The zebrafish: a new model organism for integrative physiology

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Briggs, Josephine P. The zebrafish: a new model organism for integrative physiology. Am J Physiol Regulatory Integrative Comp Physiol 282: R3–R9, 2002; 10.1152/ajpregu.00589.2001.—This brief review summarizes features of the zebrafish, Danio rerio, that make it a suitable model organism for studies of regulatory physiology. The review presents the argument that random mutagenesis screens are a valuable gene-finding strategy to identify genes of functional importance and that their utility, although well established for developmental issues, will extend to a variety of topics of interest to the regulatory physiologist. Particular attention is drawn to the range of functional responses amenable to mutagenesis screens in larval zebrafish. Other virtues of the organism, the range of genomic tools, the potential for innovative optical methods, and the tractability for genetic and other experimental manipulations, are also described. Finally, the review provides examples of functional studies in zebrafish, including studies in sensory neurons, cardiac rhythm disturbances, gastrointestinal function, and studies of the developing kidney, that illustrate potential applications. Because of the relative ease with which combinatorial studies can be performed, the zebrafish may eventually be particularly valuable in understanding the functional interaction between subtle gene defects that cause polygenic disorders.

Danio rerio; genomics

BEGINNING PHYSICIANS are frequently warned, “if you hear hoof beats, don’t think of zebras.” This reminder that common symptoms are usually caused by common diseases is generally good advice, but modern genomic biology is teaching us a different lesson. It is teaching us not to look just to the obvious, because the answers to biological questions often do not come from the expected direction, at least not from the expected species. Answers to questions such as how the horse runs, how we hear the hoof beats, may come not from studies of either the horse or the human or even the zebra, but rather from an unexpected species, perhaps even one without legs—perhaps a yeast, perhaps a fruit fly, perhaps a slime mold, perhaps a zebrafish.

The argument of this brief review is that the zebrafish (Danio rerio) is a model organism with great promise for study of physiological function and that its promise is not limited to “fishy” topics. The zebrafish has a vigorous presence in developmental biology where its scientific utility is well recognized. Increasingly, it is recognized as a useful model for study of human congenital and genetic diseases (9, 10, 18). However, the value of this model organism has been less apparent to investigators who focus on whole organ or whole organism function. I will argue it has substantial potential for the kind of complex integrative questions of interest to a regulatory or organ physiologist.

BACKGROUND

The zebrafish is a small freshwater fish, a cyprinoid teleost, that originated in rivers in India and is common as an aquarium fish throughout the world. They are easy to maintain in aquariums, almost as easy as guppies. These striped fish can be found as graceful swimmers in virtually every pet store. [The zebrafish used for the first classic screen actually originated in a
Laboratory methods for its husbandry are well established (51).

Its emergence as a model organism for modern biological investigation began with the pioneering work of George Streisinger and colleagues (47), who recognized many of the virtues of the experimental system. These include its short generation time, the large numbers of eggs produced by each mating, and the fact that, because fertilization is external, all stages of development are accessible. Streisinger developed methods to produce homozygous strains by using genetically inactivated sperm (47), performed the first mutagenesis studies, and established that complementation methods, in which heterozygous mutant fish are paired, could be used to assign mutations to genetic complementation groups (16, 20, 21). The Streisinger lab was the original core of what has become a strong scientific interest group in Oregon. The Oregon group, together with a world-wide community of investigators, has characterized the development of the zebrafish in substantial detail. Much information is available electronically (http://zfin.org/) (45) and is summarized in a number of excellent reference publications (7, 8, 23, 51, 53).

Large scale mutational screens were first undertaken by two groups, one in Tuebingen, under the leadership of Christiane Nusslein-Volhard (24), and a second at Massachusetts General Hospital led by Wolfgang Driever and Mark Fishman (11, 46). Those screens focused on early developmental events and led to the identification of ~1,800 mutants. These screens, and the hundreds of interesting mutants they produced, were the subject of a special issue of Development (December 1996; Ref. 24) and were aptly described in Science as “an accomplishment of historic proportions” (19). The issue of Development catalyzed much wider awareness of the potential of the model, which led to greater investment in the development of genetic and genomic tools for the zebrafish. Most recently, recognition of the potential of the model led to the commitment by the Wellcome Trust and the Sanger Institute to sequence the entire genome. In this review, I will expound on several major virtues of this model organism that recommend it for physiological study. First and foremost is the potential of random mutagenesis screens (11, 24) and possibly also chemical screens (41) to identify functionally important genes and gene pathways. The zebrafish is also attractive as a model because of its transparency, its tractability for a variety of experimental manipulations, and the range of genetic and genomic tools available to study it. I will provide a few examples of work in the zebrafish that illustrate the potential for physiological study of organ function. Finally, I will conclude with a series of speculations about potential areas of future scientific application.

