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THE ZEBRAFISH AS A MODEL FOR HUMAN DISEASE

William T. Penberthy, Ebrahim Shafizadeh, and Shuo Lin

Molecular, Cell, and Developmental Biology, UCLA, 621 Charles E. Young Dr. South, Los Angeles, CA 90095-1606

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1. ABSTRACT

Much of our current understanding of the function of genes modulating the normal process of embryonic development has come from mutant analysis. The availability of thousands of mutant lines in zebrafish that allows for identification of novel genes regulating various aspects of embryogenesis has been instrumental in establishing zebrafish as a robust and reliable genetic system. With the advances in genomic sequencing, the construction of several genetic maps, and cloning of hundreds of ESTs, positional cloning experiments in zebrafish have become more approachable. An increasing number of mutant genes have been cloned. Several zebrafish mutants are representative of known forms of human genetic diseases. The success of morpholino antisense technology in zebrafish potentially opens the door for modeling nearly any inherited developmental defect. This review highlights the strengths and limitations of using the zebrafish as an organism for elucidation of the

genetic etiology of human disease. Additionally a survey of current and future zebrafish models of human disease is presented.

2. INTRODUCTION

It has been 20 years since zebrafish (*Danio rerio*) was first introduced as a genetic model to study vertebrate development (1). Since then, zebrafish has proven to be a powerful animal model not only for elucidating the mechanisms involved in early embryonic patterning and development, but also as a vertebrate model of human diseases. A wide variety of reviews have recently been published describing various aspects of the zebrafish system (2-9).

Zebrafish possess several unique characteristics that make this tropical fish a suitable animal model for developmental and genetic studies. 1) The small size of zebrafish allows for maintaining relatively large numbers in a small area. 2) Zebrafish reach sexual maturity in about 2-3 months, expediting the generation of a specific line or mutant. 3) Zebrafish females are very fecund and can generate hundreds of eggs on a weekly basis. This is an ideal situation when performing genetic screening that requires a large number of siblings. 4) The fertilization of eggs occurs externally allowing for the production of haploid embryos. 5) The developing embryos are transparent, which allows the visual examination of embryogenesis and organogenesis processes, gene promoter/GFP analysis, lineage tracing, and cell transplantation experiments. 6) Water soluble chemicals and drugs (mutagens, tetrodotoxin, epinephrine, melatonin, dopamine, inhibitors of tyrosinase) are readily administered to fish by simply adding them to the water. 7) Zebrafish are amenable to injection, allowing one to generate transgenic zebrafish and knockdown or over express transcripts. 8) The extra genomic duplication in zebrafish enables greater refinement of our understanding of gene function. Where gene redundancy is involved in function, it is possible to knockdown both transcripts to determine the function of gene families (10, 11).

One of the advantages of using zebrafish is the feasibility of performing forward genetic analysis. In zebrafish the knockout technology has not been developed yet. However, there are various strategies to induce mutations in zebrafish. Three techniques, gamma irradiation (12), insertional (13), and chemical mutagenesis have been used for this purpose.

Chemical mutagenesis is the most efficient and widely used mean of inducing mutations in zebrafish. Such mutation screens have successfully been conducted in a number of invertebrate models organisms such as *Drosophila* (14) and *C. elegans* (15). Two large-scale genome wide chemical mutagenesis screens have generated hundreds of mutations affecting the entire process of development and embryogenesis in zebrafish (16, 17). Briefly, the male zebrafish are exposed to an alkylating agent, N-ethyl-N- nitrosourea (ENU) that causes single-base mutations in the spermogonia. Mutagenized males are mated with wild type females. The F1 families are then intercrossed to produce F2 families, which are screened by random mating. The F3 embryos are examined microscopically from 1dpf to 5dpf and embryos with defects in organogenesis are identified. The majority of available zebrafish mutants have been isolated from these screens. Characterization of these mutants and identification of their underlying genetic and molecular abnormalities will greatly advance our understanding of developmental pathways in vertebrates.

The zebrafish haploid genome contains 1.7×10^9 base pairs that are located on 25 chromosomes (18). They are referred as linkage groups because it is difficult to distinguish between chromosomes. Extensive phenotypical analysis, such as lineage tracing and cellular transplantation, can be used to study the function of the mutated genes at the cellular level. However, in order to fully understand the molecular mechanisms underlying the cellular functions of zebrafish mutants, it is necessary to clone the mutated genes.

Positional cloning is a multi-step process that enables the identification of mutated genes on the basis of their chromosomal location, without having prior knowledge of the biochemical and molecular nature of the genes. The availability of an increasing number of PCR based polymorphic markers (http://zebrafish.mgh.harvard.edu), such as Simple Sequence Length Polymorphisms (SSLP) (19), makes the positional cloning approach highly attainable. There are also an increasing number of cloned and mapped zebrafish ESTs (http://zfish.wustl.edu) that will greatly facilitate the identification of mutated genes by a candidate gene approach. Using the radiation hybrid map of zebrafish, it is possible to place the cloned or candidate genes on the genetic map (20). Zebrafish genomic PAC, BAC, and YAC (21-23) libraries are publicly available and can be used to isolate genomic clones containing the mutated locus. It is possible to attempt to partially rescue the mutant phenotype by injecting the genomic clones that contain the wild type gene (24).

Gene targeted knockout has not been achieved in zebrafish. The state of the art technique for studying the effects of preventing gene expression is the morpholino (MO) knockdown ((25) and entire issue of Genesis vol 30 no3 2001). MOs are chemically modified oligonucleotides that hybridize specifically with transcripts to prevent translation. Since these compounds are insensitive to RNase H, they are very stable. Zebrafish displaying the phenotype created from ectopic morpholino administration are referred to as morphants. There is a morphant database resource whereby the morpholino corresponding to a well-characterized morphant can be requested (http://beckmancenter.ahc.umn.edu/morpholino_database.html). The human diseases hepatoerythropoietic porphyria and common disorder holoprosencephaly have been simulated in zebrafish by the morpholino approach (25). Several cloned mutant genes including *chordin, no-tail, one-eyed pinhead, nacre, sparse,* and *gridlock* (26) have been phenocopied by the morpholino technique as well.

