Zebrafish as a model system for drug target screening and validation

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The zebrafish is becoming an increasingly popular model organism to study human diseases. Novel assays have been and continue to be developed to study human diseases in this model. Methods to identify novel drug targets using zebrafish include chemical mutagenesis, insertional mutagenesis and small-molecule screens. Methods to validate potential drug targets include morpholino antisense knockdown technology and target-selected mutagenesis approaches. Here we review advances in these and related technologies.

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▼ The zebrafish, a tropical fish that has long been common in home aquariums, is becoming increasingly popular among scientists from diverse fields such as developmental and evolutionary biology, toxicology and pharmacology. Researchers from academic institutions and pharmaceutical companies regard zebrafish as a new powerful tool in research and drug discovery that will accelerate screening processes and the discovery of new life-saving drugs for humans.

Indeed, zebrafish possess many features that make it a highly useful model system. Adults are relatively small (only 3–4 cm long), inexpensive, easily housed and maintained in large quantities. Zebrafish can be easily mated under laboratory-controlled conditions, with each pair typically producing several hundred eggs per mating. Embryos are transparent, allowing easy visualization of fundamental developmental processes. They develop rapidly: a one day old embryo already has formed all the major tissues and many organ precursors, such as a beating heart, circulating blood, nervous system, eyes and ears, all of which can be readily observed under a simple dissecting microscope. By five days post-fertilization (dpf), the zebrafish larva has formed the majority of internal organs including the liver, pancreas and a complex vascular network. These features, in addition to the relatively short generation time of approximately three months, makes genetic screens feasible (reviewed in [1]).

Given these attributes, experiments in zebrafish are significantly easier, faster and cheaper than in mouse, which is currently one of the main model systems for drug target discovery and validation. Can human diseases be successfully studied in zebrafish, despite the evolutionary gap? The answer is yes, for the most part.

Zebrafish as a model for human diseases

Although assay development to model human diseases in zebrafish is still in its infancy, a variety of diseases have been successfully modeled in zebrafish (Table 1). For example, mutations of the gene *tnnt2* encoding the thin-filament contractile protein cardiac troponin T are responsible for 15% of all cases of familial hypertrophic cardiomyopathy, the leading cause of sudden death in young athletes [2]. Mutations *of tnnt2* can also lead to dilated cardiomyopathy, a leading cause of heart failure [3]. The zebrafish *silent heart (sih)* mutation affects the gene *tnnt2*, and causes a non-contractile heart phenotype [4]. Functions of other muscles, such as skeletal and smooth muscle, remain intact in *sih* mutant embryos. The *sih* mutant cardiomyocytes have defective sarcomere assembly, owing to misregulation of thin-filament protein expression. Thus, *sih* zebrafish mutants serve as an excellent model for human cardiomyopathies and allow easy experimental access to study the cause and pathology of this human disease.

Another example is the myosin VIIA gene, which encodes an unconventional myosin that is expressed in vestibular and auditory hair cells of the ear and is responsible for various types of hearing disorders in humans including Usher 1B syndrome [5]. Zebrafish

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Table 1. Examples of human diseases that have been modeled in zebrafish

Disease modeled	Gene affected	Process affected	Method[a]	Refs
Erythropoietic protoporphyria	*ferrochelatase (fch)*	Heme synthesis inhibited	Mutation, MO knockdown	[37,55]
	Uroporphyrinogen decarboxylase (urod)		Mutation, MO knockdown	[36,56]
Congenital dyserythropoietic anemia	*anion exchanger 1 (slc4a1, band 3)*	Erythroid-specific cell division inhibited	Mutation	[57]
Cardiomyopathy	*Titin*	Cardiac contraction reduced due to blockage of sarcomere assembly	Mutation, MO knockdown	[58] [4]
	troponin T		Mutation	
Muscular dystrophy	*dystroglycan*	Muscle degeneration due to the separation of muscle cells from the extracellular matrix	MO knockdown	[59]
	Duchenne muscular dystrophy (dmd) gene, dystrophin		Mutation, MO knockdown	[20,60]
Holoprosencephaly	*sonic hedgehog, tiggy-winkle hedgehog*	Incomplete development and septation of midline structures in the central nervous system	MO knockdown	[36,61,62]
Alzheimer's disease	*tau*	Tau protein fails to associate with microtubules resulting in neurodegeneration	Overexpression using transgenic zebrafish	[63]
Holt-Oram syndrome	*T-box transcription factor tbx5*	Upper limb and cardiac development impaired	Mutation, MO knockdown	[64]
Hearing disorders: Usher 1B syndrome, DFNB2 and DFNA11	*myosin VIIA*	Function of the sensory hair cells in the ear impaired due to splaying of stereocilia	Mutation	[6]
Polycystic kidney disease	*nek kinase 8 (nek8)*	Kidney epithelial cell function impaired resulting in pronephric cysts	MO knockdown	[65]

