Zebrfish: From disease modeling to drug discovery
Amy L Rubinstein

Address
Zygogen LLC
520 Kell Hall
24 Peachtree Center Avenue
Atlanta
GA 30303
USA
Email: amy@zygogen.com

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The study of zebrfish, a leading model organism for developmental biology, is rapidly expanding to include human disease. Zebrfish models based on known disease mechanisms have been developed in several therapeutic areas, including blood diseases, diabetes, muscular dystrophy, neurodegenerative disease, angiogenesis and lipid metabolism. This review summarizes recent progress in disease model development, and outlines the potential of zebrfish to contribute to drug discovery through the identification of novel drug targets, validation of those targets and screening for new therapeutic compounds.

**Keywords** Disease models, drug screening, target identification, target validation, zebrfish

**Abbreviations**

dpf Days post-fertilization
gckd Glomerulocystic kidney disease
g-rcfp Green reef coral fluorescent protein
modys Maturity-onset diabetes of the young, type V
ped6 \(N^\prime\)-(6-(2,4-dinitrophenyl)amino)-hexanoyl-1-palmityl-2-BODIPY-FL-pentanoyl-sn-glycero-3-phosphoethanolamine
mptp 1-Methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine
vegf Vascular endothelial cell growth factor

**Introduction**
The zebrfish, a small teleost fish that has long been a favorite in home aquariums due to its hardy nature, has emerged as one of the leading models for studying development. Zebrfish embryos develop outside of the mother and are transparent, allowing easy visualization of tissues and organs. Furthermore, zebrfish produce hundreds of offspring per mating pair per week, and are amenable to large-scale phenotypic screening. As a vertebrate organism, the zebrfish presents many organs and cell types similar to that of mammals. Organogenesis occurs rapidly, and major organs are present in larvae by 5 to 6 days post-fertilization (dpf), as shown in Figure 1. Thus, the zebrfish has attracted researchers from various fields, such as neuroscience, hematopoiesis or cardiovascular research. In fact, the zebrfish was recently described as 'the canonical vertebrate', due to the similarities between zebrfish and mammalian biology [1]. As a result of these shared features, many laboratories have begun to exploit the unique advantages of the zebrfish system to study human disease. This drive has included the development of zebrfish disease models and disease-related assays, as well as the identification of human disease gene homologs. This review summarizes efforts over the past few years to demonstrate the relevance of zebrfish to human disease.

Modern drug discovery involves a wide variety of approaches for the identification and validation of new therapeutics, including both in vitro and in vivo assays. *In vitro* assays have been performed in cultured cells and yeast, as well as in whole animals, both invertebrates, such as *Drosophila* and *Caenorhabditis elegans*, and mammals, such as mice, rats and primates. This review examines the emerging role of zebrfish in the drug discovery process, as well as situations where zebrfish may provide a unique advantage over other model organisms.

**Zebrfish disease models**
Large-scale mutagenesis screens performed in several laboratories and published together in 1996 resulted in the identification of over 1000 mutations with visible embryonic phenotypes [2,3]. Several mutant phenotypes were later shown to be caused by genes involved in human diseases and, thus, may serve as zebrfish models for these disorders. For example, two types of porphyria were recognized [4,5], as well as mutations resulting in congenital anemia [6,7]. Blood diseases were one of the first areas in which zebrfish made advances into human disease modeling, and several excellent reviews describe this work [8-10].

Zebrfish models of human diseases with a known genetic basis have been developed in several therapeutic areas. In some cases, models are created by identifying stable mutations with phenotypes of interest from collections of chemically mutagenized fish. Specific inactivation of genes known to be involved in human disorders can also be accomplished, by injecting embryos with antisense morpholinos. Morpholinos are oligonucleotides with a stable morpholine backbone that reliably inactive gene function by either blocking translation or preventing correct splicing of pre-mRNAs [11••,12]. RNA interference mediated by double-stranded mRNA, a standard method for gene inactivation in invertebrates, causes non-specific inactivation of genes in the zebrfish, limiting its usefulness for gene function studies [13]. A third genetic method for disease model development involves overexpression of pathogenic proteins in specific cell types.

**Disease models**
Mutations causing defects in the developing heart may be useful as models for the study of cardiomyopathies and arrhythmias [14]. For example, a loss-of-function mutation found in *Tnnt2*, the gene encoding cardiac troponin T (the contractile protein of the thin-filament), causes severe heart defects in the zebrfish [15]. Mutations in the human Tnnt2 gene have been linked to familial cardiomyopathy; thus, this zebrfish mutant may represent a useful model for studying the human condition. Another example is the heartstrings mutation, caused by a deficiency of the *Tbx5* gene, a member of the T-box family of transcription factors [16]. Mutation of the human *Tbx5* gene causes a disorder known as Holt-Oram syndrome, characterized by deformities in the heart and upper limbs. Zebrfish with this mutation have analogous defects both in the heart and pectoral fins.


