylcholine are present in zebrafish brain (Rico et al. 2011). Additionally, other brain regions such as the habenula and amygdala that control behaviours in humans are also present in zebrafish, and they have a similar function as in humans. The stress-related behaviours are controlled by the habenula, similar to that of mammals. Furthermore, various neurochemical pathways involved in the alteration of zebrafish behaviour have been well characterized. Despite the lack of neocortex in zebrafish, it is capable of complex decision-making and cognitive processing (Basnet et al. 2019). Therefore, the behaviour of zebrafish could be a vital tool to assess neurotoxicity of various drugs. Both larvae and adult zebrafish models have been successfully employed for neurotoxicity assessment. In the following section, behaviour test protocol has been described.

21.4.2.1 Larvae Behaviour

The larval stages of zebrafish start after 72 hpf and last for approximately 1 month. Swimming of zebrafish starts at the time of 48–72 hpf. In the larval stages, zebrafish shows a number of behaviours which can be analysed to interpret the toxic effects of various neurochemicals. Larvae at the early stages (4–6 dpf) are generally used for neurological drug screening. As zebrafish produce a large numbers of offspring in a very short time and as they are easy to maintain, the larvae are increasingly utilized for the high-throughput screening of various neuroactive drugs. Locomotor behaviour, thigmotaxis, optomotor and the optokinetic responses of zebrafish larvae have been well studied. Locomotor behaviour of larvae in the light-dark stimuli has widely been used to screen the neurotoxicity of many compounds. Zebrafish larvae in light and dark conditions demonstrate an explicit pattern of locomotion. The light to dark transition increases larvae activity; on the other hand, the dark to light transition reduces the locomotor activity. A high-throughput automatic tracking system is required to track multiple larvae at a time, using 24 or 48 well plate module. There are various commercial systems available for larvae tracking. Open-source software such as idTracker, Ctrax and wrMTrck (Franco-Restrepo et al. 2019) are very efficient to track larvae behaviour. But, the most critical part of larval tracking is the video recording in the dark-light experiment. All these software are based on an image processing algorithm. However, unlike adult zebrafish, the tracking of larvae is tricky due to their small size and transparent body. As image processing requires high contrast difference between object and background, the arrangement of sufficient background light is required. A high-resolution IR camera is essential to track larvae in the dark. The larvae tracking setup can be designed indigenously, although commercially available setup works very accurately. A brief protocol of larvae tracking has been given below.

Larval tracking could be done using the following steps (Zhang et al. 2020):

(a) Transfer ~50 embryos per treatment group to petri dishes.
(b) Blot the system water using 1 mL pipette tip.
(c) Add neurological drugs (10–20 mL) to the petri dish gently.
(d) Similarly, add a control solution to another petri dish.
(e) Keep the petri dishes in an incubator at 28.5 °C.
(f) Replace half of the solution with fresh drug solution every day.
(g) Keep the petri dishes in the incubator until 5 dpf.
(h) Prepare 24 microwell plates, and add 8oo μL of the exposure solution to each well.
(i) Transfer a larva with 200 μL of the exposure solution to each well using 1 mL pipette tip.
(j) Keep the well plate in an incubator for 2–3 h at 28.5 °C.
(k) Put the well plate in the video recording chamber and record the videos in dark-light stimuli. For larval behaviour tracking, it is advisable to use commercial tracking systems such as ZebraBox (http://www.viewpoint.fr/en/p/equipment/zebrabox-for-embryos-or-larvae) or DanioVision (https://www.noldus.com/daniovision) that allow automated monitoring and also are capable of analysing larval behaviour in multi-well plates, up to 96 individuals simultaneously.

21.4.2.2 Adult Fish Behaviour

Adult fish tracking is easier, and custom-made video recording setup can be fabricated with easily available tools.

Video Recording

Behaviour tracking software are basically based on image processing. They process a stack of consecutive images (frames) from a video file and extract the position coordinates (two dimensional). With the help of two cameras (top view and side view), 3D tracking of fish is also possible. Good quality videos are the key to accurate video tracking. The tracking chamber should have a white background to enhance the contrast between the fish and background.

Custom-made chamber for video recording: This involves the following steps:

(a) Take a box having opening at the top and one side (Box A in Fig. 21.1, approximate length: 50 cm, height 30 cm and width 40 cm, depending on the novel tank or T-tank size).
(b) Wrap the inner side of the box with white papers.
(c) Place two web cameras, one in the front and one on the top, to record videos from separate angles.
(d) Cover the Box A with a bigger size box (Box B, cover box). The inner sides of Box B should be covered with white paper.
(e) Arrange white LED lights at the bottom of the Box A, as shown in Fig. 21.1a, to maximize diffusive light and minimize the reflection.
(f) Then, place the novel tank (or T-maze/Y-maze) inside Box A.
(g) Put the individual fish to the tank and start the video recording from two cameras.
(h) Take 5–10 min videos of every fish.
(i) Social behaviour of the fish can also be observed by putting a cohort of fish in the tracking tank. With the increasing number of fish in a cohort, the tracking efficiency will decrease.

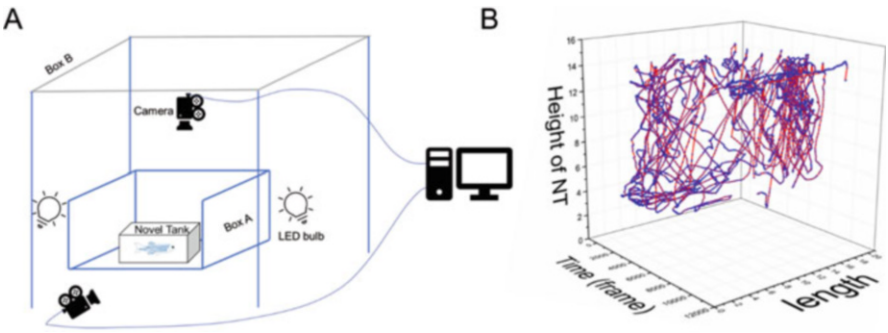

Fig. 21.1 (**a**) Schematic of video recording chamber for fish tracking and (**b**) trajectories of fish movement

(j) Save those videos according to treatment groups and number them individually as per convenience and put the individual video file in a separate folder for further processing.