[a]Abbreviation: MO, morpholino phosphorodiamidate oligonucleotides.

mariner mutant is defective in the myosin VIIA gene [6]. The mutant embryos swim in vertical loops or corkscrew paths and do not respond to acoustic or vibrational stimuli, indicating vestibular and auditory defects. The *mariner* mutants also display inner hair cell bundle defects. A similar phenotype has been observed in mouse *shaker-1* mutants, which are defective in myosin VIIA. In mice, however, sensory hair cells are located deep within the petrous temporal bone and are not accessible to direct experimental manipulation nor simple observation, whereas zebrafish sensory hair cells are located at the surface of the skin and are clearly visible. This study demonstrates the striking conservation of the function of myosin VIIA throughout vertebrate evolution and highlights the potential of using zebrafish to model human hereditary deafness.

Currently, multiple novel assays are being developed to more effectively model human diseases using zebrafish. Digestive physiology can now be observed in live zebrafish using fluorescent phospholipid reporters [7]. In this assay, zebrafish larvae ingest fluorescently quenched phospholipids. Phospholipase A2 cleaves the quencher off, resulting in detectable fluorescence. This assay will be useful to identify genes involved in diseases of lipid metabolism, such as atherosclerosis, and in disorders of biliary secretion, such as biliary atresia.

Another example is a blood coagulation assay, developed by Jagadeeswaran and Sheehan, in which zebrafish blood plasma is isolated, allowed to clot by adding one of the clotting reagents (fibrinogen, thromboplastin or factor X activator) and the clotting time is measured with a fibrometer [8]. In a vascular occlusion assay, blood flow in immobilized zebrafish larvae is observed by videomicroscopy and the time until the occlusive cell mass blocks the blood vessels is measured [9]. The development of *in vivo* assays that are related to human diseases could lead to the discovery of novel potential drug targets.

Chemical mutagenesis screens

Genetic screening is probably the most powerful tool to identify mutations in novel genes that affect a tissue or organ of interest. The first large-scale screens of a vertebrate organism for developmentally important genes were

performed in Boston [10] (led by Driever, Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA) and Tübingen [11] (led by Nüsslein-Volhard, Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany; http://www.eb.tuebingen.mpg.de/dept3/). These screens used chemical mutagenesis, which involves treatment of zebrafish males with *N*-ethyl-*N*-nitrosourea (ENU) and leads to random point-mutations. These screens resulted in the identification of over 2000 developmentally important loci, including more than 100 genes involved in heart formation and function [12,13], more than 50 involved in blood cell and vasculature formation [14,15], and more than 30 involved in ear formation [16,17]. Although only a small number of the discovered mutants have been molecularly characterized, because of the difficult and time-consuming nature of positional cloning and related methods [18], many of the mutants have become important human disease models (Table 1). For example, the zebrafish mutants *merlot (mot)* and *chablis (cha)* exhibit severe hemolytic anemia characterized by abnormal morphology and increased osmotic fragility of red blood cells [19]. Both mutations were mapped to the erythroid-specific protein 4.1R. The 4.1R protein is a multifunctional structural protein in the red-cell membrane skeleton, which interacts with both transmembrane and cytoskeletal proteins and has an indispensable role in maintaining red cell morphology, membrane deformability and mechanical stability in humans. Thus, the *merlot/chablis* mutant serves as a model of hereditary anemia owing to a defect in protein 4.1R integrity.