Table 1. Established zebrafish models of human genetic disease

Human Disease	Mutation	Zebrafish Mutant (reference)	Defect
Congenital sideroblastic anemia	Erythroid 5-aminolevulinate synthase (ALAS-E)	*sauternes* (*sau*), (29)	Anemia caused by defects in hemoglobin synthesis
Congenital hereditary spherocytosis	beta-spectrin	*reisling* (*ris*) (41)	Anemia due to defective cytoskeleton of red cell membrane
Porphyrias	uroporphyrinogen decarboxylase (UROD)	*yquem* (*yqe*) (37)	Defects in the enzymes involved in the heme biosynthetic pathway
Erythropoietic protoporphyria	ferrochelatase	*dracula* (*drc*) (40)	Defects in pathway for heme biosynthesis
Polycythemia	hematopoietic death receptor (ZH-DR)	(43)	Increase in red blood cell number and hemoglobin level, hyperplasia of kidney,
Hypochromic anemia	ferroportin1	*weissherbst* (*wes*) (36)	Defects in iron absorption and utilization
Dilated cardiomyopathy	titin	*pickwick* (*pik*) (48)	Inefficient cardiac contractility, enlarged ventricle
Familial glomerulocystic kidney disease (GKCD) maturity-onset diabetes of the young, type V (MODY5)	vHnf1	*vhnf1* (61)	Kidney cysts, underdeveloped pancreas, liver, and otic vessicles
Usher 1B syndrome	myosin VIIA	*mariner* (79)	Inherited deafness due to defects in sensory hair cell functions
Congenital fibrosis of the extraocular muscles (CFEOM2) /Duane's Syndrome	Phox2a	*soulless* (80, 82)	Strabismic disorders that are characterized by restrictive ophthalmoplegia
Waardenburg-Shah syndrome and Hirschprung's disease	sox10	*colourless* (81)	Reduced enteric nervous system
Holoprosencephaly	sonic hedgehog	*sonic hedgehog* (*shh*) and *tiggy-winkle hedgehog* (*tww*) (morpholino based) (25)]	Forebrain development

3. SURVEY OF THE STATUS OF ZEBRAFISH MODELS OF HUMAN DISEASE

The zebrafish disease models can be divided into mapped human disease-linked genes (Table 1) and those resembling human diseases for which the genes have not been cloned (Table 2). The blood disease models were the first identified disease models due to readily apparent alterations in circulation by just 48hpf. Many human blood diseases have been established at the molecular level thus assisting the candidate gene approach to gene identification. Another approach is to use zebrafish to study the disease causing orthologous gene. This can be done by BLAST searches or keyword searches containing the name of the disease against the zebrafish EST database (as illustrated in (5)). Since a disease phenotype can be established by knocking down a given gene product (25), it is within the span of this review to consider disease-linked gene research being performed in zebrafish as well. With these two approaches in mind we present a survey of the current status of various human diseases represented by zebrafish models.

3.1. Blood disease

In recent years, zebrafish have emerged as a powerful model to study the genetic and molecular biology of hematopoiesis. Critical genes involved in hematopoiesis such as *scl*, *gata* factors, and *c-myb*, have been cloned in zebrafish. Studies show that the expression and function of blood-specific genes in zebrafish resemble those in higher vertebrates (27). The rapid development of transparent zebrafish embryos allows one to microscopically monitor the process of embryonic erythropoiesis. Similar to higher vertebrates, hematopoiesis in zebrafish occurs in two successive waves called primitive (embryonic) and definitive (adult) hematopoiesis (28). Primitive hematopoiesis generates cells of the erythroid lineage that enter the circulation at about 24 hours post fertilization. Embryonic erythroid cells undergo terminal maturation and express several embryonic globin chains (29). The onset of definitive hematopoiesis is believed to be at about 96 hpf. In addition to red blood cells, definitive hematopoiesis generates cells of myeloid (30), lympoid (31), and thrombocytes (32) lineage.

Table 2. Plausible zebrafish disease models

Human Disease	Gene	Zebrafish Mutant (reference)	Description of phenotype
second-degree atrioventricular heart block	?	31J6, chemically induced state (51)	increases the ratio of atrium to ventricle contractions to 2:1, instead of the usual ratio of 1:1.
epilepsy	?	*macho* (116)	embryonic ion channelopathies
kidney disease involving cysts	?	(55)	Cysts
Senior-Loken syndrome	?	*fleer* and *elipsa* (55, 62)	combined renal-retinal dysplasia
human retinal disease	?	*no opticokinetic response* (142)	defects in photoreceptor synaptic transmission and light adaptation
Walker-Warburg syndrome	?	*glass onion* (62)	Eye and brain patterning defects
muscle-eye-brain disease	?	*nagie oko* (115, 143)	Eye and brain patterning defects
cerebro-oculo muscular dystrophy	?	*oko meduzI* (62, 143, 144)	Eye and brain patterning defects
Glaucoma	?	*archie* (75)	Degeneration of retinal ganglia

More than 50 mutants with defective hematopoiesis have been recovered (16) from the large-scale chemical mutagenesis screens (33). These blood mutants were further analyzed and subsequently categorized into several classes (34, 35). The genetic and molecular defects in several blood mutants have been characterized through positional cloning techniques or a candidate gene approach. The characterization of these mutants has been instrumental in establishing zebrafish as a genetic model of human blood disorders.

3.1.1. Hypochromic anemia

Complementation analysis of blood mutants identified five groups of mutants exhibiting embryonic hypochromic anemia. These mutants include *sauternes* (*sau*), *chardonnay* (*cha*), *weissherbst* (*weh*), *chianti* (*cia*), and *zinfandel* (*zin*). Molecular analysis showed that only the erythroid lineage was effected in these mutants, which suggested that anemia might be due to defective iron metabolism, globin gene expression, or heme biosynthesis.

