

Not Just a Fishing Trip – Environmental Genomics Using Zebrafish

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Abstract: Genetic diversity is the raw material needed by a species allowing adaptation to changing environmental conditions and thus ensuring long-term sustainability. The development of technologies for environmental genomics provides us with the opportunity to link information, at the whole genome level, with the response of an organism to its natural environment.

Over the past 15 years a small tropical fish native to the rivers of India and south Asia, the zebrafish (*Danio rerio*), has become one of the most popular vertebrate model systems. Zebrafish are abundant and many populations exist that are reproductively isolated. They evolved under distinct environments, and this has led to genetic diversity and, as a consequence, has created genotypic and phenotypic differences between the populations. For this fish species, a large number of molecular and genomic tools have been developed. As a result, the zebrafish has emerged as a popular model for the study of embryonic development and genetics as well as the study of human disease counterparts. The advantages that zebrafish possess, in addition to newly developed large scale screening assays, such as automated *in situ* hybridization and transgenics for example, has led to researchers using zebrafish to study toxicogenomics and environmental genomics. Researchers have identified molecular and biochemical pathways, which may not have been observable using standard methods, that are disrupted by some toxin exposures and environmental stressors. These studies will allow us to potentially formulate specific predictions on how vertebrate organisms and populations may be affected by both man-made and natural changes in the environment.

Key Words: Zebrafish, genomics, environment, toxicogenomics, development, physiology, genetic diversity.

INTRODUCTION

The efficiencies of scale utilized for DNA sequencing projects, genome-scale DNA sequence, and ESTs information are becoming available for a number of model organisms including zebrafish. This information has facilitated the development of resources, such as DNA microarrays, that allow us to simultaneously assay the expression of thousands of genes, and Single Nucleotide Polymorphism (SNP) chips to analyze genetic polymorphisms in expressed genes. These genomic tools have now entered mainstream science. This has spawned a number of fields resulting in collaborations between individuals from diverse biological areas. One such field is environmental genomics.

In its simplest form, the aim of environmental genomic study is to predict how an organism will respond to and how it is shaped at the genomic level by its external environment. Thus, environmental genomics integrates genetics, physiology, toxicology, ecology, and systems biology to study the relationship between genetic diversity and the environment. Sub-disciplines, such as ecological genomics, attempt to define the complement of genomes (termed a biome) that make up a given environment [1, 2]. At present, these studies are limited to bacterial ecosystems. Undoubtedly, as technologies advance, these studies should be able to encompass a

large representation of any particular biota [3, 4]. Physio-genomics studies dynamics in gene expression that occur under different physiological or pathological stimuli. A rapidly advancing discipline called toxicogenomics is defined as the study of how genomes respond to natural and man-made toxins or environmental stressors such as heat, cold, famine, desiccation, pH and salinity.

The goal of toxicogenomics is to identify the biological targets of toxic substances and to investigate the biochemical mechanisms that underlie the pathological effects of toxins [5, 6]. Comparative studies of large numbers of toxins have the potential to uncover common and unique genomic responses between and within toxin classes. SNPs can be utilized to identify variants in toxin responsive genes or to identify polymorphisms in genes that confer sensitivity to particular toxins.

Even though human cell culture systems and tissue samples can be utilized for many aspects relevant to the environmental genomics of human health, most progress is likely to come from vertebrate model systems. Therefore, the choice of which model to be used will be made based on its relevancy to the assay at hand and, in many cases, the availability of genomic resources.

THE ZEBRAFISH

Fish are the oldest, largest and most diverse group of vertebrates. They originated approximately 500 million years ago and today represent morphologically diverse animals that inhabit a variety of ecosystems [7]. Specialized

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adaptations allow fish to thrive in environments that differ significantly in terms of oxygen tension, temperature, salinity, atmospheric pressure, light intensity, predator density, and chemical concentrations, including relatively high concentrations of man-made chemicals.

The zebrafish (*Danio rerio*) recently emerged as an asset for developmental biology and genetic studies, as well as for studies of human diseases. Researchers wanting to use a vertebrate model that had utilitarian characteristics similar to invertebrate models like the fruit fly and worm began using zebrafish [8]. The zebrafish is a desirable model for its small size, simple husbandry, and short generation time (2-3 months). As well, large quantities of optically clear embryos can be readily obtained. Zebrafish embryos develop externally, thus permitting the observation of tissue and organ development, an advantage that mammalian models lack [9, 10].

Zebrafish organs have been shown to be functionally and morphologically similar to human organs. In fact, zebrafish and humans share the same tissues (except lungs) and can be afflicted with similar diseases. Like humans, zebrafish undergo complex processes of development, such as the production of definitive hematopoietic cells and the formation of a multi-chambered heart [11-14]. Many researchers have combed the zebrafish genome to find homologs of human genes. Direct modeling from zebrafish to humans is made easier through the isolation of zebrafish genes that are analogous in function to previously identified human genes [10]. Large segments of zebrafish chromosomes are syntenic with those of the human and mouse genomes, and many genes have been demonstrated to have a high degree of sequence homology [15]. Comparative analysis of syntenic relationships between zebrafish and mammalian chromosomes has added to our understanding of chromosomal evolution in fish, mammalian and vertebrate lineages and aided in the cloning of homologous genes [16]. The discovery of genetic conservation between zebrafish and humans allows researchers to study mutant zebrafish models and apply their findings to the human disorder counterparts. However, bridging the gap between fish and humans is still complicated. One complication is the fact that fish, including zebrafish, have undergone gene duplication and subsequent diversion of gene expression patterns and functions [125, 126]. As a result, mutation or molecular targeting of one paralog may not produce a predictable phenotype due to phenotypic rescue or modification by the other paralogs. Despite this complication, and our relatively poor understanding of fish pathology and disease, investigators have been able to genetically manipulate the zebrafish and use it to model human diseases.