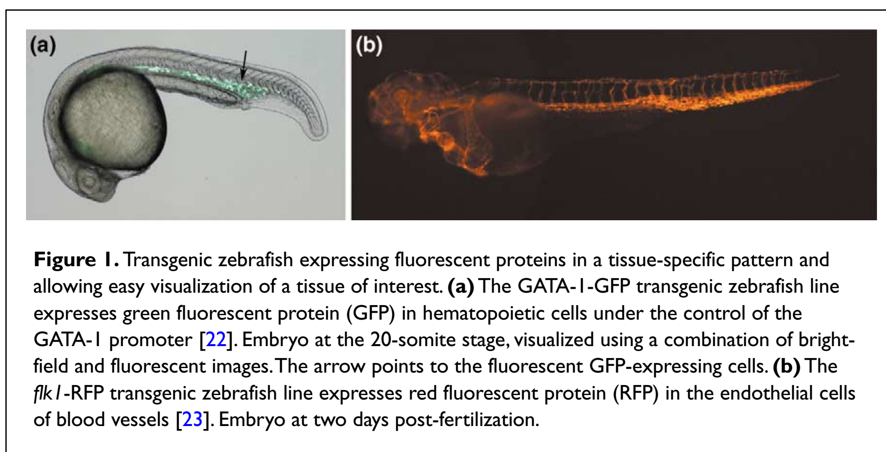

Figure 1. Transgenic zebrafish expressing fluorescent proteins in a tissue-specific pattern and allowing easy visualization of a tissue of interest. **(a)** The GATA-1-GFP transgenic zebrafish line expresses green fluorescent protein (GFP) in hematopoietic cells under the control of the GATA-1 promoter [22]. Embryo at the 20-somite stage, visualized using a combination of bright-field and fluorescent images. The arrow points to the fluorescent GFP-expressing cells. **(b)** The *flk1*-RFP transgenic zebrafish line expresses red fluorescent protein (RFP) in the endothelial cells of blood vessels [23]. Embryo at two days post-fertilization.

In a separate case, a class of recessive lethal zebrafish mutations has been identified in which normal skeletal muscle differentiation is followed by a tissue-specific degeneration that is reminiscent of the human muscular dystrophies. One of these mutations, *sapje,* has been shown to disrupt the zebrafish ortholog of the X-linked *Duchenne muscular dystrophy (DMD)* gene [20]. Mutations in this locus cause Duchenne or Becker muscular dystrophies in humans. The progressive muscle degeneration phenotype of *sapje* mutant zebrafish appears to be caused by the failure of embryonic muscle attachments, which has not been observed using the mouse model. Therefore, this zebrafish mutation may provide a model for a novel pathological mechanism of Duchenne muscular dystrophy.

Several new genetic screens using specific functional assays are in progress. Radiographic analysis, developed by Fisher *et al.* [21], allows fast analysis of skeletal bones in adult fish. During a mutant screen using this assay, a dominant mutation *chihuahua* was isolated. Heterozygous *chihuahua* fish display a general defect in bone growth, reminiscent of human osteogenesis imperfecta, a skeletal dysplasia caused by mutations in the type I collagen genes. Mapping and molecular characterization of *chihuahua* showed that the defect resulted from mutation in the gene encoding the collagen I (α1) chain. This demonstrates that mutant phenotypes analogous to human skeletal dysplasias can be discovered in the mutagenesis screen using radiography.

Analysis of a particular tissue or organ is greatly aided by using transgenic zebrafish that express green fluorescent protein (GFP) under the control of a tissue-specific promotor. This allows easy visualization of a specific organ or tissue. Thus, hematopoietic progenitor cells can be readily visualized in live transgenic embryos that express GFP under the control of the blood cell specific GATA-1 promotor [22] (Figure 1a). Similarly, formation of vasculature can be assayed using blood vessel specific expression of the red fluorescent protein (RFP) under control of the kinase insert domain receptor *(kdr, flk1)* promotor [23] (Figure 1b). Such transgenic fish lines will be invaluable tools in current and future mutagenesis screens.

Insertional mutagenesis

While ENU mutagenesis is very efficient, identification of the mutated genes is slow and labor-intensive. As an alternative to chemical- and radiation-induced mutations, insertional mutagenesis allows for rapid cloning of the gene. Large-scale screening for recessive developmental mutations with retroviral insertions has been undertaken by Hopkins' group in Cambridge, MA (http://web.mit.edu/biology/www/facultyareas/facresearch/hopkins.shtml) [24]. More than 500 insertional mutants have been identified from which 75 disrupted genes have been isolated [25].

