Zebrafish as a Genetic Model organism

REVIEW

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ABSTRACT

The study of zebrafish as a leading model organism for developmental biology is rapidly expanding. The growing interest in zebrafish research was paralleled by an increase in tools and methods available to study this fish. While research initially focused on forward genetics approaches (mutagenesis screens), in recent years the reverse genetics methods have been developed considerably (RNA interference, morpholino knock-down, TILLING). The increase in zebrafish genomic resources together with more sophisticated protocols for transgenesis and other tools referred above have contributed not only to unravel the genetic networks controlling development, but also generate zebrafish disease models with application in human biomedicine. In addition, zebrafish are increasingly used as a genetic model organism for aquaculture species and in toxicogenomics. As we describe here, this diminute fish is a versatile model organism for many fields of research.

Key words: Zebrafish, model organism, forward genetics, reverse genetics, genomics, aquaculture

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RESUMEN

El pez Zebra como modelo animal. El estudio del pez Zebra como organismo modelo líder en el campo de la Biología del Desarrollo se encuentra en rápida expansión. El creciente interés en las investigaciones relacionadas con este pez se ha desarrollado paralelamente con el incremento de las herramientas y métodos disponibles para su estudio. Mientras que las investigaciones iniciales estuvieron basadas en aproximaciones mediante genética directa (mutagénesis a gran escala y pesquizajes genéticos masivos), en años recientes los métodos de genética reversa como el ARN interferencia y el empleo de morfolinos, se han desarrollado considerablemente. El incremento de los recursos en la genómica del pez Zebra unido a métodos más sofisticados para la transgénesis y las herramientas ya descritas, han contribuido a dilucidar las redes genéticas que controlan el desarrollo y a generar modelos de enfermades con aplicaciones en la biomedicina. Se ha incrementado también el uso de este pez como organismo modelo para el estudio de especies de importancia para la acuicultura y en la toxicogenómica. Como se describe en este artículo, este diminuto pez se ha convertido en un modelo versátil en variados campos de investigación.

Palabras claves: Pez Zebra, organismo modelo, genética directa, genética reversa, genómica, acuicultura

*I*ntroduction

*A*dvantages of the Zebrafish as a Model Organism

The zebrafish (*Danio rerio*) is a tropical freshwater fish with natural habitat in rivers of South Asia, mainly northern India, as well as northern Pakistan, Bhutan, and Nepal. It belongs to the family of the cyprinids (Cyprinidae) in the class of ray-finned fishes (Actinopterygii) and within this class to the bony fishes (teleosts or Teleostei).

In the past decade, the zebrafish has become a desired vertebrate model organism, particularly for biologists studying the genetic control of embryonic development. This is due to the fact that zebrafish combine a number of key embryological and experimental advantages. They are easy to maintain and breed [1] and embryos are strong enough for experimental manipulations, such as microinjection and cell transplantation experiments [2]. They develop very rapidly (embryogenesis takes only about 24 h and organogenesis is largely complete after day 5 of development), enabling the observation of defined aspects of development as well as the completion of experiments generally within a few hours to days [3]. Moreover, the transparency of the zebrafish's chorion and its embryos and early larval stages allow the easy visualization of internal processes, such as the formation and function of internal organs inside the living animal. These also facilitate the tracking of the expression of fluorescently tagged transgenes and monitoring reporter gene activity (e.g., GFP and its derivatives, luciferase) as well as laser manipulations (e.g., cell ablation, uncaging experiments) [2]. Zebrafish also have a large number of offspring compared to other model organisms. When kept under optimal conditions, a single female can lay up to 200 eggs per week. When bred under laboratory conditions [1], zebrafish spawn throughout the year. The constant supply of large number of offspring from defined pairs makes the zebrafish ideally suited to large-scale genetic approaches aimed at identifying novel genes and at discovering their functions in a vertebrate [4].

