

Zebrafish: bridging the gap between development and disease

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The zebrafish has been the model of choice amongst developmental biologists for many years. This small freshwater species offers many advantages to the study of organ and tissue development that are not provided by other model systems. Against this background, modern molecular genetic approaches are being applied to expand the physical and genetic mapping of the zebrafish genome. These approaches complement the large-scale mutagenic screens that have led to the isolation of mutant phenotypes. Some of the phenotypes have been found to resemble human disease states, while mapping and sequencing data have revealed zebrafish genes with significant homology to human disease-causing genes. It is the realization that the zebrafish offers an amenable system for understanding disease, as opposed to development, that underpins this review. The adventitious identification of disease phenotypes amongst zebrafish mutants and the important area of deliberate disease modelling using transgenesis and gene targeting should lead to a better application of the zebrafish as a vertebrate model of human diseases.

INTRODUCTION

The zebrafish (*Danio rerio*) represents an ideal genetic system for the study of both developmental biology and disease (1–3). The many attractions of this small freshwater species include the following: it is easy to maintain at high densities in the laboratory, large numbers of embryos can be produced year round, fish grow to sexual maturity within four months and the optically transparent embryos develop externally (4). This latter attraction permits the direct observation of vertebrate tissue and organ development that the mouse, *Drosophila melanogaster* and *Caenorhabditis elegans* systems cannot provide.

Recently, the dissection of the molecular basis of developmental processes has involved large-scale mutagenic screens that have led to a comprehensive collection of mutants (2,5,6). These screens, combined with advances in physical and genetic mapping of the zebrafish genome (7–10), will contribute to our understanding of developmental pathways. In addition to the identification of developmental mutants, the genetic screens have yielded many zebrafish mutant phenotypes that resemble human disease states. However, the identification of the mutant genes underlying the disease phenotypes remains a technical challenge. As a complementary approach, the isolation of zebrafish orthologues of human disease-causing genes has also been undertaken. In contrast, the use of the zebrafish in transgenesis and targeted mutagenesis in order to model autosomal dominant and recessive disorders, respectively, is not as well advanced. Therefore, the aim of this review is to highlight what has been achieved to date with respect to human disease modelling in the zebrafish, but, more

importantly, to underscore the importance and indeed the imperative of using molecular genetic tools to develop zebrafish disease models in a targeted manner.

MODELS DERIVED FROM MUTAGENIC SCREENS

Large-scale mutagenic screens have yielded many zebrafish mutants that exhibit various developmental and physiological disorders. These disorders include haemophilia, anaemia, porphyria, neuropathies of the peripheral nervous system and diseases of the central nervous system (11–17).

Current studies indicate that haematopoiesis in the zebrafish appears to resemble haematopoiesis in higher vertebrates in terms of the conservation of expression and function of haematopoietic genes (14,18–20). Large-scale genetic screens have been undertaken to identify mutants that display an embryonic hypochromic anaemia (21). One of these mutants, *sauternes (sau)*, has been shown to encode an erythroid-specific isoform of the haem biosynthetic enzyme ALA52 (14). Mutations in the human ALA52 gene cause X-linked sideroblastic anaemia. The zebrafish *sau* mutant exhibits a similar phenotype and hence represents a vertebrate model of this human disorder.

Further large-scale chemical mutagenesis screening has also yielded a uroporphyrin zebrafish mutant, designated *yqe^{tp61}*, which is deficient in an enzyme that exhibits reduced activity in two common human porphyrias, porphyria cutanea tarda (PCT) and hepatoerythropoietic porphyria (HEP) (22). The zebrafish mutation has been localized to the *urod* gene. This mutant represents a UROD-deficient vertebrate model which

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appears to mimic the pathogenesis of human HEP and PCT (15).

Recent studies have also led to the isolation of zebrafish mutants, designated *jumbo* and *chihuahua*, which may serve as models of obesity and osteogenesis imperfecta, respectively (23). Again there is an obvious gap between the isolation of a zebrafish mutant and the identification and the gene carrying the mutation event.

