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Chapter

Zebrafish (*Danio rerio*) as a Model Organism

Farmanur Rahman Khan and Saleh Sulaiman Alhewairini

Abstract

Animals as model organisms, the silent sentinels, stand watch over the environmental health of the world. These are non-human animal species which can be used to understand specific biological processes and to obtain informations which can provide an insight into working of other organisms. Among the model organisms, the zebrafish (*Danio rerio*) is one of the best leading models to study developmental biology, cancer, toxicology, drug discovery, and molecular genetics. In addition, the zebrafish is increasingly used as a genetic model organism for aquaculture species and in toxicogenomics and also to generate zebrafish disease models for application in human biomedicines. This tiny fish is a versatile model organism for many fields of research because of its easy maintenance, breeding, and transparent body during early development.

Keywords: model organisms, zebrafish, biological process, developmental biology, cancer, toxicology, drug discovery

1. Introduction

Zebrafish (*Danio rerio*) is a prominent model organism in biological researches in recent times. Zebrafish is a tropical freshwater fish, inhabitant of rivers (Ganges mainly) of Himalayan region of South Asia especially India, Nepal, Bhutan, Pakistan, Bangladesh, and Myanmar. It is a bony fish (teleost) that belongs to the family Cyprinidae under the class Actinopterygii (ray-finned fishes).

Zebrafish was first used as a biological model by George Streisinger (University of Oregon) in the 1970s because it was simpler over mouse and easy to manipulate genetically. Streisinger's colleagues especially Chuck Kimmel in his university got much impressed by the idea of using zebrafish embryo more attractive to study the development of nervous system.

The use of zebrafish as a model organism got impetus from the 1990s when it was used to develop two large genetic mutants, one by Nobel Prize winner Christiane Nusslein-Volhard in Tubingen, Germany, and the other by Wolfgang Driever and Mark Fishman in Boston, USA. The identification of mutants is one of the most important strategies for the study in various areas of biology.

Zebrafish has a lot of physiological and genetic similarities with humans, including the brain, digestive tract, musculature, vasculature, and innate immune system [1–7]. Also 70% of human disease genes have functional similarities with those of zebrafish [8].

1.1 Salient features of zebrafish as a model organism

D. Rario is preferred by scientists because of its variety of features that make it useful as a model organism. The embryo develops rapidly outside mother and optically clear and thus, easily accessible for experimentation and observation. The embryo develops very fast, and the blastula stage lasts only for 3 h, while gastrulation gets completed in 5 h; in an embryo that is about 18 h old, very well developed ears, eyes, segmenting muscles, and brain can be viewed as the embryo is transparent. By 24 h, segmentation gets completed, and most primary organ systems are formed. By 72 h, the embryo hatches out from the eggshell and within the next 2 days starts hunting for food. In a period of just 4 days, the embryo converts rapidly into a small version of adult. The rapid development simplifies development and genetic studies.

The adult zebrafish attains sexual maturity very quickly, having generation time of about 10 weeks, and also this tiny fish has good fecundity rate. When kept under optimal conditions, the zebrafish can lay about 200 eggs per week [9, 10]. Under laboratory conditions the zebrafish can spawn throughout the year that ensures the constant supply of offspring from designated pairs that makes this transparent fish a quintessential choice for large-scale genetic approaches to identify novel genes and to discover their specific functions in vertebrates [11]. The zebrafish is a very hard fish and is very easy to raise.

In addition to the features of zebrafish mentioned above, it requires very low space and maintenance cost. These features make this fish an attractive model organism for developmental, toxicological, and transgenic studies [12].

In this chapter author summarizes some of the recent advances in the area of zebrafish research, viz., developmental biology, toxicology, transgenic studies, human disease, drug discovery, cancer, etc. This review is by no means a comprehensive one but an attempt to provide a flavor to the readers some recent advances about this wonderful creature to use in potential researches.

2. Use of zebrafish in developmental biology

Much of the pioneer works that established zebrafish as a model organism were done by George Streisinger, Charles Kimmel, and their colleagues [13]. The team of these researchers studied the embryonic axis, cell lineage analysis, embryonic formation, development of central and peripheral nervous systems, muscle development, differential regulation of gene expression, etc. [14–16].

Many of the critical pathways that control development in vertebrates are highly conserved between human and zebrafish. The zebrafish genome shares a lot of similarities with human genome. About 70% of genes associated with disease in humans have functional homologs in zebrafish [8]. Realizing the importance of zebrafish model, Grunwald and Eisen used this developmental model to study the segmental structure of the brain and characterized neurons in zebrafish for the first time in a vertebrate model [17]. Nüsslein-Volhard recognized the importance of zebrafish as a vertebrate model to study developmental biology by identifying developmentally important genes [18]. The zebrafish model has been used to see the development of various systems/processes as follows.