Genetic manipulations, such as gene knockdowns and the production of transgenics, can be easily performed on zebrafish embryos. The zebrafish is an ideal and low cost organism to develop transgenic systems. Several methods have been successfully used for the introduction of foreign DNA into zebrafish embryos: microinjection [75], electroporation of fertilized eggs [76], particle gun bombardment [77], liposome-mediated gene transfer [78] and sperm-mediated gene transfer [79, 80]. All of these techniques have been used successfully to create transgenic zebrafish. Of

these techniques, microinjection yields the highest survival rate and is the preferred method. Knockdowns are produced by the microinjection of anti-sense morpholino based oligonucleotides into fertilized eggs (<http://www.genetools.com>). These oligonucleotide constructs are designed to target and bind the translational start site of specific mRNA species. As a result, these targeted RNAs are unable to load onto the ribosome and are not translated into protein. This produces zebrafish embryos that are deficient in specific proteins. This technology can therefore be utilized to model human developmental diseases. In humans, a mutation in the gene *tbx5* results in Holt-Oram syndrome and perturbs heart and forelimb development. A zebrafish line that is mutant for *tbx5*, called *heartstring* display heart and fin defects. The functionality of the *tbx5* gene in zebrafish was confirmed by microinjection of a morpholino antisense oligonucleotide (*tbx5*-MO) into one-cell stage zebrafish embryos [17]. This antisense oligo binds to the translational start site of the *tbx5* mRNA and prevents its translation into protein. They observed that the morphant embryos displayed a progression of heart and pectoral fin defects indistinguishable from those found in *heartstring* mutant embryos and similar to those seen in individuals with Holt-Oram Syndrome.

The phenotypes of many other mutant zebrafish do resemble those of common human disorders. Many zebrafish lines exist with mutations that affect the development of the heart, vasculature, CNS, eye, limbs, and kidneys. There are zebrafish cardiac mutants called *gridlock*, which display a phenotype closely resembling the human condition of coarctation of the aorta. Some zebrafish kidney mutants also have cystic kidneys that are very similar to polycystic kidney disease in humans [8]. Additionally, two zebrafish mutants called *jumbo* and *chihuahua* have been isolated [18]. These two mutant strains have the potential to be models for human obesity and *osteogenesis imperfecta*, respectively. A Huntington's disease gene homologue has been isolated from zebrafish and investigated for its potential function in early vertebrate development [19]. Additionally, Leimer *et al.* utilized the zebrafish to clone genes known in humans to cause familial Alzheimer's disease [20].

Leroy *et al.* previously reported the identification of two siblings with a strong family history of Parkinson's Disease (PD) [21]. Both have a point mutation in the gene encoding ubiquitin carboxylase hydrolase L1 (UCH-L1). Son *et al.* wanted to determine if there was a zebrafish homologue of the UCH-L1 and if zebrafish can be used to study neurodegenerative diseases, such as PD [22]. They were able to clone and analyze the expression pattern of the zebrafish *uch-L1* gene. It was determined that this gene is expressed in the ventral diencephalon, which is functionally homologous to the substantia nigra region in humans. This is the region where dopaminergic neurons degenerate, which leads to PD. The same group is currently trying to generate germ-line transgenic zebrafish that contain the dominant mutation in *uch-L1*, the same mutation found in humans with PD. The research carried out by these, and other scientists, lends further support to the relevance of zebrafish models as a tool for studying a variety of human pathological conditions.

Due to the increased use of zebrafish as a model system, the National Institutes of Health (NIH) have set aside the

resources needed to further develop the zebrafish as a model system for embryogenesis, disease and to characterize its genome. In 1992 the number of NIH funded zebrafish research projects was less than 25, and in 2002 that number had increased to over 200 research projects. In 1997, the NIH began funding six zebrafish genomics projects that were designed to generate several types of genetic maps. These six projects have surpassed their original goals by developing a high-resolution genetic map, a physical (Radiation Hybrid) map, and a large number of ESTs. These resources are described in Table 1. Mark Fishman (Harvard) and Will Talbot (Stanford) have generated complementary genetic maps. The Fishman map was created through a cross of the AB and India strains of zebrafish and it contains Simple Sequence Length Polymorphism (SSLP) markers. The Talbot map was made using heat-shocked homozygous diploid embryos and it contains almost 4,000 gene, EST, and

SSLP markers. These two maps are cross-referenced since many of the same markers have been mapped on both, as well as ESTs and other genes [23].

In addition to genetic maps, there are many other resources available to zebrafish researchers. The Zebrafish International Resource Center (ZIRC, http://zfin.org/zf_info/stckctr/stckctr.html) located at the University of Oregon in Eugene, Oregon houses and maintains a large and growing list of wildtype, mutant and transgenic fish lines and distributes them to the scientific community (http://zfin.org/zf_info/stckctr/stckctr.html). The Zebrafish Information Network (ZFIN, <http://zfin.org>) is an online resource for the dissemination of integrated genetic, genomic, and developmental information. They also maintain definitive data reference sets of zebrafish research information. These two resources will continue to be important for the zebrafish community.

Table 1.

RESOURCE	FUNCTION	WEBSITE	REF.
T51 radiation hybrid map	Mapping, linkage group assignment, has 10,601 markers	Robert Geisler (Tuebingen), http://www.map.tuebingen.mpg.de/ Additional T51 mapping data was provided by the Children's Hospital Zebrafish Genome Initiative, http://134.174.23.167/zonrhmapper/	[27, 28]
LN54 radiation hybrid map	Mapping, linkage group assignment	http://dir.nichd.nih.gov/lmg/lmgdevb.htm	[29]
Heat Shock Diploid Cross (Talbot) map	Mapping, linkage group assignment, contains 4,199 markers	Will Talbot (Stanford), http://zebrafish.stanford.edu/genome/HeatShock99	[30]
Boston MGH Cross (Fishman) map	Mapping, linkage group assignment, contains 4,204 Simple Sequence Length Polymorphism (SSLP) markers	Marc Fishman (Harvard), http://zebrafish.mgh.harvard.edu/	[31, 32]
Gates et al. (GAT) Haploid Panel	Mapping, linkage group assignment, contains 389 markers	Will Talbot (Stanford), http://zebrafish.stanford.edu/genome/Gates99_GR/	[33]
Mother of Pearl (MOP)	Contains 556 markers with a framework of 211 random amplified polymorphic DNA (RAPD) markers and 122 simple sequence-length polymorphism (SSLP) microsatellite markers.	John Postlethwait, (University of Oregon), http://www.neuro.uoregon.edu/ionmain/htdocs/faculty/posthleth.html	[34]
SNP (variation)	Genetic linkage (meiotic) map, single nucleotide polymorphism (SNPs) mapped to the Heat Shock panel.	Will Talbot (Stanford), http://zebrafish.stanford.edu/genome/	
EST sequences in Genbank	More than 207,353 zebrafish EST sequences available	http://www.ncbi.nlm.nih.gov/	
Zebrafish Genome Resources	Incorporates information on NCBI and Community zebrafish-related resources (sequence, mapping, and clone information)	http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish/index.html	
Zebrafish International Resource Center	zebrafish strains (wildtype & lines carrying mutations)	http://zfin.org/zf_info/stckctr/stckctr.html	
Zebrafish Gene Collection (ZGC)	Contains 1,153 unique genes represented by full length clones	http://zgc.nci.nih.gov/	
Zebrafish Information Network (ZFIN)	zebrafish model organism database, contains at least 11,984 named genes	http://zfin.org	

