# Mutant and Transgenic Zebrafish in Modeling Neurobehavioral Disorders

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**Abstract**

Zebrafish have traditionally been used as effective genetic and developmental models in biomedical research. Recently, the scope and utility of zebrafish in biomedical research has been further expanded with the implementation of new genetic techniques aimed at developing translational models of human pathogenesis. Additionally, screens measuring specific neurobehavioral and developmental phenotypes have proven to be very robust. This chapter further discusses the utility of zebrafish in biomedical research and highlights some of the genetic techniques used in the creation of transgenic and mutant strains. Behavioral phenotypes of genetically altered zebrafish are also discussed with respect to both their robust stress responses and similarity to human disorders. Specific emphasis is placed on human brain pathogenesis and neurodevelopmental abnormalities, especially as they relate to stress and anxiety spectrum disorders.

**Key Words:** zebrafish, genetics, translational models

**The Utility of Zebrafish as a Model in Biomedical Research**

Biomedical research relies on animal models to elucidate the cellular and molecular basis of human pathogenesis in order to generate efficacious treatments. While many mammalian species have been utilized due their evolutionary proximity and homology to humans, zebrafish are quickly becoming a valuable model in many areas of research (Lieschke and Currie 2007).

Zebrafish (*danio rerio*) have been traditionally used to explore the genetic and physiological mechanisms involved in the functioning of a variety of systems, including the cardiovascular (Strainier 2001), immune (Sullivan and Kim 2008), and nervous systems (Pogoda and Hammerschmidt 2007). Additionally, the optical transparency of the zebrafish has been widely exploited in research on the effects of genetic manipulation on a system's (e.g., nervous, circulatory, endocrine) development and function (Dale et al 2009; Segner 2009). Ease of genetic manipulation, high fecundity, and quick development have made the zebrafish model a useful tool for screening and investigating the genetic factors involved in the disposition to and progression of pathogenesis (Lieschke and Currie 2007). The combination of transparent embryo development and the ease of embryological manipulations has fostered numerous advances in developmental biology, by allowing direct *in vivo* observation of cellular events (Lieschke and Currie 2007). These techniques (cloning, mutagenesis, transgenesis, and mapping approaches) when applied to zebrafish allow researchers to use invertebrate-style forward genetics on a vertebrate organism (Lieschke and Currie 2007). As a model of disease, zebrafish offer numerous advantages over mammalian models despite differences in anatomy. The ability to investigate the onset and progression of pathological processes in a suitable timeframe and *in vivo* is particularly notable. Contemporary research also demonstrates the robustness of behavioral phenotypes displayed by zebrafish in response to genetic manipulation and pharmacological treatment.

There are also drawbacks to the use of zebrafish in biomedical research. Zebrafish have developed a duplicate genome, although not all duplicated genes have been retained through time (Key and Devine 2003). Therefore, zebrafish possess two genes for many mouse orthologs, although the exact number of preserved duplicate genes remains unknown. It is frequently argued that further comprehension of zebrafish gene function will only uncover invalid redundant and species-specific information (Key and Devine 2003). However, duplicate genes can also provide significant advantages, particularly in relation to sub-functionalisation. In this technique, zebrafish co-orthologs represent selected expression patterns and developmental functions of mouse orthologs (Key and

Devine 2003). In this way, restricted expression of zebrafish genes, in comparison to the corresponding mouse orthologs, may lead to an improved comprehension of developmental relations in cell lineage or tissue patterning in mice (Key and Devine 2003).

**Zebrafish as Models of Brain Disorders**

A zebrafish experimental model of stress and anxiety has recently been developed to investigate the physiological mechanisms and neural biomarkers associated with anxiety-like phenotypes. When the zebrafish is exposed to a novel environment, it dives to the bottom and gradually explores as it habituates to the test apparatus. Inhibited exploratory movement, reduced speed, and increased frequency of escape behaviors are associated with higher levels of anxiety elicited by stressors (e.g., novel environment, predator exposure) (Bass and Gerlai 2008; Levin et al 2007; Speedie and Gerlai 2008). These behaviors are highly sensitive to pharmacological treatment, as exploratory behavior is markedly elevated in response to treatment with anxiolytic drug, including benzodiazepines, SSRIs, nicotine, and ethanol (Airhart et al 2007; Gerlai et al 2008; Gerlai et al 2006; Levin et al 2007). Conversely, chronic stressful stimuli (e.g., predator exposure, alarm pheromone), anxiogenic drugs, and drug withdrawal have been shown to increase anxiety-like behavior in this paradigm, as zebrafish show latency to explore the upper layer of the novel environment, spend significantly less time exploring, exhibit more erratic movements, and freeze often (Baraban et al 2005; Gerlai et al 2008; Speedie and Gerlai 2008). This simple yet high throughput test can be used as an approach in quickly and accurately identifying the biomarkers linked to a disorder and in screening the efficacy of different pharmacological treatments.

