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Forward and reverse genetic approaches to the analysis of eye development in zebrafish

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Abstract

The zebrafish has been established as a mainstream research system, largely due to the immense success of genetic screens. Over 2000 mutant alleles affecting zebrafish's early development have been isolated in two large-scale morphological screens and several smaller efforts. So far, over 50 mutant strains display retinal defects and many more have been shown to affect the retinotectal projection. More recently, mutant isolation and characterization have been successfully followed by candidate and positional cloning of underlying genes. To supplement forward genetic mutational analysis, several reverse genetic techniques have also been developed. These recent advances, combined with the genome project, have established the zebrafish as one of the leading models for studies of visual system development. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Forward genetic analysis

The hallmark of forward genetic analysis is a mutagenesis screen (Fig. 1). Although exceptionally successful in invertebrates, this approach has been historically of limited use in vertebrate model systems. Since its early days as a research organism, the appeal of the zebrafish has relied on its potential use in genetic screens. The expectation that the zebrafish model will introduce screens as a standard tool of vertebrate genetics has certainly been fulfilled. Today, the repertoire of zebrafish mutagenesis tools, breeding strategies and mutant selection approaches has no match in any other vertebrate.

The complexity and scale of screening experiments in zebrafish vary greatly. The simplest screening protocols relied on morphological inspection of mutant phenotypes with a dissecting microscope; the most involved ones employed, for example, sophisticated dye injection devices to label axonal projections of a specific cell class. While some screens inspected just several hundred adult individuals, others analyzed hundreds of thousands of embryos (Baier et al., 1996; Malicki et al., 1996; Li & Dowling, 1997). A rich repertoire of techniques is currently available to search for mutant phenotypes affecting eye development. First, mutant selection approaches have been developed based on a broad range of morphological, histochemical, and behavioral criteria. Similarly, several breeding schemes have been in use. Some of them take advantage of the ability to produce zebrafish haploid or parthenogenetic diploid embryos on a scale sufficient to support a mutagenesis screen. Finally, at least two types of mutagens, chemical and retroviral, have been successfully applied on a large scale. The advantages and drawbacks of particular screening tools have been discussed in several recent reviews (Malicki, 2000a,b).

The progress of genetic analysis in zebrafish has greatly benefited from a thorough description of the wild-type embryonic and larval eye. Morphological transformations leading to the appearance of the optic cup have been described in detail (Schmitt & Dowling, 1994; Li, Joseph, & Easter, 2000b) and so has the timing of neurogenesis and cell class differentiation (Nawrocki, 1985; Hu & Easter, 1999). These studies revealed that one of the initial events in retinal neurogenesis is the appearance of postmitotic ganglion cells in the "ventral patch" area of the retina between

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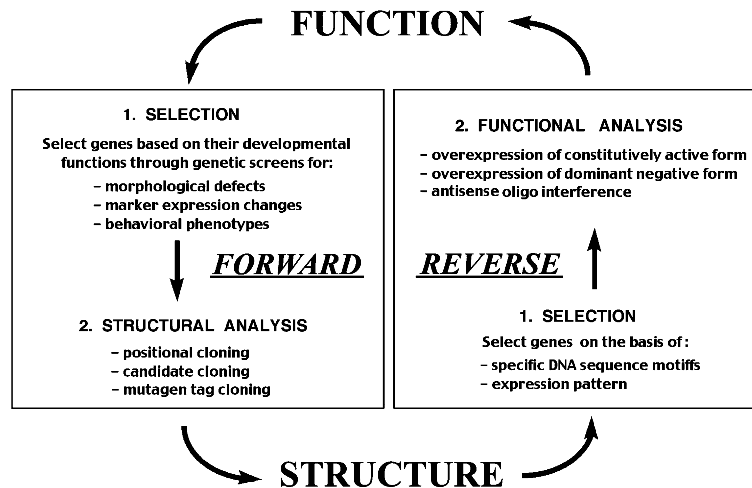

Fig. 1. Forward genetic and reverse genetic approaches in zebrafish. Both approaches consist of selection and analysis steps. Positional and candidate cloning are used to identify chemically induced alleles. In the case of retroviral mutagenesis, retrovirus itself is a molecular tag that facilitates cloning. Three general approaches to reverse genetic function analysis are available in zebrafish: overexpression of a constitutively active form, overexpression of a dominant negative form, and antisense oligonucleotide interference.