Fish Tracking and Analysis of Data

The success of the behaviour model lays in the accuracy of tracking. Earlier, behaviour tracking was done manually, which was inefficient. However, with the help of high-end automated tracking systems, behaviour parameters can be analysed with very high accuracy. There are several commercial software in this field, such as Noldus (https://www.noldus.com/) and Viewpoint (http://www.viewpoint.fr/), which provide high-throughput and high-content behaviour analysis. These commercial software provide a ready-made solution. However, they come with a higher economic cost. Interestingly, there are several open-source software based on MATLAB, Python or ImageJ that provide a very efficient and low-cost solution for behaviour tracking. With a basic knowledge of MATLAB or Python, several behaviour parameters can be calculated. idTracker is one of the most efficient behaviour tracking software developed by Pérez-Escudero et al. (2014). It is a MATLAB-based, user-friendly software. Besides individual fish tracking, idTracker is also capable of tracking the social behaviour of a fish cohort.

Tracking Using idTracker

We have used idTracker for fish tracking, which is a very efficient software for behaviour tracking. In the following section, we have provided a description of fish tracking using this software (here we take front side camera's video).

(a) Open the idTracker.exe (see Fig. 21.2).
(b) Load one of the recorded videos from the experiment. User interface of idTracker will be opened.
(c) Set the number of individuals (number of fish in the video) in the user interface of idTracker. Set one for single fish novel tank test; for social behaviour, it depends on the cohort size.
(d) Select region of interest (one can choose rectangular, circular or polygon depending on the test tank shape). In Fig. 21.2, polygon was chosen.
(e) Set the intensity threshold value such that only the fish are marked with green colour. Typically, the values are in the range of 0.7–0.9.
(f) If there are other backgrounds marked with green colour, then adjust the minimum size and/or threshold value to remove the disturbances.
(g) Set the frame number from start to end. It depends on your frame-per-second captured videos. (Normally videos are recorded with 30 fps; therefore, for a 5-min tracking, set the initial frame as 1 and the final frame as 9000).
(h) Click on the remove background option.
(i) Start the tracking.
(j) After finishing, idTracker generates trajectories.m files for every video.
(k) The trajectories.m files comprise the two-dimensional position (X-Y position) of a fish along with time (frame number).

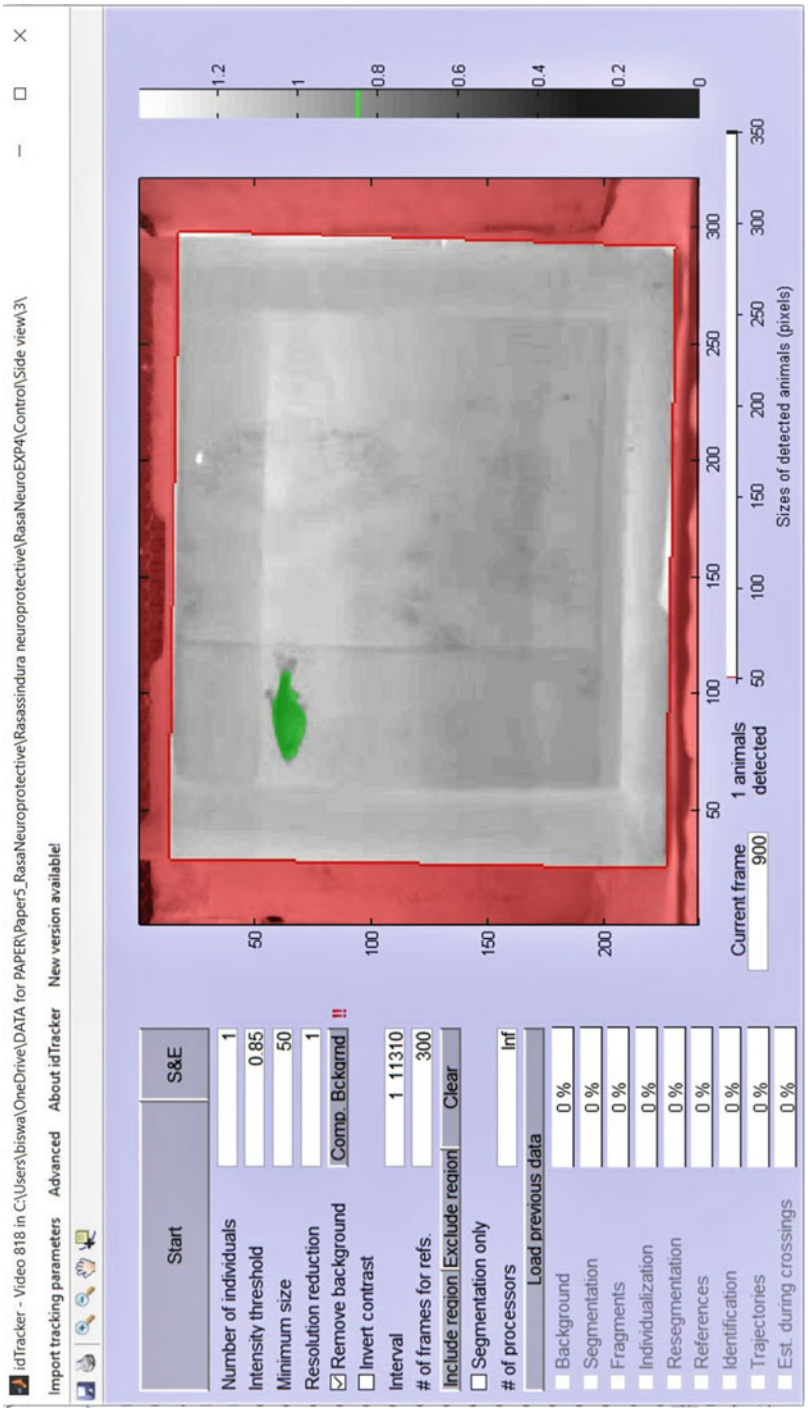

Fig. 21.2 User interface of idTracker. Image is generated using idTracker software (http://www.idtracker.es/home)