Moreover, the small size of early developmental stages facilitates high-throughput approaches, for example, in genetic mapping approaches and screening of chemical compound libraries in high throughput pipelines. This latter approach can be applied to toxicology, and with mutants that are models for diseases, to drug discovery and development. Commercial initiatives that offer *in vivo* zebrafish high-throughput screening assays for

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drug and toxicology validation are emerging (e.g. Phylonix Pharmaceuticals) [5].

These advantages, combined with recently established protocols and tools for genetic manipulation and analysis, make the zebrafish uniquely suited to uncovering the genetic regulatory networks underlying the development and function of vertebrates [6] (Figure 1).

Genetic Tools and Methods Available for Studying Gene Function in the Zebrafish

In the 25 years since it was first proposed as a model organism to study embryonic development [7], the list of tools and techniques available to study zebrafish has continuously extended. Among the first protocols developed were protocols for efficient mutagenesis. The first large-scale genetic screens were most notably employed in the early 1990s and have led to the identification of several hundred characterized mutations [8-10]. More recently, also reverse genetics techniques (RNA interference, morpholino knock-down and TILLING) were established in zebrafish to target known genes.

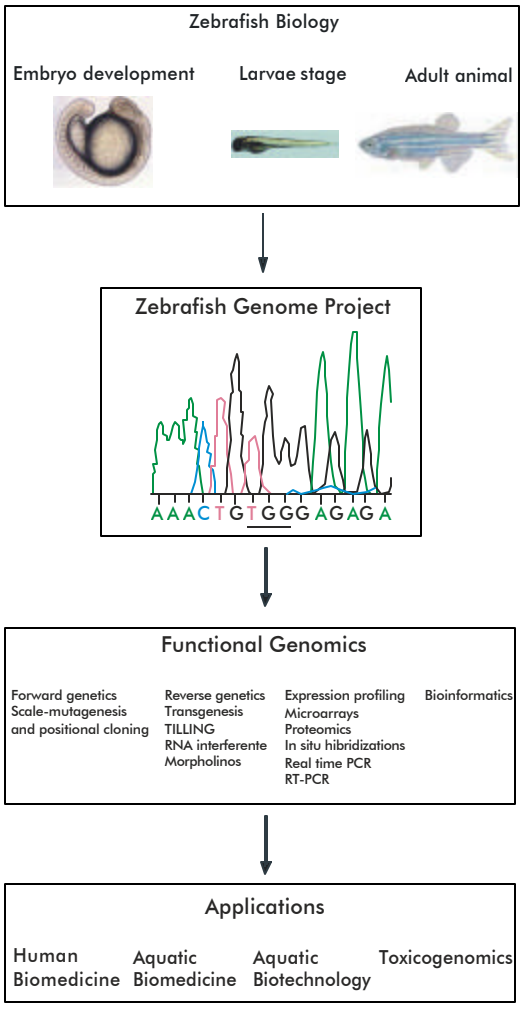

Figure 1. Knowledge of zebrafish biology, full genome sequences and functional genomics opens the possibility of the application of this fish as a model organism.

Forward Genetics

Genetic screens are still the method of choice for identifying genes with essential functions. They are based on the random induction of mutations in the genome and the subsequent screening for individuals displaying mutant phenotypes. Genetic screens have an advantage over reverse genetics approaches, in that they are comparatively unbiased with respect to prior knowledge and thus also allow the identification of novel genes. However, the ability to identify mutants for a given morphological structure, process, or behavior requires that specific assays be developed, which allow the detection of the corresponding phenotypes. Moreover, since genetic screens rely on the ability to detect phenotypes, they can identify only those genes with unique or at least partially non redundant functions. Genes with redundant functions, by contrast, will go undetected [6].

Zebrafish has several advantages to carry out large-scale genetic screens like the relatively moderate space needed to keep large number of fish, the large number of offspring, the speed of zebrafish development and the ease with which it can be observed. Besides, mutations can be preserved in sperm samples which can easily be used for *in vitro* fertilizations [4].

