

Fishing for Drugs? Zebrafish in Drug Discovery

a report by

Michael Schebesta

Post-doctoral Fellow, Developmental and Molecular Pathways Department, Novartis Institutes for Biomedical Research, Cambridge, Massachusetts

The Birth of a New Model Organism

As early as the 1930s, the development of the small teleost fish species *Danio rerio*, commonly known as zebrafish, was studied. However, it was not until five decades later that George Streisinger, a scientist at the University of Oregon, pioneered the use of zebrafish as a vertebrate model organism. His team studied the early development of zebrafish in detail and laid the foundation for introducing random mutations into the genome of this species. In 1996, the results of two large-scale chemical mutagenesis screens were published: the Boston screen and the Tuebingen screen. These forward genetic screens generated vast collections of mutant lines, providing numerous insights into early embryonic development, organogenesis, neural development and behaviour. More recently, in 2000, the first small-molecule screen in zebrafish was published.¹ Stuart Schreiber and his group treated embryos in a 96-well format with 1,100 samples from a small-molecule library. They identified compounds specifically affecting development of the central nervous system (CNS), the cardiovascular system, the neural crest and the ear.

Why Fish?

Zebrafish are small in size (up to 3cm) and their embryonic development is rapid. Their body plan is laid out within 24 hours after fertilisation (see *Figure 1*). The optical clarity of the embryo enables *in vivo* observation of organogenesis, in particular combined with expression of fluorescent proteins in transgenic animals. External fertilisation and development together with high fecundity allow for the collection of several hundred embryos per week and breeding pair, while the cost of maintenance for fish is relatively low (less than 1% of the cost of mice). In addition to the low cost and ability to maintain large numbers of animals in a small space, multiple genetic and molecular tools have been established over the last decade. Chemical and retroviral insertion mutagenesis provide the basis for forward genetic screens.^{2,3} Morpholino-modified oligonucleotides (morpholinos) enable specific and efficient gene knock-down during the first days of embryonic development.⁴ Tilling technology allows the isolation of stable fish lines with mutations in genes of interest.⁵ Microarrays have been well established, high-frequency generation of transgenic animals is performed routinely^{6,7} and the zebrafish genome sequence is nearly complete.



Michael Schebesta is a Post-doctoral Fellow in the Developmental and Molecular Pathways Department at Novartis Institutes for Biomedical Research (NIBR), where he is developing disease models and genetic screens in zebrafish. Before joining Novartis, he received post-doctoral training at Harvard Medical School, where he investigated tissue regeneration in zebrafish. He obtained his doctorate from the Institute of Molecular Pathology (IMP)—University of Vienna, where he studied early B-cell development in the mouse immune system.

Examples of Highly Conserved Biological Systems

As a vertebrate, the zebrafish body plan shares many similarities with mammals, e.g. the cardiovascular system consists of a two-chambered heart and a vascular system containing arteries and veins. In humans, interference with the human ether-a-go-go (hERG)-related ion channel causes arrhythmias, and new molecular entities are routinely screened for potential interaction with hERG.⁹ Cloning of the zebrafish orthologue of hERG has demonstrated its high conservation to the human protein (99% identity in the pore domain).¹⁰ In a 2003 study, 23 drugs known to cause arrhythmias in humans were tested for their effect on zebrafish heart rhythm.¹¹ Eighteen of those drugs caused similar arrhythmias after submerging the animals in drug-containing solution. Four of the remaining five drugs were effective after injection into the fish, demonstrating that 95% of the tested arrhythmia-causing drugs showed efficacy in zebrafish.

Another striking example of conservation was the finding that 'golden', a zebrafish strain with reduced black pigmentation, carries a mutation in the putative cation exchanger solute carrier family 24, member 5 (slc24a5).¹² Polymorphisms in the human orthologue of slc24a5 are also strongly correlated with skin colour. Therefore, although the composition of fish and mammalian skin is different, similar molecular and cellular mechanisms are responsible for skin pigmentation in fish and humans.

As a final example, a study in 2007 investigated the effects of known neurotoxins for zebrafish.¹³ They observed that all tested compounds demonstrated similar effects in fish as shown in humans (e.g. 6-hydroxydopamine (6-OHDA) caused dopaminergic neuron loss and acrylamide induced de-myelination). Furthermore, 11 out of 14 known mammalian neuroprotectants showed efficacy against oxidation-induced neurotoxicity.

Fishing for Drugs?

The identification of new drugs can be divided into two approaches: organism-based and target-based discovery. Historically, the former process, in which a whole organism is treated with a compound, has been the prevailing method of research. For example, in 1775 William Withering observed the beneficial effects of foxglove on a patient suffering from heart failure, and years later isolated digitalis, which is still an important therapy for heart failure.¹⁴ More recently, target-based discovery has become the method of choice in drug discovery. With this approach, the molecular causes of a disease are identified first and compounds that specifically interfere with these causes are then isolated using high-throughput screening (HTS). The zebrafish model may prove useful for both approaches. Forward genetic screens have generated numerous insights into the molecular and cellular

Figure 1: Zebrafish Embryos



Top left: a zebrafish embryo three hours after fertilisation; top right: three-day-old embryos in a single well of a 96-well plate; bottom: a 24-hour-old embryo.

mechanisms of normal and pathological development, thereby supplying potential new genes and pathways for target-based discovery. On the other hand, recent small-molecule screens in zebrafish embryos are prime examples of organism-based drug discovery.