IDENTIFICATION OF CANDIDATE DISEASE GENES IN THE ZEBRAFISH

The studies described above highlight the potential of modelling human diseases in the zebrafish. In this context, the identification of conserved genes among vertebrates offers a means of using mutants in zebrafish orthologues of human genes in order to model human disorders. It is important to stress here that, without supporting synteny data, the comparison of genes between the zebrafish and humans is complicated. In the case of directed modelling of disease processes in the zebrafish, we should rely on the isolation of genes that are analogous (in function) to genes previously identified in humans. Such genes may have similar evolutionary origins and are thus orthologues, or may encode proteins of similar function and hence should be considered analogues. This discrimination is further complicated when there are several members of a gene family (paralogues) and therefore identification of the true orthologue is difficult in the absence of evolutionary analysis (24). However, with regard to disease modelling, the isolation of analogous genes could be considered appropriate.

Davidson *et al.* (1) have reported the zebrafish orthologues of the mouse *Growth/differentiation (Gdf) 5* and *6* genes, designated *contact*, *radar* and *dynamo* based on a comparison of synteny shared among zebrafish, mouse and human genomes (25). Mutations in the human orthologue of the mouse *Gdf5* (zebrafish *contact*) gene causes Hunter–Thompson type chondrodysplasia (26). The study of steroidogenesis and human disease associated with steroid imbalance has been suggested by Lai *et al.* (27). These workers isolated two zebrafish genes that share significant amino acid similarity with the P450 scc and 3 β -HSD genes. In addition, Ardouin *et al.* (28) have reported the isolation of two orthologues of the human X chromosome-linked *KAL-1* gene, which is impli-

cated in Kallmann syndrome. The expression pattern of the two orthologues is similar to those reported for the human *KAL-1* and chicken *kall* genes. These workers suggest that the zebrafish offers a means of studying the function of *KAL-1* in normal vertebrate development as well as the developmental failure characterized by the olfactory defect in Kallmann syndrome.

Karlovich *et al.* (29) have reported the isolation of a gene in the zebrafish that exhibits 70% identity to human huntingtin. These workers suggested that gamma ray mutagenesis and subsequent PCR-based approaches should lead to the isolation of mutants in this gene and hence to the subsequent characterization of any associated developmental defects. We suggest that, in line with HD models that have been developed in the mouse, fly and worm (30–33), transgenesis of the zebrafish using disease-causing CAG repeat expansions may lead to a deliberate zebrafish model of this neurodegenerative disorder (see below).

Wulf *et al.* (17) have reported the isolation of a peripheral myelin protein 22 (PMP22)-related cDNA from the zebrafish. Human hereditary neuropathies are associated with mutations in the gene encoding PMP22. This protein is a component of compact myelin of the peripheral nervous system (PNS). *In situ* hybridization studies of the zebrafish analogue of *PMP22* indicate that the expression of this gene occurs prior to the onset of myelination. Wulf *et al.* (17) speculated that this gene may play a role in early PNS development and that mutations in this gene may mimic PNS-restricted defects seen in mammalian mutants of the *PMP22* gene. Finally, Manickam *et al.* (34) have reported the isolation of the zebrafish orthologue of *MEN1* and mapped it to a region that appears to show synteny to the *MEN1* locus in humans. Mutations in the human *MEN1* gene lead to the autosomal dominant disorder, multiple endocrine neoplasia type 1 (MEN1).

Screening of zebrafish genome and EST databases can also yield many analogues of human disease-causing genes. Tables 1 and 2 present the search outcomes of several genes implicated in human neurological disorders. These disorders were selected from the recent publication of the complete sequence of the *D.melanogaster* genome and the identification of all its genes (35).