3.1. Congenital sideroblastic anemia

The zebrafish *sau* mutant suffers from a microcytic, hypochromic anemia. Further characterization of the mutants by Brownlie *et al* (29) revealed the presence of immature circulating erythroid cells, along with abnormal regulation and persistence of embryonic $beta_{e2}$ *globin* and *gata1* expression. Using positional cloning strategies, it was demonstrated that the *sau* gene was located on linkage 8 and encoded the erythroid-specific isoform of delta-aminolevulinate synthase (ALAS2), a critical enzyme in the heme biosynthesis pathway. In humans, mutations in *ALAS2* cause sex-linked congenital sideroblastic anemia with similar characteristics to the *sau* phenotype. The zebrafish *sau* mutant represents the first animal model of congenital sideroblastic anemia.

3.1.3. Anemia due to a defective iron transporter

Another zebrafish mutant characterized by embryonic hypochromic anemia is *weissherbst* (*weh*), whose defective gene was determined to be *ferroportin1* located on linkage group 9 (36). Ferroportin1 is a multiple-transmembrane domain protein whose function in zebrafish embryos is to transport iron from yolk sac to the circulation and is required for synthesis of hemoglobin. Zebrafish *weh* mutants exhibit no abnormality in the number of circulating red cells up to 48 hpf; however, mutant red cells have very little hemoglobin compared to wild type cells. As the mutant embryos develop, there is a progressive decrease in the number of circulating red cells, when at 96 hpf only 20% of red cells remain in circulation. Positional cloning strategies were performed to identify the *weh* locus. Sequence analysis revealed a premature stop codon in one of the two alleles of *weh*, suggesting the mutant phenotype is due to a defect in *ferroportin1*. In zebrafish embryos, *weh* is expressed in the yolk syncytial layer, whereas in adult fish the expression of *ferroportin1* is detected in the intestine. In humans and mice, *ferroportin1* transcripts are detected in various tissues, and it functions as a basolateral iron transporter. In human placenta ferroportin1 transcripts are also detected on the basal side of syncytiotrophoblasts, which may function similarly to *weh* to transport iron from maternal to embryonic circulation.

3.1.4. Porphyrias

A group of four mutants identified based on photosensitivity or auto-fluorescent blood includes the mutants *yquem* (*yqe*) and *dracula* (*drc*). These are characterized by defective enzymes in the heme biosynthesis pathways.

3.1.4.1. Hepatoerythropoieitc porphyria

The heterozygous mutant *yqe* can develop to adulthood without apparent abnormalities.However, homozygous *yqe* embryos die when exposed to light due to photo-ablation of their blood cells. Wang *et al* (37) used a candidate gene approach and demonstrated that *yqe* encodes uroporphyrinogen decarboxylase (UROD), a critical enzyme in the heme synthesis pathway. Sequence analysis revealed a missense point mutation in UROD that reduces its activity three fold in homozygous embryos. Human UROD has been isolated and mutations causing reduced activity of this enzyme have been identified in porphyria patients (38, 39). Zebrafish *yqe* is considered to be the first animal model of human hepatoerythropoieitc porphyria.

3.1.4.2 Erythropoietic protoporphyria

A similar screen for light sensitive blood disorders was used to demonstrate that zebrafish *dracula* encodes ferrochelatase, the last enzyme in the heme biosynthesis

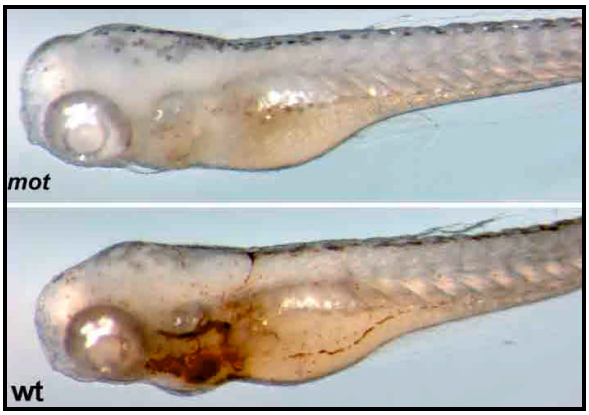

Figure 1. Whole-mount *o*-dianisidine staining of hemoglobin on wild type and *mot* embryos at 96 hpf shows lack of circulating blood cells in the cardiac sinus of *mot* compared to wild type.

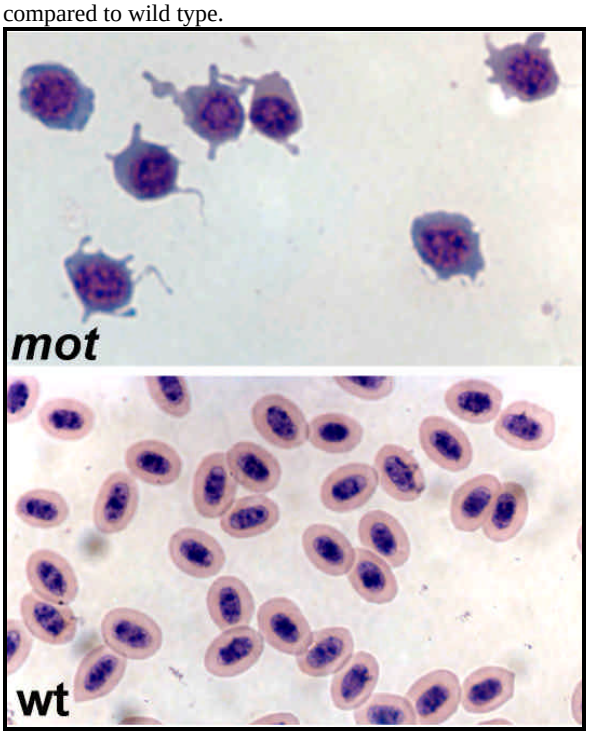

Figure 2. Wright-Giemsa staining of peripheral blood from wild type and homozygote adult *mot* reveals presence of differentially arrested red cells with abnormal morphology with membrane projections.