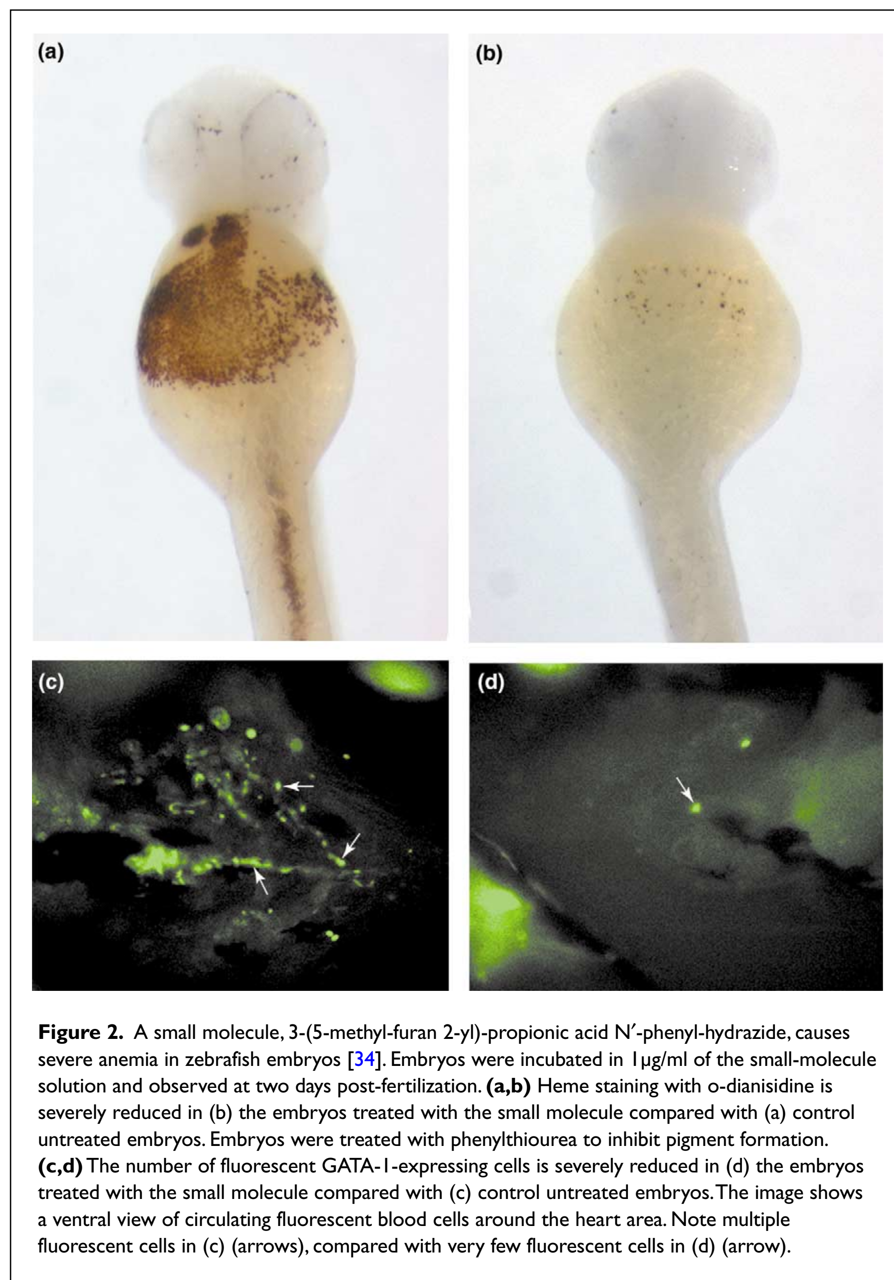

Figure 2. A small molecule, 3-(5-methyl-furan 2-yl)-propionic acid N′-phenyl-hydrazide, causes severe anemia in zebrafish embryos [34]. Embryos were incubated in 1 μg/ml of the small-molecule solution and observed at two days post-fertilization. **(a,b)** Heme staining with o-dianisidine is severely reduced in (b) the embryos treated with the small molecule compared with (a) control untreated embryos. Embryos were treated with phenylthiourea to inhibit pigment formation. **(c,d)** The number of fluorescent GATA-1-expressing cells is severely reduced in (d) the embryos treated with the small molecule compared with (c) control untreated embryos. The image shows a ventral view of circulating fluorescent blood cells around the heart area. Note multiple fluorescent cells in (c) (arrows), compared with very few fluorescent cells in (d) (arrow).