2.1 Development of the enteric nervous system

Recently advances have been made to study the development of the enteric nervous system (ENS) using the zebrafish model. Like other vertebrates, the zebrafish

gastrointestinal tract is a complex organ composed of multiple cell types like epithelial, muscular, vascular, neural, and immune cells. The gut of the zebrafish (teleost) and amniotes have structural similarities, but in teleost it is less complex as compared to amniotes [19]. The zebrafish GI tract has no distinct stomach but an enlarged area of the anterior intestine that is known as the intestinal bulb. This intestinal bulb displays patterns of motility as well as goblet cells that produce acid and neutral mucins like the stomach of mammals [20, 21]. The gut epithelium of zebrafish is simpler than that of amniotes; it lacks crypts and is arranged in an irregular broad fold rather than forming villi [21, 22]. The genes (*sox2*, *barx1*, *gata5*, *and gata6*) which are responsible for the formation of the stomach of zebrafish also resemble with that of amniotes [23].

Like that of all vertebrates, the enteric nervous system of zebrafish is also derived from neural crest [24], but it differs potentially from amniotes wherein the enteric nervous system is derived from both the vagal and sacral crests, while in the case of zebrafish, it is derived from the vagal crest only [25–28]. Enteric neural crest cell (ENCC) migration along the gut in zebrafish is also similar with that in amniotes. It takes place in two parallel chains along the length of the developing gut [25, 27, 28]. Afterward the precursors of the ENS voyage circumferentially around the gut and differentiate into the enteric neuron and glia. The final organization of the zebrafish ENS is also very simple as compared to that of amniotes; it is composed of single neuron or small group of neurons rather than more complex ganglionated myenteric and submucosal plexuses [27, 29].

2.2 Angiogenesis

Zebrafish model also has been used in the study of angiogenesis and regeneration. Angiogenesis is the process through which new blood vessels originate from preexisting vascular structures which play essential role in healthy physiological and pathological conditions. It is achieved through interaction between endothelial cells and their niche. Inadequate maintenance leads to the development of many disorders like tissue ischemia, inflammatory disorders, retinopathies, excessive vascular growth, or abnormal remodeling that promotes cancer [30].

Being a transparent vertebrate, the zebrafish has emerged as a convenient alternative to study the early development of the cardiovascular system and observe the flow of blood [31]. In zebrafish larvae the vessels and blood flow can easily be visualized by using simple dissecting microscope and also by using fluorescent proteins; the development of the blood vascular system could be examined in great details. By using confocal microscopy and time-lapse imaging, the detailed morphogenetic movements and cell shape changes can be carried out in live specimens [31].

Vascular anatomy development of zebrafish using molecular tracers during early embryonic stages has high level of similarities with other vertebrates [1, 32, 33]. In one of the experiments, the injected fluorescent microsphere was detected when lumenization and anastomosis of the vascular network were complete [34]. The same approach was adopted to compare the development of blood and lymphatic vasculatures in zebrafish [35]. The individual cell growth during vascular development also can be tracked.

For vascular development and growth, angiogenesis plays a very important role. During embryonic development, the intersegmental vessels are formed by angiogenic sprouting from the dorsal aorta, and they have been the target of studies using genetic perturbations or drugs [36]. It has been reported that mammalian malignant cells can be xenotransplanted into zebrafish embryos, and they can form tumors [37], and thus models for tumor angiogenesis have been developed [38].

2.3 Regeneration

The zebrafish exhibits remarkable capacity of regeneration even in adult stages. The caudal fin especially provides an ideal tissue for vascular regeneration studies due to its simple and fine architecture and relative transparency [39]. After successive amputations the full regeneration of the caudal fin used to take place within couple of weeks [40]. The regenerating vessels in the regenerating caudal fin originate from vein-derived cells that have angiogenic potential [41]. These cells migrate individually or in groups and assemble into the vessel in response to chemokine signaling [42].

The zebrafish as an alternative model for angiogenesis and regeneration studies provides the relevance of in vivo assays with simplicity and versatility of in vitro assays. In larvae, access to developing vasculature through fluorophore-tagged strains and small size of zebrafish makes the use of high-throughput strategies possible. In adults, the caudal fin is equally convenient as a model tissue as regenerating vessels can be observed at all stages, and the animals (zebrafish) are suitable for experimental drug manipulations [31].