pathway. Childs *et al* (40) showed that mutant red cells are strongly fluorescent when exposed to UV light with a rhodamine filter and undergo hemolysis within minutes. The photosensitivity results in a severe anemia in fish that are raised under ambient light condition. In addition, mutant fish showed abnormal crystal accumulations in their liver. Biochemical analysis showed accumulation of protoporphyrin in the mutant embryos, suggesting a defective ferrochelatase activity. Molecular cloning of the *drc* revealed a point mutation in a splice donor sequence that caused a premature termination codon. Similarly, some forms of human erythropoietic protoporphyria are caused by mutations in ferrochelatase, which results in clinical symptoms resembling the *drc* fish. Photosensitive red cells and liver disease are a common feature in human porphyria.

3.1.5. Hemolytic anemia

Another group of zebrafish blood mutants have been characterized by normal onset of primitive hematopoiesis followed by a progressive decrease in number of circulating red blood cells at various stages of larval development. These include *retsina* (*ret*), *cabernet* (*cab*), *merlot* (*mot*), and *riesling* (*ris*).

3.1.5.1. Hereditary spherocytosis

The mutant *ris* is characterized by an onset of anemia and abnormal red cell morphology after 3-4 days of development (41). Hematological analysis of adult homozygous *ris* fish showed a severe and partially compensated hemolytic anemia. Liao *et al* showed that circulating red cells undergo apoptosis, and have a defective microtubule marginal band. Molecular analysis revealed a normal expression of hematopoietic genes in *ris* embryos. Using positional cloning strategies, it was demonstrated that *ris* encodes spectrin. Spectrin is the major component of the membrane skeleton in red blood cells, which in association with actin filaments and other structural proteins, forms a protein network underlying the lipid membrane. Spectrin deficiency in humans is associated with a group of red cell membrane disorders called hereditary spherocytosis, which is characterized by reduced membrane deformability and abnormal morphology.

3.1.5.2. Hereditary elliptocytosis

The zebrafish *merlot/chablis* mutant is characterized by the onset of a severe hemolytic anemia at 4 days post fertilization (Figure 1). The analysis of peripheral blood from homozygous embryos and adults revealed erythroid cells with a profoundly abnormal morphology, exhibiting a maturation arrest at the basophilic erythroblast stage as seen in figure 2 (42). Further analysis showed an increase in the osmotic fragility of the *mot* cells, indicative of decreased red cell membrane deformability and lack of membrane integrity. Positional cloning techniques and candidate gene strategies were applied to demonstrate that *mot/ cha* encodes the erythroid specific isoform of protein 4.1R, a critical component of the red blood cell membrane skeleton. Sequence analysis of *4.1R* cDNA from both alleles of *mot* and *cha* revealed two different nonsense point mutations resulting in a premature stop codon. Protein 4.1 is a crucial component of the red cell membrane that anchors the spectrin-actin based cytoskeleton to the plasma membrane, and plays an important role in maintaining red cell membrane deformability and morphology. In humans, hereditary elliptocytosis is due to dysfunctional or absent of 4.1R in red cell membrane cytoskeleton and is characterized by abnormal red blood cell morphology with a decrease in red cell deformability. The zebrafish mutant *merlot* is the first nonmammalian vertebrate model of hereditary elliptocytosis.

3.1.5.3. Congenital dyserythropoiesis

Mutations in the erythroid cytoskeletal protein anion exchanger AE1 (band 3) have been detected in the

mutant *retsina* (B. Paw, and L. Zon, personal communication). The mutant fish is characterized by a normal embryonic erythropoiesis for the first 72 hours and an onset of a severe anemia at 96 hpf. The hematological examination of peripheral blood from adult homozygotes revealed maturation arrest at the late erythroblast stage and presence of 40% binucleated erythroid cells, which is similar to congenital dyserythropoietic anemia type II in humans. The *ret* gene was mapped to the linkage 3 and subsequently was shown to encode the zebrafish *erythroid anion exchanger 1* (band 3). In humans, mutations in band 3 are also associated with a heterogeneous group of erythroid membrane disorders including hereditary spherocytosis and congenital dyserythropoietic anemia.

3.1.6. Polycythemia

Transgenic techniques have also been applied to generate mutant phenotype to study the normal function of novel hematopoietic genes. A zebrafish erythroid specific cDNA library was screened and a novel gene encoding a protein containing death domain was identified (43). Further studies showed that mRNA transcripts of the zebrafish hematopoietic death receptor (ZH-DR) were restricted to the site of embryonic hematopoiesis, the intermediate cell mass. A construct containing *gata1* promoter and a dominant negative form of ZH-DR lacking the death domain was used to generate germ-line stable transgenic zebrafish. The transgenic fish developed normally, but showed phenotypical abnormalities after 2-3 months of age. Hematological and histological examination of homozygous transgenic fish revealed an increase in the number of circulating red cells, an increase of total hemoglobin, a erythroid hyperplasia of kidneys (site of definitive hematopoiesis), and an accumulation of excess red blood cells in the trunk muscles. The polycythemic phenotype of transgenic fish is due to the inhibition of the erythroid specific pro-apoptotic receptor. In humans, polycythemic patients suffer from similar symptoms. In some cases mutations in erythropoietin are shown to be the etiologic cause of human polycythemia, but in the majority of patients the underlying molecular abnormalities are undefined.

3.1.7. Hemostasis

Extensive morphological, functional, and immunological analysis have been applied to identify zebrafish thrombocytes and to confirm that they are equivalent to the mammalian platelets (32). The results of functional assays showed that chemical agonists could induce aggregation, adhesion, and secretion in zebrafish thrombocytes in a similar fashion to human platelets. Immunofluorescence experiments also revealed that the membrane of zebrafish thrombocytes contain the major platelet-specific glycoproteins GpIb and GpIIb/IIIa, which are von Willebrand factor binding sites and a fibrinogen receptor, respectively.