Some of the isolated mutations are in the homeobox gene *vHnf1*, which is associated with human diseases MODY5 (maturity-onset diabetes of the young, type V) and familial GCKD (glomerulocystic kidney disease). Phenotypes of the isolated zebrafish *vHnf1* mutants include the formation of kidney cysts and underdevelopment of the pancreas and the liver, which is similar to the symptoms observed in human patients [26].

Although retroviral insertions facilitate the rapid cloning of genes, this approach is hindered by potential limitations in size, regulatory sequences, complexities in preparation and safety concerns [27]. Some of these problems can be circumvented by using the transposon-mediated systems. Transposons provide stable gene expression from a single-copy integration event, and they can tag the integration loci to facilitate subsequent molecular analyses. Two different transposon-mediated systems have been created in fish. The *Sleeping Beauty* transposon belongs to the *Tc1/mariner* transposon family and has been constructed by replacing non-functional codons from an evolutionary inactive fish transposon [28]. Another system uses the *hAT* transposon family member Tol2, which was originally derived from the medaka fish and has been adopted for transposition in zebrafish [29,30]. Efficient genome integration via transposition and germ-line transmission has been demonstrated for both of the transposons [29,31]; however, the effectiveness of these transposon systems in insertional mutagenesis remains to be established.

Small-molecule screens

An alternative to mutagenesis is the use of small-molecule developmental screens. Chemical compounds of relatively small molecular weight (small molecules) can bind to specific proteins, thus modulating their functions and resulting in changes in an organism's phenotype in a nonheritable manner. Subsequently, the proteins that interact with the compound can be isolated using affinity chromatography methods. The structure of the small molecule can be directly used in the drug design and testing. Small molecules have been used in several pilot screens in zebrafish to assess their effects on general development and on disease-related problems. Small molecules have been discovered that specifically affect the brain, notochord, heart, ear development and pigment cell formation in zebrafish embryos [32,33]. A novel compound that induces hemolytic anemia has also been discovered [34] (Figure 2). In a separate study, a triazine compound library was screened and a compound affecting anterior development was identified. Different modifications of this compound were tested to find the one that exerts the strongest biological effect. Subsequently, the candidate compound-interacting proteins

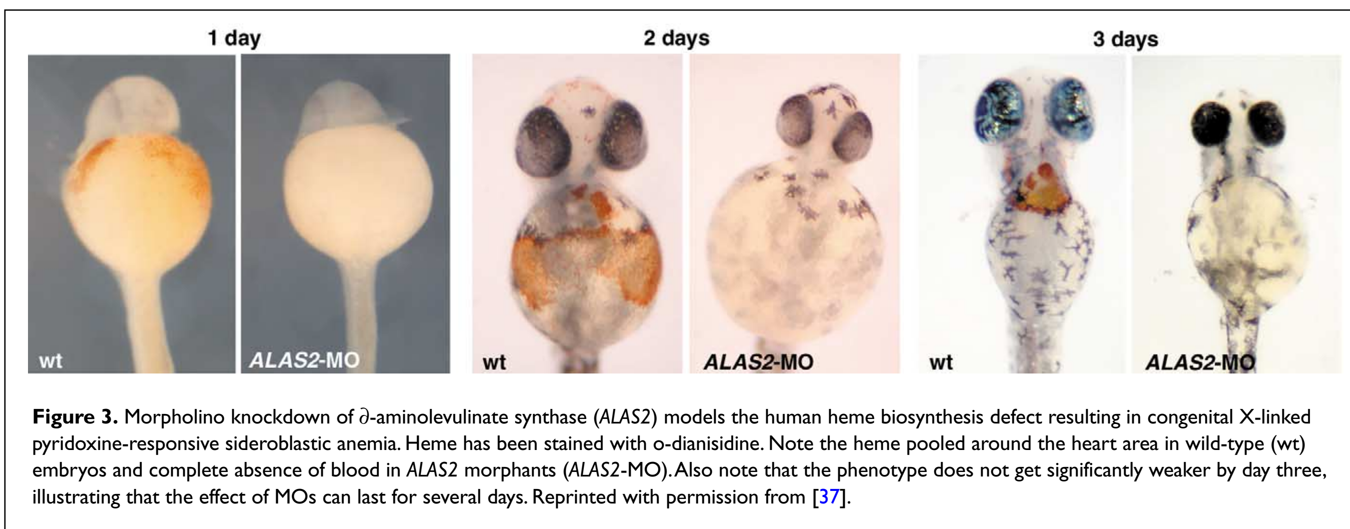

Figure 3. Morpholino knockdown of ∂-aminolevulinate synthase (*ALAS2*) models the human heme biosynthesis defect resulting in congenital X-linked pyridoxine-responsive sideroblastic anemia. Heme has been stained with o-dianisidine. Note the heme pooled around the heart area in wild-type (wt) embryos and complete absence of blood in *ALAS2* morphants (*ALAS2*-MO). Also note that the phenotype does not get significantly weaker by day three, illustrating that the effect of MOs can last for several days. Reprinted with permission from [37].