Genomic synteny and resulting cellular morphology and neurophysiology are largely conserved in zebrafish (Detrich and Yergeau 2004), supporting the translatability of data to advance research on psychiatric disorders in humans. Transgenic zebrafish show much promise for developing high throughput animal models of complex brain disorders, such as Huntington's, Alzheimer's, and possibly schizophrenia (Diekmann et al 2009; Lee and Cole 2007; Wood et al 2009). The common application of transgenic zebrafish models has been as a screening assay to identify the genetic and physiological mechanisms involved in brain disorders; however, development of behavioral models may provide further insight towards the diagnosis and pharmacological treatment of these disorders. Research using the zebrafish model of stress and anxiety must incorporate transgenic zebrafish models of brain disorders, to correctly assess the genetic factors that precipitate abnormal neurobiological, physiological, and behavioral phenotypes.

**The Generation of a Transgenic or Mutant Zebrafish**

The most frequently utilized methods of transgenic line formation include plasmid DNA or bacterial artificial chromosome (BAC) injections into the cytoplasms of one-cell stage embryos (Amsterdam and Becker 2005). However, these procedures yield germline transgenics with just 5-20% efficiency (Amsterdam and Becker 2005). Therefore, transposon-mediated systems are becoming increasingly popular, due to their higher approximated efficiency of 50-80% (Lieschke and Currie 2007). This method is rapid, effective, and produces lines that facilitate genetic screening. Other advantages of transposon-mediated transgenesis include vectors that are more simply engineered than in other methods, and the potential for the remobilization of existing insertions to generate new insertions (Amsterdam and Becker 2005).

Transposon-mediated transgenesis relies on transposon vectors, which use the *cis*-acting elements from naturally occurring transposons in juxtaposition with the gene generally encoded by the transposon in *trans* (Amsterdam and Becker 2005). It is this gene that encodes transposase, the catalyzing enzyme in both the excision of the vector from plasmid, and the assimilation into the host genome (Amsterdam and Becker 2005). To express a gene of interest or create a gene trap, researchers may engineer specific sequences into the vector rather than utilize the transposase gene (Amsterdam and Becker 2005). Plasmids containing the transposon vector and the mRNA, which encodes the transposase, are coinjected into embryos at the one-cell stage (Amsterdam and Becker 2005). The injected embryos will be mosaic for integration events, due to rapid divisions during the early stages of zebrafish development.

Although transgenic zebrafish may be created through the use of transposons, or the injection of plasmid DNA or pseudotyped retroviruses, retroviruses are the only efficient insertional elements in the generation of mutant zebrafish (Amsterdam and Becker 2005). This insertional mutagenesis allows for the mutated gene to be easily identified with the help of a molecular tag at the mutagenic lesion site (Amsterdam and Becker 2005). Sequencing of DNA flanking the insertion has been shown to be sufficient in the identification of the mutant gene in roughly 80% of mutants (Amsterdam and Becker 2005).

Retroviral-mediated insertional mutagenesis requires the injection of a virus into embryos between the 512 and 2,000-cell stage (Amsterdam and Becker 2005). These embryos will then grow

into founder fish (F0), which have mosaic germ lines and upwards of 25-30 different insertions (Amsterdam). Quality control assays are typically performed on viral stocks and founder injections before breeding founders. F1 fish, however, are not mosaic, and transfer their insertions in a Mendelian manner (Amsterdam and Becker 2005). When F1 pools reach 6 weeks of age, tail DNA can be isolated from 30 fish in order to identify subjects with the most insertions. F1 zebrafish with the most insertions may then be crossed with other multiple insertion fish to produce an F2 pool. At minimum, 6 crosses are required to screen at the F2 level, resulting in almost every insertion being homozygosed in at least one of the crosses (Amsterdam and Becker 2005).

In addition to retroviral methods, chemical mutagens may also create random mutagenesis. Zebrafish mutants can be generated through the exposure to ethylnitrosourea (ENU), a popular mutagen that typically produces point mutations (Lieschke and Currie 2007). Due to the relative resistance of zebrafish to ENU toxicity, higher levels of mutagenesis can be achieved and defined loci can be affected with greater accuracy than in other vertebrates (Lieschke and Currie 2007). Chemical mutagenesis generally supersedes retroviral mutagenesis in efficiency.