3. Zebrafish as a cancer model system

Cancer is a cursed reality for millions of humans worldwide and in fact for all vertebrates. The invertebrates such as flies and nematodes also can develop anomalies in cell proliferation. Clinically and pathologically this dreaded disease is present almost exclusively in all vertebrates, from fish to humans. To understand better the formation, growth, and spread of malignant tumors, vertebrate models are imperative. Being a vertebrate the zebrafish is an ideal model to study cancer, though humans and fishes are separated from their common ancestry but biology of the cancer in both groups of organisms is the same [43]. Because of the variety of benefits to use zebrafish as a model organism which are mentioned in "Introduction," the zebrafish is adroitly exploited to carcinogenic treatment, transplantation of mammalian tumor cells, and transgenic regulations [44].

3.1 Zebrafish as a model for carcinogen effects and development of cancer studies

Fishes are exposed to many waterborne carcinogens in the wild that lead to the development of a variety of benign and malignant tumors in teleosts, and these tumors have similar histology as in humans [45, 46]. As like humans, cancer is a genetic disease in fishes as shown by melanomas which develop in *Xiphophorus* hybrids [47]. Choosing zebrafish for modeling cancer studies has many advantages. Highly conserved cancer pathways can be screened genetically using zebrafish. Primarily cancer is a disease of adults, but through mutagenesis screens, cell cycle phenotype could be examined in rapidly developing transparent embryos of the zebrafish. The genes regulating cell cycle, cell proliferation, and apoptosis have already been screened in yeast, *Drosophila* and *C. elegans*, in the similar way gene functions for these biological pathways can be screened in zebrafish to understand the events that lead to the development of cancer in any vertebrate species [43].

By inducing different gene mutations or stimulating signaling pathways through chemicals, the tumors can be induced in different organs of the zebrafish like the pancreas, liver, GI tract, vasculature, muscles, skin, and testes [46, 48–51]. It is

possible to identify the interacting oncogenes via suppressor and enhancer screens which cause the formation of specific type of tumor. The mammalian tumor cells can be transplanted into the zebrafish, dispensing a novel way to study the interactions between transplanted tumor cell and vasculature of host.

3.2 Tumorigenesis

Tumorigenesis is a multistep process induced by chemical carcinogen [52], with accumulation of both epigenetic aberrations and mutations in regulatory regions of genes and disorder of signaling pathways [53, 54]. Methylation of DNA at CpG dinucleotides is an important component of epigenetic gene expression regulation [55] that causes the modulation of protein-DNA interactions [56, 57]. Aberrant methylation of CpG islands (CGT) takes place in the exonic and promoter regions [58, 59] and changes in gene expression associated with tumorigenesis. Hypermethylation of tumorigenetic genes has negative impact (regulation) over tumor suppressor genes (TSGs), DNA repair genes, and antiangiogenic genes, and it is a common quality of neoplastic cells [55, 60–63].

A variety of fishes have been used as model to study tumors induced by environmental carcinogens. Among all the zebrafish proved best for investigating embryogenesis, organogenesis, and impact of environmental carcinogen for the development of cancer [64]. Chemically induced tumors in zebrafish and humans are histopathologically similar [43, 65], and orthologous oncogenes and tumor suppressor genes (TSGs) have been identified in fishes and humans [65]. Hepatic gene expression in humans and zebrafish has revealed conservation of gene expression profiles at different stages of tumor aggressiveness between these two phylogenetically distant species [66, 67].

3.3 Xenotransplantation

Xenotransplantation represents another novel way to induce tumor in zebrafish. The most important feature of xenotransplantation is that tumor cells can be stained/marked by fluorescent stain that distinguishes transplanted cells from normal cells and helps in clear observation of developmental process of the tumor [68]. Several other types of tumor, such as pancreatic cancer, lung cancer, ovarian carcinoma, breast cancer, prostate cancer, retinoblastoma, leukemia, etc., have also been transplanted in the zebrafish [7].

3.4 Angiogenesis

Angiogenesis is the most important factor in tumor growth and subsequent metastasis. The importance of angiogenesis has been discussed well in the previous section on development. The vascular network is helpful to transport oxygen and nutrients to the cells; likewise tumor cells also get the supply of all these materials. Because of this reason, the development and the capability of the formation of blood vessels within the tumor determine the malignancy of the cancer as well as influence the therapeutic effects and prognosis. The endothelial cells of the vascular system can be stained by fluorescent dye/protein that helps to visualize the neovascularization of tiny tumor at the earliest stage, and metastasizing tumor cells can be tracked explicitly at cellular level [7]. The vascular system of tumor has always been the target of antitumor therapies; it is evident from research and clinical observations that if angiogenesis inhibitors are used in combination with chemotherapy, it can improve the outcome in cancer patients [69].