Calculation of Various Parameters

The idTracker provides a position coordinate of fish in a two-dimensional coordinate (Fig. 21.3) with respect to each frame (time). Therefore, for a 5-min tracking, there will be 9000 frames (videos recorded at 30 fps/s). And for an 'n' number of fish, the size of data will be enormous. The fish trajectories do not provide much insight about neurotoxicity. Thus, it is necessary to transform the trajectory data into various behavioural parameters. To evaluate behavioural parameters, very basic MATLAB code should be developed.

Behaviour Parameters

Several motor and endpoints behavioural parameters are listed here that are common in the behavioural study.

(a) Distance travelled.
(b) Speed.
(c) Maximum speed.
(d) Angular velocity.
(e) Meander.
(f) Freeze points.
(g) Distance travelled with fast speed.
(h) Time spent in the upper or lower zone.
(i) Wall distance.
(j) Social behaviour such as schooling.

Typical results of various behaviour parameters of zebrafish after drug treatment ($HgCl_2$) are shown in Fig. 21.4.

21.4.3 Biochemical Assays

21.4.3.1 Sample Preparations

Embryo and Larvae

Embryo/larvae (around 30–50 numbers) should be taken out from the exposure medium at endpoint after treatment. They should be euthanized with 0.1% MS-222 for 5 min. Then, the embryo should be washed with PBS (pH = 7.4) for two to three times and transferred into a 2 mL centrifuge tube, and then snap frozen with liquid nitrogen and stored in −80 °C.

Extraction and Storage of the Brain (for Adult Fish)

Extraction of the brain from the fish is the key factor for generating reliable data. Carefully, the brain should be completely extracted from the skeleton; otherwise, tissue loss will cause erroneous data in the downstream analysis. Extraction of tiny zebrafish brain (~4 mm size) is critical and requires experienced personnel. An example has been given by Lopez-Ramirez et al. to dissect zebrafish brain (Lopez-

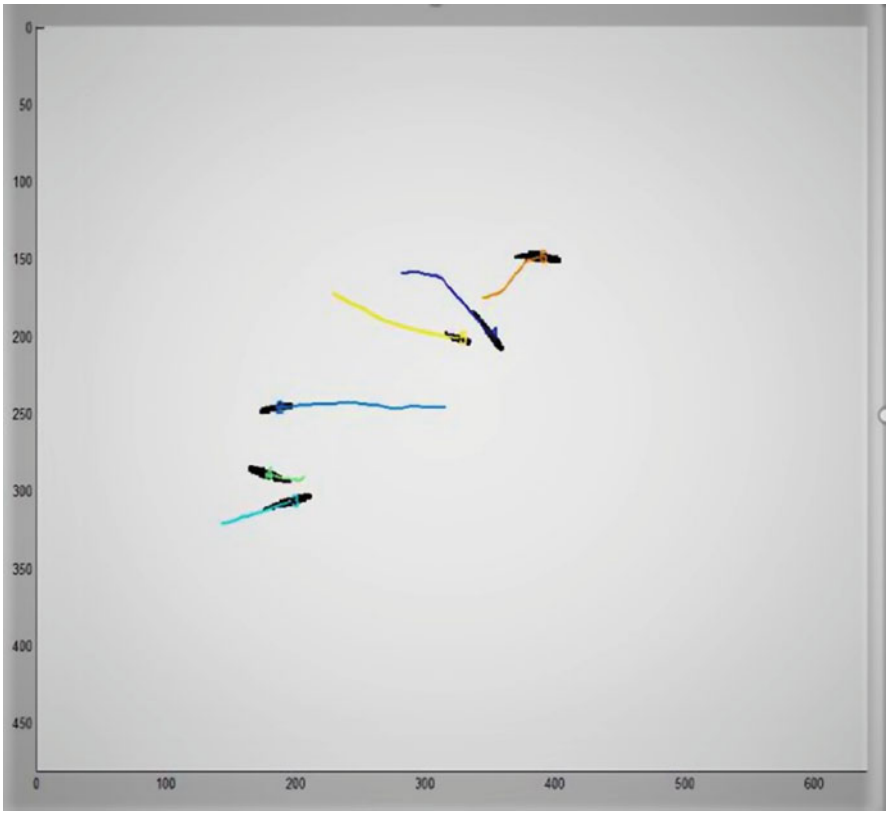

Fig. 21.3 Tracking of fish using idTracker (http://www.idtracker.es/home), which provides the 2D position coordinate of every fish with time. Here, the tracking of six fish has been shown

Ramirez et al. 2016). Extract the fish brain and then wash the brain with PBS to remove any blood that is present. The extracted brain should be snap frozen immediately with liquid nitrogen and then kept in −80 °C refrigerator.

