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REVIEW ARTICLE

Developmental Biology: Model Systems - A Series of Reviews

In this the second of five review articles focusing on model organisms currently being studied to understand developmental mechanisms Drs. Veldman and Lin examine the Zebrafish model. This model is a particularly useful system for advancing our understanding of the genetic mechanisms involved in vertebrate development.

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Zebrafish as a Developmental Model Organism for Pediatric Research

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ABSTRACT: Zebrafish has many advantages as a model of human pediatric research. Given the physical and ethical problems with performing experiments on human patients, biomedical research has focused on using model organisms to study biologic processes conserved between humans and lower vertebrates. The most common model organisms are small mammals, usually rats and mice. Although these models have significant advantages, they are also expensive to maintain, difficult to manipulate embryonically, and limited for large-scale genetic studies. The zebrafish model nicely complements these deficiencies in mammalian experimental models. The low cost, small size, and external development of zebrafish make it an excellent model for vertebrate development biology. Techniques for large-scale genome mutagenesis and gene mapping, transgenesis, protein overexpression or knockdown, cell transplantation and chimeric embryo analysis, and chemical screens have immeasurably increased the power of this model organism. It is now possible to rapidly determine the developmental function of a gene of interest *in vivo*, and then identify genetic and chemical modifiers of the processes involved. Discoveries made in zebrafish can be further validated in mammals. With novel technologies being regularly developed, the zebrafish is poised to significantly improve our understanding of vertebrate development under normal and pathologic conditions. **(*Pediatr Res* 64: 470–476, 2008)**

The zebrafish, *Danio rerio*, is a fast expanding and extremely valuable model system for studying vertebrate developmental biology. Because its introduction as a modern experimental model organism in the early 1980s, many new tools have been developed for imaging and manipulating early developmental processes in zebrafish. Given the high genomic and molecular similarities between zebrafish and other vertebrates including humans, many of the important discoveries in zebrafish development are applicable to humans. This review will summarize the advantages of the zebrafish model system for exploring vertebrate developmental biology under normal and pathologic conditions and highlight examples where experimental results from zebrafish can lead to a greater understanding of human biology.

Zebrafish provide multiple significant advantages over mammalian vertebrate model organisms such as mice and rats. Early development of zebrafish embryos is external, rapid, and visually accessible. The embryo and chorion, the protective membrane surrounding the embryo, are transparent. This allows the scientist to follow development from the first moments of fertilization through to free swimming, actively feeding larval fish. Baby zebrafish hatch 2 d after fertilization and begin actively feeding 5 d after fertilization. During the first 5 d, all major organ systems develop and begin functioning. In addition, zebrafish are able to breed year round with females capable of producing several hundred embryos each week. The abundance of offspring is especially advantageous for genetic mapping studies and experiments that demand large sample sizes. Zebrafish can be raised and maintained in

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Abbreviations: ENU, *N*-ethyl-*N*-nitrosurea; **IMPDH,** inosine monophosphate dehydrogenase; **ISH,** in situ hybridization; **HSC,** hematopoetic stem cell; **MPA,** mycophenolic acid; **TILLING,** targeting induced local lesions IN genomes; *UROD*, *uroporphyrinogen decarboxylase*

high-density aquarium systems requiring much less space and cost than the animal facilities necessary for mammals. These traits are what initially drew researchers to work with zebrafish as an experimental model. More recent technological advances have significantly enhanced these initial advantages.