3.5 Skin cancer

Skin or dermal cancers represent the most common type of cutaneous malignancy globally, which includes melanoma and carcinoma of squamous cells [70]. Melanoma is the most pernicious form of skin cancer among all types of skin cancers and has mortality rate over 80% [71, 72]. Melanoma usually develops in the pigmented epidermal cells (melanocytes), which are responsible for the production of melanin. In the beginning of melanoma, it is restricted to the epidermis because of the radial growth phase (RGP) of melanoma, and it can be removed by surgical excision [73]. In later stages of tumor progression, the melanoma cells invade the subcutaneous tissues due to vertical growth phase (VGP) of melanoma and eventually lead toward the metastatic phase. At this stage, very limited therapeutic options are available, and melanoma frequently deteriorates and becomes untreatable [73, 74].

Cutaneous squamous cell carcinoma (cSCC) mostly develops due to UV radiation exposure of epidermal cells, namely, keratinocytes, in which uncontrolled proliferation starts [75]. cSCC accounts for the most frequent type of non-melanoma cutaneous cancer and constitutes about 20% of all skin malignancies [75, 76].

SCCs are curable in situ by surgical excision. Metastatic SCCs are responsible for majority of deaths due to non-melanoma skin cancer [70]. Head and neck squamous cell carcinoma (HNSCC) develops in various places such as the oropharynx and laryngopharynx which is very common worldwide [77]. Especially oral squamous cell carcinoma (OSCC) accounts for about 24% of HNSCC with a mortality rate of 2 million deaths every year [76, 78].

Zebrafish is a powerful in vivo tool to study pathologies and treatment for skin cancer (melanoma and SCC). The zebrafish can be used to study melanoma development, progression, drug screening, and treatment. The zebrafish model has been exploited recently to recognize the key molecules which are responsible for the development of cutaneous squamous cell carcinoma (cSCC) and head and neck squamous cell carcinoma (HNSCC) [72] as well as for SCC target therapies [79].

3.6 Tumor metastasis

Metastasis is a multistep and complex process in which tumor cells penetrate in the vascular system and spread deep in parenchymatous tissues [80]. For better therapeutic practices like development of antitumor drugs and advancements of clinical treatments, the insight into mechanism of tumor metastasis is very helpful. Because of many significant disadvantages in the previous studies using in vivo mouse model, the metastasis process cannot be abstracted properly, but zebrafish cancer model has overcome the drawback of previous models and has shown exceptional strengths. The adaptive immune system in larvae of zebrafish usually develops after 14 DPF, which provides very conducive environment for survival of transplanted cancer cells and metastasis [81], and the process of tumor metastasis can be observed through the transparent body of zebrafish under microscope. To better understand the process of metastasis, the transplanted tumor cells can be stained/treated by dye like CM-Dil or may be labeled by red fluorescent protein (RFP) [82]. Mammalian tumor cells treated with red fluorescent protein when injected into transgenic zebrafish, the process of tumor cell metastasis and angiogenesis can be viewed well after 48 h of transplantation [83]. By using zebrafish, the suppressing or promoting factors for metastasis can be identified. In RFP treated U87 glioma stem cells (GSCs), when transplanted into the yolk sac of the zebrafish embryo, the various invasive stages of GSCs like approaching, cluster formation, invasion, migration, and transmigration can be observed clearly at 48 h postinjection [83].

4. Toxicology and drug discovery

As discussed previously in Section 1, because of many advantages, the zebrafish has recently emerged as a prominent model for toxicological studies and drug discovery. The effects of drugs on growth and development can be examined visually through length and shape of the zebrafish body as well as the morphology of internal organs such as the brain, liver, cardiovascular system, pancreas, intestine, kidney, notochord, etc. The zebrafish model also has been used to know the organ function assays and assessment of drug effect [84].

Zebrafish embryos are used as predictive model to assess the toxicity in mammals. The lethal concentration (LC_{50}) of different chemicals has been determined in embryos of zebrafish and has been compared with the mammalian LC_{50}, and it has been found that median lethal dose of zebrafish is lower than mammals [84]. The effects of drugs on specific organs have also been studied, and it has been found that organ toxicity is similar in both zebrafish and mammals. The drugs that were used to evaluate the organ toxicity were gentamicin, cisplatin, vinblastine, quinine, neomycin, doxorubicin, dexamethasone, cyclosporin A, caffeine, camptothecin, MPA, fluorouracil, etc. [85–90].