21.4.3.2 ROS Study of Zebrafish Brain

ROS of larvae or adult zebrafish brain can be done after homogenizing the sample as follows (Huang et al. 2018):

(a) Remove the centrifuge vial containing brain/larvae samples from −80 °C freeze and keep it in the ice.
(b) Add 300 μL of ice-cold phosphate-buffered saline (1× PBS at pH = 7.4) to one brain sample (30–50 larvae) and homogenate with a mechanical homogenizer (bead mill homogenizer/rotor-stator homogenizers) for 5 min.
(c) Centrifuge it at 12,000 × *g* for 10 min at 4 °C.

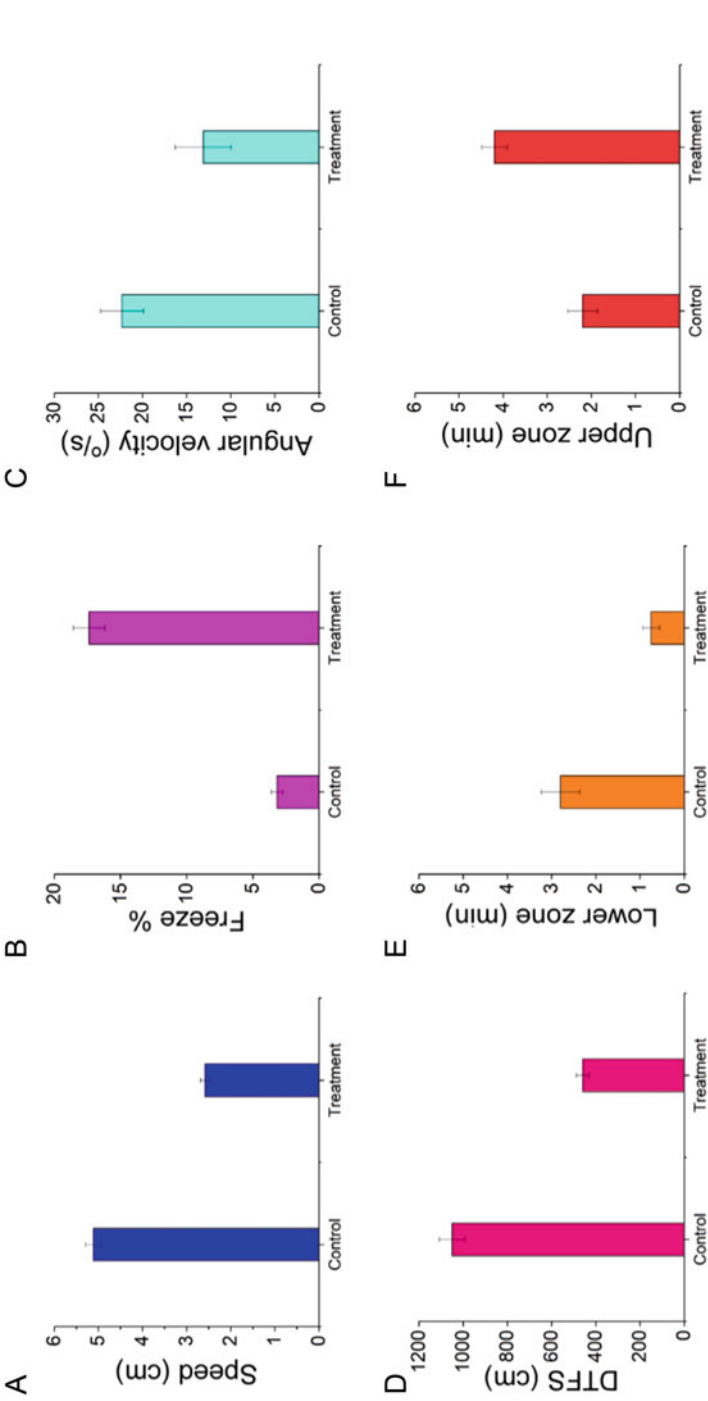

Fig. 21.4 Typical results showing alteration of various behavioural parameters due to exposure of neurotoxic drug ($HgCl_2$) as compared to the control group. (**a**) Speed, (**b**) freeze%, (**c**) angular velocity, (**d**) DTFS-distance travelled with fast speed, (**e**) time spent in the lower zone and (**f**) time spent in the upper zone

(d) Discard the precipitate and keep the supernatant at 4 °C (can be stored at −80 °C).
(e) Place 100 μL of respective sample in a well of black-bottom 96 well plate. Keep a blank with 100 μL buffer (homogenizing buffer).
(f) Add 50 μL of DCFDA solution (20 μM) and keep it for 30 min in 37.5 °C.
(g) Measure the fluorescent with an excitation wavelength of 480 nm and emission at 537 nm.

21.4.3.3 TBARS (MDA)

Thiobarbituric acid reactive substances (TBARS) analysis indicates oxidative stress. The following protocols are used to express MDA of zebrafish tissue samples:

(a) Make a pool of three brains per sample.
(b) For larvae, make a pool of 50 larvae per sample.
(c) Homogenate the sample with 300 μL PBS.
(d) Then add 500 μL of 0.25 N HCL to 250 μL of the homogenate.
(e) Add 500 μL 10% w/v trichloroacetic acid (TCA).
(f) Keep the reaction mixture for 1 h with occasional shaking.
(g) Centrifuge the reaction mixture at 5000 × *g* for 10 min.
(h) Take 1 mL supernatant and add 1 mL 0.67% w/v TBA (thiobarbituric acid) in a 5–10 mL glass test tube.
(i) Cover the vial with aluminium foil.
(j) Keep the reaction mixture in boiling water for 10 min.
(k) The solution will turn pink, which can be measured at 535 nm wavelength in a 96 well plate or in cuvette.
(l) For blank, instead of the sample in step (d), use 250 μL Milli-Q water.
(m) Prepare a standard of MDA from 2 to 10 μM (standard curve should be linear).