Zebrafish Genome Project and Microarray Tools

The genome of zebrafish is being sequenced by the Sanger Center in collaboration with the zebrafish research community. The current version, April 2007 Zv7, has five and a half fold coverage and over 45,000 predicted genes. This invaluable database provides DNA sequence information for genetic mapping studies as well as data mining tools for identification of conserved as well as novel genes. In parallel, several gene microarrays have been developed to study the expression of thousands of genes under normal and experimental conditions. Previously, our laboratory and several others used these tools to examine gene expression changes in the *cloche* mutant embryo which lacks blood and endothelial cell lineages (1–3). These studies led to the discovery of many novel genes involved in the development of these tissues and confirmed the importance of previously identified genes. One gene in particular, *etsrp*, an ets family transcription factor was found to be critical for myeloid and endothelial cell development in zebrafish (4–6). A recent study suggests that the mammalian homolog, *ER71*, is an important mediator of these same processes in mice, demonstrating the power of genomic information, microarray analysis, and conservation of gene function between fish and mammals (7).

Gene Expression Analysis by *In Situ* Hybridization

The developmental transparency of zebrafish embryos makes examination of endogenous gene expression in the whole embryo by RNA *in situ* hybridization (ISH) possible. Several methods exist for staining embryo by ISH; the most common involve hybridizing the embryo with digoxigenin-labeled antisense RNA probes, then antibody staining with antidigoxigenin antibody conjugated to alkaline phosphatase followed by an enzymatic reaction that forms a colored precipitate or fluorescent product (8,9). This technique provides spatiotemporal information about expression of the gene(s) of interest and can be used to detect relationships between different genes through double ISH or ISH coupled to whole mount immunostaining. This method can be used as a powerful screening tool for forward genetic screens.

Forward Genetic Screens

Although the zebrafish was occasionally used in toxicological or developmental biology studies before the 1980s, an article published by Streisinger *et al*. (10) on the ability to produce homozygous diploid fish thrust the zebrafish to the forefront of vertebrate molecular genetics. This technique allowed for the rapid identification of recessive mutations and more efficient mapping of mutant loci. Thus, forward genetic approaches formerly available only for invertebrate species was now possible in vertebrates. The Streisinger *et al*.'s laboratory also played an instrumental role in establishing common laboratory techniques for zebrafish husbandry and embryological observation. These have been extended by fellow researchers at the University of Oregon and published in *The Zebrafish Book,* which is available in print and in a free online version (http://zfin.org/zf_info/zfbook/zfbk.html) (11). Inspired by these advances, a pair of large-scale chemical mutagenesis screens were performed by the Driever and Fishman laboratories and Nusslein-Volhard laboratory (12,13). In these experiments, adult male fish were treated with the mutagen *N*-ethyl-*N*-nitrosurea (ENU). This chemical efficiently creates point mutations in the germline (14,15). Through a simple breeding scheme, mutations are bred to homozygosity and recessive phenotypes can be readily observed. These studies were the first systematic, large-scale genetic screens done in a vertebrate species. More recently, retroviral and transposable element insertional mutagenesis techniques have been developed for zebrafish, significantly decrease the time necessary to go from mutant phenotype to identification of the mutant genetic loci (16–18). It is now possible for individual laboratories to perform their own focused, small-scale mutagenesis screens using methods developed by these pioneering researchers.

Random mutagenesis strategies in zebrafish have generated thousands of distinct mutant phenotypes, many of which are consistent with human genetic disorders. For example, a mutant fish identified in the original Nusslein-Volhard screen with hematopoetic defects was mapped by our laboratory to the *uroporphyrinogen decarboxylase* (*UROD*) gene (19,20). *UROD* is involved in human porphyria cutanea tarda and hepatoerythropoietic porphyria (21). These syndromes present as sensitivity to sun exposure, including blistering, scarring, and liver problems. The *UROD* enzyme performs the fifth step in heme production and decrease in its function results in build up of uroporphyrinogen and light sensitivity. *UROD* mutant zebrafish exhibit light dependent lysis of erythrocytes and this defect can be rescued by overexpression of the wildtype allele of the *UROD* gene. The identification of this mutant zebrafish and its rescue by gene therapy suggests a possible route to treat human patients and also provides a tool for screening pharmacological agents for treatments to alleviate symptoms of these diseases.