were isolated by competitive affinity chromatography [35]. These studies illustrate that small-molecule screening can be a powerful method to find novel drug targets.

Screens using antisense molecules

Reverse genetics approaches can be used to test the function of a gene of interest. There are two main methods under development in this area: the use of antisense molecules to knockdown the function of a gene of interest; and the genetic knockout of a gene of interest.

The fastest way to analyze the function of a gene of interest is by using antisense methods. From several different antisense molecules that have been tested, morpholino phosphorodiamidate oligonucleotides (morpholinos [MOs], available from Gene Tools, Eugene, OR; http://www.gene-tools.com), and, more recently, peptide nucleic acids (PNAs, available from Active Motif, Carlsbad, CA; http://www.activemotif.com) have been demonstrated to effectively knockdown a specific gene function in zebrafish [36–38]. MOs have received particularly wide usage owing to their high efficacy, specificity and commercial availability. Translation-blocking MOs are designed to target the 5′ untranslated region (UTR) of mRNA or the first 20–25 nucleotides of the open reading frame (ORF) of an mRNA of interest, thus preventing initiation of translation [39]. MOs can also be designed against splice-acceptor or splice-donor sites of pre-mRNA (reviewed in [40]). The splice-blocking MOs interfere with the function of the spliceosome leading to exon-skipping or usage of cryptic splice sites and resulting in aberrantly spliced mRNA.

MOs can be easily delivered into zebrafish embryos by simple microinjection at early stages of development, when they uniformly distribute throughout the embryo. During the past three years, the function of more than 100 genes has been studied using MOs, several of them being clinically relevant (Table 1). For example, morpholino knockdown of *ALAS2,* which encodes ∂-aminolevulinate synthase (an enzyme catalyzing the first step of heme biosynthesis), results in heme biosynthesis defects, similar to the symptoms of human X-linked sideroblastic anemia (Figure 3) [37,41]. In another case, MO knockdown of a blood coagulation factor VII and a coagulation inhibitor VIIi results in the prolonged or shortened time to occlusion of blood in zebrafish, modeling their function in thrombosis in humans [9]. The disadvantages of MOs include a relatively short period of action of up to 3–5 dpf, and the necessity of appropriate specificity controls. Although MOs cause fewer non-specific effects than other antisense compounds, several non-specific defects caused by high doses of MOs have been described. Proper specificity controls are therefore essential in MO- and other antisense-based studies [37,42].

The ease of delivering MOs and observing the resulting phenotypes in zebrafish has led to the idea of MO-based screens. A consortium of researchers, coordinated by Ekker at the University of Minnesota, is performing a pilot MO knockdown screen for the functions of secreted proteins and their receptors (http://beckmancenter.ahc.umn.edu/html/morphalino.html) [43]. Commercial companies such as Discovery Genomics, Inc. (http://www.discoverygenomics.net) are also using MO technology for the screening and validation of potential drug targets in angiogenesis, cancer and other disease-related areas. The unexpected function of a chaperone protein GP96 in ear development [44] is just one of the discoveries to come out of these screens.

Another inhibitory technique widely used in several developmental model organisms, from invertebrates such as the worm *Caenorhabditis elegans* to mammalian cell cultures,

is the use of small interfering RNA (siRNA) and RNA interference (RNAi) (reviewed in [45,46]). Unfortunately, RNAi has been shown to produce many non-specific effects in zebrafish [47]. Effectiveness of siRNA in zebrafish has not been completely explored yet. In the only published report so far, the *dystrophin*-specific siRNA caused nearly twofold reduction in the transcript level of the *dystrophin* gene, which resulted in the disruption of myosepta and muscle fiber organisation, the phenotype observed upon morpholino knockdown of *dystrophin* and in *sapje/dystrophin* mutants [48].