4.1 Drug toxicity

In drug development, the toxicity plays a major role. Due to the toxicity problem, many new drugs have been declined by the FDA. The evaluation of toxicity of drug is very essential to know the end points of toxicity, dose-response relationships, and mechanism of toxicity and also to determine the toxicodynamics of the drug [91].

The zebrafish is acquiring the reputation rapidly as a promising model animal to study drug and chemical toxicology [92, 93]. The toxicity of some of the important drugs has been examined using the zebrafish model, for instance, Amanuma et al. [94] developed a test in which susceptible zebrafish was used to detect small molecule-induced mutagenesis. The embryos of zebrafish were utilized to compare the developmental toxicity resulting from the exposure to ethanol or acetaldehyde [95]. Toxicity of antirheumatic drug like diclofenac was evaluated by using zebrafish. Now, zebrafish has got the status of a successful animal model to study drug toxicity and toxicology caused by environmental contaminants [91].

4.2 Zebrafish and drug discovery

The zebrafish model has been used potentially in drug discovery and to know the effects of neurotoxic, ototoxic, and neuroprotectant drugs. The process of drug discovery is divided into four main components: screening of lead compounds, target identification, target validation, and assay development [96]. The process of target identification involves the recognition of target gene or protein which when modulated by a drug can have positive effects on the progression of disease. After identification of possible target, the validation process of target begins through determination of protein function and assessment of the druggability of the target [84, 97–99]. Zebrafish has great role in each of these areas of drug discovery.

4.3 Angiogenesis

The angiogenesis has already been discussed earlier in detail in previous sections on development and cancer. The impact of various proangiogenic compounds like simvastatine or penicillamine[20] or antiangiogenic compounds like vandetanib

or PTK787 can be assessed well and visualized through the development of the vascular system in transparent zebrafish embryo [84].

4.4 Cardiotoxicity

In drug development, cardiotoxicity is one of the major concerns. Through the transparent zebrafish embryo, various cardiac functions like heart rate, rhythm, contraction, circulation, etc. can be assessed directly. It has been demonstrated well that toxic effects of ten cardiotoxic agents in zebrafish embryos have similar impact as in humans [100]. Treatment with terfenadine and clomipramine caused severe impairment of cardiac functions, edema, hemorrhage, arrested heartbeat, and even death. These results in zebrafish exhibit similarities with humans [101]. Another group of researchers proposed to use a transgenic model for high-throughput testing of small molecules that modulate the heart rate of the zebrafish embryo [102]. Thus, zebrafish is a suitable model for preliminary screening of molecules which have potential therapeutic or toxic effects.

5. Human disease and zebrafish

Most of the tissues and organs found in humans and zebrafish are the same except lungs and prostrate and mammary glands. The cloning of mutated genes screened for specific phenotypes in zebrafish has similarities in humans and thus serves as model for human disease and to study underlying mechanisms. The first human disease identified using zebrafish was a blood disorder involving specific defect in hemoglobin production through ALAS2 mutated gene [103].

Many other mutants which show phenotypic similarities to human disease have been screened and identified. These include neurological disorders [104], hematological disorder [105, 106], cardiovascular diseases [107], muscle disease [108] and cancers [109, 110], Parkinson's disease [111], anxiety, and posttraumatic stress disorder [112].

6. Zebrafish as a model organism for aquaculture species

Among different fish species of interest to aquaculture, zebrafish is genetically more tractable. The zebrafish model is used commercially in many areas of aquaculture such as in the identification of genes involved in the development of the muscles, bones, and fats, the metabolism of nutrients, disease, and stress pathways and also behavioral traits. The drugs which affect the physiology of the fishes can be tested easily in zebrafish especially their effect on a range of alleles to assess their genetic property [113]. Many researches have been done regarding the improvement of diet and their husbandry to improve the growth rate and reduce stress and disease in many fish species like gilthead seabream, seabass, rainbow trout, Atlantic salmon, tilapia, catfish, cod, etc. [10]. The zebrafish disease models are being used in various infections of aquaculture, for instance, tuberculosis and streptococcal and salmonella infections [114].

7. Conclusion

Zebrafish is a successful and versatile animal model system, offering a tool to model gene function, development of various organ systems, cancer studies,

toxicology, drug discovery, human disease and disorders and also in aquaculture, etc. because low cost and easy maintenance, transparent embryo, easy manipulation, high fecundity, and rapid embryonic development favor the zebrafish as an attractive model for in vivo assays with simplicity and versatility of in vitro assays over mammalian models which lack all of these benefits. The future of zebrafish as model organism is very bright. In coming years, an increased number of reports are expected on the application of zebrafish as an effective bioindicator.

Conflict of interest

The author declares that there is no conflict of interest.

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