21.4.3.4 Catalase Activity

Catalase activity of zebrafish brain sample can be determined by the rate of H_2O_2 degradation.

(a) Homogenate brain (a pool of two to three brains)/larvae (50 nos.) in 300 μL 1× PBS.
(b) Centrifuge the homogenate at 10,000 × *g* for 10 min at 4 °C.
(c) Collect the supernatant.
(d) Take 100 μL supernatant of brain/larvae sample in a quartz UV vial.
(e) Add 850 μL PBS to the vial.
(f) Add 50 μL 10 mM hydrogen peroxide to it and measure the absorption at a wavelength of 240 nm.
(g) One unit of CAT is equivalent to that of elemental 1 mM H_2O_2 ($\varepsilon = 40\ M^{-1}\ cm^{-1}$).

21.4.3.5 AChE Activity

AChE activity can be analysed by the following methods (Ellman et al. 1961; Paul and Borah 2017):

(a) Add 300 μL of ice-cold PBS to the brain or larvae sample (alternative buffer 50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid (Senger et al. 2011)) and homogenate with a mechanical homogenizer (bead mill homogenizer/rotor-stator homogenizers) for 5 min.
(b) Centrifuge it at 10,000 × *g* for 10 min at 4 °C.
(c) Collect the supernatant.
(d) **Reagent:** Prepare 2.5 mM DTNB (5,5-dithio-*bis*-2-nitrobenzoic acid). Use fresh solution every time.
(e) **Substrate**: The substrate is acetylthiocholine iodide, 0.075 M (21.67 mg/mL). This solution can be stored in the refrigerator for several days.
(f) Take 100 μL of sample and add 900 μL of buffer.
(g) Then add 800 μL of DTNB.
(h) Pre-incubate the solution at 25 °C for 2–5 min.
(i) Add the 200 μL of the substrate into the sample solution to initiate the reaction.
(j) Measure the absorption data in UV-Vis spectroscopy at 412 nm from 0 to 3 min with an interval of 30 s.
(k) Similarly, controls without the homogenate preparation should be performed.

21.4.4 Whole-Body Cortisol Measurement

Cortisol indicates the stress level in zebrafish, which is a marker for stress related to any neurotoxicity in zebrafish. The protocol followed here is adopted from Cachat et al. (2010).

(a) Euthanize the fish in tricaine solution (100–120 mg/L) for 60 s.
(b) Dissect the head part and remove it.
(c) Weigh the body part of each fish.
(d) Cut the whole body into pieces for better homogenization.
(e) Put each fish in a 10–15 mL centrifuge glass tube.
(f) Add 3 mL ice-cold PBS (1×, pH = 7) buffer and homogenate with a motor-driven homogenizer (rinse the homogenizing rotor blade and probe with ethanol every time after one sample).
(g) Then, add 3–5 mL diethyl ether to every vial and vortex for 1 min, and then centrifuge it at 5000–7000 × *g* for 20 min.
(h) Collect the upper organic layer carefully with a glass pipette containing cortisol and place it in a glass tube.
(i) Repeat the (g) and (h) steps three to four times and collect the organic layer in the same vial for the same sample.
(j) Allow the diethyl ether to evaporate completely under the fume hood overnight.
(k) After complete removal of diethyl ether, add 1 mL of PBS and vortex for 1 min.

(l) Keep the sample at 4 °C overnight.
(m) After incubation, vortex the samples and allow them to settle at room temperature.
(n) Measure the cortisol using commercial ELISA kit.

For larval cortisol analysis, take a pool of 30–50 larvae. Euthanize by heat shock and collect the larvae in a 10 mL glass vial. Add 1–2 mL PBS and homogenate the larvae by sonication. For fish larvae, do the experiment by following the procedures from step (g).

Human salivary cortisol ELISA assay kit could be used for cortisol detection.

21.4.5 Metabolomics

Metabolomics is the study to identify and quantify a wide range of small molecules associated with specific cells, tissues or a whole organism (Blaženović et al. 2018). The metabolomics study can help to classify numbers of biomarkers that associate with the neurotoxicological changes in any organism, after exposure to the toxicant. With the help of bioinformatics, metabolomics data can deliver pathway analysis of various metabolic processes. A number of techniques can be utilized for the analysis of metabolomics such as liquid chromatography-mass spectrometry (LC-MS/MS), gas chromatography/time-of-flight mass spectrometry (GC-TOF) and nuclear magnetic resonance (NMR) (Dhillon et al. 2019). NMR is a powerful tool for metabolomics study; however, strict conditions are required for NMR measurements (Raftery 2014). NMR analysis is very sensitive to numerous factors. For instance, a change of pH or ionic strength of solvent might influence major shifts in characteristic NMR peaks associated with metabolites. In our studies, we used LC-MS/MS technique for neurochemical study of zebrafish brain samples due to enhanced identification capabilities, greater sensitivity and availability of huge database for molecule identification (Gómez-Canela et al. 2017).

In the following section, we will discuss the LC-MS/MS technique for the metabolomics study of zebrafish model.

21.4.5.1 Sample Extraction for LC-MS/MS-Based Metabolomics Study

Embryo or larvae should be collected in 2 mL Eppendorf tubes after the endpoint of any exposure experiment. Then, with the help of a pipette, the excess water is to be removed. The samples should be then snap frozen by dipping them in liquid nitrogen for 30 s and stored at −80 °C.

For adult fish, euthanize animals from each experimental group in ice, extract brain samples and keep them in 2 mL Eppendorf tubes. Snap freeze the brain samples immediately in liquid nitrogen for 30 s and store them at −80 °C until further experiments.

