Zebrafish (Danio Rerio) Inmodeling Brain Disorders

Tianzhi Yang¹, Duy Tran¹, Leanne Lai² and Shuhua Bai*¹

¹Department of Basic Pharmaceutical Sciences, Husson University, USA
²Department of Sociobehavioral and Administrative Pharmacy, Nova Southeastern University, USA

Abstract

Zebrafish (Danio rerio) has been considered as an increasingly popular model organism for biomedical research since 1980s. Due to highly conserved nature of both genetics and cell biology as higher vertebrates, Zebrafish is a suitable animal model in screening leading compounds and identifying drug targets. Small body size, ease of care, rapid development, and transparency of the Zebrafish embryo allow researchers to visualize the processes of morphogenesis in early developmental stages with the high throughput screening and in the cost-effectiveness of producing and maintaining a large number of larvae in the laboratory. Moreover, variety of gene editing tools including chemical and insertional mutagenesis, morpholino antisense knockdown, and recent target-selected mutagenesis approaches have been available to model human diseases in Zebrafish. By reviewing current studies, we highlight the use of Zebrafish in representing depression, brain tumor, epilepsy, and anxiety brain disorders. As a relatively simple and feasible vertebrate species, Zebrafish provides new promises in defining disease pathway and discovering specific and powerful therapies.

Keywords: Zebrafish; Gene editing; Depression; Brain tumor; Epilepsy; Anxiety

Introduction

The interest in Zebrafish (Danio rerio) has been rapidly growing in the drug development and discovery over the past few decades [1,2]. Zebrafish models have been widely used ranging from drug screening, identification, target confirmation, to toxicity assessment [3,4]. The use of Zebrafish essentially bridges translational gaps to the clinic. With scale and throughput advantages just like cell study, Zebrafish provide a unique in vivo system to validate new drugs with the full anatomical and physiologic permeability and enzymatic barrier characteristics of physiological systems [4,5]. Multiple advantages come from Zebrafish models in the drug discovery including high fecundity, rapid development, and transparency during embryonic and larval stages, available genetic editing tools, pharmacological manipulations, and cost-effectiveness [6]. As good breeders, female fish lay large numbers of eggs per week and the eggs/embryos develop quickly and externally through six stages: embryonic pre-hatching (0-72 hpf, hours post fertilization), post-hatching (72-120 hpf), larval (5-29 dpf, days post fertilization), juvenile fish (30-89 dpf), adult fish (90 dpf-2 years), aged fish (from 2 years) [7]. All the major tissues and organs such as heart, circulating blood, eyes, ears, and nervous system are formed at1 dpf; by 3 dpf, the blood-brain (BBB) is observed; and fish larva develop the liver, pancreas, and a complex vascular network after 5 dpf [8,9]. These rapid embryonic development and external fertilization make experimental manipulations and monitoring much easier [7]. Transparent fish eggs and embryos also make Zebrafish experiments significantly easy as higher vertebrates, Zebrafish is a suitable animal model in screening leading compounds and maintaining large numbers of larvae at a low cost. About 3 mm of larvae at 5 dpf and 3 cm size of adults of Zebrafish enable large numbers of these vertebrates to be maintained in a relatively small laboratory space [2]. Therefore, many specific studies in the Zebrafish can be automatically assessed in a 96-well plate assisting with multichannel pipettes or robotic delivery machines. While each well of the plate can be manually scored for phenotype in low-throughput assays, images or videos are recorded by using transgenic fluorescent fish with automated stage microscopes and laser cytometers [11]. High-throughput examinations of Zebrafish embryos bring a great potential to automatically document real-time physiological processes such as heart beat, blood flow, and behavior. These types of dynamic studies have never been screened in in vitro, in cell, or in vivo animal models [3]. With those unique features, Zebrafish have been used most successfully and extensively in the different phases of drug development (Figure 1).
Genetic Tools for Modeling Human Diseases in Zebrafish