27 and 28 h postfertilization (hpf). This is followed 10 h later by the emergence of postmitotic interneurons which form the inner nuclear layer, and finally at ≈43 hpf, the photoreceptor neurons in the outer nuclear layer. Differentiation of ganglion and photoreceptor cells has received particular attention. Axons of the first ganglion cells leave the eye by 36 hpf and reach the optic tectum by 48 hpf (Stuermer, 1988; Burrill & Easter, 1995). Similar analysis determined that the photoreceptor outer segments become distinguishable first by 55 hpf while the photoreceptor synaptic termini differentiate synaptic ribbons first between 60 and 62 hpf (Schmitt & Dowling, 1999). Finally, it has been established by behavioral criteria that the zebrafish retina becomes functional between 60 and 80 hpf (Easter & Nicola, 1996). All these studies of wild-type development have been invaluable in the analysis of mutant phenotypes.

Defects of nearly every aspect of eye development, from optic lobe morphogenesis to the differentiation of photoreceptor synaptic termini, have been found in mutagenesis screens performed so far (Malicki et al., 1996; Brockerhoff, Dowling, & Hurley, 1998; Neuhauss et al., 1999). Mutational analysis has been particularly revealing in four areas: specification of the eye field, neuronal patterning in the retina, differentiation of photoreceptor cells, and retinotectal pathfinding. For example, over 30 mutant alleles produce defects in photoreceptor cell development and function alone (Malicki et al., 1996; Fadool, Brockerhoff, Hyatt, & Dowling, 1997; Becker, Burgess, Amsterdam, Allende, & Hopkins, 1998; Brockerhoff et al., 1998; Drummond et al., 1998; Neuhauss et al., 1999). While some of them affect photoreceptor morphogenesis, others do not produce any obvious structural abnormalities. This group of mutants is also particularly easy to relate to human retinal disorders such as retinitis pigmentosa, cone dystrophies or macular degenerations. The mutant phenotypes of *elipsa* (*eli*), *fleer* (*flr*) and *oval* (*ovl*) loci display both photoreceptor loss and kidney defects bearing a striking resemblance to a syndromic form of retinitis pigmentosa: the Senior–Loken syndrome (Warady, Cibis, Alon, Blowey, & Hellerstein, 1994; Satran, Pierpont, & Dobyns, 1999). Because the zebrafish larval retina is cone rich, it may be particularly useful as a model of macular degeneration in the human retina. This is potentially of great importance as age-related forms of macular degeneration account for approximately 50% of blindness in the western world (Klein et al., 1992; Vingerling et al., 1995; Stone et al., 1999).

One particularly informative form of phenotypic study in the zebrafish embryo is mosaic analysis. Mosaic zebrafish are generated by transplanting blastomeres from one embryo to another (Ho & Kane, 1990; Halpern, Ho, Walker, & Kimmel, 1993; Malicki & Driever, 1999; Doerre & Malicki, 2001). If the genotypes of host and donor individuals differ, the resulting embryo will consist of mutant and wild-type cells intermingled together. In some embryos, mutant cells will be entirely surrounded by wild-type tissue. In such cases, if a mutant defect is caused by defective cell–cell communication, interaction of mutant cells with wild-type tissue will restore their normal phenotype. The value of mosaic analysis is that it allows one to identify instances of cell–cell interactions.

Mosaic experiments have been performed on selected eye mutants and revealed several cell nonautonomous phenotypes. Interestingly, all four neuronal patterning loci identified so far, *glass onion* (*glo*), *mosaic eyes* (*moe*),

nagie oko (*nok*), and *oko meduzy* (*ome*), display noncellautonomous phenotypes in the retinal neuroepithelium (Malicki & Driever, 1999; Jensen, Walker, & Westerfield, 2001; Pujic & Malicki, 2001). At least in the case of *mosaic eyes*, but possibly also in some of the other mutants, aberrant retinal neuroepithelial patterning is caused by defective cell–cell interactions with the retinal pigment epithelium.