Several mutagenesis protocols have been established for the zebrafish such as chemical mutagenesis (employing N-ethyl-N-nitrosourea, ENU, as a mutagen); radiation, mainly gamma rays and insertional mutagenesis using retroviruses or transposable elements [11]. However, to date the vast majority of zebrafish mutants currently studied carry ENU-induced mutations [8-10].

For a list of published mutants with brief phenotypic and, where available, molecular characterizations see Frohnhoefer [10] or refer to the searchable database at http://www.zfin.org.

Reverse Genetics

The genome sequencing projects completed in recent years have resulted in a number of novel genes predicted by genome annotation. The main task of biological research in the future will be to determine the functions of these genes. Similarly, the biological functions of many studied genes are only incompletely characterized. Reverse genetics approaches (the specific knock-out or knock-down of genes of interest) provide a rapid elucidation of the functions of these predicted or known genes.

Recently, it was discovered that double-stranded RNA (dsRNA) can be a potent and specific inhibitor of gene activity in the nematode *Caenorhabditis elegans* [12] and now similar results have also been reported in other species, including *Drosophila melanogaster* [13], planaria [14], mouse [15], and zebrafish [16, 17]. This effect of dsRNA, called RNA interference or RNAi, happens at a posttranscriptional level at which endogenous mRNA is targeted to degrade [18]. Experimental evidence in plants, suggests that dsRNA may have a more general role in gene regulation and antiviral processes, while in vertebrate systems dsRNA has been shown to induce a global antiviral response leading to general translational arrest and RNA degradation [19].

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Li *et al.* [17] claimed that more than 80% of the embryos injected with dsRNA produced specific defects. They did not report any toxicity in their studies, establishing RNAi as an ideal approach for sequence specific gene inactivation in zebrafish. In addition, Wargelius *et al.* [16] reported embryos injected with no tail or lacZ dsRNA showed a reduced level of the endogenous mRNA and 20-30% of the embryos displayed specific defects reflective of no tail phenotypes. In their studies, approximately equivalent numbers of the injected embryos had several defects. In our laboratory, we obtained good results by microinjection of dsRNA, corresponding to biologically active C-terminal domain from aminoacid 268 to end codon of tilapia myostatin protein, into zebrafish embryos producing an increased body mass in treated fish [20]. In contrast, some authors describe nonspecific defects in zebrafish embryos microinjected with dsRNA [21, 22]. It has been shown that dsRNA has a number of profound effects on cells, but these effects differ depending on the nature and location of the duplexes [23].

Some data indicate that siRNA (small interfering RNA) prepared by endoribonuclease digestion (esiRNA) is unable to cause specific developmental defects in zebrafish, while siRNA should be an alternative for downregulation of specific gene expression in zebrafish in cases, where RNAi techniques are applied to zebrafish reverse genetics [24, 25].

Morpholino-mediated knock-down, a variation of dsRNA technique, proved to work excellently in zebrafish. Morpholinos are short oligonucleotides (generally 18 to 25 bases long) whose backbone is chemically modified, rendering them resistant to degradation by nucleases. A morpholino-mediated knock-down acts through sequence-specific binding of a morpholino oligonucleotide to the targeted mRNA, generally the START codon (ATG) or a crucial splice site. When injected into an embryo (usually into the yolk of a one-cell stage embryo), the morpholinos bind to and immediately block the translation of the endogenous mRNA transcripts into protein. The resulting effect of a morpholino injection is a phenotype that closely resembles or is identical to that of a loss-of-function mutant. Varying the amount of morpholino injected makes it possible to attain different dose-dependent strengths of a phenotype [26].