Animal models, ranging from small vertebrates to large animals, have long been important for recapitulating almost all the processes involved in the development of a disease, thereby providing the only feasible system known today to test new drugs for therapeutic interventions from bench to bedside [3]. Because of the striking homology between mammalian genomes and the many similarities from anatomy to cell biology and physiology, mouse has been preeminent in mimicking human diseases [4]. Sophisticated transgenic approaches using gene knockdown techniques have allowed the creation of mouse models as the most widely used model of human diseases. However, a number of factors such as high-throughput screening must be considered when an animal disease model is chosen. Despite large-scale and long-generation time of mutagenic stages in mouse animals, Zebrafish have been extraordinarily successful strategies in the drug screening, providing considerable insight into how to treat human diseases in regulating similar processes [1,3,4]. As Zebrafish genome has recently been fully characterized, more than 70% genes in Zebrafish are found to possess functionally homologous similarity with human, and play important roles in the development of diseases [12,13]. Several independent alleles with varying phenotypes have established the patho-physiology of human diseases [12,13]. More importantly, sophisticated genetic editing tools including forward-genetic screens, morpholino oligo-nucleotide (MO) knockdown, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system have been used to develop diseases in Zebrafish (Table I) [4,14]. A number of human diseases including cancers, renal disorders, infections, cardiovascular diseases, hearing disorders, and neurological degenerative diseases have been modeled in Zebrafish on a large scale and with an economic cost. Traditionally chemical and insertional mutagenesis tools have been firstly used to model human diseases in Zebrafish for small-molecule screening since 1980s [6]. Facilitated by the transparency of embryos and larvae, these forward-genetic screens attribute the ease of phenotypic screening, allowing studies to be done on a large scale without sophisticated infrastructure or equipment [4]. Several chemical mutagenesis techniques have been utilized to screen and link random point-mutations in Zebrafish with their corresponding genes [4]. N-ethyl-N-nitrosourea (ENU), a mutagen, was successfully used to randomly generate hundreds of point mutations in Zebrafish, resulting in a high frequency of mutant phenotypes as a forward genetic model system [15]. These screens resulted in the identification of over 2,000 developmentally important loci, including more than 100 genes involved in heart formation and function, more than 50 involved in blood cell and vasculature formation, and more than 30 involved in the early body formation [16]. While chemical mutagenesis is very efficient, identification of the mutated genes is slow and labor-intensive after induced mutations by chemicals. As an
A new transposon-based mutagenesis approaches was successfully utilized for "gene trapping" and "gene breaking" in Zebrafish [17]. Beside forward genetics, reverse genetics is another viable tool to observe effects in the injected larvae immediately [23]. Up to date, TALENs gene-editing tools has proven to be revolutionary in Zebrafish research and many disease models including hematological disorders, malignancy, and neurological syndromes have been rapidly possible to observe effects in the injected larvae immediately [23].

Table 2: Examples of modeled brain diseases in zebrafish.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Target</th>
<th>Method</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Depression</td>
<td>GR</td>
<td>Cloning mutation</td>
<td>[31]</td>
</tr>
<tr>
<td>Brain tumor</td>
<td>Xenograft</td>
<td></td>
<td>[29; 30; 32]</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>p53</td>
<td>Cloning Mutation</td>
<td>[33]</td>
</tr>
<tr>
<td>Anxiety</td>
<td>rb1</td>
<td>TALEN</td>
<td>[34]</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>STXBp1</td>
<td>CRISPR/Cas9</td>
<td>[36]</td>
</tr>
<tr>
<td>Anxiety</td>
<td>NMDA receptor</td>
<td>Chemical</td>
<td>[37]</td>
</tr>
<tr>
<td>AHDS</td>
<td>mtr1 gene</td>
<td>Cloning Mutation</td>
<td>[38]</td>
</tr>
<tr>
<td>AHDS</td>
<td>Mct8</td>
<td>ZFN</td>
<td>[39]</td>
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GR: Glucocorticoid Receptor; rb1: tumor suppressor retinoblastoma1; TALEN: Transcription Activator-Like Effector Nucleases; PTZ: Pentylenetetrazole; STXBp1: Syntaxin Binding Protein 1; NMDA: N-Methyl-D-Aspartate; mtr1: fragile X mental retardation 2; AHDS: Allan-Herndon-Dudley Syndrome; Mct8: Monocarboxylate Transporter 8; ZFN: Zinc-Finger Nucleases; CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats Associated Protein 9.