Molecular cloning of mutant genes is the most informative experimental approach to follow mutagenesis screens. In this area the progress has been breathtaking. Only five years ago, shortly after the completion of the first large-scale screening projects, positional cloning of any of the hundreds of chemically induced mutant alleles bordered on the impossible. This was mostly due to the paucity of basic genomic tools such as genetic maps of appropriate density or large-insert genomic libraries. Over the last several years, however, the density of microsatellite-based genetic markers in the zebrafish genome increased more than 30-fold (Knapik et al., 1996; Shimoda et al., 1999, for the most recent count see: http://zebrafish.mgh.harvard.edu/mapping/ssr_map_index.html). In parallel, yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), and phage artificial chromosome (PAC) large-insert genomic libraries, radiation hybrid panels, and cDNA arrays became available (Zhong et al., 1998; Amemiya & Zon, 1999; Geisler et al., 1999; Hukriede et al., 1999). These technical advances greatly improved the success rate of positional cloning endeavors.

In the initial period following large-scale mutagenesis screens, the vast majority of successful cloning experiments involved the candidate gene approach. These efforts led, for example, to the discovery that the *no isthmus* (*noi*), *sonic you* (*syu*), and *bozozok* (*boz*) mutations affect the pax-2.1, sonic hedgehog, and dharma genes respectively (Lun & Brand, 1998; Schauerte et al., 1998; Fekany et al., 1999). The contribution of the positional cloning experiments has markedly increased in the recent years. Several groups have now published positional cloning of ENU-induced mutant alleles. *One eyed pinhead* (*oep*) and *foggy* (*fog*), two of the genes identified using this approach, are necessary for proper eye development. Mutations in *oep*, which encodes a membrane associated EGF–CFC type factor, lead to severe cyclopia (Zhang, Talbot, & Schier, 1998). *Fog*, on the other hand, plays a role in cell differentiation during retinal neurogenesis. Mutations of *fog*, a phosphorylation-dependant transcription elongation regulator, inhibit cell differentiation in the inner nuclear layer and the photoreceptor cell layers (Guo et al., 2000). More recently, positional cloning of several other mutant genes, including *knypek* (*kny*) and *nagie oko* (*nok*) has also been completed (Topczewski, J. & Solnica–Krezel, L., unpublished results; Wei, X. & Malicki, J., unpublished results).

2. Reverse genetics

Reverse genetic approaches have played a fundamental role in the analysis of vertebrate eye development. The majority of genes known to regulate retinal neurogenesis were first identified using a variant of a reverse genetic approach. Reverse genetic analysis can be subdivided into two phases:

1. Selection of candidate genes that are likely to play a role in a given developmental process;
2. Functional analysis of candidates (Fig. 1).

The first step usually identifies a particular spatiotemporal pattern of expression, or the presence of a specific DNA sequence motif or both. As transcription factors are known to play important roles in cell fate decisions, several groups screened retina-specific cDNA libraries for evolutionarily conserved DNA-binding domains. These efforts led to the identification of brn, rx and crx genes, for example (Xiang et al., 1993; Furukawa, Kozak, & Cepko, 1997a; Furukawa, Morrow, & Cepko, 1997b). The arsenal of tools available to select genes based on their expression patterns has recently been enriched by gene chips and Serial Analysis of Gene Expression (SAGE) (Velculescu, Zhang, Vogelstein, & Kinzler, 1995; Livesey, Furukawa, Steffen, Church, & Cepko, 2000; Velculescu, Vogelstein, & Kinzler, 2000). Both have been successfully applied to the study of retinal neurogenesis.