During the Sanger Institute Zebrafish Workshop in 2000, the Zebrafish Genome Project was announced. It was to begin in February 2001, a collaboration between the Sanger Institute and the Zebrafish Community. By November 2003 the version 3 assembly of the zebrafish whole genome shotgun was released (http://www.ensembl.org/Danio_rerio/). The assembly comprises a total sequence length of 1,459,522,189 bp in 58,336 supercontigs. This is a preliminary draft and contains a high level of misassembly. Despite the quality of the preliminary draft, there is an enormous amount of useful sequence information available.

Researchers have begun to study gene expression changes using zebrafish DNA microarrays. Lo *et al.* tested the usefulness of a microarray, containing 11,480 EST sequences from the Z1 library, to investigate gene expression during zebrafish embryogenesis [24]. These EST sequences represent 3100 unique cDNA clusters. Ton *et al.* used a zebrafish cDNA microarray containing 4512 unique genes from embryonic and adult hearts, and skeletal muscle cDNA libraries [25]. They wanted to study gene expression changes and adaptive molecular responses to hypoxia during development. SNP chips that will be used to evaluate polymorphisms in expressed genes are currently being developed to complement cDNA and oligo microarrays [26].

Commercial microarray chips are also available from MWG Biotech, Compugen, Qiagen and Affymetrix. MWG Biotech (<http://www.mwg-biotech.com/>) offers a 14k array comprised of at least 14067 zebrafish genes (50 mer) spotted onto two arrays. The oligonucleotides were calculated using 1800 zebrafish gene sequences from the NCBI and a database of 12768 putative open reading frames (ORF). The ORFs were constructed in-house using NCBI zebrafish EST sequence information. Compugen/Sigma-Genosys (<http://www.cgen.com/>; <http://www.genosys.co.uk/>) offer a 16K zebrafish oligonucleotide library array representing 16,288 unique gene clusters (65 mer). Qiagen (<http://www1.qiagen.com/default.aspx>) offers The Zebrafish Genome Oligo Set Version 1.0, which contains 3,479 (70 mer) probes representing 3,479 zebrafish genes. Finally, Affymetrix has constructed a GeneChip to detect 14,900 genes and was designed with 16 oligonucleotide pairs to detect each transcript.

With the recent completion of the human genome sequencing project, we are now faced with the daunting task of assigning function to the approximately 35,000 genes found within this genome. Functional information can be obtained from human genetic studies, disease causing genes and even bioinformatic analysis. Most studies of gene function are conducted using various animal model systems, such as zebrafish.

BIOLOGICAL VARIATION

In addition to the genomic resources available, zebrafish possess a number of characteristics that make it a good model for environmental genomics and for studying how genetic polymorphism may affect behavior, physiology, and morphology.

Wild populations of zebrafish exist across a wide geographic area. Native populations are found in south and south East Asian countries including Pakistan, Bangladesh,

Myanmar and Bhutan [35-38, 120]. The most surveyed populations are from the Indian subcontinent where they are found in the tributaries of the Ganga and Brahmaputra Rivers, streams of Bengal and the eastern Coromandel Coast, and commonly in rice fields [39-41]. In addition, introduced populations have been found in California, Florida, New Mexico and Columbia [42-46]. At present it is uncertain if these American populations are reproducing; however basic environmental parameters in these areas should make this possible. These expatriate populations of zebrafish offer an interesting opportunity to test how an organism may evolve after being displaced into completely different environmental conditions.

Given the widespread distribution of zebrafish, it is not surprising that there is considerable variation in gene loci and phenotype at both inter and intra-population levels. Wright *et al.* analyzed variations in shoaling and boldness in laboratory-reared F1 offspring of 4 different populations of wild zebrafish [47]. Experiments indicated a genetic component to shoaling behavior and that the 4 populations have genetically based differences in boldness. In addition, the wild zebrafish strain, Nadia, displays different shoaling behaviors and predator responses compared to a more domesticated strain called TM1 [48]. Compared to laboratory strains, the Nadia strain displays enhanced sexual dimorphism, faster growth rates, and spends more time swimming near the water's surface (Barrie Robinson, University of Idaho, personal communication).

Life history data collected on two populations of zebrafish indicate that there can exist significant differences in the mean and maximum life spans between the two groups despite being raised under identical conditions [49]. Skeletal abnormalities and loss of muscle that is so often observed in aging human populations were also observed in senescent zebrafish and, thus, zebrafish may be utilized as a model for human aging conditions, such as sarcopenia [50].

Genetic polymorphisms have been well characterized in wild and laboratory raised strains. The Darjeeling strain that was collected from the wild differs genetically from the domesticated Oregon AB stocks [51]. Genetic polymorphisms have been evaluated in five different zebrafish strains (C32, SJD, AB, WIK and Florida wild-type) by CA-repeat markers and SSCP polymorphisms [52], in addition to using RAPD primers [51]. Finally, wild caught zebrafish populations have been assessed for the presence of recessive mutations that affect embryonic development [53]. It was demonstrated that there is a high effective population size in wild zebrafish groups sampled. Within these zebrafish populations recessive mutations are rare but can affect nearly all aspects of development.