Initial forward genetic screens in zebrafish were performed at the visible anatomical level (12,13). Labeling specific cell populations by ISH allows the visualization of cells that is not normally distinguishable in the live embryo. One such cell population is the early insulin positive pancreatic islet. Insulin is a critical hormone that regulates blood sugar levels and dysfunction of insulin signaling results in the disease diabetes. To identify developmental mechanisms of insulin expression and β-cell identity, our laboratory performed an ENU mutagenesis screen using insulin ISH as a screening tool (22). Out of 900 genomes screened, 11 mutant lines were identified representing nine genes. Three general classes of mutant phenotypes were described: I) decreased insulin expression or fewer cells with normal islet morphology, 4 lines; II) fewer insulin positive cells with abnormal islet morphology, 1 line; and III) abnormal split and scattered insulin positive cells, 4 lines (Fig. 1). These mutations likely represent genes involved

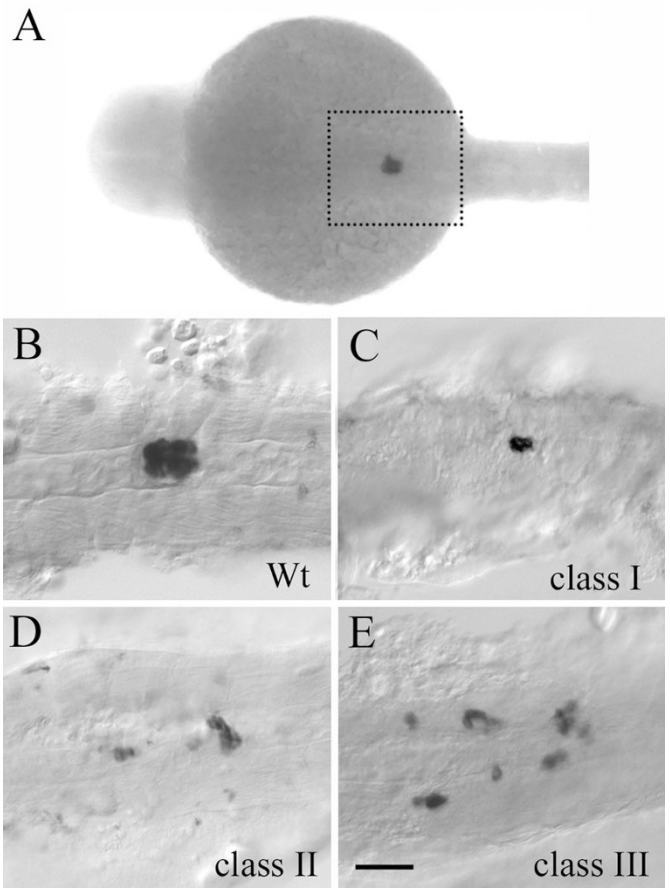

Figure 1. *In situ* hybridization (ISH) for insulin mRNA identifies distinct classes of mutant genes. *A*, Whole mount ISH of 1-d-old zebrafish embryo for insulin expression identifies a single distinct cluster of cells, the islet. Dorsal view with anterior to the left. *Dotted box* identifies region shown in *B-E* in flat mount. *B*, Wild type fish shows a single cluster of insulin positive cells. *C*, Class I mutants display decreased insulin expression or fewer cells with normal morphology. *D*, Class II mutants display fewer insulin positive cells with abnormal islet morphology. *E*, Class III mutants display abnormal split and scattered insulin positive cells. Scale bar in $E = 50$ μm. Adapted from Mol Endocrinol 20:194–203, Copyright © 2006 by The Endocrine Society, with permission.

in cell specification, differentiation, and migration. Identification of the mutant loci will improve our understanding of β-cell and islet development, which should provide insight into the dysfunction of these cells in diseases like diabetes.