MUTAGENESIS SCREENS

Mutations, spontaneous or induced, targeted or random, are central to determination of gene function. In the mouse, the power of homologous recombinant mutation induction is clear. Gene knockouts yield frequent surprises. Sometimes an organ system or function changes unexpectedly. Sometimes inactivation of a gene product previously considered critical has no obvious biological consequences. In the first round of knockout studies, mice were largely examined for dramatic anatomic or pathological defects. Increasingly, however, physiologists have been recruited to study these animals; with more subtle and probing physiological methods, interesting functional effects are being detected, often in animals with no obvious morphological defects.

Knockout methods using homologous recombination are not yet possible in the zebrafish, but random mutagenesis methods are well established and have been used for more than a decade (20–22). In contrast to targeted mutagenesis, which focuses on known genes, random mutagenesis looks for unknown genes. Random mutagenesis screens are increasingly recognized as a good large-scale strategy to find functionally important genes.

The first large zebrafish mutagenesis screens were the conceptual outgrowth of the Drosophila work of Nusslein-Volhard and colleague (39); her Nobel prize-winning studies used saturation mutagenesis of the fruit fly genome to identify genes important for early development. The first screens in the zebrafish followed the fruit fly model (24). The method was as follows. Step 1: male fish were treated with ethylnitrosourea (ENU), a chemical mutagen that produces multiple small defects; the dose and administration schedule produced mutations, usually point mutations, at a rate of about one or two per haploid genome. The first generation offspring of this mutagenized male became the founder, F1 males. Step 2: a founder male was mated with a wild-type female, and the offspring producing the F2 “family” were expected to include both normal fish and fish heterozygous for the induced mutations. Step 3: brother-sister matings, about 15 pairs per family, were set up, and the offspring of these pairings, the F3 generation, were screened. One-fourth of these matings were predicted to yield homozygous offspring (24).

As powerful as the early screens were, it has been apparent that the approaches used missed many genes of interest. The screens looked only at early developmental stages. Although some functional screening was included, the investigators primarily concentrated on mutants that produced morphological rather than functional defects. The vast majority of mutants were recessive in phenotype. Interestingly, very few mutations detected in the initial screens were compatible with viability to adulthood. For example, from the Tuebingen screen only 79 of 4,264 were viable as homozygotes; an additional 19 are described as semi-viable (24).

The Tuebingen group is currently undertaking a second-generation large-scale mutagenesis screen, the Tuebingen 2000 screen, together with Artemis Pharmaceuticals (43). National Institutes of Health-spon-
sored investigators are also undertaking a series of smaller screens in individual laboratories using a variety of strategies for phenotypic characterization.

Advances in insertional mutagenesis are another important new development (1, 47). This strategy uses a vector that inserts a sequence tag into the genome at random sites, disrupting gene function. The vector sequence can later be used as a PCR tag to pull out and potentially identify the mutated gene, an approach that dramatically decreases the work and time needed to determine the site of the mutation. Initial attempts with this type of strategy reported a mutation efficiency that was too low to be useful, several logs lower than that with chemical mutagens (24), but recent data suggest that with newer approaches the mutation rate, although lower than with ENU, is still high enough to be very effective (47).

The future power of mutagenesis approaches will depend in part on the imagination of investigators in developing new methods for phenotypic screening. One approach being used to clarify gene pathways important in development is to screen embryos for changes in expression of a potential target gene, using either whole mount in situ hybridization or transgenic lines expressing green fluorescent protein driven by a specific promoter (2, 33, 43).