Alternative method, insertional mutagenesis allows for rapid cloning of the gene with large-scale screening for recessive developmental mutations. More than 500 mutations and about 350 loci with 335 cloned to date can be quickly characterized after the insertional nature of the mutagen using retroviral vectors [17]. However, inability to develop a similarly mutagenesis and high-titer retrovirus with robust expression hinders the generation of expression based mutagenic for the Zebrafish [17]. A new transposon-based mutagenesis approaches to produce many non-specific effects in Zebrafish. Management of off-target effects such as neural toxicity and validation of potential gene specific effect are critical [18]. Targeted mutagenesis, which has greatly advanced gene function studies in mouse, is currently exploited towards site-specific mutagenesis and gene knockout in Zebrafish. Some of first Zebrafish mutants have been generated and characterized by using Targeting Induced Local Lesions in Genomes (TILLING). As a reverse genetics strategy, TILLING methodology has been used to identify more than 150 loss-of-function mutations and the effectiveness of TILLING is continued to be improved both forward and reverse gene mutations in Zebrafish [20]. A Zebrafish TILLING consortium has been established to facilitate the isolation of specific mutant lines through the Zebrafish International Resource Center. Recently, advances in nucleases-based genome editing technologies, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system, have allowed researchers to generate diverse genetic modifications in cultured cells and even in whole animals [21] (Figure 3). ZFNs and TALENs are chimeric proteins fusing the DNA-binding domains required for the protein-DNA interaction. Their programmable and sequence-specific protein link to a non-specific DNA cleavage domain, induce DNA double-strand breaks, and enable a broad range of genetic modifications. This results in stimulating error-prone non-homologous end joining or homology-directed repair at specific genomic locations [21,22]. First reported that ZFNs were engineered in the Zebrafish ortholog of the vascular endothelial growth factor-2 receptor and encoded ZFNs into one-cell-stage Zebrafish embryos led to mutagenic lesions at the target site with high frequency [22]. As a cheaper and more efficient gene editing tool, TALENs have several advantages over ZFNs and morpholinos: while ZFNs can only target specific sequences, TALENs have the potential to work on any DNA sequence; morpholinos are temporary modifications, but the effects of TALENs are permanent. Importantly, TALENs allow faster analysis of induced mutations because it is possible to observe effects in the injected larvae immediately [23]. Up to date, TALENs gene-editing tools has proven to be revolutionary in Zebrafish research and many disease models including hematological disorders, malignancy, and neurological syndromes have been rapidly and successfully constructed in Zebrafish [14]. Compared to ZFNs and TALENs, CRISPR/Cas system has more programmability using binding domains -single guide RNA (sgRNAs). This advantageous feature makes the system the most amenable approach to high-throughput mutagenesis projects. Moreover, an increasing number of tools designed for CRISPR/Cas system in Zebrafish are website or software designed to assemble sgRNAs with minimized off-target effects based on the wild type genomic sequences, including CRISPR Multi Targeter, CRISPR direct, CCTop, CHOPCHOP, sgRNAcase9, CRISPR scan [14]. Altogether, these gene-editing tools are opening new doors for engineering knock-outs in Zebrafish and...
providing great promising disease models for high-throughput drug target and validation.

**Brain Disorder Models in Zebrafish**

Modeling brain disorders remains a challenge due to the complexity of the diseases. Most animal models (rodents) use adults, which have less potential for high-throughput screening than larvae and embryos [10]. Zebrafish’s brain morphology shares many similarities with human’s and rodent’s brains in terms of both cellular morphology and macrostructure [12]. Brain neurochemistry, including transmitters, receptors, transporters, and enzymes of synthesis and metabolism, is also highly conserved across human and Zebrafish [12]. As Zebrafish affective behaviors are involved with the amygdala and habenula, their brain is very relevant to human brain functioning and structures. Similar to humans, cortisol activated by the cascade of hypothalamo-putitary hormones mediates stress responses in Zebrafish [24,25]. These advantages make Zebrafish become more and more popular in the studies of neuroscience and pharmacology [12]. Moreover, Zebrafish demonstrate easily assessed multiple social behaviors. Those anxiety/fear-related behavior, mood/depression reduced activity, impaired memory, increased/decreased shoaling startle response, impulsive hyperactive locomotion, reward-related behaviors, and pain responses, have been proven very useful in characterizing the development of neuro-cognitive disorders [12,26]. Furthermore, since larvae are less than a few millimeters in length, Zebrafish are particularly amenable to high throughput screening in 96-well plate format for drug delivery across the blood-brain barrier (BBB). Studies have shown that tight-junction proteins, such as Claudin-5 and ZO-1, and P-glycoprotein efflux proteins, can be detected in some cerebral vessels in Zebrafish after 3 dpf [27,28]. Brain distribution of anticancer drugs and small interference RNAs have been evaluated in transgenic Tg (flii:GFP) fish [29,30]. Those unique brain features in addition to many available genetic methods, rapid development, cost-effectiveness, large-scale quantitative behavioral assessment, and advanced quantitative anatomical evaluation, give Zebrafish an optimal organism for studies on brain diseases [4]. Major brain disorders, including depression, brain tumor, epilepsy, and anxiety have been modeled in Zebrafish (Table II).