21.4.5.2 Extracting Solution

The extracting solvent plays a major role in the metabolic detection from tissue samples. Metabolites or a group of metabolites are generally solvent sensitive, and their detection is largely dependent on the extraction procedure.

We have listed some of the extraction solutions below:

A: Methanol/water (90:10, v/v).
B: Methanol/water (50:50, v/v).
C: Deionized water with 0.1% formic acid.
D: Acetonitrile with 0.1% formic acid.
E: MPA solution for acidic extraction.
F: Methanol/chloroform/water (5:1:1, v:v:v %) (Xu et al. 2017).

21.4.5.3 Internal Standard

Internal standards are essential to get accurate results in the metabolomics analysis. One can add the stable isotope-labelled internal standards of each neurochemical (such as GABA-d_6, dopamine-d4, glutamate-d_5, DOPA-d_3, serotonin-d_4, etc.) intended for analyses (Tufi et al. 2015). On the other hand, several researchers used single internal standards such as methionine sulfone (Gómez-Canela et al. 2017), 3,4-dihydroxybenzylamine (DHB) (Kashem et al. 2016) and L-aspartic acid-^{15}N (Gómez-Canela et al. 2018). However, using multiple isotope-labelled internal standards is expensive. Thus, it is better to use a single internal standard. In our work, we used L-aspartic acid-^{15}N as internal standard, which could be detected consistently in the LC-MS/MS analysis.

21.4.5.4 Sample Preparations for LC-MS/MS Study

The sample should be taken out from the fridge and kept in ~4 °C.

(a) Add 500 ng or 1000 ng internal standard(s) to the centrifuge tube containing the sample.
(b) Add cold 300 μL of extracting solution (A/B/C/D or E) to the sample (we used extracting solution A for our experiments).
(c) Homogenise for 5 min with the help of either the bead mill homogenizer or mechanical homogenizer or sonication in ice.
(d) Vortex or shake the sample for 5–10 min.
(e) Centrifuge the solution at 14,000 rcf at 4 °C for 20 min.
(f) Remove the supernatants from the centrifuge tube and transfer to a chromatographic vial after filtration with 0.20 μM PTFE filters.
(g) Store the vial at 4 °C (neurotransmitters are stable for a month in this condition).

21.4.5.5 Analytical Instrument

There are a number of LC-MS/MS analytical instruments. Among them, the following instruments are widely used for metabolomics studies:

(a) Liquid chromatography connected to a triple quadrupole detector (Xevo TQD, Waters, USA) (LC-MS/MS).
(b) Liquid chromatography connected to a quadrupole time-of-flight (QTOF) (Agilent 6546 LC/Q-TOF).
(c) Liquid chromatography connected to linear trap quadrupole (LTQ)-Orbitrap (Thermo Fisher Scientific).

21.4.5.6 Chromatographic Column

The selection of chromatographic column is important for the analysis. The retention time and selectivity of any molecule largely depend on the LC-column. Several neurochemicals are hydrophilic and polar in nature, especially neurotransmitters (Chirita et al. 2010). Therefore, their analysis using normal reversed-phase chromatography (RPLC) is difficult due to insufficient retention. Hydrophilic interaction chromatography (HILIC) and polar reversed-phase chromatography column appear to be interesting alternatives for the analysis of these types of polar compounds (Gómez-Canela et al. 2018; Chirita et al. 2010).

21.4.5.7 Chromatographic Conditions

Use a binary mixture of 0.1% of formic acid in water (A) and 0.1% formic acid in methanol (B). At the initial condition, run with 95% A and 5% B for 2–3 min. A gradient increase of B is normally used. For the next 5 min, increase B up to 30%. Lastly, increase B further up to 95% within 13 min and keep it at this condition for 5 min. The injection volume of the solution should be 10–20 μL. Keep the flow rate at 500 μL min^{-1}.

21.4.6 Proteomics

Proteomics is the technique for identification and quantification of overall protein of any organism, tissue or cell. MALDI-TOF/TOF and LC-MS/MS are widely used techniques for proteomics study. However, the proteomic study involves knowledge from several disciplines such as biochemistry, equipment operation, software and bioinformatics. Hence, skilled personnel are required for this technique. On the other hand, the proteomics study involves a very high economic cost. There are several sample preparation and analysis techniques even in LC-MS/MS, such as isotope-coded affinity tag (ICAT) labelling, isobaric tag for relative and absolute quantitation (iTRAQ) techniques, stable isotope labelling with amino acids in cell culture (SILAC) and label-free quantification (LFQ) (Itze-Mayrhofer and Brem 2020). LFQ is an economical and efficient technique, which can handle several samples at a time. The sample preparations of LFQ proteomics are easy, and each sample is analysed separately by MS. The sample preparation steps are described below.

21.4.6.1 Sample Preparation

(a) Extract the brain samples and homogenate them in 400 µL of 8 M urea, 400 mM ammonium bicarbonate and 10 mM dithiothreitol (DTT) (Faria et al. 2018) at 4 °C.
(b) Measure the amount of protein in each sample.
(c) Take approximately 20 µg protein from each sample.
(d) Add 50 µL DTT (25 mM) and keep it for 30 min on a shaker at a speed of 300 rpm and 60 °C temperature.
(e) Alkylation should be done by adding 20 µL (25 mM) iodoacetamide for 30 min in the dark at room temperature.
(f) Dilute four times with 50 mM ammonium bicarbonate.
(g) Then, trypsinize the samples (1:20 enzyme to substrate ratio) overnight at 37 °C in constant shaking at 300 rpm.
(h) Dry the samples completely and reconstitute in 50 µL 5% acetonitrile (with 0.5% trifluoroacetic acid) solution.
(i) Desalt each sample using C18 spin column (Pierce® C18 Spin Columns, Thermo Fisher Scientific, USA).
(j) After desalination, reconstitute it in 20 µL 0.1% formic acid.
(k) Then the peptide concentration should be analysed by the UV-Vis spectrophotometers (Implen™ NanoPhotometer, German).
(l) Dilute the samples to the final concentration of 0.1 µg/mL with 0.1% formic acid solution.