The morpholino approach is particularly useful for generating phenotypes for uncharacterized genes or to test candidate genes for mutants identified in mutagenesis screens. Morpholinos can also overcome the problem of redundancy in gene function, which often precludes the identification of the function(s) of a gene in mutagenesis screens. By co-injecting morpholinos targeted to related genes, genes with redundant functions can be inactivated simultaneously, eliminating the need for generating double or triple mutants through time-consuming breeding regimes. Moreover, morpholinos can be used to knock down additional genes in a mutant background to test for the effect of multiple gene loss. Despite its advantages, the morpholino knockdown method also have some limitations: the phenotypes can be reliably identified only up to the first 3 to 4 days of development, the injection of morpholinos may result in non-specific phenotypes, a morpholino tagged to a specific gene may bind to a related sequence in the mRNA of another gene and the knock down by morpholinos is transient and not transmitted to subsequent generations [6].

Many of these downsides of morpholinos have been overcome by the recently established TILLING technique to generate knock-out mutants in zebrafish. TILLING (from targeting induced local lesions in genomes) is an effective technique to isolate mutants with defined mutations, including putative loss-of-function alleles (knockouts).

TILLING relies on the random generation of mutations (generally via chemical mutagenesis) and a subsequent screening for mutations in target genes. In a first published TILLING experiment in zebrafish, a library of 4.608 zebrafish derived from ENU-mutagenized founder fish was screened for mutations in 16 different genes [27, 28]. This approach resulted in a total of 255 mutations being identified. A 10% of these mutations are likely to result in complete loss of function or knock-out phenotypes for the affected genes. This would correspond to a rate of null mutant production significantly better than that achieved by homologous recombination in mice. It must, however, be noted that in contrast to methods relying on homologous recombination in mice, TILLING cannot be used to introduce designed mutations. Instead it relies on the spectrum of mutations inducible randomly by chemical mutagenesis.

This is now exploited on a large scale by a consortium of European zebrafish groups who generate zebrafish knock-out on request (for details please see http://www.zf-models.org/KO).

Transgenesis is another important method to study the functions of genes *in vivo* and to generate organisms with novel properties. In the past, techniques to achieve either a transient or a stable expression of transgenes in the zebrafish have been developed. Transient expression is generally achieved by an injection of DNA constructs into fertilized eggs or early embryos [2]. However, genes transiently expressed in zebrafish often do not faithfully mimic the expression pattern of an endogenous gene. To achieve this, a stable transgenic line has to be produced.

Several protocols have been established to create stable transgenic zebrafish lines. These include the infection with retroviral vectors, penetration with DNA-coated microprojectiles, electroporation as well as microinjection of plasmid DNA [29]. Of these, microinjection is the most commonly employed technique. Generally, the DNA is injected into embryos at the one-cell stage [2]. This technique is fast and it results in high rates of integration. In recent years, several tools and techniques have become available to achieve better control and analysis of transgenes in zebrafish such as: the GAL4-UAS system [30], the employment of inducible promoters [31] and the use of reporter genes like GFP and its derivative forms under cell-type specific promoters for the tracing of specific cells during development [2].

Cell Biological Methods

As the zebrafish has become a favorite model organism for vertebrate embryology, a huge range of cell

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biological methods originally developed in other organisms has been adapted to it. Cell lineage tracing with dyes (Cell labelling) is carried out extensively in the zebrafish. Labeling of cells is either achieved by pressure-driven microinjection (with a pneumatic microinjector) in early embryos or by voltage-driven microinjection (which involves depolarizing the cell membrane to create a hole) after the 256-cell stage.

An initial difficulty was posed by the fact that up to the fifth cell division zebrafish blastomeres are connected by cytoplasmic bridges, resulting in leakage of the injected dye. This was overcome by the introduction of high molecular weight dextran dye conjugates that cannot pass through the bridges, enabling fate mapping of the early embryo [32]. Other fluorophores employed in zebrafish are: Membrane-specific dyes such as DiI and DiO, mainly used for the tracing of axons [33], photoactivatable dyes, in particular 4,5-dimethyloxy-2-nitrobenzyl ester (DMNB)-caged fluorescein. After labeling, individual cells or small groups of cells can be targeted with a laser, resulting in uncaging of the dye [34] and quantum dots, these recently introduced semiconductor crystals are available in a multitude of colors, can be targeted to specific subcellular compartments and offer higher fluorescent yield and photostability than organic dyes, making them the first fluorophores suitable for long-lasting time-lapse studies [35].