The presence of clotting factors in zebrafish has also been investigated. Zebrafish prothrombin has been cloned (44), and there is biochemical and functional evidence for the presence of other major coagulation factors in zebrafish such as factor X, and protein C. This data is strongly suggestive of a significant similarity between the zebrafish and mammalian coagulation pathways. Zebrafish with chemically induced hemophilia have been generated (45) and novel assays to identify defective clotting factors have been developed (45, 46). The characterization of thrombocytes and coagulation pathways in zebrafish has shed light into the future application of zebrafish to study a broad range of coagulation disorders such as hemophilia and thrombosis.

3.2. Cardiovascular disease

In zebrafish embryos, the heart is the first developed organ. At 22 hpf, the heart starts beating and shortly after that circulation starts. By 36 hpf, the embryonic heart generates a strong circulation in the head and trunk. The heart of a zebrafish embryo at 36 hpf resembles the human embryonic heart at 3 weeks gestation (47), and by the 6th day after fertilization, the chambers and the valves in the embryonic heart are fully developed. Several characteristics make zebrafish an amenable model for studying the formation and function of the cardiovascular system. In the transparent embryos, one can easily scrutinize the formation of the heart chambers, monitor the heartbeat, blood pressure, and the initiation of circulation. Furthermore zebrafish embryos can survive during the first week of gestation even in the absence of a functional heart as oxygen can get to the tissues of the small embryo by diffusion.

The results of large-scale mutagenesis screens identified more than 50 mutations that affect functional and organogenesis aspects of the cardiovascular system. Zebrafish heart mutants can be classified into two groups with defective cardiac function or formation. The first group includes mutants such as *passive aggressive*, *hal* , and *pickwick* (*pik*), which show defects in cardiac contractility. The heart development in zebrafish mutant *pickwick* is normal; however, the heart contracts poorly and has a reduced systolic pressure. Xu *et al* applied positional cloning techniques and showed that *pik* encodes titan, a protein required for sarcomere assembly (48). The thin-walled poorly contractile heart of *pik* embryos resembles hearts of human patients with dilated cardiomyopathy. Several other mutants with cardiac dysfunction have also been isolated. These mutants include *slow mo*, *tremblor*, and *reggae*, which have defective heartbeat rhythm. The *slow mo* fish have a slowed heart rate (bradycardia) that persists throughout life and can be attributed to a decrease in pacemaker current (49, 50); however, the *slo mo* gene has not been isolated. Mutants displaying morphological abnormalities include enlarged (*santa*) or shrunken hearts (*heart and soul*). Specific defects in the formation of heart valves (*pandora*) and ventricles (*jekyl*) have also been isolated.

Peterson *et al* used zebrafish to screen for small molecules that modulated different stages of embryonic development and organogenesis (51). Several small molecules were identified that affected the cardiovascular system. A compound was identified that could alter the ratio of atrial to ventricular contractions from the usual 1:1 to 2:1, a phenotype resembling the human cardiovascular

condition called second-degree atrioventricular heart block. One of the mutations affecting the vascular system is *gridlock* mutation, which resembles a human cardiovascular disorder involving defects in a coarctation (narrowing) of the aorta (52). The *gridlock* encodes a basic helix-loop-helix protein that is a member of the *Hairy/Enhancer* family of transcription factors (26). Coarctation has a high sibling recurrence, thus it will be of interest to examine the human gridlock-related gene as a candidate gene for this disease.

3.3. Kidney diseases

Wilm's tumor, polycystic kidney disease, and renal carcinoma are all believed to result from abnormal early development (53). Understanding the molecular etiology of renal disease may provide significant understanding of the pathogenesis. The basic development of the zebrafish pronephros is similar to higher vertebrate at several levels. In both cases the kidney arises from intermediate mesoderm and is vascularized by the dorsal aorta. Zebrafish have been shown to express some of the same genes essential to kidney development in the same basic locations as in humans. These include *pax2.1, wt1,* and *sim1* (54, 55). Additionally, Na/K ATPase transporters are present at the basolateral membrane of the glomerulus. Zebrafish pronephros consist of a pair of fused glomeruli that receive and filter blood before sending it via a pair of tubules to ducts ultimately releasing the waste near the anus (55). The vascularization of pronephros and initiation of glomerular filtration in zebrafish occurs between 40-48 hpf.

Zebrafish mutants with defective pronephros development have been isolated. These include 15 recessive mutations causing the development of visible pronephric cysts.. Autosomal recessive polycystic kidney disease (ADPKD) is a very common human disease (56), and in the majority of cases mutations in the *PKD1* gene have been identified (57). Injection of truncated forms of the *PKD* into zebrafish suggest that the protein is involved in Wnt signaling (58).

Zebrafish may be useful for determining genes involved in regeneration of an injured kidney as well. There is evidence to support the idea that renal stem cells are still present in the adult zebrafish. This includes the observation that regeneration has been observed following chemically induced tubular degeneration in goldfish (59).

The true zebrafish models for a form of human kidney disease includes familial glomerulocystic kidney disease (GCKD) and possibly Senior-Loken syndrome. It has been shown in patients that mutations in *HNF1-beta* are associated with GCKD (60). Zebrafish harboring a mutation in *vhnf1* resulting in the formation of cysts in the pronephros, have been isolated from insertional mutagenesis screens (61). The mutation also results in patterning defects in the pancreas, liver, and hindbrain. There is reason to believe that these mutations may affect the proper functioning of the pancreas as well. Another potential model for human renal disease includes zebrafish cyst mutants, *fleer* (55) and *elipsa* (62), that show evidence of combined retinal and renal dysplasia as seen in a condition in humans called Senior-Loken syndrome.