**Genetically modified zebrafish express marked behavioral phenotypes**

Although zebrafish models of stress and anxiety have only recently been developed, research focusing on the effects of genetic manipulation on behavioral phenotypes is being conducted in conjunction with physiological analyses. Using forward and reverse genetic engineering, transgenic zebrafish strains have been established to model brain disorders and to screen the genetic factors that induce or intensify pathogenesis (Amsterdam and Becker 2005; Zon and Peterson 2005). Typically, zebrafish models are used to analyze the physiological mechanisms disrupted by genetic mutation that lead to neurobiological morphology relevant to psychiatric disorders. However, recent research has also assessed the behavioral phenotypes associated with these neurological abnormalities.

Several transgenic zebrafish exhibit robust aberrant behavioral phenotypes linked to the knockout of specific target genes. For example, *nevermind* (nev) gene mutant zebrafish display severe disruption of optic nerve innervation (Solnica-Krezel 1999). While muscle morphology is normal, *nev* dorsal retinotectal axon projections terminate on both the dorsal and ventral side of the tectum, resulting in atypical swimming phenotypes (Patton and Zon 2001). Specifically, these mutants display a corkscrew swimming pattern, in which they rotate around their long body axis (Patton and Zon 2001). Similarly, sphingosylphosphorylcholine (SPC, a lipid mediator) KO zebrafish perform

spontaneous erratic movements and escape behaviors (e.g., rapid turning) without provocation from stressful stimuli (Patton and Zon 2001).

Many of the transgenic zebrafish models in current use focus on abnormal developmental patterns that prevent proper innervations between nuclei and in turn disrupt neurophysiology. One example of this is the mutation of the Lhx2 homolog, *bel*, a transcription factor involved in retinotectal axonal growth. The optic chiasm does not form in these zebrafish mutants resulting in a robust phenotype that simulates a related human disorder, non-decussating retinal-fugal fiber syndrome (NDRFF). In zebrafish, achiasmatic-induced oculomotor deficits generate spontaneous eye oscillations that may model congenital nystagmus in humans, in addition to causing reversed perception of visual stimuli, misappropriated eye movements, and circling swimming behavior. Research utilizing this strain may help investigate developmental factors and possible treatments in patients suffering oculomotor and visual impairment (Huang et al 2006).

Vertebrate optokinetic nystagmus (OKN) is a compensatory oculomotor behavior that is evoked by movement of the visual environment. Its primary function is to stabilize visual images on the retina. It is possible to evoke this response from many vertebrate species, including zebrafish, in an experimental environment. This enables for the screening of mutations affecting visual system development. Rick and collegues (2000) have shown that belladonna (bel) mutant larvae exhibit abnormal functioning of the OKN with about 40% displaying reversal of the OKN upon experimentally induced visual stimulation. The behavioral deficit observed in OKN function is caused by defective, non functional optic chiasm formation in the achiasmatic mutants displaying a reversal of OKN (bel mutants). This study highlights how a forward genetic approach can be used in the zebrafish to uncover very specific behavioral deficits. Further, the analysis of behavioral mutations in combination with neuroanatomical analysis links behavioral abnormalities with their underlying structural deficits. This combination approach of analyzing neural circuits by genetic means allows researchers to establish structure–function relationships of morphological defects and elucidate the resulting behavioral consequences (Rick et al 2000).

Studies employing genetic classification in conjunction with behavioral analysis have been used to further examine defects in the visual system of zebrafish. In particular, the problems of retinal degeneration and abnormal visual system development have been examined. Behavioral assays, along with morphological, electrophysiological, cellular, and molecular methods have proven to be powerful tools for examining this complex system (Li 2001). Advances have been made in regards to the

isolation and classification of zebrafish with visual system deficits (Brockerhoff et al 1995). Indeed, behavioral screens have been developed with the ability to isolate specific disturbances in the visual processing system of zebrafish (Brockerhoff et al 1998). Advances in our understanding of disorders related to visual system development and visual system processing in zebrafish may lead to novel treatments for human visual system disorders, and highlight the translatability of zebrafish as genetic models of human pathogenesis.

In another transgenic model, *nacre* zebrafish that lack melanophore stripes were used in conjunction with wild-type zebrafish to examine the effects of altered morphology on social behavior and mating (Engeszer et al 2007). Analyzing the distance between subjects within a shoal and the preference to swim near morphologically similar zebrafish showed no selectivity in regard to visual characteristics. Despite the fact that shoaling behavior in other fish is influenced by body color, pattern, and size of shoal-mates, the absence of melanophore development had no effect on the shoaling behavior of zebrafish (Lister et al 1999), supporting that morphological alterations used in physiological and developmental analysis do no confound behavioral responses in zebrafish.