Forward Genetic Screens to Locate Potential Drug Targets

Based on the seminal work from the Boston and Tuebingen screens, a wide array of screens have been performed. Forward genetic screens provide an unbiased approach to identify genes critically involved in a biological process of choice. In contrast to cell-culture-based screens (e.g. RNA interference (RNAi) screens), whole-organism-based screens allow the study of highly complex biological processes in the context of a living animal. For example, to observe lipid processing in the digestive tract, zebrafish larvae were fed with fluorescently labelled lipids and the accumulation of the tracer in the gall bladder was observed. In a forward genetic screen, mutants were identified in which lipid processing is absent even though morphologically the digestive system looks normal.¹⁵ In the Boston and Tuebingen screens, mutants with anatomical and/or functional defects in the heart were characterised.¹⁶⁻¹⁸ To gain insight into host-pathogen interactions, assays have been established to observe *Mycobacterium marinum* or *Salmonella typhimurium* infections in zebrafish larvae.^{19,20} Currently, screens are under way to identify host factors modulating this host-pathogen relationship. Another highly complex field is the CNS and its functions. To find genes modifying chemical processes in the brain, a screen for resistance to chemically induced seizures was performed in larvae, identifying six recessive mutants.²¹ Also, complex behavioural patterns can be studied in zebrafish, as shown by screens for circadian rhythm mutants²² and screens for modulators of cocaine-induced addiction.²³

Developing the right aquatics system is more Picasso than paint-by-numbers.



Great engineering is an art.

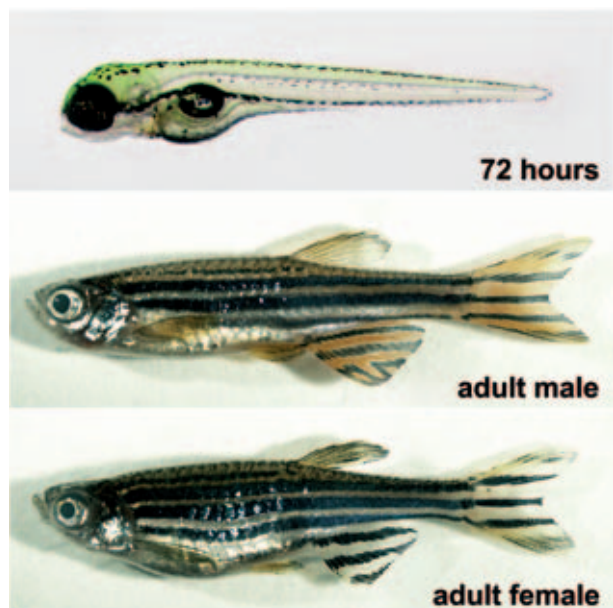
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Figure 2: Developed Zebrafish



From top to bottom: a three-day-old embryo; adult male; adult female.

Small-molecule Screens in Zebrafish

In addition to providing better understanding of diseases and target discovery through forward genetic screens, zebrafish are also an excellent model for organism-based discovery. They are particularly well suited for compound screening due to their small body size and aquatic habitat. Embryos can be conveniently raised for several days in 96-well or even 384-well plates, drastically reducing the amount of compound necessary for screening (see *Figure 1*, top right). In addition, unlike invertebrates, many chemicals are readily taken up through the skin by larvae, which also tolerate dimethylsulfoxide (DMSO) concentrations of up to 1% in the water. Peterson et al.¹

performed the first small-molecule screen, looking for compounds that cause developmental defects. Approximately 2% of the 1,100 tested compounds were toxic for the embryos, but about 1% of the compounds demonstrated specific effects on the CNS, the cardiovascular system, the neural crest or the ear. In a similar screen, a compound (concentramide) was pin-pointed causing a defect in heart formation similar to a genetic mutant, known as heart-and-soul, identified in a forward genetic screen.²⁴ Recent small-molecule screens include screening for cell-cycle inhibitors during early development,²⁵ screening for compounds interfering with larval fin regeneration²⁶ and screening for compounds that influence haematopoietic stem cell numbers.²⁷ In addition to screens looking for compounds interfering with normal development, suppressor screens have been carried out to rescue a phenotype caused by a defined genetic mutation. In gridlock mutants, vascular development is abnormal, resembling aortic coarctation in humans.²⁸ Screening a library of 5,000 small molecules, two structurally similar compounds were identified to suppress the gridlock phenotype.²⁹ The second example of a chemical-suppressor screen was the search for compounds rescuing genomic instability and mitotic arrest in crash-and-burn mutants carrying a mutation in *bmyb*.³⁰ Sixteen thousand compounds were screened identifying persynthamide and two previously known DNA synthesis inhibitors as potent suppressors of crash-and-burn.