Thus, native zebrafish populations, expatriate populations and isolated laboratory strains offer opportunities to study how the environment can shape the genome of an organism and how genetic variation can influence an organisms response to environmental stimuli.

TOXICOGENOMICS

In 1920, H. Winkler coined the term "genome" to describe the complete set of human chromosomes and their

genes [54]. In 1996 the term “genomics” came into existence for the discipline that studies chromosomes and their genes [68]. The National Institute of Environmental Health Sciences (NIEHS) coined the phrase “environmental genomics” with the announcement of the Environmental Genome Project (EGP) in 1997 [55-57]. The EGP investigates how genetic variation affects response to environmental exposures and represents the second generation of the Human Genome Project [58]. In September 2000 the NIEHS formed the National Centre for Toxicogenomics (NCT, <http://www.niehs.nih.gov/nct/home.htm>). The center facilitates the application of genomics and proteomics to the study of environmentally induced diseases and coordinates the nationwide research into this newly emerging field. The NCT coordinates a nationwide research effort for the development of a toxicogenomics knowledge base.

Typical toxicological studies focus on determining the effects of environmental toxicant exposure on the health of the exposed animal or human. These toxicological experiments would use physical parameters, such as body weight, organ weight, blood pressure, activity level, histological features of tissue samples or blood chemistry indicators to determine any adverse effects of toxin exposure. Similar assays have also been developed for zebrafish embryos [124]. The effects of both short term and long-term exposure of zebrafish to a wide range of toxins including carcinogenic agents were carried out with relative ease. Since the late 1970's the accumulation and effects of a number of chemicals have been tested on the zebrafish [59, 60]. Compounds tested include zinc, cadmium, selenium, mercury, copper, nickel, iron, cobalt, lead, chromium, aluminum, as well as organics such as phenol, aniline, cyclohexane and their derivatives [61]. The toxic properties of complex chemical mixtures [62], the bioaccumulations of certain chemicals [63], and wastewater and effluent from industrial sources have all been assayed for toxic properties using zebrafish [64].

Although these methods of study yielded sufficient diagnostic indicators of health, they do not shed any light on the molecular mechanisms of the toxicity or the pathogenesis and progression of the disease state induced by the toxicity. As well, typical toxicological studies are usually not sensitive enough to detect the effects of low-level toxicity that may affect the individual at the molecular level, nor are they useful for determining the early pre-clinical stages of the disease. Therefore, it is essential to have methods available to determine, understand and prevent the effects of toxicity and disease pathology at the molecular level.

Today, researchers have developed a new area of study—toxicogenomics. Toxicogenomics is the study of the effects of environmental stressors and toxicant on the genome. This area of research combines the fields of molecular biology and toxicology with the study of chemical and physical interactions and their role in diseases. One of the important aspects of toxicogenomics is the development of bioinformatics tools and databases that facilitate the analysis, mining, visualization, and sharing of the vast amounts of biological data that can be generated. Environmental toxicogenomics is a relatively new approach to studying environmental biology. It allows researchers to identify and

characterize genomic signatures of environmental toxicants as gene and protein expression profiles. A major application of gene expression profiling is to understand human and animal genetic variability and susceptibility to disease. Toxicants usually affect a cascade of genes and gene interactions rather than a single gene [65]. The knowledge of changes in gene expression is critically important in developing a complete understanding of toxicological processes. This is because gene expression can be altered either directly or indirectly as a result of toxicant exposure [66].

The goals of toxicogenomics include achieving a better understanding of the mechanisms of toxicity and to identify gene expression patterns that are representative of adverse outcomes. Toxicogenomics also aims to improve the predictive accuracy of extrapolating from animal models to human and *in vitro* to *in vivo* through a better understanding of molecular mechanisms; and to identify gene expression patterns that accurately reflect and predict specific and quantifiable toxicological end points [67].

The specific mode of action of a toxicant is a prerequisite to predicting the hazards associated with exposure to that toxicant. Therefore, analyzing gene expression changes in biochemical pathways could lead to a more detailed mechanism for the biological effects of the toxin. The promise of toxicogenomics is that it may one day be used to test the entire human genome and to identify all the genes that respond to certain environmental chemicals. Granted it may be years before this technology is available for routine screening of chemical toxicity, but it is nevertheless an excellent tool to identify biochemical pathways that are affected by chemical exposure [68].

Traditionally Northern Blot analysis was used to study the changes in gene expression. However, this method is quite labour intensive and is only useful in examining the expression changes in a limited number of genes rather than a whole genome. Recently, DNA microarrays have been used to study genome-wide gene expression changes due to toxicant exposure. These arrays are based upon cDNAs or oligonucleotides spotted, through the use of a high-speed robot, onto a glass microscope slide. Control and experimental RNA is extracted from cells or tissues of the desired organ(s)/organism for which the array was generated. Fluorescent cDNA probes are usually made from the extracted RNA using reverse transcriptase in the presence of fluorescently tagged dUTP (for example: Cy3-dUTP and Cy5-dUTP) so that the control RNA is labeled with one dye and the experimental with the other dye. These two batches of cDNAs are then mixed and hybridized to the glass slide using a desired buffer and temperature to ensure optimal binding. The fluorescent signal is detected through the use of a custom-designed scanning confocal microscope equipped with a motorized stage and lasers for excitation fluorescent dye. Data is then analyzed with custom digital image analysis software that determines, for each spot, the ratio of the experimental and control dyes corrected for the local background.