There are numerous functional screens possible as well. At ∼48 h postfertilization, the zebrafish embryo hatches and becomes a free-swimming larva. The larva shows a wide range of behaviors: it swims, swallows, shows escape responses (32), is touch sensitive (42), and, by day 5, it appears able to hear hoof beats—or at least show startle responses to acoustic stimuli (29). Its regulatory capacity, nutrient requirements, responses to oxidant, osmolar, or thermal stress, to name a few examples, are incompletely defined. In adult fish, an even wider array of regulatory and behavioral functions, environmental responses, nutritional and metabolic effects, reproductive function, could possibly prove susceptible to development of high-through-put screening methods, with the potential of detecting more subtle gene defects. A mainstay of studies in yeast, the identification of gene defects that are conditionally lethal under selected environmental circum-
stances, is another approach that has not been explored in the zebrafish. Characterization of regulatory functions in both the larval and adult fish could provide the basis for eventual mutagenesis screens to look for gene defects that produce either enhanced or reduced regulatory function.

It is worth noting that mutagenesis screens have also been undertaken in the mouse. A mutagenesis screen is a major undertaking; it requires large numbers of animals, demanding animal husbandry, meticulous record keeping, and a dedicated screening team. However, as daunting as the requirements are in zebrafish, they pale compared with the needs and costs to carry out similar projects in the mouse.

**GENOMIC RESOURCES**

A second virtue of the zebrafish is the availability of a large and growing array of molecular information and reagents. Some of the major publicly maintained, web-based data sites are listed in Table 1. In addition, a number of individual labs maintain valuable web sites that facilitate access to additional information.

The most powerful genomic resource will unquestionably be the availability of the sequence of the complete genome, expected within a year or two. The genome is estimated to contain 1.7 gigabases, making it about one-half the size of the human genome. A draft sequence is expected to be available in late 2002 or early 2003. As sequencing proceeds, data are being made publicly available in weekly releases. At the time of writing of this review, data from ∼4 million sequencing reads, which corresponds to close to 1.7 gigabases (1× coverage) have been released into publicly searchable National Center for Biotechnology Information (NCBI) and Ensembl-EBI databases (for links, see Table 1). Sequence for 117,276 expressed sequence tags (as of dbEST release 092101) is also available through GenBank or dbEST. In addition, NCBI is providing curated and annotated information about zebrafish genes through the inclusion of the zebrafish in LocusLink, Unigene, and HomoloGene (see www.ncbi.nlm.nih.gov/genome/guide/D_rerio.html). These data are already facilitating identification of homologs to

### Table 1. Public web-based resources for zebrafish genomics

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gene products known in other species and the identification of candidate genes within regions of interest in the genome.

A variety of mapping tools exists to facilitate the positional cloning of gene defects, including high-density genetic maps (i.e., Ref. 44) and radiation hybrid panel maps (17, 26, 31). The NCBI Map Viewer presents a convenient graphical integrated view of the zebrafish radiation hybrid maps and the principal genetic maps. Access to the individual maps is available through web-based resources linked through either Zebrafish Information Network (ZFIN) or the NCBI catalog of zebrafish resources.

TRANSPARENCY

The third virtue of this organism is the extraordinary range of optical methods possible in the developing embryo and larval fish. There is a substantial degree of transparency through about 5 days. Individual living cells and even cellular organelles can be observed directly with no staining and little preparation of the fish and no tools other than a good microscope. With the use of an optical sectioning technique, usually differential interference contrast or Nomarski, it is possible to visualize many structures, even subcellular organelles, with exceptional clarity. For example, in studies discussed below, direct patch clamping of sensory neurons was performed in a virtually intact preparation of 2-day-old embryos (42).

With a little more work, it is possible to visualize patterns of gene expression. Whole-mount in situ methods produce excellent images of the entire embryo through about 3 days, and, after this point, gene expression can still be well delineated in more superficial structures, although deep structures, such as the developing pronephros, are best characterized in sectioned material (13). The visualization of expression patterns of green fluorescent protein, under control of lineage or cell-specific promoters, promises to have great value, both to track the gene product of interest and to identify and potentially isolate specific cell populations (2, 33).

The reach of the eye can be dramatically extended with fluorescent probes. Fluorescent markers have been very effectively applied in zebrafish studies to classic developmental issues such as tracking cell lineage (28, 30). Particularly visually arresting images have come from studies that use fluorescent probes to trace the development of neural pathways (4, 52) or vascular development in developing larvae (48). However, the use of fluorescent probes to address the issues more typically studied by physiologists, such as assessing organ and metabolic function or monitoring intracellular ions concentrations, has received much less attention.

A recent study by Farber and coworkers (15), described below, using fluorescent lipid reporter molecules elegantly illustrates this potential.