The resultant peptides should be analysed with the help of liquid chromatography-mass spectrometry (Q-Exactive Plus Biopharma-High Resolution Orbitrap, Thermo Fisher Scientific, USA). Use C18 reverse-phase chromatographic column for chromatographic separation. Inject 2 µL of the sample to the column with a flow rate of 300 µL/min. The elution of peptides should be done by 120 min of linear gradient from 5 to 28% ACN (with 0.1% formic acid). Then, 40 min of linear gradient from 28 to 95% ACN (with 0.1% formic acid) should be passed through the column. The Orbitrap generally operates in the positive mode in data-dependent acquisition mode. Use an isolation window of 3 Da. Acquisition of data could be controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific).

21.4.6.2 Data Processing and Analysis

The data processing of proteomics data could be done by commercial software such as proteome discoverer (Thermo Fisher Scientific); however, open-source software such as MaxQuant (https://www.maxquant.org/) is a very powerful tool in proteomic analysis. MaxQuant is a quantitative proteomics software package which can handle large mass spectrometric data sets. MS/MS spectra were analysed by MaxQuant (https://maxquant.net, v1.6.10.43) software using the zebrafish UniProt sequence database. Label-free quantification could be carried out on the extracted ion intensity of precursor ions. More than three biological replicates from each treatment group should be analysed. Only proteins present at least in three samples per group should be further processed for statistical analysis using Perseus software (https://maxquant.

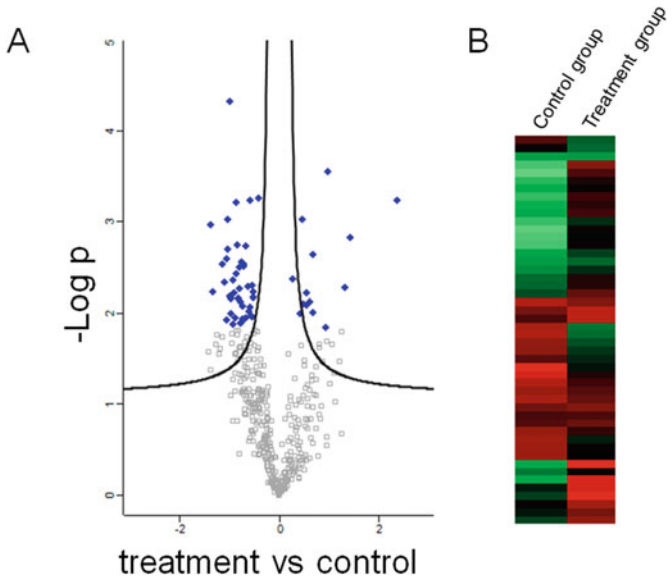

Fig. 21.5 (**a**) Volcano plot of differentially expressed proteins of fish brain samples for the treated group as compared to the control group and (**a**) heat map of differentially expressed proteins of fish brain samples for the treated group as compared to the control group. Plots are generated using Perseus software (https://www.maxquant.org/perseus/)

net/perseus). Welch's *t*-test along with *p*-test ($p < 0.05$) should be carried out to identify differentially expressed proteins (Fig. 21.5).

For bioinformatics study, STRING (https://string-db.org/), DAVID (https://david.ncifcrf.gov/home.jsp) and PANTHER (http://www.pantherdb.org) could be used to identify various KEGG pathways and/or gene ontology (GO) biological process, cellular components and metabolic process from the differentially expressed (DFE) proteins of those treatment groups (Mi et al. 2019). An example of protein-protein network is shown in Fig. 21.6.

21.4.7 Immunohistochemistry of Brain Tissue

After drug exposure, euthanize fish in tricaine solution and remove the head using a razor blade.

(a) Place the head quickly in 4% paraformaldehyde in PBS (use 10 mL solution).
(b) Store the sample at 4 °C for 18–24 h.
(c) Discard the paraformaldehyde and subsequently place the sample in 10%, 20% and 30% sucrose solution for 6 h each at 4 °C (use 10 mL sucrose solution for each concentration, prepare sucrose solution in PBS).