Transplantation of cells has similarly been established as a standard technology in the zebrafish field [34]. Transplantations can simply be carried out using a compound or dissection microscope and a hydraulic microinjector, usually at late blastula stages. Transplantation between mutant and wild-type embryos is commonly used to establish the cell autonomy of mutant phenotypes [36], while transplantation of genetically tagged cells allows assessing the state of commitment of cells [37], and offers yet another way of tracing lineages.

Genomics in the Zebrafish

Global expression profiles

Zebrafish microarrays have been produced that contain either DNA fragments derived from expressed sequence tag (EST) and cDNA libraries [38], or from oligonucleotide libraries based on all the genes or transcriptional units predicted from bioinformatic analysis of the entire zebrafish genome. At present, 14 000-22 000 zebrafish genes are included on commercially available arrays (Agilent, Affymetrix, Compugen/Sigma-Aldrich, MWGBiotech and Qiagen/Operon) offering a standardized toolset for zebrafish transcriptional profiling. Among academia-based oligonucleotide microarrays are slides printed with a zebrafish 16k 65-mer oligo library - chips printed with a regularly updated version of this library are available to the scientific community from Alestrom's laboratory (http://www.mikromatrise.no)[39] and microarrays for single nucleotide polymorphism (SNP) mapping in zebrafish are also emerging [40]. Recently, microRNA expression profiles have been characterized [41] adding this new family of gene expression control factors to the zebrafish toolbox repertoire.

Maps of the zebrafish genome

Several genetic maps have been constructed for the zebrafish. The one with the highest density, the MGH map, is based on a panel of 48 diploid full-sib F2 fish from a cross of the lines AB and India. The initial map was constructed from 705 microsatellite markers (CA-repeats) covering 2.350 cM, and a total of 3.845 microsatellites were subsequently mapped [42]; http://zebrafish.mgh. harvard.edu/). This map is now used as the common reference for all mapping efforts in the zebrafish. Several hundred mutant loci have been mapped on this panel. Two radiation hybrid maps have been generated for the zebrafish, the T51 and LN54 maps [43, 44]. It is anchored to the MGH map by 624 microsatellites (http://wwwmap.tuebingen.mpg.de). An effort has been made to produce an integrated map (ZMAP) from all the mapping data available (http://zfin.org/).

BACs libraries

To simplify positional cloning of zebrafish mutations and as a basis for the zebrafish genome project, several libraries of bacterial artificial chromosomes (BACs) have been created and ordered in contigs. In addition to two pilot libraries, two libraries with approximately 10-fold coverage were created from sperm of Tubingen fish (the line used in most mutagenesis experiments) by the Children's Hospital Oakland Research Institute (CHORI) and the company Keygene, respectively [45]. The libraries, BAC pools suitable for screening, and individual clones can be obtained from the RZPD resource center (http://rzpd.de). The great majority of the BACs were physically mapped by restriction fingerprinting.

A sequenced genome

A genome sequencing project for the zebrafish has been initiated by the Sanger Institute. This project uses a combination of whole-genome shotgun sequencing and sequencing of BAC contigs derived from the fingerprinting project. The current genome coverage is 6.5- to 7-fold and 0.4-fold, respectively. The sequence is assembled on the basis of the T51 radiation hybrid map and is available with annotations through the ENSEMBL (whole-genome shotgun) and VEGA (BAC-based) genome browsers (http://www.ensembl.org/Danio_rerio/index.html; http://vega.sanger.ac.uk/Danio_rerio/). The combination of a relatively high repeat frequency and a high degree of polymorphism relative to mammals poses special problems for the sequence assembly, exacerbated by the fact that the starting material for the BAC and shotgun libraries is derived from several fish of the non-isogenic Tubingen line. Therefore an additional fosmid library has recently been prepared from a single double-haploid Tubingen fish and is being sequenced to a low coverage to aid in assembly of the genome. One chromosome, LG20, has been finished to date. It is planned to finish the entire genome within the coming years [6].