Mutations in the homeobox gene variant hepatic nuclear factor 1 (vHnf1) are associated with two human diseases, maturity-onset diabetes of the young, type V (MODY5) and glomerulocystic kidney disease (GCKD). Zebrafish with mutations in vHnf1 exhibit defects in the liver and pancreas, suggesting that the vHnf1 protein plays a role in their development [17]. vHnf1 Mutants should be useful models for delineating the mechanisms of MODY5 and GCKD.

A model for muscular dystrophy was designed by using morpholinos to inactivate the gene encoding dystroglycan, a component of the dystrophin-glycoprotein complex [18]. While mouse dystroglycan mutations result in early embryonic lethality, loss of dystroglycan function in the zebrafish is less severe. Embryos survive for at least 2 days, exhibiting muscle degeneration that resembles human muscular dystrophy. Genes coding for proteins linked to the familial Duchenne muscular dystrophy (DMD), dystrophin and Dp71, have also been cloned in zebrafish [19].

A recent study explored the use of human microtubule-associated protein tau, which has been implicated in Alzheimer’s disease, in the development of neurodegenerative disease models [20]. In particular, a mutant form of tau, found in patients with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), was expressed specifically in neurons, resulting in neurofibrillary tangles similar to those observed in the brains of patients with Alzheimer’s disease.

Another method for designing disease models in zebrafish involves the use of chemicals to induce disease-like states. This is illustrated by a zebrafish model of hemophilia created by treating embryos with copper chloride, which resulted in a prolonged clotting time [21]. The chemical mutagen ethylnitrosourea, a known carcinogen in mammals, induces a variety of skin cancers in the zebrafish [22]. Our research team at Zygogen (USA) is developing a model for Parkinson’s disease in the zebrafish using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a chemical compound that causes parkinsonism in humans and has been used in many animal models of Parkinson’s disease. We have shown that MPTP destroys clusters of dopaminergic neurons in the zebrafish, suggesting that its mechanism of action in zebrafish and mammals is conserved [McKinley E, Cato C, Rubinstein A, unpublished data].

Recent work has also begun to establish zebrafish models of bacterial pathogenesis. Zebrafish are susceptible to strains of streptococcus, including one that normally infects humans [23]. Injection of *Streptococcus iniae* into zebrafish muscle leads to a rapid systemic infection that resembles streptococcal disease. *Mycobacterium marinum* is another common pathogen that causes tuberculosis in adult zebrafish. Macrophages in the zebrafish embryo can also be infected with *M. marinum*, a process visualized *in vivo* by labeling the bacteria with green fluorescent protein [24].

### Zebrafish disease gene homologs

While the size of the zebrafish genome is approximately half of that of the human genome, it may actually encode a greater number of genes, due to gene duplication events [25]. Because the regulatory regions of gene duplicates may diverge, they will often, together, fulfill the function of a single mammalian gene, allowing a more detailed dissection of gene function [26]. Most gene families present in mammals are represented by one or more orthologs in the zebrafish. The identification of numerous human disease gene orthologs has further confirmed the relevance of the zebrafish for the study of human disease. Identification and characterization of human disease gene orthologs should facilitate the development of new zebrafish disease models. For example, genes linked to a number of neurological disorders, such as Alzheimer’s disease, Huntington’s disease and amyotrophic lateral sclerosis, have been cloned in the zebrafish. In addition, genes involved in thrombosis, inflammation, leukemia and diabetes have been cloned. Examples of human disease genes that have been identified in the zebrafish are presented in Table 1 [27-32,34-39].

### Integrating zebrafish into preclinical drug discovery

The modern drug discovery process can be divided into three major components: target identification, target validation and drug screening. Target identification refers to the process of identifying gene products that, when modulated by a drug, can have a positive impact on disease state progression. Once a possible gene target is identified, the target validation process begins by determination of protein function and assessment of the ‘druggability’ of the
Since zebrafish are amenable to large-scale mutagenesis, a Target identification tested against validated targets for their ability to modulate target. Furthermore, small molecule compounds can be used in each of these areas of drug discovery.