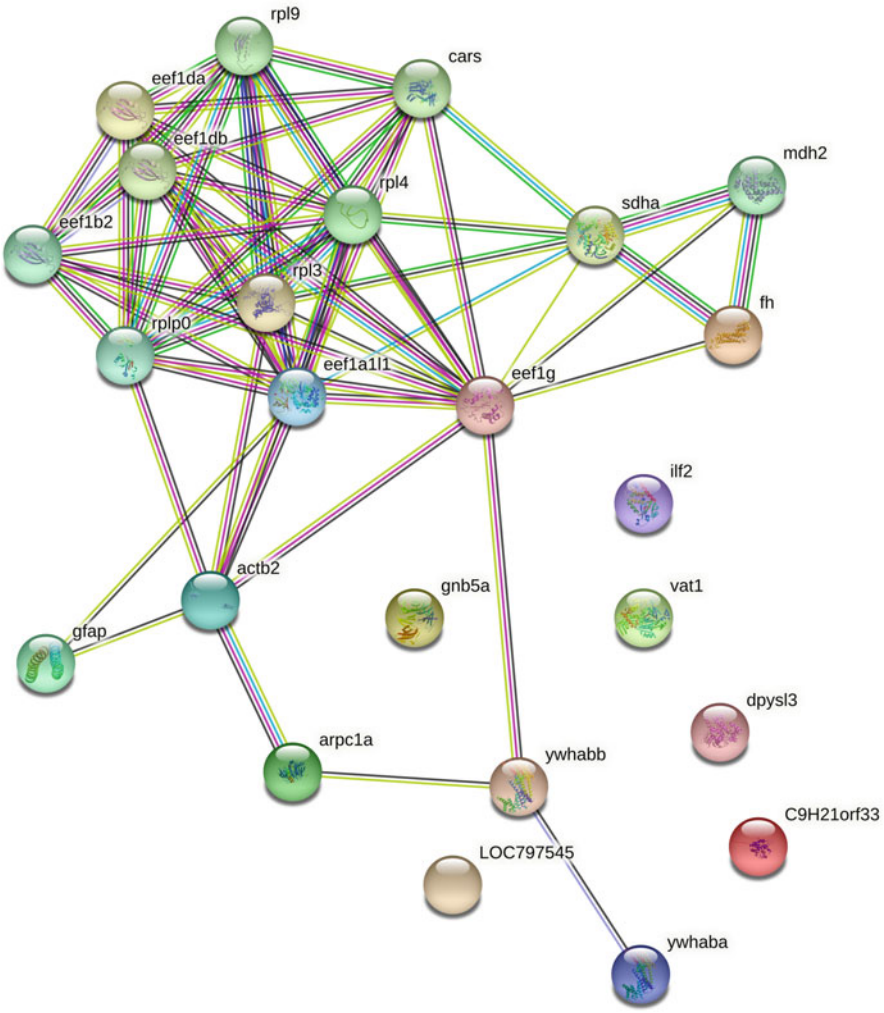

Fig. 21.6 Protein-protein network using STRING database of differentially expressed proteins between control and treatment groups (representative figure, https://string-db.org/)

(d) Mount the head in HistoPrep (Thermo Fisher Scientific, USA) or Tissue-Tek O.C.T. (Sakura, USA) frozen tissue embedding media.
(e) The head should be placed vertically or horizontally as per section of interest.
(f) Place the embedded tissue block in liquid nitrogen for 30 s.
(g) Store the block in −80 °C for 24 h.
(h) Section the fish head (12–15 μm) using cryostat at −25 °C.
(i) Put the section on a gelatine-coated glass slide.
(j) Store slides at −80 °C until immunohistochemistry experiment is performed.

(k) Before staining the slides, dry them at 37 °C for 2 h.
(l) To reduce background autofluorescence, incubate slides in sodium borohydride in ice-cold PBS for three times (10–15 min each).
(m) Rinse the slides thrice with PBS.
(n) Incubate the slides in 5% normal donkey serum in PBS containing 0.3% Triton-X (blocked with 20% NGS and 2% BSA in 0.3% PBS/Triton X-100) for 1 h. https://doi.org/10.1089/zeb.2015.1147.
(o) Incubate the slides in the primary antibody (1:100 dilution) for 24 h in 4 °C.
(p) Wash the slides four times with PBS.
(q) Incubate the slides in secondary antibody coupled to any fluorescent compound for 2 h in the dark.
(r) Wash the section thrice with PBS.
(s) Counterstain with a suitable fluorescent dye (such as DAPI) for 10 min in the dark.
(t) The slide should be mounted using coverslips and PermaFluor Mountant (Thermo Fisher Scientific, USA).
(u) Dry the slides in the dark and store them in 4 °C.
(v) Use confocal microscopy for imaging.

21.4.8 Accumulation of Drugs

For the heavy metal-related neurotoxicity experiment, the accumulation of metal ion in brain tissue or larvae can be analysed using the ICP-MS technique.

(a) Extract the fish brain carefully and make a pool of five to ten brain tissues per sample in a 20 mL glass tube.
(b) For the larvae study, take 50 larvae after drug exposure, and then euthanize them in ice-cold water. Decant the water completely. Wash the larvae five to ten times with Milli-Q water.
(c) Add 2 mL of ultrapure nitric acid (Nitric acid 65% Suprapur, Merck, Germany) to the sample and heat at 60 °C for an hour in a fume hood. Use glass test tubes with volume marking.
(d) Make up the volume up to 10 mL by adding Milli-Q water.
(e) Filter each sample with a syringe filter (0.22 μm) carefully, with protective gears.
(f) Measure the metal concentration with the help of ICP-MS.
(g) Prepare the blank with 2 mL nitric acid and 8 mL Milli-Q water.
(h) Prepare a standard of metal ions from 0 to 20 ppb (0, 1, 5, 10 and 20) in 20% nitric acid.
(i) Run the standard and plot the standard curve. A linear curve is expected.
(j) Check the reliability standard curve, and run two known standards (say 1 and 10 ppb).
(k) If the value of the known standard is <5%, then go for the sample run. Otherwise, prepare the standard curve again.

(l) Run a known standard after every three samples.
(m) For mercury, add ionic gold (add known gold concentration to samples and Hg standards). Use ICP-MS standard of gold to reduce the memory effect. For mercury, it is better to run a known standard between every two samples.

21.5 Summary

Animal studies are tedious and require a very experienced hand. However, the zebrafish model is one of the less complex animal models due to easy maintenance. Therefore, the zebrafish model is gaining lots of interest among the research community. Drug screening using zebrafish behaviour model is highly successful, and the numbers of drugs showed alteration of neurobehavioural parameters. For instance, caffeine induces stress-like behaviour in zebrafish. On the other hand, mercuric compounds reduce motor behaviour. Therefore, the behaviour of zebrafish can provide us with a primary indication of neurotoxicity. However, biological studies, as described here, are necessary to explain the alteration of those factors in the molecular and cellular level. As zebrafish is a comparatively new animal model, several experimental protocols are yet to be optimized. This chapter provides detailed protocols to carry out the neurotoxicity experiment.

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