The Next Step

Zebrafish has become a common vertebrate model offering numerous new understandings of normal and pathological development. Despite its popularity for studying embryonic development, less emphasis has been placed on establishing disease models in general, and models in adult fish in particular. Adult fish models will become essential in particular for common diseases, such as cardiovascular disease, type 2 diabetes and neurodegenerative diseases. The years to come will determine whether current approaches and new strategies using the zebrafish model will contribute valuable new insights to the development of new therapies. ■

- Peterson RT, Link BA, Dowling JE, Schreiber SL, Small molecule developmental screens reveal the logic and timing of vertebrate development, *Proc Natl Acad Sci U S A*, 2000;97:12965–9.
- Golling G, Amsterdam A, Sun Z, et al., Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development, *Nat Genet*, 2002;31:135–40.
- Patton EE, Zon LI, The art and design of genetic screens: zebrafish, *Nat Rev Genet*, 2001;2:956–66.
- Nasevicius A, Ekker SC, Effective targeted gene 'knockdown' in zebrafish, *Nat Genet*, 2000;26:216–20.
- Wienholds E, Schulte-Merker S, Walderich B, Plasterk RH, Target-selected inactivation of the zebrafish *rag1* gene, *Science*, 2002;297:99–102.
- Grabher C, Joly JS, Wittbrodt J, Highly efficient zebrafish transgenesis mediated by the meganuclease I-SceI, *Methods Cell Biol*, 2004;77:381–401.
- Kawakami K, Takeda H, Kawakami N, et al., A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish, *Dev Cell*, 2004;7:133–44.
- Parinov S, Kondrichin I, Korzh V, Emelyanov A, Tol2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes *in vivo*, *Dev Dyn*, 2004;231:449–59.
- Sanguinetti MC, Tristani-Firouzi M, hERG potassium channels and cardiac arrhythmia, *Nature*, 2006;440:463–9.
- Langheinrich U, Vacun G, Wagner T, Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia, *Toxicol Appl Pharmacol*, 2003;193(3):370–82.
- Milan DJ, Peterson TA, Ruskin JN, et al., Drugs that induce repolarization abnormalities cause bradycardia in zebrafish, *Circulation*, 2003;107:1355–8.
- Lamason RL, Mohideen MA, Mest JR, et al., SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans, *Science*, 2005;310:1782–6.
- Parrng C, Roy NM, Ton C, et al., Neurotoxicity assessment using zebrafish, *J Pharmacol Toxicol Methods*, 2007;55:103–12.
- Hauptman PJ, Kelly RA, Digitalis, *Circulation*, 1999;99: 1265–70.
- Farber SA, Pack M, Ho SY, et al., Genetic analysis of digestive physiology using fluorescent phospholipid reporters, *Science*, 2001;292:1385–8.
- Chen JN, Haffter P, Odenthal J, et al., Mutations affecting the cardiovascular system and other internal organs in zebrafish, *Development*, 1996;123:293–302.
- Haffter P, Granato M, Brand M, et al., The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*, *Development*, 1996;123:1–36.
- Stainier DY, Fouquet B, Chen JN, et al., Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo, *Development*, 1996;123:285–92.
- Davis JM, Clay H, Lewis JL, et al., Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos, *Immunity*, 2002;17: 693–702.
- van der Sar AM, Musters RJ, van Eeden FJ, Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections, *Cell Microbiol*, 2003;5:601–11.
- Baraban SC, Dinday MT, Castro PA, et al., A large-scale mutagenesis screen to identify seizure-resistant zebrafish, *Epilepsia*, 2007;48:1151–7.
- DeBruyne J, Hurd MW, Gutierrez L, et al., Isolation and phenogenetics of a novel circadian rhythm mutant in zebrafish, *J Neurogenet*, 2004;18:403–28.
- Darland T, Dowling JE, Behavioral screening for cocaine sensitivity in mutagenized zebrafish, *Proc Natl Acad Sci U S A*, 2001;98:11691–6.
- Peterson RT, Mably JD, Chen JN, Fishman MC, Convergence of distinct pathways to heart patterning revealed by the small molecule concentramide and the mutation heart-and-soul, *Curr Biol*, 2001;11:1481–91.
- Murphey RD, Stern HM, Straub CT, Zon LI, A chemical genetic screen for cell cycle inhibitors in zebrafish embryos, *Chem Biol Drug Des*, 2006;68:213–19.
- Mathew LK, Sengupta S, Kawakami A, et al., Unraveling tissue regeneration pathways using chemical genetics, *J Biol Chem*, 2007; epub ahead of print.
- North TE, Goessling W, Walkley CR, et al., Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis, *Nature*, 2007;447:1007–11.
- Weinstein BM, Stemple DL, Driever W, Fishman MC, Gridlock, a localized heritable vascular patterning defect in the zebrafish, *Nat Med*, 1995;1:1143–7.
- Peterson RT, Shaw SY, Peterson TA, et al., Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation, *Nat Biotechnol*, 2004;22:595–9.
- Shepard JL, Amatrudda JF, Stern HM, A zebrafish *bmyb* mutation causes genome instability and increased cancer susceptibility, *Proc Natl Acad Sci U S A*, 2005;102:13194–9.