**Depression**

Pathophysiology of depression involves in genetic, biological (neurochemical), and environmental factors. In Zebrafish, these factors have been observed to have a strong correlation with certain Zebrafish phenotypes’ behaviors that resemble clinical depression in human [12]. Cortisol level is increased in response to stress in human. Cortisol signals through glucocorticoid receptor (GR) and mineral corticoid receptor (MR) to activate a cascade that ultimately disrupts the stress reaction and assists recovery. There has been evidence that GRs have positive effect on stress, and thus glucocorticoid resistance might play a role in clinical depression [31]. A Zebrafish mutant, gr-s357, has been identified with defective GR transcriptional gene. This phenotype expresses behaviors that resemble depression, i.e. decreased exploratory behavior and decreased habituation to anxiogenic environment [31]. Additionally, treatment with selective serotonin reuptake inhibitors restores normal behaviors. This evidence suggests that Zebrafish is a potential candidate for modeling depression in human and also a promising drug screening tool for antidepressants [32].

Besides genetic models, other tools, such as the chronic unpredictable stress (CUS) that has been widely used in rodent models, has recently been successfully adapted in Zebrafish model. Zebrafish with CUS were shown to increase anxiety levels [33], impair cognitive function, increase corticotrophin-releasing factor [34], and decrease GR expression [35-39], which lead to decrease in shoaling, exploration, and increase in anxiety behaviors. These changes resemble depression in humans [40]. Pharmacological models have also been used to trigger depression-like behaviors in Zebrafish. For example, reserpine, which depletes monoamines and causes depressive behaviors in humans and rodents, has been shown to reduce Zebrafish activity after 7 days treatment, resembling depression in humans [41].

**Brain Tumor**

There have been studies utilizing Zebrafish for studying metastatic potential, tumor-induced angiogenesis, extravasation, and tumorigenicity [13]. Despite the evolutionary gap between fish and human, Zebrafish shares many similarities with human in terms of genetic pathways [42]. Vasculature remodeling, cancer invasion, and metastasis has been specifically effective in widely studied in Zebrafish. Brain tumors in Zebrafish developed by transplantation are used to evaluate therapeutic efficacy of optimized formulations in which such dynamic process can be followed in cancer invasion, and metastasis. Zebrafish developed by transplantation are used to evaluate therapeutic efficacy of optimized formulations in which such dynamic process can be followed in real time [28]. For the transplantation of brain cancer model, after fluorescent labeled human glioblastoma-astrocytoma U-87 MG cells were injected into the brain ventricles at 2 dpf, brain tumors developed with the aggregated cells at 5 dpf [30] (Figure 4). A similar study developing an orthotopic transplant into the vitreous cavity of a Zebrafish did observe that human retinoblastoma cells could be injected into the vitreous cavity about 2dpf and maintain stability and size for about 4 dpi [43].

Many genes and pathways related to onco-genesis are conserved in Zebrafish. For example, the tumor-suppressor gene tp53 mutation related cancers has been extensively studied in human. Almost half of human tumors are found with loss-of-function mutation of the tp53 gene [33]. was able to isolate 3 Zebrafish lines that express mutations in the tp53 gene. Two of those had mutations that are similar to those found in human cancers [33]. These facts suggest that Zebrafish can provide a good model for studying cancers. Not surprisingly, Zebrafish has already been used for modeling certain brain cancers, such as glioblastoma, neuroblastoma, and melanoma, using transplantation methods [13,44]. Although Zebrafish has a low rate of spontaneous gliomas, transgenic techniques can induce rapid development of tumors [45]. Has demonstrated that several cancer types, including glioblastoma, can be induced in Zebrafish using transgenic approach. By inducing the expression of Zebrafish Smo1-EGFP combining with the expression of human AKT1, Zebrafish were able to form several tumor types [45]. Adult Zebrafish, unlike mammals, retains abundant amount of embryonal epithelial tissue surrounding the ventricular system of the brain. Zebrafish, therefore, has a higher rate of developing embryonal carcinoma of the central nervous system (CNS). This makes Zebrafish a good candidate for modeling pediatric neoplasia of the brain and eye [46]. However, with transgenic approaches, oncogenic Zebrafish has decreased survivability, thus loses usability [45]. In addition to the advantages provided by forward genetics, Zebrafish’s high rate of reproduction
Epilepsy