**Target identification**

Since zebrafish are amenable to large-scale mutagenesis, a forward genetics approach can be used to identify novel disease-relevant targets. For example, one assay used in such a screen involved whole-mount in situ hybridization to analyze catecholaminergic neurons in mutated fish [40]. Genes that disrupt the formation of specific neuronal classes may represent important targets for neurodegenerative and psychiatric disorders. Another screen directly examined the behavior of zebrafish exposed to cocaine to identify mutants with reduced sensitivity to cocaine [41]. Cocaine-treatment of normal fish increased their aggressive behavior, and changed their conditioned place preference, an indication of the rewarding effect of cocaine. Analysis of mutations that affect these cocaine-induced behaviors may help identify targets for modulation of addictive behavior. In a third screen, zebrafish mutations with defects in blood clotting were identified by measuring time to clotting prolonged by a drug [42].

A fluorescent lipid assay was used to identify genes that may interfere with normal lipid processing [42]. Zebrafish larvae ingested the quenched fluorescent phospholipid substrate N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-sn-glycero-3-phosphoethanolamine (PED6) that fluoresces after cleavage by phospholipases in the intestine. Since the larvae are translucent, the resulting bright fluorescence in the intestine and gall bladder was easily visualized, reflecting hepatobiliary transport of PED6. The PED6 assay was used to identify the fat free mutation, which appears to interfere with normal lipid processing [42]. Because no obvious abnormalities were observed in fat free, this mutation would not have been identified through conventional screens based on morphological observation.

Genes that are expressed specifically in a tissue affected by a disease process may also represent novel targets for that disease. The ability to generate tissue-specific fluorescent transgenic fish can lead to the production of exquisitely tissue-specific cDNA libraries through the use of fluorescence-activated cell sorting for tissue collection [43]. Sequencing the genes from these libraries can be useful for the identification of novel tissue-specific genes that may represent targets for drugs intended to affect a single tissue or cell type. For example, a novel death receptor expressed specifically in erythrocytes was found in a zebrafish erythrocyte cDNA library [44]. Inhibition of this death receptor resulted in zebrafish with more red blood cells, an outcome with possible therapeutic implications.

Zebrafish presents a clear advantage over other vertebrate systems in the study of thrombosis. Mature mammalian platelets contain relatively small amounts of RNA [45] because they lack nuclei and are no longer actively transcribing genes. Furthermore, megakaryocytes, the nucleated precursors of platelets, can be difficult to isolate and purify. This has hampered efforts to take a functional genomics approach to new target identification in this field. In contrast, mature zebrafish thrombocytes, which appear to be functionally equivalent to mammalian platelets, retain their nuclei [46]. Thus, a zebrafish thrombocyte-specific cDNA library may provide a rich source of novel genes involved in platelet aggregation and blood clotting. A transgenic zebrafish with fluorescent thrombocytes would make the recovery of these cells and the synthesis of a thrombocyte-specific library straightforward.

**Drug screening**

The small size of zebrafish embryos and larvae (ranging from 1.5 mm in length at 1 dpf to ~ 4 mm at 6 dpf),

Table 1. Examples of disease-related genes cloned in zebrafish.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Genes</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>presenilin-1</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>presenilin-2</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>acetylcholinesterase</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td>amyloid precursor protein</td>
<td>[30]</td>
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<td></td>
<td>apoE</td>
<td>[31]</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Huntingtin</td>
<td>[32]</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>sod-1</td>
<td>[33]</td>
</tr>
<tr>
<td>Muscular dystrophy</td>
<td>dystroglycan</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>dystrophin</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>Dp71</td>
<td>[19]</td>
</tr>
<tr>
<td>Leukemia</td>
<td>runx1</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>cbfb</td>
<td>[35]</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>Factor VII</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>COX-1</td>
<td>[37]</td>
</tr>
<tr>
<td></td>
<td>COX-2</td>
<td>[37]</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>cardiac troponin T</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>titin</td>
<td>[14]</td>
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<tr>
<td>Diabetes</td>
<td>insulin</td>
<td>[38]</td>
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<tr>
<td></td>
<td>IA-2 autoantigen</td>
<td>[39]</td>
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<tr>
<td></td>
<td>IA-2β autoantigen</td>
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combined with the fact that they live in an aqueous environment, makes them ideal for drug screening. Embryos can be arrayed in 96-well plates in ~50 µl of water, where they can survive for several days without feeding. In one of the first screens of a compound library using zebrafish, compounds were identified that induced specific developmental phenotypes in early zebrafish embryos [48]. One of these compounds, concentramide, caused a phenotype similar to a heart mutation called heart-and-soul, which fails to form a functional atrium [49]. More recently, zebrafish embryos were used to screen a small library of triazine compounds for their ability to inhibit tubulin polymerization in vivo as a first step toward identification of new anticancer drugs [50].