Owing to its high fecundity, transparency of embryos, and extrauterine development, the zebrafish has proven to be an excellent medium for a variant of a reverse genetic screen that relies on direct observation of in situ hybridization expression patterns in whole embryos (Thisse & Thisse, unpublished). This experiment is a reverse genetic equivalent of a large-scale mutagenesis screen. Similar to the large-scale forward genetic screens performed in Boston and Tuebingen (Driever et al., 1996; Haffter et al., 1996), it does not focus on a specific tissue or organ or on a particular part of the genome. The number of transcripts analyzed so far places it in the category of large-screen endeavors. To analyze expression patterns, embryos are fixed at nine stages of development and hybridized in batches of 60 with cDNA probes derived from a normalized, embryonic cDNA library. So far, $\approx$7200 cDNAs have been analyzed and this number will increase in the future (Thisse & Thisse, unpublished results). These cDNAs are estimated to represent $\approx$5800 genes and constitute 12–15% of the entire zebrafish transcriptome. About 1.3% of all cDNAs screened are expressed in the retina in a cell type-restricted manner between 24 and 72 hpf. Their expression patterns were analyzed further on transverse sections through the retina at 36, 48, 60, and 72 hpf. This analysis revealed that the expression of some genes

is confined to the proliferating cells of the retinal neuroepithelium or the proliferative retinal–marginal zone, while the expression of others is present in differentiated neurons (Pujic & Malicki, unpublished results). The most promising expression patterns are confined to a distinct cell class or cell type and correlate with the appearance of these cells during neurogenesis.

The impact of reverse genetic approaches ultimately depends on ways to assess the function of genes identified in the initial screening protocols. Two best established approaches in this area, neither unique to the zebrafish model, are the overexpression of constitutively active or dominant negative variants of the genes of interest. More recently, additional technologies have become available: double-stranded RNA (dsRNA) interference, and morpholino-modified antisense oligonucleotides. dsRNA interference, although initially proclaimed effective in zebrafish, has not withstood a more extensive scrutiny (Wargelius, Ellingsen, & Fjose, 1999; Li, Farrell, Liu, Mohanty, & Kirby, 2000a). Its usefulness appears to be limited by frequent nonspecific effects. Morpholino-modified antisense oligonucleotides, on the other hand, appear much more promising. In these nucleic acid analogues, the pentose ring is replaced with a morpholine (hydro-1,4-oxazine) moiety (Summerton & Weller, 1997). They were shown to effectively and specifically induce phenotypes closely reminiscent of chemically induced loss-of-function alleles of several genes (Nasevicius & Ekker, 2000; Wei & Malicki, unpublished). Morpholino-modified antisense oligonucleotides have to be targeted to either the translation initiation codon or the sequences located immediately upstream and are presumed to interfere with translation. So far this new form of antisense technology has generated much enthusiasm among its users.

To test morpholino-modified antisense oligonucleotides as a tool of gene function analysis during eye development, we chose to block the expression of rodopsin—a gene expressed in the retina at a high level after neurogenesis is largely complete. The zebrafish rodopsin is detectable first at $\approx$50 hpf. By 60 hpf, it is expressed at a high level in cells of the ventral retina (Raymond, Barthel, & Curran, 1995). Injection of anti-rodopsin morpholino-modified oligonucleotides into 1–4 cell-stage embryos abolished rodopsin expression entirely in 16 out of 18 embryos analyzed at 60 hpf (Fig. 2). This experiment demonstrates that this form of antisense technology can be used to block gene expression during neurogenesis in the zebrafish retina.

A bonus of antisense knockdown approach is that it allows one to downregulate the expression of multiple genes at a time. This is particularly important in the case of functionally redundant loci. The necessity to manipulate two genes in parallel to reveal their developmental role has been recently demonstrated in the case of the zebrafish hedgehog genes. Although both mouse and human sonic hedgehog mutations result in cyclopia, loss-of-function alleles of *sonic you*, a zebrafish sonic hedgehog ortholog, do not produce this phenotype (Chiang et al., 1996; Schauerte et al., 1998; Wallis & Muenke, 2000). Cyclopia only becomes apparent in zebrafish when both *sonic hedgehog* and its paralog, *tiggy–winkle hedgehog*, are downregulated. This has been recently demonstrated using morpholino-modified oligonucleotides for these two loci (Nasevicius & Ekker, 2000). Thus morpholino-modified antisense oligonucleotides offer an efficient alternative to the generation of double mutants—a time consuming endeavor even if chemically induced alleles of two functionally redundant loci are available.