Recently the results of preliminary genomics and proteomics studies have been published describing the use of microarray and 2D gels for identifying genes whose expression in zebrafish larvae is affected by exposure to the

estrogenic contaminant 4-nonylphenol (4-NP). Nonylphenol is an alkylphenol ethoxylate compound that is widely used in agriculture and industry. It is considered a ubiquitous environmental contaminant that has the potential to disrupt the endocrine system of both wildlife and humans. Of the 230 zebrafish genes that were assayed on the array it was observed that nine of them were consistently repressed following different levels of exposure to 4-NP [69]. These genes were elongation factor 1-alpha 1, elongation factor 1-alpha 2, laminin receptor, CCAAT-binding transcription factor I subunit A, 16S ribosomal RNA, 60S acidic ribosomal protein, cytochrome P450, P62 Ras-Gap associated phosphoprotein and DNA polymerase alpha. Even through these putative repressed genes had not been validated in another system or by any other means, they may still be useful as biomarkers for exposure to this particular toxin. Concurrent proteomics experiments by the same group reveal a number of protein features on 2D protein electrophoresis gels that were different between proteins isolated from 4-NP treated animals and controls [70]. Work is currently being conducted to identify these proteins and to determine their function in this toxicological response.

The toxicological significance of gene expression changes must be validated, which includes an assessment of the robustness of microarray results between or across different laboratories, species, individuals, tissues, and time periods. It is important to know the time course of gene expression changes following toxic exposures. Some alterations might be transient and others might lead to permanent changes [71]. One of the most difficult issues using DNA microarrays for toxicological studies will be in differentiating the gene expression changes that represent the normal adaptive response of cells to external stimuli to that of those gene expression changes that truly represent the early stages of disease progression. Many changes in gene expression could simply represent the nonspecific and fully reversible response of the cell to stress or a response with no biological consequence. Undoubtedly there will be a massive quantity of data produced by DNA microarrays and much of this will likely include many false positive results. Therefore, the results obtained need to be verified through other molecular biological techniques [71]. Presently, several studies have been able to provide a direct link between abnormal phenotypes produced by chemical exposures and the specific gene targets of these chemicals.

Martin *et al.* [120] observed that treatment of zebrafish embryos with the cancer therapy drug 5-azacytidine, a known DNA methyltransferase inhibitor, resulted in global DNA hypomethylation, a shortened body axis of the embryo, and abnormal expression of the gene *no tail* in the notochord. Work by Yamakoshi and Shimoda [121] has indicated that normal expression of the *no tail* gene during early embryonic development is regulated by specific methylation of CpG islands associated with the *no tail* gene. Thus the abnormal phenotype observed in the 5-azacytidine treated embryos may be the result of improper DNA methylation at the *no tail* CpG islands. Dong *et al.* [122, 123] exposed zebrafish embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and observed increased levels of apoptosis in the midbrain and reduced blood flow in the mesencephalic vein. This affect was inhibited by concurrent treatment with

cytochrome P450 inhibitors, and therefore these researchers have suggested that induction of cytochrome P450 by TCDD is the biochemical mechanism responsible for the chemically induced phenotype.

Presently, a number of large-scale screens have been conducted to assay the effects of various chemicals on the behavior or embryonic development of zebrafish [72]. The advantages of using adult zebrafish for large-scale screens include their small size (3-4 cm long) and simple husbandry, resulting in large numbers of fish that can be housed in small spaces. As well, zebrafish embryos have their own advantages for use in large-scale screens. These advantages include: large numbers of embryos that can readily be obtained, the embryos are permeable to small chemicals, and the chemicals are absorbed through diffusion. Since zebrafish embryos develop *ex utero*, there are no maternal effects caused by the chemical exposure that could complicate the experiment. As well, the embryos are transparent during early development, which allows for the observation of all embryonic development, including the visualization of heartbeats and blood circulation. Finally, due to their small size, embryos can live in a single well in a standard 384 well plate for a few days, surviving on the stored nutrients in their yolk.

Large-scale screening methodologies for zebrafish were first established during the first mutagenesis screens that selected for mutations that affect embryonic development. Similar strategies were subsequently used in a wide variety of high throughput assays and selection processes [131]. Through the screening of families of mutagenized fish it was possible to identify mutations in genes affecting dopaminergic signaling and cocaine sensitivity pathways [73]. In addition, a collection of small molecules have been screened and shown to modulate gene products and metabolic pathways [74]. These small molecules offer the ability to identify novel genes and dissect genetic function.

Because the embryos are transparent, fluorescence reporter systems can be used to monitor cell, tissue, or metabolic changes that may occur as a result of gene mutation, environmental change, or toxin exposure. For example, Farber *et al.* [127] measured lipid metabolism by using a phospholipid substrate containing a fluorescent molecule and quencher combination. Metabolism and cleavage of the phospholipid molecule resulted in separation of the fluorophore and the quencher, which could be visualized by fluorescence microscopy. Fluorescent activation of this molecule was not observed in a mutant zebrafish line called *fat free*, indicating that it possessed a defect in lipid metabolism. This was supported by the fact that fluorescent activation was prevented in wildtype zebrafish embryos by the addition of an inhibitor of cholesterol synthesis called atorvastatin. Transgenics containing green fluorescent protein (GFP) reporters have also been used to visualize cells and tissues in developing embryos. A transgenic line containing the promoter sequences of the zebrafish gene *fli-1* linked to enhanced green fluorescent protein (eGFP) has been produced that displays eGFP in the vascular endothelial cells of zebrafish larvae. These fish have been used to screen for mutations that affect vascular development [128, 129]. In addition, Pelster [130] has indicated that hypoxic exposure in

embryos can influence blood vessel formation (see later section on hypoxia). Both of these fluorescent reporter systems could be utilized to screen for toxic substances that disrupt either lipid metabolism or vascularization.