Targeted mutagenesis

Targeted mutagenesis, which has greatly advanced gene function studies in the mouse, is currently not a viable technology in zebrafish. Nevertheless, encouraging advances have been made towards site-specific mutagenesis and gene knockout in zebrafish. Cell cultures from fibroblasts, neurons and germ cells have been established [49,50]. Several groups including that of Lin at UCLA (http://www.mcdb.ucla.edu/Research/ShuoLin/) are currently trying to develop the technology for site-specific recombination in zebrafish cell cultures. Nuclear transfer technology already allows embryos to be generated from the nuclei of the cultured cells [50]. Advances in all of these technologies could soon allow the generation of mutations of interest.

Yet, even in the absence of site-specific mutagenesis, it is possible to find a null mutation in a gene of interest by employing the targeting-induced local lesions in genomes (TILLING) approach. In this approach, a library from the progeny of randomly ENU-mutagenized males is generated. Genomic DNA is isolated, and testes are cryopreserved. If the library is comprehensive enough, most genes will be represented by at least one null allele. Mutations in the gene of interest can be screened by amplifying a gene-specific region from fish DNA samples and sequencing it. The zebrafish *rag1* null mutant was generated this way [51]. Another approach to screen the genomic DNA library is based on cleavage of DNA heteroduplexes by the endonuclease Cel I [52]. With this method, gene segments are amplified using fluorescently tagged primers, and products are denatured and reannealed to form heteroduplexes between the mutated sequence and its wild-type counterpart. These heteroduplexes are substrates for cleavage by Cel I. Following cleavage, products are analyzed on denaturing polyacrylamide gels using the LI-COR DNA analyzer system (http://www.licor.com). By using this approach, 255 different mutations were identified in 16 different zebrafish genes just within a few months [52]. Among them, 14 resulted in a premature stop codon, 7 in a splice donor/acceptor site mutation, and 119 in an amino acid change. This illustrates that TILLING can be used to detect a variety of mutations, including null and hypomorphic alleles in a gene of interest.

Gene misexpression using transgenesis

Although many human diseases are caused by the loss-of-function of a certain gene and are inherited in a recessive fashion, some arise owing to gain-of-function mutations. In particular, many cancers are caused by the increased activity of oncogenes. The potential of zebrafish in cancer-related studies is only beginning to be exploited, but several achievements in this area have already been accomplished. Many different types of cancer, including pancreatic carcinomas, intestinal and hepatocellular carcinomas, neuroblastoma, retinoblastoma and angiosarcoma, have been observed in fish treated with different chemical mutagens (reviewed in [53]). To develop a fish model for T-cell leukemia, a transgenic zebrafish was created expressing mouse *c-myc* under the control of the zebrafish *rag2* promoter, which targets gene expression specifically to lymphoid cells. This transgenic zebrafish developed T-cell leukemias that could be observed *in vivo,* because the *c-myc* protein was fused to a GFP reporter. After leukemic cells were harvested from the transgenic fish and injected into the peritoneum of wild-type recipients, GFP-positive tumors were evident at the site of injection and had spread throughout the recipient fish two weeks after injection [54]. These data confirm the malignant nature of the T-cell neoplasm and illustrate that tumors can be monitored and studied in live fish using fluorescent protein markers.

The bright future

A big advantage of zebrafish over some other model systems is their potential to perform genetic analyses. The success of positional cloning of mutants depends on the density of markers on the genetic map, as well as knowledge of the genome sequence. At this point, the zebrafish genome sequencing project at the Sanger Centre (http://www.sanger.ac.uk/Projects/D_rerio) is under way and is expected to be completed in 2005. More than 400,000 expressed sequence tags (ESTs) have been sequenced, 12,000 of which have been placed on two radiation-hybrid maps. Analysis of gene function is greatly aided by microarray analysis. Two different zebrafish oligonucleotide-based microarrays are currently available, one designed by Compugen (http://www.labonweb.com) and the other by Affymetrix (http://www.affymetrix.com), and each contain over 14,000 unique sequences.

Zebrafish has come a long way from an aquarium fish to becoming a favorite model of choice in developmental biology. With the advances of zebrafish-based assay development,

novel mutagenesis tools and progress in genome analysis, zebrafish is fast gaining popularity for modeling human diseases and drug development. The potential for understanding the underlying mechanisms of many human diseases and development of novel drugs to fight them will soon be a reality with the help of zebrafish.

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