The studies described above have been limited to the identification of mutant lines with disease-like phenotypes, or the

Table 1. Comparative analysis of selected human disease genes against *Drosophila melanogaster* (F), *Caenorhabditis elegans* (W), *Saccharomyces cerevisiae* (Y) and *Danio rerio* (Z) genome database

Neurological disorder	OMIM no.	GenBank identifier for the human sequence	BLAST e-values				Database identifiers			
			F	W	Y	Z	F	W	Y	Z
Amyotrophic lateral sclerosis	147450	338276	9.00e ⁻⁵¹	1.00e ⁻⁴⁶	2.00e ⁻⁴⁴	2.00e ⁻⁶²	CT36899	C15F1.7	YJR104C	Y12236
Fragile X syndrome	309550	544328	9.00e ⁻⁸³	3.10e ⁻⁰²	4.50e ⁻⁰²	1.00e ⁻³⁶	CT17036	–	–	AF169146
Huntington's disease	143100	1170192	3.00e ⁻²¹	2.00e ⁻⁰³	3.10e ⁻⁰¹	0.00e ⁺⁰⁰	CT28171	–	–	AF052602
Spinal muscular atrophy 1	600354	624186	5.00e ⁻⁰⁷	1.00e ⁻⁰⁴	1.00e ⁺⁰⁰	1.00e ⁻³⁴	CT13648	–	–	Y17256

OMIM numbers and GenBank identifiers for each of the disease genes are included in the analysis. The BLAST e-value columns (<http://www.ncbi.nlm.nih.gov/BLAST>) have the e-value for the top BLAST match. The database identifiers are the top BLAST match and likely orthologues for the *Danio rerio*, *C.elegans*, *S.cerevisiae* and *Drosophila melanogaster* matches. The identifiers represent the protein IDs from the original sequencing centre and these identifiers can be accessed at CeleraScience. Adapted from Table 1 in ref. 89.

Table 2. Keyword search of zebrafish EST database

Neurological disease	OMIM no.	DNA sequence	GenBank identifier for the human protein sequence	EST match using keyword search of zebrafish EST database
Ceroid-lipofuscinosis (CLN2)	204500	NP_000382.3	572	Keyword = Ceroid; EST Match = fd57h10.y1 EST: 1 SNVFPMPDYQVDVAVRAYLKSQSLPPQTYFNTTGRAYPDLAALSDNYWVVSNRVPIPWVS 180 SNVFP P YQ +AV +L S LPP +YFN +GRAYPD+AALSD YWVVSNRVPIPWVS CLN2: 413 SNVFP RPSYQEEAVTKFLSSPHLPSSYFNASGRAYPDVAALSDGYWVVSNRVPIPWVS 472 EST:181 GTSASTPVVGGILSLINDQRFLKGLSRLGIHQ 279 GTSASTPV GGILSLIN+ R L G LGP CLN2: 473 GTSASTPVVGGILSLINEHRILSGRPPLGLNP 505 Score = 136 bits (339), Expect(2) = 6e ⁻³⁸ , Identities = 65/93 (69%), Positives = 71/93 (75%), Frame = +1
Machado-Joseph disease (MJD1)	109150	S75313	833928	Keyword = Machado; EST Match = fe14b03.y1 EST: 2 IFHEKQEGSLCAQHCLNNLLQGECCSFVELSSIAQQLDEEERMRM-AEGGVQTEEYRTFLQ 181 IFHEKQEGSLCAQHCLNNLLQGE S VELSSIA QLDEEERMRMAEGGV +E+YRTFLQ MJD: 4 IFHEKQEGSLCAQHCLNNLLQGEYFSPVELSSIAHQQLDEEERMR-MAEGGVTSSEYRTFLQ 63 EST: 182 QPSGNMDDSGFFSIQVINSALGVWGLEIVLNFNSREYHKLQMDP-MHEKAFICNYKEHWFTV 361 QPSGNMDDSGFFSIQVINSAL VWGLE++LNFNS EY +L++DP++E++FICNYKEHWFTV MJD: 64 QPSGNMDDSGFFSIQVINSALGVWGLELILFNFSPEYQRLRIDP-INERSFICNYKEHWFTV 123 EST: 362 RKLGGQWFNLSLLTGPELISDTYLALFLAQLQQEG 469 RKLGG+QWFNLSLLTGPELISDTYLALFLAQLQQEG MJD: 124 RKLGGQWFNLSLLTGPELISDTYLALFLAQLQQEG 159 Score = 285 bits (722), Expect = 2e ⁻⁷⁶ , Identities = 136/156 (87%), Positives = 148/156 (94%), Frame = +2