Transgenesis

The transparency of zebrafish was a major reason for its rise in importance in experimental biology. Transparency allows direct experimental observation of basic developmental processes such as cell division, migration, and differentiation in real time. Many of these processes are accessible through a simple stereo dissecting microscope. However, the ability to generate tissue-specific fluorescent transgenic fish and observe them using fluorescent confocal microscopy has significantly enhanced the zebrafish's utility. The first stable transgenic zebrafish with tissue-specific expression using a fluorescent protein marker was the *gata1:EGFP* line (23). This fish displays green fluorescing erythrocytes recapitulating the expression pattern of the endogenous *gata1* gene. These fish are a valuable tool for examining erythrocyte development and circulation defects in the developing embryo. This initial transgene was generated using a plasmid vector. It is also possible to use bacterial artificial chromosomes to generate fluorescent transgenic lines (24). This method is especially advantageous when transcriptional regulatory elements are a long distance ($\sim>10$ kb) from the transcription start site. Bacterial artificial chromosomes have the ability to hold up to 300 kb of genomic sequence. Hundreds of fluorescent transgenic zebrafish lines have been generated with tissue-specific expression in various organs and tissues. Recently, large-scale enhancer trapping using transposon-based fluorescent reporters have generated large resources of tissue-specific transgenic fish (25,26). The creation and discovery of new fluorescent proteins with different excitation and emission spectra has also benefited the zebrafish model as it is now possible to generate and intercross multiple transgenic lines with different colored fluorescent tissues (27). The use of fluorescent proteins in transgenic zebrafish allows high-resolution developmental studies of cellular interactions in real time. In addition, fluorescence activated cell sorting can be used to isolate specific cell populations from the labeled tissue giving researchers the ability to examine changes in cellular response to experimental conditions (28,29).

Protein Overexpression

Several options are available to experimentally manipulate gene functions in zebrafish embryos. Overexpression of genes of interest can be accomplished through microinjection of synthetic mRNA or DNA for the desired protein into the 1–2 cell stage embryos (30). Injection of mRNA results in uniform expression in all embryonic tissues. Duration and level of expression is dependent on RNA concentration and RNA and protein stabilities. If spatial or temporal regulation of protein overexpression is desired, DNA transgenes can be created that drive expression at the required time and place. Embryos transiently injected with transgene DNA express protein in a mosaic pattern. If more uniform expression is desired, germline transgenic fish should be established. A valuable tool for zebrafish transgenesis is the heat shock protein, 70 promoter (31). This promoter enables temporal induction of transgene expression by increasing the environmental temperature from 28 to 38°C resulting in ubiquitous transgene induction. In addition, localized heating by use of a laser or heating iron can be used to spatially restrict transgene expression (31,32). Another transgenic technology available to zebrafish researchers is the GAL4-upstream activating sequence system which was initially developed for *Drosophila* and adapted for zebrafish by the Campos-Ortega laboratory (33). In this system, the desired promoter is used to drive expression of the yeast GAL4 protein in a transgenic or enhancer trap line in a spatiotemporally restricted manner. This line is then crossed into a separate transgenic line containing multiple GAL4 upstream activating sequence binding sites driving expression of the protein of interest. An additional technology adapted for use in zebrafish is the Cre-lox system commonly used to

create conditional misexpression or gene knockouts in mice (34–36). In this system, the Cre recombinase enzyme is expressed in the desired tissue by a transgene. Another transgene is introduced, which normally has a fluorescent marker flanked by Cre recombinase sites followed by the effecter protein of interest. The recombinase removes the fluorescent marker protein shifting the effecter protein into the open reading frame allowing it to be translated.