The size and optical characteristics of the zebrafish embryo make it an excellent sample for screens using whole mount *in situ* hybridization analysis. This process labels cells with a colored precipitate that contains mRNA that is complimentary to an anti-sense gene probe. Traditionally this process was very labor intensive and as a result could not be utilized effectively as a high throughput method of screening. Our laboratory is exploiting recent advances in automation technology. By utilizing high throughput, automated *in situ* hybridization we are conducting a large-scale screen of toxins that affect the embryonic development of the zebrafish brain. We have selected a group of gene markers that are expressed at different locations in the brain. Our ability to test the expression patterns of a large number of genes, on a large sample size gives us the ability to identify compounds whose effects may not be grossly pathological but that can only be identified using statistical methods. In addition, the use of computer aided microscopy and image analysis allows us to also automate the process of collecting morphometric measurements of the expression domains of each of the gene probes. Imaging strategies, such as pixel threshold techniques, allow measurements to be taken of the expression domains to be done in an unbiased fashion. The large numbers of individuals that can be analyzed facilitates the need for statistical significance and

permits the identification of subtle differences that might not be observed using qualitative methods. One example of this analysis is found in Fig. (1). We have shown using this technique that treatment of zebrafish embryos with environmentally relevant concentrations of cadmium chloride results in abnormal brain development. Our analysis revealed a significantly larger expression domain of the gene *eng2* in the midbrain/hindbrain boundary compared to control group animals. These results suggest that there may exist a neuropathology associated with cadmium exposure during vertebrate embryogenesis. We have also used this method to demonstrate differences in the *eng2* pattern in embryos treated with arsenic and nickel compounds (unpublished data). The efficiency of this technique allows unprecedented rapid screening of teratogenic compounds not only for phenotypic effects but also genomic effects. This strategy could be made more powerful by combining the tissue specific analysis of gene expression with the comparative power of DNA microarray analysis.

Researchers have begun to use transgenic zebrafish for studying toxicology. By producing transgenic zebrafish that contain environmentally responsive enhancer or promoter sequences coupled with reporters such as GFP, researchers have been able to generate living sentinels or beacons to detect the occurrence of stresses or environmental contaminants [81]. Transgenic zebrafish containing GFP-coupled retinoic acid response elements have been produced that show reporter expression upon environmental exposure to retinoids [82]. Evidence from zebrafish cell cultures

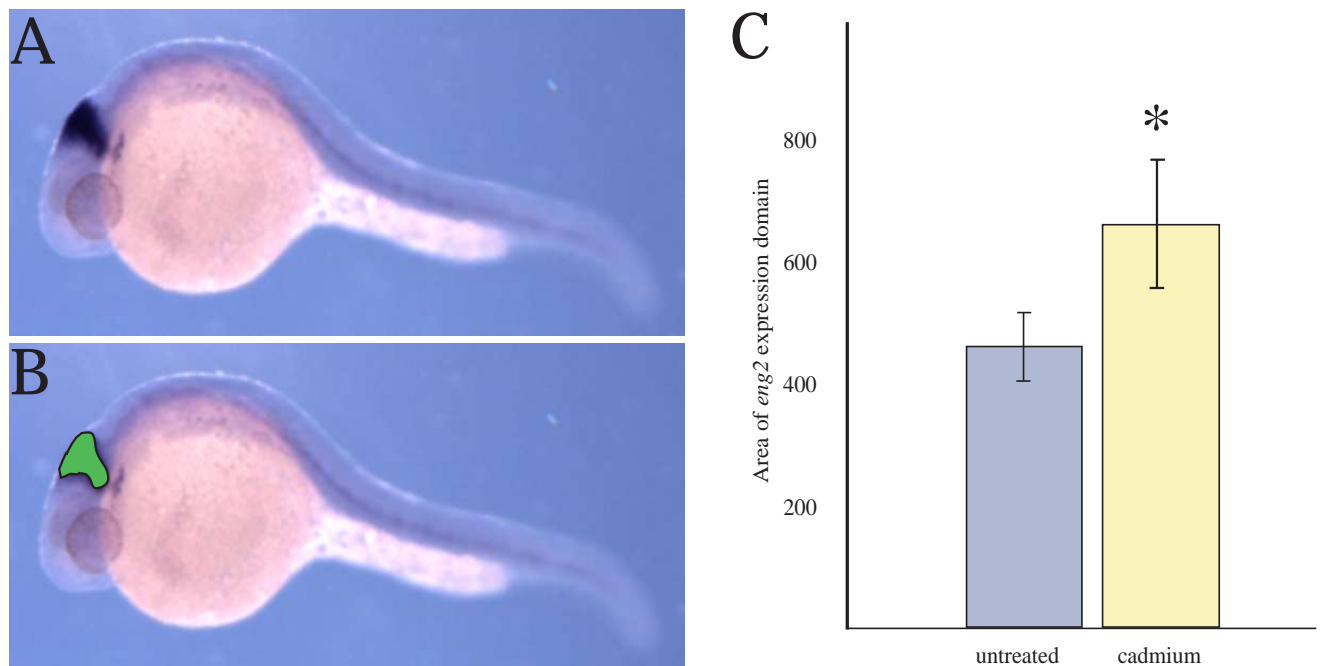


Fig. (1). Treatment of zebrafish embryos with cadmium chloride results in abnormal brain development. (A) Whole mount *in situ* hybridization of a zebrafish embryo using a *eng2* antisense probe. *eng2* mRNA is localized to the midbrain/hindbrain boundary (purple stain). (B) Pixel thresholding techniques are utilized to define the expression domain of *eng2*. (C) A histogram showing the area (in arbitrary pixel units) of the *eng2* expression domain in cadmium chloride treated and untreated 24 hour post fertilization zebrafish embryos. (* = $p < 0.05$).

transfected with metal regulatory elements (MREs), isolated from metallothionein genes, coupled to a reporter indicate that similar transgenic fish could be produced to serve as bioindicators for the presence of heavy metal contamination of aquatic environments [83].

Utilizing a transgenic zebrafish line containing an *hsp70* promoter coupled to GFP, researchers at the University of Saskatchewan were able to use these fish as living beacons for the presence of cadmium in water [84]. In addition to observing tissue specific expression of the GFP reporter in the olfactory neurons of fish larvae exposed to cadmium, this chemically induced expression was observed at low concentration exposures despite the fact that no gross phenotypic or survival effects were evident. Thus, the use of these transgenics for detecting the presence of cadmium in an aquatic environment is more sensitive than classical toxicological assays such as LC50 and EC50. It was further suggested that the tissue specific expression of the transgene in the olfactory neurons indicates that these tissues are targets for cadmium toxicity and that cadmium exposure could potentially affect later survival of adults by disruption of the sensory system.