The keyword was used to search the zebrafish EST database at Washington University Zebrafish Genome Resources Project (http://www.genetics.wustl.edu/fish_lab/frank/cgi-bin/fish/home_est.html). A comparison of the protein sequences of two human neurological disease genes with the predicted protein sequences of two sequences from the Zebrafish EST database is shown.

isolation of zebrafish orthologues/analogues of human disease-causing genes. The next step in fully utilizing the potential of the zebrafish system is the development of robust methods for the directed manipulation of genes and their expression. First, we will consider transgenesis and then current novel approaches to achieve gene down-regulation and gene knock-outs.

TRANSGENIC STUDIES IN THE ZEBRAFISH

Methods developed for the introduction of DNA into zebrafish embryos have included microinjection, retroviral infection, the use of microprojectiles and electric field-mediated gene transfer (36). The microinjection of plasmid DNA has proven to be a reliable means of producing transgenic zebrafish (36) and remains the most widely employed method. Transgenic fish have been generated by the injection of both supercoiled and linearized plasmids. However, linear DNA is more likely to integrate stably into the genome (36). Following injection, linear plasmid DNA is generally rearranged into a head-to-tail concatamer and integrates at a single site (4,37–39). The rapid cell division in the zebrafish embryo favours integration of foreign DNA during later stages of development, resulting in a mosaic expression pattern in founder fish (40).

The transmission of foreign DNA through the germline is highly variable because integration occurs in only a proportion of cells. Although up to 40% of progeny can inherit the transgene, frequencies are usually <10% (36,41). In subsequent generations, however, foreign sequences are inherited in a Mendelian fashion (37). The low efficiency of this system in the initial stages is counterbalanced by the ease of screening if foreign sequences are fused with those encoding the green

fluorescent protein (GFP) (41). The advantages of this reporter gene are discussed below.

Transgenic zebrafish expressing hygromycin resistance were first described a decade ago (37). Subsequently, numerous lines expressing reporter genes under the control of both exogenous and endogenous promoters have been produced (36). A range of transformation methods has been utilized; however, the most common approach involves microinjection of plasmid DNA into the fertilized egg.

Naked DNA can be microinjected into the cytoplasm of single-cell stage embryos, at a rate of 200/h (38). Approximately 23% of these fish survive to sexual maturity (38). Germline founders are invariably mosaic, presumably due to the uneven distribution of DNA in the embryo (41). The transgene is transmitted to between 2 and 50% of the F₁ generation (4); however, subsequent generations display true Mendelian inheritance (38,41). This low level of transmission to F₁ limits the rate at which transgenic fish can be generated. However, when combined with a simple and rapid means of identifying transgenics, the zebrafish system becomes extremely efficient (42). Collas and Aleström (43) have reported the cytoplasmic injection of DNA–nuclear localization signal (NLS) complexes in zebrafish eggs as a means of enhancing the uptake of DNA by embryonic nuclei. These authors suggest that the complexes may increase the efficiency of germline transmission. The transparency of the zebrafish embryo can be exploited by using GFP as a reporter for transformation. This protein, derived from the cnidarian *Aequorea victoria*, can be used to screen live fish harmlessly and to study the distribution of fusion proteins (41,44–46). Our own unpublished work with fusion proteins encoded by CAG repeat expansions and the *GFP* gene and expressed from the presumptive zebrafish hunt-

ingtin gene promoter suggest that the modelling of HD in the zebrafish could be achieved using a fluorescent reporter approach for ease of screening.

The major application of transgenic technology in the zebrafish to date has been the analysis of promoter activity through reporter gene expression (36,45–49). This focus has led to the development of techniques for the modification of bacterial artificial chromosomes using homologous recombination (50), with subsequent transient expression in microinjected zebrafish embryos. The aim of these studies has been the identification of *cis*-acting regulatory elements for proper spatial and temporal gene expression patterns. Insertional mutagenesis in the zebrafish has also been achieved using retroviral vectors (51). This development complements chemical mutagenesis, and offers the advantage of improved cloning of mutant genes.