Several technical advances have made the production of transgenic zebrafish more efficient and rapid. The discovery of the Tol2 transposon by the Kawakami laboratory has led to the development of transgene vectors with efficiencies of germline integration greater than 50% *versus* the less than 10% efficiency of naked DNA injection (37,38). This increased efficiency has inspired large-scale gene trap and enhancer trap screens in zebrafish. In addition, recently described Gateway system transgene tools for zebrafish should greatly simplify the process of building transgenes (39,40). This system uses modified recombination sites instead of restriction enzymes to cut and paste promoter sequences to the coding sequence of the protein or fluorescent marker of interest. Fusion protein tags can also be easily added and the resulting transgene placed in a Tol2 vector for efficient transposition into the zebrafish genome. The modularity of this system with promoter modules, open reading frame modules, and fusion protein modules easily switched in a single reaction provides a simple and rapid method for generating transgenes without the limitation of multiple restriction enzyme cutting sites being required. As more researchers adapt this new technology, its power will increase exponentially with the availability of new promoters and protein coding sequences.

Transgenic zebrafish are most easily adapted for use in modeling human diseases with dominant disease gene phenotypes. Several laboratories have developed models of cancer through the overexpression of oncogenes. Overexpression of the mouse *c-myc* gene in the zebrafish thymus under control of the *Rag2* promoter causes the development of T-cell acute lymphoblastic leukemia (41). This model was later improved upon by making conditional *c-myc* expression using the Cre-lox system (34). Expression of the human oncogenic *kRASG12D* protein in muscle precursors results in embryonal rhabdomyoscarcoma (42). Heat shock conditional expression of the *AML1-ETO* oncogenic fusion protein results in an acute myelogenous leukemia like phenotype in zebrafish embryos (43). In these latter two models, microarray analysis was performed on oncogenic zebrafish tissue and compared with human patient samples. Many genetic similarities were identified which support the validity of the zebrafish model in cancer research. Zebrafish transgenic models should be a valuable research tool for genetically dominate human diseases.

Gene Knockout in Zebrafish

Thus far, directed gene knockout technology, as developed for mice, does not exist for zebrafish because of the lack of suitable embryonic stem cell lines in which to perform the necessary recombination reactions. However, two technologies, morpholino antisense oligos and targeting-induced local lesions IN genomes, do exist which can accomplish gene knockdown in zebrafish. Morpholino antisense oligos are 25-base long molecules that bind complementary RNA sequences and either block translation when targeted to the 5′-untranslated region or inhibit spilicing when targeted to splice junction sites (44,45). The mechanism of morpholino action is distinct from short hairpin RNA or micro-RNA. Morpholinos are thought to work through steric hindrance whereas short hairpin RNA and micro-RNA work through RNA degradation in concert with cellular enzymes. Morpholinos are very stable because of phosphorodiamidate linkages and morpholino rings replacing the sugar backbone present on oligonucleotides. The unnatural structure of these linkages prevents cellular enzymes from degrading them. When injected into 1–2 cell stage zebrafish embryos, morpholinos can dose dependently effect gene expression for up to 3–5 d after fertilization. Although care should be taken when choosing a targeting site, because morpholinos can have sequence-specific toxicity and cross react with similar target sequences in multiple genes, morpholinos when properly controlled are capable of knocking down any gene of interest (46,47). Recently, it has also been demonstrated the morpholinos can be used to study micro-RNA function in zebrafish (48).

A common use of morpholinos is to quickly validate human disease genes identified through genetic linkage analysis. Traditionally, these validation experiments were performed through the laborious process of generating of knock-in or knockout mice, which takes 1 or 2 y. By using morpholinos in zebrafish the homologous experiment can be accomplished in a days to weeks. For example, one of the genes responsible for nephronophthisis (NPHP), an autosomal recessive cystic kidney disease common to children and young adults, was linked to the *CEP290* locus (49,50). These patients display chronic renal failure, retinal and cerebellar defects. Expression of the zebrafish homolog of this gene was examined by ISH and found to be elevated in the retina and cerebellum at 2 d after fertilization. Morpholino mediated knockdown of this gene resulted in retinal and cerebellar defects and cystic kidneys consistent with the patient phenotype (50). This not only confirms the *CEP290* gene's role in NPHP, but also provides a model from which genetic modifiers and therapeutic agents can be tested.