PHYSIOGENOMICS

Rainbow trout, carp, eel, goldfish, salmon and tilapia have been the most popular model systems to study fish physiology. In addition to their large size, making them easy to manipulate, these fish possess a number of desirable properties. Salmon, for example, migrate between fresh and salt water making them of interest in the field of ion exchange and excretion. These fish spawn seasonally allowing endocrinologists to study the hormonal regulation of fish reproduction. Since these fish are of interest to the growing worldwide aquaculture industry considerable research has gone into producing a vast literature on the growth, metabolism, biochemistry and energy requirements of these species.

In contrast, the adult zebrafish is small, spawns continuously and there is little public interest in these fish except as a traditional or exotic genetically modified glowing zebrafish pets [85]. As a result, the fish physiologist research community has only recently embraced zebrafish as a model system. The development during the last couple years of genomic tools for zebrafish and the availability of mutations that appear to affect physiological processes has spawned new interest and collaborations between developmental geneticists, molecular biologists, and traditional physiologists. The development of microtechniques, small physiological sensors as well as increasingly sensitive biochemical assays now makes the zebrafish a more suitable model for physiology and physiogenomics [86-88].

Several areas that have received attention, at both the physiological and genomic levels, are the response of zebrafish to hypoxia and heat shock (stress).

HYPOXIA

Unlike terrestrial organisms that live in environments where oxygen levels are relatively high and stable, aquatic organisms often survive under oxygen conditions that can be

low (hypoxia) or extremely variable. As a result, the genomes of aquatic vertebrates, such as fish, have evolved to elicit various physiological, biochemical, and even behavioral responses that allow them to adapt to such variations in the oxygen availability of their habitats.

A number of research groups are beginning to use zebrafish as a model system for cardiovascular physiology. Metabolism and cardiac output has been measured under various conditions including hypoxia in both embryos and larvae [89-92]. Cardiovascular function has also been studied using traditional pharmacologic methods [93, 94].

Pre-exposure of zebrafish to hypoxia can result in acclimation where a second exposure to hypoxia shows less detrimental effects [95]. Using microarray analysis Ton *et al.* conducted gene expression profiling of zebrafish embryos exposed to hypoxic conditions in order to understand the molecular events that follow exposure [25, 96]. Of 4,512 genes assayed using this DNA microarray, 138 genes were differentially transcribed following the exposure of 24-hour embryos to 5% oxygen. During this twenty-four hour exposure, glycolytic enzymes such as phosphoglycerate mutase, kinase, aldolase and enolase are up regulated suggesting a transition from aerobic to anaerobic glycolysis. In addition, genes responsible for cellular protection and DNA repair are induced upon hypoxic exposure. These include heat shock proteins HSP30 and HSP70, stress-inducible immunoglobulin binding protein (BiP), and DNA enzymes such as RAD52 and MSH6. Finally, it was noted that expression of a transcription factor called hypoxia inducible factor 1 alpha (HIF1 α) is up regulated. This protein is very important because of its known binding to hypoxia response elements (HRE) near the promoter region of genes, drastically increasing expression levels. For example, the vascular endothelial growth factor (VegF) gene has been found to contain HREs. VegF is required for angiogenesis in most organisms studied thus far including zebrafish and is also important in the vascular infiltration of human tumors [97, 98].

Even though the accumulation of HIF1 α in the cell is primarily regulated by oxygen-dependent degradation, these results suggest that transcriptional upregulation of HIF1 α may also be important in the hypoxic response of the zebrafish. A curious observation was that there was no increase in the expression of globin and erythropoietin because in both adult rainbow trout and mammals these proteins increase the affinity of hemoglobin to oxygen during hypoxia [99, 100]. Studies of mutant zebrafish that possess defects in the vascular and hematopoietic systems and embryos whose oxygen transport has been disrupted have indicated that early stages of embryonic development (48 hours post-fertilization and earlier) do not require a functional circulatory system and blood cells to survive [101]. Evidently, at these stages of development, oxygen is delivered to the cells by diffusion. Many genes are down regulated when zebrafish embryos are exposed to low oxygen. Coordinate with changes in metabolism there are significant reductions in the transcription of genes encoding enzymes such as succinate dehydrogenase, citrate synthase, malate dehydrogenase and muscle creatine kinase. Indeed, the cellular response of early zebrafish embryos to hypoxia

seems to be to enter a state of suspended animation or quiescence with halted developmental progression and cellular arrest in S and G2 phase [102]. The transcription of basic cellular components such as the cytoskeleton and extra-cellular matrix is markedly decreased. As well, there are decreases in the expression of genes that are components of the translational machinery such as ribosomal proteins, G1-cyclin and PCNA of the cell cycle regulatory system, and the GTPase family of nuclear pore transport molecules.

To further understand the molecular mechanisms involved in establishing the hypoxic response, particularly the role of HIF1 α , our laboratory is combining microarray analysis with our ability to produce, by antisense morpholino injection or mRNA injection, zebrafish embryos that are deficient in HIF1 α protein (HIF1 α morphants) or embryos that over-express HIF1 α respectively. By comparing genes that are expressed in hypoxia treated HIF1 α morphants with genes expressed in normal embryos exposed to hypoxia we will be able to differentiate between HIF1 α regulated genes and genes whose expression is modified indirectly or mediated by means other than the HIF1 α transcription factor. Additionally, by comparing the genes expressed in normal embryos to the genes expressed in embryos over-expressing HIF1 α in the absence of hypoxia we can potentially identify genes whose expression is directly modified by HIF1 α .

Some of the most revealing studies of molecular cardiovascular physiology have utilized mutants that have been recovered from the large mutagenesis screens of the zebrafish genome (see Special Issue of Development December 1996) [13, 103-105]. We have discussed some of these already in this review.

STRESS AND HEAT SHOCK

When cells of living organisms are exposed to elevated temperatures or various stresses there occurs a relatively stereotypical transcriptional up-regulation of a diverse group of genes encoding the heat shock proteins (Hsp). Hsps, also called molecular chaperone proteins, function to maintain protein homeostasis, and guide the assembly, folding and stability of proteins that have known involvement in cell differentiation, cellular signaling, growth, and cell death. Stresses that can induce the heat shock response include exposure to some pesticides, and heavy metals such as arsenic and cadmium [106-108]. Indeed Hsp genes are regularly included in microarrays used for toxicological analysis [109] and can act as biomarkers for environmental toxicology [110]. In humans, Hsps are over-expressed in the tumors of many types of cancers and tissues, particularly brain neurons that have been exposed to hypoxic insult as in the case of a stroke or temporary heart failure. As a result, these proteins have received much attention as possible targets for cancer treatment or as mediators of neuronal protection [111, 112].