In a similar experiment, mutant strains of zebrafish possessing either truncated (*short-fin*) or elongated (*long-fin,* Fig. 1) fin genes were generated and social/sexual interactions were observed. While there was no preference of the WT or *short-fin* female zebrafish, *long-fin* female zebrafish showed increased social interaction with *long-fin* over WT male zebrafish. Since female zebrafish are reported to select mates based on body size rather than fin size, and because the *short-fin* mutant showed no preference, the *long-fin* mutation is thought to be involved in social perception (Gumm et al 2009).

Additionally, a zebrafish model of Huntington's disease (HD) was generated through a reverse genetics technique, using a morpholino (antisense morpholino-modified oligonucleuotides) to inhibit translation of the protein huntingtin (Diekmann et al 2009; Nasevicius and Ekker 2000). The huntingtin (Htt) *knockdown* zebrafish displayed abnormal physiological phenotypes associated with HD in humans, including reduced expression of brain-derived neurotrophic factor (BDNF), widespread neuronal apoptosis, and other developmental disruptions linked to HD. Unfortunately, no behavioral testing was performed in this experiment; however, a spatial alternation screen using reward stimuli as described by Williams and colleagues (Williams et al 2002) could illuminate the learning and memory deficits associated with HD. In addition, assessing anhedonia, motor

coordination, and anxiety in Htt *knockdown* zebrafish may help in fully defining a zebrafish transgenic model of HD.

Expression patterns in genes related to deafness are beginning to provide a basis for understanding the roles of various cell types within the cochlea and are bringing to light the causes of both syndromic and non-syndromic forms of deafness (Ernest et al 2000). Recently a large scale mutagenesis screen in zebrafish identified 8 genes that are essential for proper balance and hearing (Ernest et al 2000). Zebrafish have proven to be effective models with respect to studying the molecular basis of development and function of sensory hair cells (Ernest et al 2000). Importantly, anatomical studies have shown that the structure of the vestibular inner ear is highly conserved between teleost fish and higher animals. Additionally, fish sensory hair cells are morphologically and physiologically similar to hair cells in higher vertebrates (Ernest et al 2000). As such, zebrafish are emerging as a novel translational model for examining the pathogenesis that occurs as a result of mutations in deafness genes (Ernest et al 2000).

Zebrafish are also proving useful as genetic models to examine the complex processes involved in the development of addiction, which is a principle health issue throughout the world. The use of forward genetic manipulation in conjunction with a selective behavioral screen for the classification of cocaine sensitivity in mutant zebrafish has helped to identify candidate genes responsible for sensitivity to addiction. In the highlighted experiment a cocaine-induced conditioned place preference developed in a sensitivity-dependent manner. Sensitivity to cocaine, and the resulting behavioral phenotypes observed in the conditioned place preference paradigm, was shown to be dependent on dominant mutations in single genes (Darland and Dowling 2001). Indeed, certain mutagenized zebrafish exhibited abnormally low responses to cocaine. Performance profiles of these zebrafish in additional behavioral screens measuring visual adaptation and learning suggested that their low response to cocaine was the result of mutations in genes that affect dopaminergic signaling in both the retina and the brain (Darland and Dowling 2001). This forward genetics approach in which the genome is mutagenized, resulting phenotypes characterized through behavioral screening, and underlying genes classified and cloned using a vertebrate species represents a valuable tool to advance research. Zebrafish in particular are an idea choice for forward genetic manipulation due to their ability to reproduce quickly, and their low maintenance cost (Darland and Dowling 2001). These benefits along with the fact that zebrafish lend themselves well to behavioral observation paradigms further expands their role as translational models in biomedical research.

## Conclusion

Zebrafish represent effective models for translational research, spanning the developmental, genetic, and behavioral level. Their utility within the field of biomedical research is unquestionable. However, in order to fully realize their scope of impact researchers must combine approaches in order to create effective models that exhibit translatable validity towards the treatment of human pathogenesis. The development of new genetic techniques and their application towards the creation of mutant and transgenic lines of zebrafish, merged with proper behavioral phenotype classification of genetically modified and mutant zebrafish, will allow researchers to parallel human disorders with much greater accuracy. These developments will lead to a better understanding of the complex nature of neurobehavioral and neurodevelopmental disorders. Through the combined efforts of biomedical researchers in all areas of research the development of novel, effective treatments for neurobehavioral and neurodevelopmental disorders will be achieved.

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**Figure 1. Wild type (top) and long-fin mutant (bottom) zebrafish.**