A zebrafish gene homolog of *NROB1* which is involved in human adrenal hypoplasia congenita (AHC) has been identified and used to model aspects of this disease using morpholino technology (51). This gene and its protein product, DAX1, are important for the proper development and function of steroidogenic tissues such as the gonads and adrenal gland (52). Loss of function mutations in this gene result in hypogonadal hypogonadism and adrenal insufficiency. Morpholino knockdown of this gene in zebrafish resulted in impaired osmoregulation and reduced or lost expression of the steroidogenic genes *cyp11a* and *star* in the interrenal organ, the fish equivalent of the adrenal cortex (51). Because the phenotype of the DAX1 knockdown was similar to that described for *ff1b*, a steriodogenic factor 1 homolog, epistasis experiments were performed to determine whether the factors acted in the same developmental pathway. Morpholino knockdown of

DAX1 has no effect on *ff1b* expression whereas knockdown of *ff1b* prevents DAX1 expression. This suggests that *ff1b* functions upstream of DAX1 in adrenal development. Morpholino technology can quickly establish epistatic interactions between genes through multigene knockdown and rescue experiments.

Targeting-induced local lesions IN genomes is a recently developed technology designed to identify specific genetic mutations in libraries of ENU treated germ cells (53,54). To identify mutations in your gene of interest, DNA from the germ cells of the F_1 generation of mutagenized fish is scanned for mutations using the CEL-1 enzyme to detect hetero-duplex DNA generated from a gene-specific PCR reaction. The presence of hetero-duplex DNA means a noncomplementary base pair caused by a nucleotide difference exists suggesting a heterozygous F_1 fish. The DNA of these fish is then sequenced to verify the mutation's location in the gene of interest. The mutation can then be bred so that the F_3 generation is homozygous for the mutation and the recessive phenotype can be observed. This method has been used to generate libraries of mutant alleles available through the Sanger Institute and a service to identify mutations in your gene of interest is available. Although not a true gene targeted knockout technology, the result is the same with a mutation in the gene of interest being generated.

Cell Transplantation and Cell-Cell Interactions

A technique for creating chimeric zebrafish embryos through cell transplantation was developed over a decade ago (55). This technique has been used to test for cell autonomous or cell nonautonomous gene functions, cell fate commitment in developmental time, identify signaling molecule functional properties, observe single cell behaviors, and more. Chimeric fish generation involves transplanting a few experimentally manipulated cells into wildtype embryos at late blastula stage and following their developmental fate. Most commonly, transplanted cells are labeled with fluorescent dextran conjugates and then fate is determined through anatomical location, ISH, or immunohistochemical staining. The converse experiment can also be performed with wildtype cells being placed in an experimentally manipulated embryo. In addition, cells from multiple donors can be transplanted into a single host. Common sources of donor cells and host embryos include mutant, morpholino treated, mRNA injected, or transgenic embryos. The coupling of cell transplantation with other zebrafish experimental tools greatly enhances its value (56).

This method was used to describe the role of the *tbx1* gene in the zebrafish *van gogh* mutant and describe its possible role in human DiGeorge syndrome (57). DiGeorge syndrome is defined by malformations of the thymus, aortic arch, ear, and cranial facial structures (58). *Van gogh* mutant fish display phenotypically similar defects (59). To determine whether the mutation was cell autonomous in cranial facial development, Piotrowski *et al.* transplanted wildtype cells into *van gogh* mutants and examined pharyngeal cartilage development. The rescue of cartilage development in genetically mutant tissues suggested that craniofacial defects are cell nonautonomous, and the authors suggest *van gogh* mutants have defective signaling from the endoderm to the developing branchial arches. Also, they noted that wildtype cells integrated into the semicircular canal of the ear directly rescuing the ear defect and suggesting that *tbx1* acts cell autonomously in ear development (57). Therefore, using chimeric zebrafish assays, a better understanding of the potential and diverse developmental mechanisms of DiGeorge syndrome were discovered.