What are the future prospects for reverse genetic analysis in zebrafish? In situ screens for promising expression patterns may be supplemented with enhancer trap analysis. Enhancer traps are reporter constructs

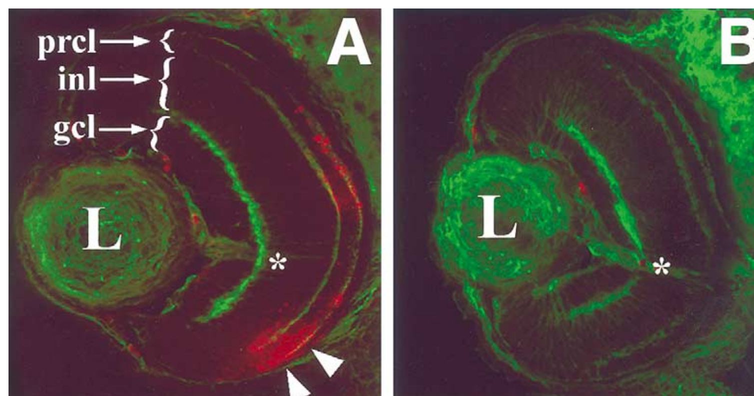

Fig. 2. Morpholino-mediated knockdown of rodopsin expression. Cryosections through the central retina stained with an antibody to rodopsin (Fret 11), a gene expressed in photoreceptor cells at a high level starting at 50 hpf. Asterisks indicate the optic nerve. (A) At 60 hpf, the strongest rodopsin expression (red) is present in the ventral portion of the photoreceptor cell layer, in the so-called "ventral patch" (arrowheads). The overall cellular architecture in the retina is visualized with Alexa 488-phalloidin (green). (B) Injections of antisense oligonucleotides into early embryos efficiently block rodopsin expression at 60 hpf.

randomly inserted throughout the genome to detect activity of neighboring regulatory elements (O'Kane & Gehring, 1987). Similar to in situ screens, they identify loci characterized by specific expression patterns. Enhancer trap screening proved to be very productive in Drosophila leading to the expectation that this approach could be equally fruitful in a vertebrate model organism (Klambt & Goodman, 1991; Sentry et al., 1994; Pignoni, Hu, & Zipursky, 1997; Mollereau et al., 2000). The advantage of using enhancer traps is that they lead to the generation of stable transgenic lines. The use of such lines is at least 2-fold. First, they can be incorporated into the UAS-GAL4 system to study the consequences of gene overexpression in well-defined cell populations (Brand & Perrimon, 1993; Scheer & Campos-Ortega, 1999). Second, transgenic lines expressing vital reporter genes in small cell populations may be useful in genetic screens because they allow one to search for subtle phenotypes undetectable by morphological criteria. Enhancer trap constructs can be delivered to the zebrafish genome by injecting retroviral constructs into early embryos or by inducing transposable elements to jump in the genome. As the first of these approaches has already been used in an extensive mutagenesis screen, a precedent exists to validate its efficiency (Amsterdam et al., 1999). Although the transposon-based system is at an earlier stage of development, ultimately its use may be less labor-intensive than retroviral approaches (Raz, van Luenen, Schaerringer, Plasterk, & Driever, 1998).

Reverse genetic approaches will also benefit from improved tools of gene function analysis. The existing methodologies certainly need refinement. In the case of modified antisense oligonucleotides, tissue or organ-specific delivery would most certainly be an important improvement. Methods to accomplish this goal are not yet available. Ultimately, the best way to perform reverse genetic functional analysis it to introduce heritable changes in the sequence of specific genes. Although the first steps towards this goal have already been taken (Ma, Fan, Ganassin, Bols, & Collodi, 2001), this type of technology has been elusive in zebrafish so far.

Both forward and reverse genetic analyses will benefit enormously from the zebrafish genome project. Once the genome sequence is available, the tedious tasks of chromosome walking and sequence analysis will be eliminated. This will greatly shorten the period necessary to clone a chemically induced mutant allele using the positional approach. Similarly, functional analysis using morpholino-modified oligonucleotides will become easier because 5′ untranslated regions of transcription units will be available in the genome sequence. The combination of forward and reverse genetic approaches in the zebrafish model is becoming a particularly effective strategy to dissect the genetic circuitry that regulates the development of the vertebrate eye.

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