TARGETED MODELLING OF HUMAN DISEASE IN THE ZEBRAFISH

There has been little work that extends the zebrafish system to the deliberate and targeted modelling of human disease in the zebrafish. A useful approach to achieve this aim is to adopt techniques that have been reported in other modelling systems, in order that they may be replicated in the zebrafish. The tools that have been reported to date encompass those that fall into three broad classes. First, antisense and RNA interference, which bring about transient down-regulation of gene expression through translational inhibition. Secondly, triple-helix forming molecules, which affect transcription of the targeted gene, and, thirdly, the possibility of targeted mutagenesis of genes. These three classes are described below.

Antisense and RNA interference

Antisense oligonucleotides have been used widely for the study of gene function in cell culture systems and to a limited extent in *in vivo* applications (52–54). In contrast, only a small number of studies have documented the use of antisense technologies in the zebrafish (55,56). The success of antisense approaches in the zebrafish is predicated on a physiologically significant concentration of antisense oligonucleotide which needs to be delivered at the one cell stage of embryo development. Although the approaches to date have concentrated on transient effects that may be suitable for the study of development, long-term analysis may require a continuing non-toxic effect by the antisense oligonucleotide even when the embryo consists of many thousands of cells. Barabino *et al.* (55) circumvented this potential problem by bathing dechorionated zebrafish embryos in a solution of antisense oligonucleotide and demonstrated a concentration-dependent effect. This approach has many attractions; however, further work is needed to determine the level of oligonucleotide uptake and distribution. Recent developments in antisense oligonucleotide design have focused on the structure of the oligonucleotide backbone leading to an increase in binding stability and persistence *in vivo* (53,57,58). These types of development may significantly increase the usefulness of this technique in the zebrafish.

More recently RNA interference has gained significant attention as a technique for down-regulating the expression of

genes (59–62). RNA interference is a process by which introduced double-stranded RNA (dsRNA) that are homologous to a gene will strongly down-regulate the expression of that gene with an efficiency far beyond any possible antisense mechanism. This down-regulation may operate in a way that is similar to gene silencing that has been reported in plants (59,63,64). RNA interference has been shown to be effective in down-regulating the expression of genes in model systems like *C.elegans* (65–67) and *D.melanogaster* (68–70). Recently two groups have described the use of RNA interference in the zebrafish. Wargelius *et al.* (71) microinjected dsRNA directed against three genes of known function into zebrafish embryos. The embryos exhibited the same developmental defects as those observed in mutants of the same genes. In addition, Li *et al.* (72) obtained the down-regulation of GFP expression following the co-microinjection of a recombinant plasmid expressing GFP, together with dsRNA against the transcript of the GFP gene. These approaches raise important questions regarding the duration and extent of the effect of dsRNA and antisense oligonucleotides on gene expression in the developing zebrafish. The answers to these questions bear on the use of these approaches for disease modelling in the zebrafish. Although the modulation of gene expression early in development may suffice for the study of some genes, sustained reduction in expression will be necessary to model effectively the majority of inherited disorders. Recently, Tavernarakis *et al.* (73) demonstrated vectors expressing dsRNA encoded as an inverted repeat. This approach would allow animals that are transgenic for these constructs to achieve constant expression of specific dsRNAs, and thus continuous down-regulation of gene expression. An alternative approach for modelling diseases would be the targeted disruption or mutagenesis of the target gene. This approach should facilitate the production of stable mutant lines that would then greatly improve the analysis of mutational effects over the life span of the fish.

Targeting transcription and mutagenesis

Although simple in concept, the specific disruption and manipulation of genes in organisms has proved a difficult and demanding task. Producing knock-out mice using embryonic stem cells has become the paradigm for producing an organism deficient in one or more genes (74). The lack of embryonic stem cells for the zebrafish (75), requires a radically different approach to achieve whole organism gene targeting and disruption. In this context, significant progress has been made during the last 10 years in developing synthetic reagents that recognize and bind desired targets in dsDNA (76).