Another cell transplant technique applied to zebrafish is xenotransplantation of tumor cells into embryonic or juvenile fish (60–63). Mammalian tumor cells can survive, divide, metastasize, and induce angiogenesis in the zebrafish embryo before the immune system develops or in immunosuppressed juvenile fish. This technique can be combined with transgenic cell labeling to visualize the interaction of tumor cells with their microenvironment *in vivo* (60,63). In addition, mutagenic or chemical screens can be devised to identify genes or chemicals that modify tumor metastasis or angiogenesis. Recently, a transparent adult line of zebrafish, termed *casper*, was described (64). This line is double homozygous for the mutations *nacre* and *roy* resulting in a loss of melanocytes and iridophores and an adult fish with visible internal organs. These fish can be applied to tumor transplant studies of growth, metastasis, and angiogenesis with high resolution, *in vivo*, imaging techniques (64). The visibility of internal organs in an adult organism will also make this fish a valuable tool for the general study of physiologic processes under normal and pathologic conditions.

Testing Chemical Compounds in Zebrafish Embryos

The zebrafish embryo represents a convenient model for studying interactions between chemical compounds and animal physiology. The value of the zebrafish model is derived from its small size, chemical permeability, ease of observation, and physiologic similarity to other vertebrates including humans (65). The small size of zebrafish embryos allows them to be arrayed in 96- or 384-well plates. Chemical compounds can then be added to the wells and experimental end points measured after the desired incubation period. This process can be mechanized so that large libraries of compounds can be screened efficiently. The aqueous environment of zebrafish is advantageous, because chemical compounds can be directly added to the fish's water and freely diffuse into the developing embryo. Observation of morphologic changes is simple in zebrafish embryos, as described previously, and the use of transgenic zebrafish can significantly enhance the ability to view changes in specific organs or tissues. Some researchers have even developed sentinel transgenic zebrafish which induce fluorescent proteins when exposed to toxic stressors (66). Although there are metabolic and physiologic differences between fish and mammals, the similarities in terms of response to chemical treatment appear to be largely conserved. A survey of drugs known to prolong the cardiac QT interval, the time interval between ventricular depolarization and repolarization, in humans did the same in zebrafish in 22 of 23 cases (67). Langheinrich (68) summarized many chemicals involved in diverse processes such as angiogenesis, hemostasis, apoptosis, lipid metabolism, and inflammation which have

similar effects on zebrafish and mammals. All these advantages make zebrafish an excellent vertebrate model system in which to examine environmental toxins or identify new candidate drugs.

An interesting application of this chemical screen method was performed by Chiu *et al.* (69). The lateral line system of the zebrafish is an external extension of the hearing and balance organs with hair cells analogous to those found in the inner ear present on the skin. It is known that a possible side effect of some drugs, especially some antibiotics, is ototoxicity and hearing loss. Because no systematic study of Food and Drug Administration approved drugs on ototoxicity has been performed, Chiu *et al*. used the power of the zebrafish embryo to screen over 1000 compounds. They were aided in this screen by the use of a fluorescent vital dye for hair cells. As a proof a principle, seven compounds known to be toxic in mammals were tested and confirmed to be toxic to the lateral line. Importantly, 14 previously unreported drugs were found to destroy hair cells in zebrafish embryos. These drugs might cause a previously unappreciated side effect and should be further examined in mammalian models and human patients.

Our laboratory has performed a screen for chemicals which effect embryonic angiogenesis (70). Using the *flk:EGFP* transgenic line with fluorescent vasculature, several chemicals with antiangiogenic activity were discovered. One compound, mycophenolic acid (MPA), strongly inhibited vessel growth in the trunk (Fig. 2). This drug is used as an antirejection drug, in patients who have undergone organ transplants, because of its antiproliferative effect on B and T cells. The target of this drug is inosine monophosphate dehydrogenase (IMPDH) of which zebrafish has three genes. Morpholino knockdown of one of these genes, *IMPDH2*, phenocopied the effect of MPA administration suggesting the antiangiogenic effect of MPA is mediated through inhibition of IMPDH2 activity (Fig. 2). This discovery implies that MPA or its derivatives may be a useful drug for blocking angiogenesis in diseases such as cancer or macular degeneration.