EXPERIMENTAL TRACTABILITY

Another virtue of the zebrafish is the array of genetic and experimental manipulations possible. Current established experimental methods, which include methods of obtaining haploid and homozygous fish, techniques for primary cell cultures, and techniques for transient and stable transgenesis, are well described in several excellent reference texts (7, 8, 23, 51). Since publication of these texts, there have been some significant breakthroughs in the development of methods for manipulation of gene expression.

Ekker and colleagues (14, 38) recently demonstrated downregulation of gene expression for at least 4 days after injection into the developing blastula of antisense oligonucleotide constructs modified by addition of a morpholino group. The method was validated by showing that morpholinos for several known gene products produced the phenotype expected from mutation studies (14). Another new innovative approach involves using photomediated activation of caged mRNA molecules (3), permitting temporal control of gene inactivation.

Chemical screening techniques also show great unexplored potential. Another recent report describes an approach to zebrafish studies that illustrates the potential of being able to monitor an array of complex biological events on large numbers of small animals. In these studies, panels of small molecules were screened for developmental effects using fish embryos arrayed in 96-well plates (41). In each experiment, several hundred molecules could be screened for specific developmental effects, with the fish monitored at frequent intervals for specific anatomic alterations. The investigators identified several small molecules that altered specific aspects of zebrafish ontogeny without producing toxic or nonspecific effects. In one case, a molecule that altered otolith formation, the approach allowed identification of critical intervals for differentiation events. The investigators propose that this technique, similar to mutagenesis methods, may be useful to find genes; the protein targets of the small molecules would be identified through protein binding or other affinity methods. The power of chemical screening methods in the zebrafish is just beginning to be explored, but clearly this approach may help elicit function of a variety of target molecules, lipids, and sugars, as well as proteins and nucleic acids.

Targeted gene inactivation or knockout strategies are still not established for zebrafish and have acquired somewhat a “holy grail” status. Pluripotent embryonic stem cell lines and fish feeder lines that support their maintenance in a dedifferentiated state exist from a closely related species, the medaka (25). Recently, investigators demonstrated that zebrafish cells obtained from short-term cell cultures from gastrula embryos could result in germ line transmission when introduced into host embryos (34). Their demonstration of germ line transmission relied on a strategy similar to that used to produce the agouti mouse: the donor gastrula came from a pigmentation defective line.
and the host embryo from a normally pigmented fish. A number of additional steps are necessary to develop gene knockout methodology; most critical will be the need to determine whether it is possible to maintain gastrula cells in a undifferentiated state long enough to select for homologous recombinants. Nonetheless, the recent report may prove to be an important breakthrough.

A FEW APPLICATIONS

Function of sensory neurons. Three mutant lines, macho, alligator, and steifftier, have been identified in which the withdrawal response to touch was reduced or absent. Ribera and Nusslein-Volhard (42) used whole cell recordings from mechanosensory Rohan Beard neurons in semi-intact preparations of 2- to 4-day-old embryos to characterize electrical membrane properties in these mutant lines and in normal wild-type embryos (42). The studies correlated the development of excitability in these cells with the development of the behavioral response. In the touch-insensitive mutant fish, the sodium current amplitudes were reduced and action potentials either had diminished overshoots or were not generated. In macho mutants, the action potential showed no overshoot and the sodium current remained small; alligator and steifftier showed similar but weaker effects. The effects were specific to sodium channel function, and resting membrane potentials were unaffected. In wild-type fish, sodium current expression in mechanosensory neurons developed during the transition from a touch-insensitive to a touch-sensitive embryo. However, in macho, developmental changes in action potential overshoot and sodium current were absent although action potential duration was normal. The investigators concluded that the maturation of voltage-dependent sodium current was critical for the behavioral response to touch. The gene defects in these three mutants are still unknown, but once characterized are likely to contribute to understanding of molecular interactions that regulate membrane expression of sodium channels.