Two reagents, helix-invading peptide nucleic acids (PNAs) and triplex-forming oligonucleotides (TFOs), appear to be potent inhibitors of transcription and potential agents for site-specific gene mutagenesis. The action of these reagents is based on the targeting of double-stranded homopurine DNA, resulting in the formation of a triple helix structure. Structures that can inhibit transcription through steric hindrance of the transcription complex offer alternatives to antisense and RNA interference for knocking down the expression of genes. Lower concentrations of oligonucleotides would be required per cell in order to exert an effect via targeting the chromosomal copies of the gene, rather than mRNA copies. Several studies have

demonstrated specific reduction in gene expression via this method, but there are issues regarding binding stability and accessibility of genomic sequences within the chromatin structure that need to be addressed before this can be considered a viable alternative to targeting mRNA (76).

PNAs are DNA mimics in which the deoxyribose phosphate backbone is replaced by an uncharged polyamide backbone. This structure gives PNAs some unusual and interesting properties with many potential applications (77–79). PNAs can bind to DNA or RNA via Watson–Crick binding, with binding affinities significantly higher than those of the corresponding DNA oligomers, and they are nuclease and protease resistant. Homopyrimidine PNAs can bind to complementary sequences in duplex DNA by strand invasion resulting in the displacement of one DNA strand and the formation of a D-loop. Dimeric PNAs in which two PNA strands are connected by a flexible linker can form highly stable PNA–DNA–PNA triplexes at polypurine DNA sequences with extremely high melting temperatures. In these molecules, one strand forms Watson–Crick base pairs to the DNA strand in an anti-parallel orientation, whereas the other strand forms Hoogsteen base pairs to the PNA–DNA duplex in parallel orientation relative to the DNA strand in the duplex. The high binding affinity of PNAs for double-stranded DNA underscores its importance as an anti-gene agent. Several studies have demonstrated the blocking of transcription by PNA binding and subsequent steric hindrance of the transcription complex (80,81). A study by Faruqi *et al.* (82) demonstrated that PNA binding to duplex DNA could introduce mutations into a chromosomal gene in mouse cells. Although the frequency of introduced mutations was low this study illustrated the potential of PNAs as a gene targeting tool.

TFOs are based on more traditional phosphodiester or phosphorothioate backbones and form triple helices by binding to the major groove of double-helical DNA and forming Hoogsteen-type hydrogen bonds with purine bases of the Watson–Crick base pairs. TFOs have also been shown to interfere specifically with transcription, but do not offer the binding stability and thus the steric hindrance of PNAs. However, TFOs have recently received considerable attention for their potential in delivering DNA reactive agents such as psoralen to specific sequences in chromosomal DNA. Psoralen is a bifunctional photoreagent that introduces a covalent crosslink into the target sequence following irradiation at 365 nm. These crosslinks have been shown to be highly mutagenic in eukaryotic cells by inhibiting DNA repair mechanisms (83). Several groups have reported the successful use of TFO–psoralen conjugates in introducing mutations in mammalian cells (84–87) and yeast (88). Current work is aimed at increasing the binding stability and persistence of TFOs and thus maximize the potential of TFO–psoralen-based gene targeting in *in vitro* and *in vivo* systems.

CONCLUSION

The use of mutagenic screens and the isolation of zebrafish orthologues/analogues of human genes are providing excellent resources for the molecular dissection of normal and perturbed vertebrate development. Although the advances in physical mapping and indeed sequencing of the zebrafish genome will complement the above research, it remains necessary to adopt

strategies that will enable directed disease modelling to be achieved in the zebrafish. It is in this context that transgenesis and targeted mutagenesis protocols should be applied. The use of NLS-complexed DNA molecules and GFP reporter constructs may offer an enhanced means of achieving and screening for transgenic zebrafish. In addition, PNA- and TFO–psoralen-based gene disruption strategies appear ideal tools to achieve zebrafish models of human disease states. We therefore envisage that the use of transgenic and gene targeting technology in the zebrafish will open up an exciting new system for the modelling and analysis of disease, as well as provide a means of addressing the important area of functional genomics.

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