3.4. Diabetes

Screens for zebrafish mutants affecting the pancreas have lagged behind mutants affecting organs such as the heart and kidneys by virtue of the lack of an obvious visual phenotype. Nonetheless expected cell types of the endocrine pancreas have been observed and insulin expression has been detected at 19 hpf of development (63). Transgenic zebrafish expressing GFP under the control of an insulin promoter have been generated (64). To date, only one mutant has been isolated with manifestations in the pancreas. As described above, the gene *vhnf1* (61) is involved in maturity onset diabetes of the young (MODY5) caused by mutations in *HNF1-beta* (60, 65, 66). This form of MODY is distinguishable from the others in that there is also a renal dysfunction as described in the kidney disease section of this review. However the diabetic aspects of this mutant fish have not been reported significantly.

Zebrafish have been considered for the study of one of the genes possibly involved in type I diabetes, the type IA-2 autoantigen (67). This gene is a member of the protein tyrosine phosphatase family however it does not possess phosphatase activity. It has been shown that roughly 70% of patients with type I diabetes possess autoantibodies to this protein (68, 69) and the presence of these autoantibodies is considered as an early predictive marker for type I diabetes (70, 71). The true function of the type IA-2 autoantigen remains a mystery, however it may be possible to determine the functional roles this tyrosine phosphatase family member has in development by using the zebrafish.

3.5. Diseases of the eye, blindness

Although no zebrafish eye mutants resembling genetically identified human eye diseases have been cloned, the process of eye development and early mutations in zebrafish eye show remarkable similiarities to those in humans. There are over 120 different classified genetic conditions related to blindness many for which the gene has been identified. The most common cause of blindness in the western world involves retinal degeneration. Similarly in zebrafish the most frequently observed association with blindness observed by chemical mutagenesis has been outer retina dystrophy (72). In humans the traditional model of rod-cone photoreceptor loss is retinitis-pigmentosa. Potential disease models for retinitis-pigmentosa and other types of eye disease are represented by several zebrafish mutants (62, 72, 73).

Human photoreceptor degeneration can be grouped into several categories. Interestingly, there are several zebrafish mutants that exhibit similar patterns with the initial degeneration of peripheral (*brudas*, *elipsa* and *fleer*), central (*mikre*, *oko*, *niezerka*), or patchy areas (*krenty* and *discontinuous*) of photoreceptors (62, 74). The identification of the various genes that cause blindness in zebrafish shall increase the list of candidate genes to be examined for human patients.

The zebrafish mutant *lakritz* is blind at 4 days after fertilization. The mutated gene in *lak* encodes for the

zebrafish homolog of *Drosophila* atonal *ath5*, which is a basic helix-loop-helix transcription factor. In humans, *lak* is now considered a prime candidate gene for the rare condition of inherited optic-nerve aplasia (75). . Given that the retinal ganglia cells fail to develop in *lakritz*, this fish may be a model of some forms of glaucoma involving ganglion cell degeneration. Another zebrafish mutant, *archie*, may also be a model for glaucoma, because in *archie* the ganglion cells degenerate rather than completely failing to develop (75).

A zebrafish mutant exhibiting a reverse optokenetic nystagmus has been described (76). Studies showed that some homozygous *belladonna* embryos are achiasmatic, resulting in a reverse eye movement in response to visual stimulation. Similar behavior has been identified in humans; however, the molecular defects remain unidentified (77).

Dominant mutations are involved in retinal degeneration in humans as well. The *night blindness* fish have a dominant mutation whereby retinal degeneration occurs slowly over a 2-5 month period. Homozygous *night blindness* fish die within 8 days of development due to degeneration of the central nervous system (73, 78). The isolation of the *night blindness* gene will contribute to the list of candidate genes involved in dominant forms of human retinal degeneration.

3.6. Diseases of the ear, deafness

The zebrafish mutant *mariner* is considered a model of human hereditary deafness (79).The mutant fish was originally identified based on their circling swimming pattern, defective inner ear hair cell bundle, and the lack of respond to acoustic vibration. Based on phenotypical similarities between the *shaker-1* mouse and *mariner*, such as defective apical endocytosis in sensory hair cells, the zebrafish *myosin VIIA* was isolated and identified as the candidate gene. Further experiments revealed that myosin VIIA is expressed in the otic vesicle and the developing inner ear of zebrafish embryos. Sequence analysis revealed 3 nonsense and 2 missense mutations in 5 alleles of *mariner*, establishing *myosin VIIA* as the defective gene in *mariner*. Zebrafish *myosin VIIA* is mapped on linkage 18. There is a high degree of homology between mammalian and zebrafish myosin VIIA proteins. Mutations in *myosin VIIA* are associated with several human balance and hearing disorders such as Usher 1B syndrome, DFNB2, and DFNA11.

3.7. Neural disorders

There are three zebrafish disease models for human neural disorders including congenital fibrosis of the extraocular muscles (CFEOM2 (80)), Waardenburg-Shah syndrome/Hirschsprung disease (81), and holoprosencephaly (25).

The congenital fibrosis of the extraocular muscles (CFOEM) syndromes results in restricted monogenic isolated strabismic disorders characterized by restrictive ophthalmoplegia. The zebrafish mutant *soulless* (80) has eyes that are projected parallel to the body instead of angled to view what is in front of the fish. These fish harbor a mutation in the *phox2a* gene and may be a model for congenital fibrosis of the extraocular muscles. For both fish and mice, a mutation in the *phox2a* gene results in defects in motor nuclei in the midbrain (oculomotor cranial nerve III and abducens cranial nerve IV). Patients with CFEOM2 have been identified with mutations in the *phox2a* gene as well (82).

Hirschsprung disease is the main cause of functional intestinal obstruction (83). Mice models for Hirshprung's and the related Waardenburg-Shah syndrome (84) had been characterized and the gene had been isolated (85, 86) prior to the candidate gene cloning in zebrafish (81). The defects had the distinguishing dual characteristics of affecting melanocytes and the enteric nervous system, leading to enteric aganglionosis. The gene disrupted in the zebrafish mutant *colourless* and mice is *sox10* (81). This mutation results in extensive loss of pigment cells and enteric nervous system, along with large reductions in sensory and sympathetic neurons and certain glial cells (87). Studies in zebrafish have enabled delination of the absent cell types (non-ectomesynchymal crest cells) in sox10 deficient embryos (81).