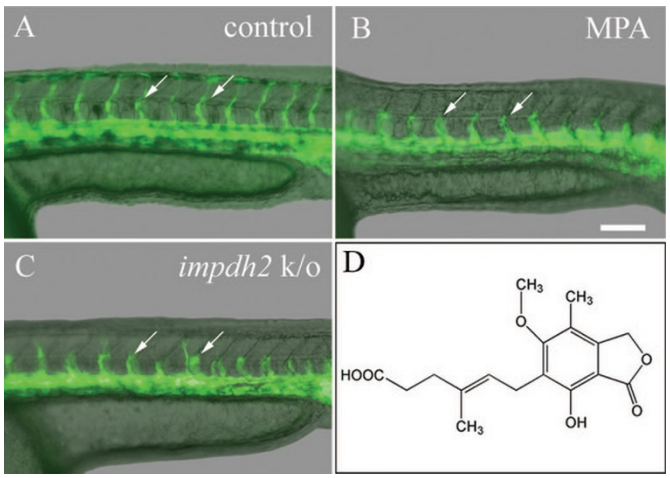

Figure 2. *Flk:GFP* transgenic fish with green fluorescent blood vessels was used to screen a chemical library for antiagiogenic activity. *A*, Control embryos exhibit uniform GFP expression in intersomitic vessels identified with *white arrows*. *B*, Mycophenolic acid (MPA) inhibits angiogenic sprouting in the zebrafish trunk. Notice the truncated intersomitic vessels. *C*, Morpholino mediated knockdown of *impdh2*, the likely target of MPA, results in a similar deficit in angiogenesis to MPA treated embryos. Compare intersomitic vessels in *B* and *C*. *D*, The chemical structure of MPA. Scale bar in $B = 100\ \mu$m. Adapted from Arterioscler Thromb Vasc Biol 26:2414–2416, Copyright © 2006 American Heart Association, Inc., with permission.

An exciting discovery made using zebrafish chemical screen methodology was the role of prostaglandin E2 in hematopoetic stem cell (HSC) homeostasis (71). Definitive HSCs arise from the dorsal aorta of zebrafish embryos around 36 h after fertilization and are defined by the expression of *runx1* and *cmyb*. Using ISH for these genes to screen 2357 compounds, 82 were found to effect the distribution of HSCs. Ten of these compounds altered the prostaglandin pathway. A combination of chemical, morpholino, and transgenic technologies were used to demonstrate the function of the prostaglandin E2 pathway in HSC homeostasis in zebrafish. Further, these findings were confirmed *in vitro* and *in vivo* in mice. If human HSC behave in a similar way to prostaglandin signaling, this discovery has important implications for bone marrow transplantation and future stem cell technologies. This example nicely demonstrates how application of zebrafish chemical screen can lead to basic discoveries in animal biology with clinical applications.

SUMMARY

The zebrafish model has significantly improved our ability to study vertebrate developmental biology. The strengths of this model system lie in its external, visually accessible development, ease of experimental manipulation, and common genetic underpinnings with other vertebrates including humans. The powers of the experimental techniques described above are significant by themselves. However, when applied in combination the scientific value of these methods is exponentially increased. The ability to identify a mutant phenotype, map the mutated gene, study its function through protein overexpression, protein knockdown, chimeric embryo studies, and microarray analysis, and then to screen for chemical and genetic modifiers of the phenotype on a large scale at reasonable costs is an attractive scientific proposition. When taking into account the biomedical implications of these tools for discoveries of clinical value, the zebrafish model looks to have a very bright future.

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