The zebrafish heart. The beating heart is one of the most prominent features of the developing fish embryo. Mutations that result in alterations in the formation of the cardiac chambers were among the first to attract attention of investigators (10), and study of these mutations is contributing to identification of sets of genes essential for formation of the key structural elements (6). Elegant hemodynamic studies have been performed in larval fish (see references in Ref. 48). Perhaps more interesting to the physiologist, however, may be study of other mutations that do not result in abnormal heart formation but rather influence either contractility or heart rhythm. One such mutation is called slow mo. This mutation was identified because it was seen to result in a slowed heart rate in the embryo; in patch clamp studies of cardiac cells taken from mutant embryos, the inwardly directed hyperpolarizing pacemaker current ($I_h$) was found to be reduced (5). Recent studies report that this mutation is compatible with survival to adulthood, but bradycardia persists; in wild-type fish, $I_h$ expression is present in atrial adult cardiomyocytes but current density is reduced in ventricular cells, whereas in slow mo, low-current density is observed in both chambers (50). The investigators conclude that the pacemaker current continues to be an important determinant of heart rate even after neural and humoral influences are established in the adult.

The zebrafish gastrointestinal tract. Farber and coworkers (15) recently reported studies using lipid reporter molecules that are substrates for phospholipase A2 (PLA2). One such reporter, PED6 (15) is fluorescently labeled with a BODIPY conjugate that is covalently linked adjacent to the PLA2 cleavage site, with the consequence that PLA2 cleavage modifies the emission frequency—orange (568 nm) emission in the unmodified state, green (515 nm) emission after PLA2 cleavage. When this molecule is swallowed by 5-day-old larvae, it initially labels the pharynx orange; the metabolized green product is absorbed into the hepatobiliary system, then secreted into the gall bladder, and shortly thereafter it produces green intestinal luminal fluorescence. The probe could potentially be used to screen for a variety of defects in gastrointestinal function. Farber and coworkers report its use to identify defects in the initial step of lipid processing and report the identification of a mutant, which they call fat free, that has normal digestive organ function but defective lipid metabolism (15).

The zebrafish nephron. Over the last four years Drummond and colleagues (12, 13, 27, 35–37) have published a series of meticulous studies characterizing the development of the zebrafish pronephros. This simple kidney consists of a pair of midline glomeruli, proximal tubule-like pronephric tubules, and paired collecting duct-like pronephric ducts; the cellular components, podocytes and tubular epithelia, are strikingly similar in general appearance to those of the mammalian kidney. The pronephros functions as an excretory organ for the developing larval fish for about the first 30 days of its life. The majority of kidney mutations identified to date cause cystic malformations of the tubule, reflecting the screening methods used; none of these mutations have been cloned to date, but it is to be anticipated that the nature of the molecular defects resulting in cyst formation will be of substantial interest. Of particular interest are recent studies in double-mutant animals that carry a cyst-inducing mutation but lack a beating heart; findings in these fish suggest that glomerular filtration is not a prerequisite for the fluid accumulation that drives cyst formation (40). Other informative studies have examined pronephros formation in fish with known mutations. These studies suggest that podocyte formation is not dependent on midline no-thorched factors (36) or the presence of endothelial cells (35).
WHAT IS IT GOOD FOR?

The zebrafish is not a simple system. The critical reader will note that my functional examples all describe studies on mutants where the gene defects are, in fact, not yet known. Despite the array of genomic tools, positional cloning of a zebrafish mutation is still a tough business. In the five years since the landmark edition of Development appeared, the genes are still not identified for the vast majority of these defects. A recent tabulation indicates that genes for between 60 and 70 mutants have been identified by positional cloning (N. Hopkins, personal communication). However, the pace of identification is clearly accelerating, and the availability of genomic sequence and progress in insertional mutagenesis approaches will certainly facilitate the still laborious process of linking a defect with a gene. The reality is that the work I have described here, and functional studies in the zebrafish in general, is very much in its infancy, just a beginning of the complex task of understanding how genomic DNA sequence information translates into integrated biological function. The hard work of molecular characterization of these mutations, and development of functional methods necessary to understand them, is justified by the surprising extent of evolutionary conservation, not just of molecular building blocks, but also of genetic pathways and modules of interacting genes. This evolutionary conservation is the dominant lesson emerging from the study of whole genomes.

The fish will be particularly valuable, I speculate, in understanding the functional consequences of interacting gene defects. This scientific inquiry is at the heart of understanding the regulatory defects that cause polygenic disease. Production of double mutants is a much more straightforward experiment in the zebrafish than in the mouse. For the next few years, work in the zebrafish will concentrate on understanding single gene defects. This will be an informative process. But the zebrafish may particularly come into its own when we move on to the even tougher problem of trying to understand how two subtle molecular defects combine to produce the chronic malfunction that causes some of the important polygenic diseases.

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REFERENCES