The last remaining established zebrafish model for human neural disease involves the most common developmental defect of the forebrain in humans, holoprosencephaly (HPE) (88). The severity of HPE ranges from a complete lack of separation of the telencephalic ventricles to the less severe forms with only partial separation of the ventricles. Several genes have been identified as culprits involved in the development of human HPE including *sonic hedgehog* (*shh*) (89-91). With regards to *shh* it has been shown that morpholino knockdown of *shh* does not result in HPE for zebrafish, rather it also is necessary to knockdown the related family member, *twiggy-winkle hedgehog*, by morpholino (25). In the said fish the eyes are immediately juxtaposed with very little brain between them. Thus it is described as being cyclopic. These results make it clear that both of these genes are working together in zebrafish to perform the function of *shh* in mammals. Other genes being considered for HPE include the *one-eyed pinhead* (*oep*) gene which was originally isolated using zebrafish (88). This gene became a candidate for HPE patients given the cyclopic phenotype observed in *oep* fish.

Many researchers are exploiting zebrafish for the study of known disease-linked genes. Given the potential to use morpholinos for the study of developmental diseases, these studies which focus on known human disease-linked genes, may be pertinent to this review. Disease-linked genes being studied in the zebrafish include: Huntington's gene in Huntington's disease (92), *kal-1* in Kalman's syndrome(93), peripheral myelin protein-22 (PMPP) in human hereditary neuropathies(94), Apolipoprotein E (95, 96), and presenelin in Alzheimer's disease (97). The zebrafish Huntington's gene has been isolated and characterized with respect to its expression (92). The large protein is reportedly expressed at high levels early in development. It will be of particular interest to learn

whether this gene is important during zebrafish development. Individuals with Kallmann's syndrome are unable to smell due to a mutation in *Kal-1* (98, 99). The zebrafish ortholog for this gene has been isolated and is being studied (93). It has been observed that the Kal-1 is specifically expressed in the olfactory bulbs of zebrafish. Nothing, however, is known about the function of Kal-1. Other researchers have isolated the zebrafish ortholog for PMP22 (94). PMP22 has been shown to be involved in the developmental initiation of myelination and maintenance of axons (100-102). Mutations in PMP22 can result in human hereditary neuropathies. PMPP22 gene dosage has been shown to affect myelination (103-108). Clearly the zebrafish can be useful to study early stages of myelination given the optical clarity of the zebrafish nervous system. Thus morpholino knockdown of PMP22 may enable the zebrafish to become a potential model for human neuropathies.

Apolipoprotein E (ApoE) and A-1 have been isolated and examined with respect to expression patterns in zebrafish (95, 96). Expression of the ApoE isoform was localized to distinct areas within the zebrafish brain. With the recent discovery that ApoE:cholesterol is required for synapse formation, the importance of this gene for basic neural functions of the brain became apparent (109, 110). ApoE polymorphisms have been associated with increased susceptibility for late onset Alzheimer's, which is the more common form. It is suggested that the zebrafish is potentially useful for studying tissue specific transcriptional and post-transcriptional regulation of ApoE.

Several neurodegenerative diseases including Alzheimer's (111), Parkinson's (112), and Huntington's (113), arise from the accumulation of protein plaques including. One of three genes shown to be fully penetrant for familial Alzheimer's disease (FAD) is the *presenilin* gene (114). Zebrafish presenilin has been analyzed biochemically and with regards to expression (97). Surprisingly, wild type zebrafish presenilin promoted secretion of A-beta-42 peptide, typical of mutated human presenilin observed in FAD. The unexpected wild type, Aspartate 374, of zebrafish presenilin is homologous to a known fully penetrant substitution mutation in human presinilin involved in A-beta-42 secretory activity and FAD pathogenesis. Furthermore the zebrafish studies suggested that presinilin may serve a housekeeping function since it was observed to be maternally expressed. While this is not a disease model it does present an interesting paradox given that zebrafish seem able to survive with a wild type presinilin that would be expected to produce Alzheimer like effects in people. Additionally, there is a group of clinical correlates for the eye and brain patterning defects observed in zebrafish *glass onion, nagie oko*, and *oko meduzi* (115). This group includes: Walker-Warburg syndrome, muscle-eye-brain disease, and cerebro-oculo muscular dystrophy. Lastly, developmentally normal, but touch insensitive zebrafish mutants have been isolated that display decreased sodium channel amplitudes (116). These may represent disease models for embryonic ion channelopathies that have been implicated in human developmental disorders such as epilepsy (117, 118).

3.8. Cancer

There are no reported zebrafish models for cancer as of yet. However researchers have presumably screened and isolated zebrafish mutants displaying enhanced susceptibility to cancer (119). There have been studies describing the cellular details of skin cancer formed in zebrafish treated with ethylnitrosourea (120). Here it is assumed that zebrafish can be used as a model for skin cancer since treatment with high concentrations of ENU can result in all three forms of skin cancer.

Surprisingly, rather limited studies have been performed on zebrafish as related to chemical sensitivity in carcinogenesis (120-124). N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz [alpha]anthracene (DMBA) have both been shown to affect embryos more than juveniles in resulting in tumor formation (123, 124). These studies emphasize the potential role of zebrafish as a model system for studying the mechanisms of chemical carcinogenesis. Recently the National Cancer Institute requested further studies to establish zebrafish model systems for cancer research (PA-01-095). Additionally researchers at Phylonix are screening for anti-apoptotic drugs by taking advantage of whole body zebrafish apoptosis assay (NCI.NIH.GOV).