Zebrafish as a model in human diseases

While the size of the zebrafish genome is approximately half of that of the human genome, it may actually encode

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a greater number of genes, due to gene duplication events [46]. Because the regulatory regions of gene duplicates may diverge, they will often together fulfill the function of a single mammalian gene, allowing a more detailed dissection of gene function [47]. Most gene families present in mammals are represented by one or more orthologs in the zebrafish. The identification of numerous human disease gene orthologs has further confirmed the relevance of the zebrafish for the study of human disease.

The usefulness of zebrafish as a model for human medicine has been reviewed recently [48]. Several groups have investigated zebrafish as a model for a variety of topics in human biomedicine, including: molecular mechanisms of skin [49] and neurological diseases [50]; muscular dystrophy [51]; cardiac muscle regeneration [52, 53]; eye disorders [54]; acute renal failure [55]; hematopoetic and immunological diseases [56]; cancer [57, 58] and odontology [59].

Zebrafish are susceptible to strains of *Streptococcus* [60]. In infectious disease research, zebrafish have been used as a model for one of the most frequent pathogens of humans, *Streptococcus pyogenes* [61]. Besides, the fact that astronauts develop osteoporosis in the microgravity encountered during space missions has led the European Space Agency (ESA) to support a project using a transgenic fish model to study gene expression profiles on earth compared with those in microgravity environments [62] (ESA ENFORM Consortium; http://www.pvi.uni-bonn.de).

Zebrafish as a model organism for aquaculture species

Zebrafish is genetically more tractable than the majority of fish species of interest to aquaculture. Thus, the aquaculture industry will likely be able to benefit significantly from insights gained from studying zebrafish. Possible areas where zebrafish research could yield commercially applicable results are, for example, the identification of genes involved in the development of certain organs (e.g., muscle, fat, or bone tissue), the metabolism of nutrients, disease and stress pathways, as well as behavioral traits. Moreover, novel drugs and their effects on fish physiology can be easily tested in zebrafish, particularly when their effect on a range of alleles for a genetic property is to be assessed [6].

Up to now, aquaculture is still less solidly founded on genetic research than the breeding of farm animals. Large-scale initiatives are therefore underway to gain a deeper understanding of the fish species bred and to use this understanding for improvements, for example, the European projects BRIDGE-MAP, BASSMAP, and AQUAFIRST, targeted primarily at gilthead seabream and seabass; genome projects for the rainbow trout, atlantic salmon, Japanese flounder, and tilapia; and breeding programs for the channel catfish and cod. Main areas of interest are the improvement of diets and husbandry with respect to optimizing growth rates and reducing stress and disease, and the prospect, yet unfulfilled, of improving the fish themselves by marker-assisted breeding. In each of these areas the zebrafish community may be able to offer results of interest. For example, recently, a Spring Viremia of Carp virus (SVCV) zebrafish challenge model was established [63], which opens the door for studies of the functional genomics and molecular details connected with infection, pathogenesis and the effects of candidate vaccines, in addition to non-vaccine strategies, to combat viral infections. Of the SVCV vaccines tested, a DNA vaccine expressing the viral G-protein was shown to be effective [64]. A recent review covers other zebrafish disease models, including tuberculosis and lethal streptococcal and salmonella infections [65]. The efficient systems for conducting challenge experiments, together with the functional genomics research potential on both host and pathogen, aids the study of 'host-pathogen crosstalk' and places the zebrafish at the forefront of studies on basic infection and disease biology, and the biotechnological strategies to combat these.

Huge advances have been made in zebrafish research and extraordinary efforts have been focused on the genetics, genomics and developmental biology of this fish. Thus, in the coming years, one can expect to see an increasing number of reports on the application of zebrafish as biomonitors. As described here, the zebrafish is a versatile and well-characterized model with applications in many fields of study.

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