The value of the zebrafish system for drug screening will depend on whether they react to drugs in a way that will be predictive of the action of these drugs in humans. Conservation of drug action between humans and zebrafish has been reported. The cholesterol synthesis inhibitor atorvastatin (Lipitor), which has a reproducible effect in the zebrafish model of lipid metabolism described above [42], is one such example. When zebrafish are treated with Lipitor, PED6 fluorescence is not observed in the intestine and gall bladder, which is likely due to the lack of cholesterol-derived biliary emulsifiers. This change in fluorescence can be quantified [42], suggesting that the PED6 assay is amenable to automation.

Another example involves common mammalian platelet agonists, such as ADP, collagen and thrombin, which cause similar clotting events in zebrafish blood [46]. We found that antithrombotic drugs currently used in humans are effective in preventing blood clots induced by ADP in living zebrafish larvae [Doan T, Cross L, Rubinstein A, unpublished data]. In conclusion, while current data suggest that zebrafish can be of value for drug screening, further studies will be necessary to determine the degree to which drug effects in humans can be predicted by testing in zebrafish.

**Assay development**

To fully realize the potential of zebrafish for both target validation and drug screening, the development of disease-relevant assays, particularly those that are amenable to automation, will be necessary. The zebrafish is especially suitable for identification of angiogenesis inhibitors, since development of blood vessels in early embryos is well characterized and easily monitored [51]. For example, two research teams have shown that tyrosine kinase inhibitors targeting VEGF receptors can prevent angiogenesis in the early embryo [52,53]. Angiogenesis was measured using either endogenous alkaline phosphatase staining of blood vessels or microangiography. Transgenic lines of zebrafish with fluorescent blood vessels have been developed, which simplifies the process by which blood vessels are visualized [54]. An example of a transgenic zebrafish larva with fluorescent blood vessels is shown in Figure 2. This transgenic line was designed by driving expression of green reef coral fluorescent protein (G-RCFP, Clontech Laboratories Inc, USA) with the VEGFR-2 promoter. Embryos and larvae with fluorescent blood vessels can be used for angiogenesis assays. For example, we have shown the inhibition of angiogenesis in transgenic fluorescent embryos with the VEGF-specific tyrosine kinase inhibitor SU-5416 (semaxanib; SUGEN Inc), as well as with the more broadly active tyrosine kinase inhibitor, SU-6668 (SUGEN Inc) [Cross L, Lin S, Rubinstein A, unpublished data]. The ability to perform angiogenesis assays in fluorescent zebrafish should facilitate automation.

![Figure 2. Transgenic zebrafish embryo (3 dpf) expressing G-RCFP under the control of the VEGFR-2-promoter.](image-url)
Assays can also be designed by exploiting the typical zebrafish behavioral repertoire. For example, specific assays have been designed to examine drug and alcohol abuse using the zebrafish [55]. Other assays have been designed to test for hearing defects, by examining either defects in normal swimming behavior, or the response of zebrafish to loud sounds [56,57]. The visual system is especially amenable to study in the zebrafish, due to the large size of the eyes and the ease with which visual assays can be established [58,59]. Thus, diseases such as retinal pigmentosa and macular degeneration can be modeled.

Conclusions
A large body of evidence is accumulating that shows that zebrafish can accurately represent human biology, and that drugs currently used by humans can have a predictable effect in zebrafish assays. Since mammalian disease models are expensive and generally not conducive to high-throughput target validation and drug screening, the zebrafish system has an opportunity to provide a crucial link between high-throughput in vitro assays and in vivo mammalian disease models. However, development of disease-relevant assays and disease models in the zebrafish is still in its infancy. The success of zebrafish as a component of the drug discovery process will hinge on the quality of novel assays and models. In particular, it will be necessary to show that these models recapitulate the underlying mechanisms of human disease. While the ability of zebrafish models to predict drug responses in humans has been demonstrated, additional drugs should be tested to more firmly establish the value of zebrafish in drug discovery.

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References
This paper describes a novel fluorescent assay to monitor lipid metabolism in the zebrafish, and shows that Lipitor has a predictable effect in this assay.

This study constitutes the first example of a transgenic zebrafish with tissue-specific expression of a fluorescent reporter, and describes the use of these fish to generate a tissue-specific cDNA library.

This paper contains the first detailed characterization of zebrafish platelets, and describes some of the similarities between zebrafish and mammalian platelets.

A thorough description of vascularization in zebrafish development.

A comprehensive review of human disease genes that have zebrafish homologs. The review includes a search of zebrafish-expressed sequence tags for disease gene homologs.

This study constitutes the first example of a transgenic zebrafish with tissue-specific expression of a fluorescent reporter, and describes the use of these fish to generate a tissue-specific cDNA library.

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