Multiple endocrine neoplasia type 1 (MEN1) is a well characterized form of cancer resulting in tumors of the parathyroid, pituitary, and pancreatic tissues. The gene was positionally cloned from patients (125). Studies of the zebrafish *menin* gene have shown that the protein functions in a similar manner to human MEN1 in the transcriptional repression of the transcription factor jun. Furthermore, sequence conservation was evident for all residues shown to be important in carcinogenesis.

3.9. Diseases of addiction

3.9.1. Alcoholism

There has been a great amount of biochemical and genetic research, which has ultimately resulted in the identification of trait markers for subgroups of alcoholism (for a review (126)). The only reliable negative estimator has been functional polymorphisms of dehydrogenase enzymes for alcohol and acetaldehyde leading to averse reactions to alcohol in Asian populations (127). However a comprehensive trait marker has not been identified and screens are underway to isolate genes associated with alcoholism using zebrafish (128). Zebrafish are a schooling fish (129-131) showing a preference for social groups. Low amounts of alcohol have been observed to result in aggressive behavior while higher amounts cause the opposite effect. By monitoring the behavior of zebrafish under the influence of alcohol it is expected that it will become possible to isolate mutant fish that display notably unique responses to alcohol due to mutations.

3.9.2. Street drug

Similar screens to those investigating alcoholism have been performed looking for locus association with cocaine addiction. Fish generally prefer to go to a chamber containing the cocaine in the water (132). Already three groups of insensitive fish have been isolated in a

preliminary screen. One of the common mechanisms in drug addiction involves an increase in dopamine concentration within the nucleus acumbens (133, 134). It shall be interesting to determine if this pathway is disrupted in cocaine unresponsive fish. This genetic approach represents the first mutagenesis screen involving behavioral response using large numbers of vertebrate organisms.

3.10. Digestive diseases

Another realm of possibility involving zebrafish includes the study of digestive diseases. By making use of an engineered molecule with phospholipase A_2-dependent fluorescence during normal lipid processing, a mutant (*fat free*) was isolated in zebrafish that possessed a defect in bile synthesis or secretion (135). The fish displayed apparently normal digestive organ morphology, however *fat free* had a defect in lipid processing. Furthermore, incubation of zebrafish embryos with a potent inhibitor of cholesterol metabolism, atorvastatin, caused an inhibition of cholesterol metabolism in the zebrafish. Therefore, zebrafish may be useful therefore in screening or testing for cholesterol lowering drugs. This type of *in vivo* enzymatic screen represents a completely novel and quite exciting approach for identifying significant genes involved in proper digestion. Combining this approach with morpholino knockdown will enable greater elucidation of gene function as it relates to digestion and metabolism.

3.11. Other diseases

Zebrafish are also being exploited by industry to develop assays for drug screening (136). This includes attempts to look for anti-angiogenic compounds at Phylonix (Cambridge, MA). There are advantages to using zebrafish for drug screening. The zebrafish can inherently take into account overt toxicity considerations as it relates to development better than cell based assays. The zebrafish also enables analysis of a greater number of organisms at an earlier step in the drug testing better than mammalian based assays. It shall be very interesting to see how successful this approach is for drug development.

Muscular dystrophy is being studied as well using zebrafish (137, 138). The disease Duchenne Muscular Dystrophy (DMD) occurs due to mutation in the dystrophin protein (139, 140). Many of the genes encoding the proteins known to be present in the typical dystrophin-associated glycoprotein complex have been identified by zebrafish EST searches. Zebrafish researchers hope to determine the role of alternatively spliced dystrophins and take advantage of the optical clarity of zebrafish for *in vivo* examination of the dystrophin function. Given the motility defects isolated in the original screen this gene is also considered as a candidate gene for various zebrafish mutants.

Other possible disease models include *jumbo* and *chihuahua* that may represent obesity and osteogenesis imperfecta (141). *Jumbo* fish were isolated via a food based screen whereby fish were allowed to eat as much as they wanted at which point they were transferred to a new tank and offered more food. The *jumbo* fish continued eating. *Chihuahua* was isolated in an x-ray based screen. The fish was particularly small in the head and jaws. Further analysis revealed that the ribs were a mass of broken bones. Thus far the locus has been mapped to region close to a member of the collagen family of genes similar to osteogenesis imperfecta. For humans with osteogenesis imperfecta and these particular zebrafish the bones develop normally but become very fragile. Mice without the *LEPTIN* gene have defects in temperature homeostasis. Thus zebrafish mutagenesis screens have been performed relying on temperature gradients in tanks. Since fish are cold blooded they generally stay at a certain position. The *hot body* fish prefers water several degrees warmer and does not exhibit satiety similar to *LEPTIN* knockout mice. These disease models enable a greater survey of potential culprit disease genes and more refined analysis of cellular pathobiology.

4. CONCLUSIONS AND FUTURE PROSPECTS

The most common diseases (heart disease, cancer, and diabetes) are polygenetic disorders. For such complex diseases there is a need for quantitative trait loci mapping. This is a very difficult venture that requires considering multiple genes at a time. The zebrafish system is currently not ready for this challenge given that the zebrafish genomic infrastructure is still in development. However, the zebrafish has already proven to be a valuable organism for elucidating single gene function to date. Most of the genes isolated from zebrafish mutagenesis screens have not turned out to be medically relevant. However, these studies have increased the list of candidate genes potentially relevant to the human geneticist. The list of identified mutated genes in zebrafish will explode over the next several years given the increase in zebrafish laboratories and the completion of the sequencing of the zebrafish genome later this year. Furthermore, the increase in screens should result in the isolation of more zebrafish mutants resembling human diseases. For these reasons it is important that clinicians consider the results of zebrafish mutation research in their search for genetic links to disease. The many favorable developmental attributes of zebrafish will facilitate greater understanding of the genetic etiology of disease.

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Send correspondence to: Dr Shuo Lin, Molecular, Cellular, and Developmental Biology, UCLA, 621 Charles E. Young Dr. South, Los Angeles, CA 90095-1606, Tel: 310-267-4970, Fax: 310-206-9184, E-mail: shuolin@ucla.edu