Animal models for epilepsy such as the maximal electroshock seizure and pentylentetrazole (PTZ) induced seizure in rodents have been utilized for more than 6 decades as the main methods for antiepileptic drug (AED) screening [47]. In fact, many AEDs have been discovered in the last 20 years by using these models. However, about 30 percent patients still experience refractory epilepsy. made an interesting point in a review article in 2011 that drug-resistant epilepsy could be due to the old screening models, which only identify agents that are similar to existing drugs [47]. As knowledge on Zebrafish and its genetic toolbox expands, this animal model has become more and more viable as a novel AED screening method. Various methods have been developed to induce and characterize epilepsy in Zebrafish. For example, similar to rodent models, PTZ can be used to induce epileptic seizures in Zebrafish [35]. PTZ levels in the Zebrafish brain can be determined by high-performance liquid chromatography method. Epileptic seizure is characterized based on phenotypic behavior of the Zebrafish e.g. erratic movements, burst swimming, circular movements, abnormal whole-body rhythmic, loss of body posture, and death. Increased expression of c-fos in the brain can also be measured [35].

The models discussed earlier may not represent chronic epilepsy, instead that they may only mimic acute seizure episodes in humans. To overcome this, forward genetics has been utilized. Several Zebrafish mutants have been identified and used as models for chronic epilepsy [48-50]. One example is the Scn1a Zebrafish mutant, which has a defective voltage-gated sodium channel [48]. Because of the defect, these Zebrafish experience spontaneous seizures characterized by using electroencephalography and also drug-resistant seizures, which may better represent epilepsy in humans. Another example is the mind-bomb mutant, which has a mutation in the ubiquitin E3 ligase gene [49,50]. This mutation leads to failures in the Notch signaling, excessive numbers of neurons, and depletion of neural progenitor cells. These mutants also experience spontaneous seizures [50]. Moreover, these mutants are responsive to AED treatment [48], which further supports the use of Zebrafish in modeling epilepsy in humans.

Anxiety Disorders

Unlike brain cancer and epilepsy, anxiety disorders are associated with a combination of genetic factors and complex changes in neuro-activity/memory in response to stress. Anxiety, although, represents one of the most common health problems, remains poorly understood and inadequately treated [51]. Mouse models of anxiety disorders have been extensively studied and utilized for the discovery of therapeutic compounds [51]. Recently, Zebrafish has also emerged as a promising model for anxiety disorders [37]. Not long ago, Zebrafish was thought to be primitive and would not be a candidate for studying complex affective disorders in humans. However, recent evidence has shown that Zebrafish can display many “emotional” behaviors, including anxiety-like behaviors. These are triggered by dangerous or potentially dangerous environmental stimuli. These Zebrafish have reduced exploration, reflected as diving, thigmotaxis, scototaxis, freezing, opercular movements, body color change, and erratic movement [26]. Moreover, Zebrafish also share several similar neuro-chemical pathways and processes with humans and rodent models [13]. For example, Alsop et al. has shown that adult Zebrafish has fully developed corticoid stress axis, similar to humans [44]. As discussed earlier, the corticoid stress axis is believed to be the core element in the stress response, which strongly associates with anxiety disorders. Several rodent paradigms for assessing anxiety-related behavior have been adapted in Zebrafish. Some examples include the novel tank test, which is similar to open field test in rodents; light-dark boxa a measurement of scototaxis; social preference test; shoaling; boldness and novel object approaching; and predator avoidance[37]. These tests, combining with observational tools, such as automated camera and analyzing software, make Zebrafish a much more efficient model for anxiety disorders [13,37]. Additionally, Zebrafish also respond to anxiogenic or anxiolytic agents, further increasing the usability of Zebrafish in drug screening [37].

Conclusion

The knowledge and available tools for researching Zebrafish continue to grow. The interest in Zebrafish has increased rapidly in the past decade due to its numerous advantages over the traditional rodent models for human diseases. As the need for new therapeutic agents rises, Zebrafish becomes more and more attractive as an excellent model for brain disorders.

References