Northern blot and *in situ* hybridization analysis using zebrafish reveals that some heat shock protein genes are expressed at specific times and in specific tissues during embryonic development in the absence of Heat or stress [113, 114]. This is surprising because it was previously held that most hsp expression was ubiquitous and inducible. The *hsp90 α* gene, for example, is expressed in differentiating

muscle. Pharmacologic inhibition of the Hsp90 α protein by geldanamycin resulted in abnormal muscle development confirming its importance in muscle differentiation [115].

In addition to the expression of some Hsps under non-stress conditions in zebrafish, the stress response can be activated by exposure of cells, embryos, or whole adults to 37°C for one hour. The promoter of heat shock protein genes contains a consensus sequence called the heat shock element (HSE). Heat shock induces the dimerization of a protein called heat shock factor 1 (HSF-1) that subsequently binds to the HRE facilitating the transcriptional up-regulation of the heat shock protein genes. The promoter of the zebrafish *hsp70* gene has been isolated and utilized to drive inducible expression of both reporters and endogenous proteins within transgenic zebrafish [116, 117]. In these transgenic fish, a laser could be used to gently heat individual cells and, thereby, activate transgene expression in any cell or population of cells. In a remarkable demonstration using an *hsp70-sema3a1* transgenic it was shown that semaphorin has a repulsive effect on growing motor axons. Evidently, transgene expression could be activated by laser heat applied to single muscle cells in the vicinity of growing motor axons. Transgene expression of the semaphorin gene in this muscle cell caused the path of growing axons to divert away. Thus, inducible transgenes offer a novel system for testing gene function *in vivo*.

In zebrafish the *hsp10 (cpn10)* and *hsp60 (cpn60)* genes (the chaperonin genes) is separated by a bidirectional promoter that contains an HRE. These genes exist in a head to head genomic configuration and are transcribed in opposite directions. We have isolated this promoter and characterized the expression of both of these genes under normal and heat shock conditions [118, 119]. Our expression analysis suggests that the chaperonin bi-directional promoter can also serve as a useful inducible promoter for transgene constructs. An additional benefit to promoter inducibility is that this promoter can drive the expression of two different genes simultaneously. For example, a reporter such as GFP, could be placed on one side of the promoter. This reporter will act as an internal marker for transgene activation. By cloning into the other side of the promoter, the functions of other genes can be tested. Practical uses aside, the head to head genomic organization of the chaperonins were found by database mining to be conserved in all vertebrates and in some invertebrates. Furthermore, the bacterial homologs of Hsp10 and Hsp60 genes occur in a single operon. This high degree of conservation suggests that there may be positive selection to maintain tight coordinate regulation of these two genes.

SUMMARY

Environmental genomics provides information on how an organism responds to environmental conditions at the level of the whole genome. Model organisms are the best subjects for these types of studies and data obtained from vertebrate models, such as the zebrafish, are likely to be directly relevant to other aquatic vertebrates and also humans. A number of characteristics and resources of the zebrafish make it an excellent model for environmental genomic studies. These include:

1. Genomic information
2. Well characterized biology
3. Genetic diversity
4. Ease of husbandry

Despite its apparent bright future, much of the past research using the zebrafish has only served to lay the foundations for truly novel expeditions to come. This review has given overwhelming praise for the zebrafish despite the fact that a number of characteristics and information shortcomings may never make it an ideal system for modeling mammalian (including human) conditions. Zebrafish develop externally and therefore aspects of placental embryonic development can never be investigated using the zebrafish model system. Our understanding of tissue pathology, disease and health in fish has long been a neglected aspect of veterinary medicine. As a result those researchers using zebrafish to model human disease are often faced with developing basic tools and knowledge that have been long established in systems such as the mouse. Unique aspects of excretion and respiration in fish, combined with differences in cutaneous absorption, may make toxicology data difficult to extrapolate to potential effects in humans.

However, the zebrafish is no longer only considered a model for genetics and developmental biology. Investigators are now recognizing that features that have made it a primary model for genetics and development also make it an excellent model for toxicology, physiology and ecology. This fish has come a long way from its origins in the rivers of India and the many years it spent as a home aquaria pet.

ACKNOWLEDGMENTS

Some of the research discussed in this manuscript was supported by grants to C.C. Martin and D. Lean from the Natural Sciences and Engineering Research Council of Canada. We thank Tammy Wakin-Martin and Mary Ann Steggle for editorial comments. Victoria Karimi conducted some of the *in situ* analysis discussed in this review.

REFERENCES

- [1] Gibson, G. Microarrays in ecology and evolution: a preview. *Mol. Ecol.* **2002**, *11*: 17-24.
- [2] Feder, M.E.; and Mitchell-Olds, T. Evolutionary and ecological functional genomics. *Nat. Rev. Genet.* **2003**, *4*: 651-657.
- [3] Ye, R.W.; Wang, T.; Bedzyk, L.; Croker, K.M. Applications of DNA microarrays in microbial system. *J. Microbiol. Methods.* **2001**, *47*: 257-272.
- [4] Zhou, J. Microarrays for bacterial detection and microbial community analysis. *Curr. Opin. Microbiol.* **2003**, *6*: 288-294.
- [5] Amin, R.P.; Hamadeh, H.K.; Bushel, P.R.; Bennet, L.; Afshari, C.A.; Paules, R.S. Genomic interrogation of mechanism(s) underlying cellular responses to toxicants. *Toxicology* **2002**, *182*: 555-563.
- [6] Orphanides, G. Toxicogenomics: challenges and opportunities. *Toxicol. Lett* **2003**, *140-141*: 145-148.
- [7] Powers, D.A. Fish as model systems. *Science* **1989**, *246*: 352-358.
- [8] Barut, B.A.; Zon, L.I. Realizing the potential of zebrafish as a model for human disease. *Physiol. Genomics* **2000**, *2*: 49-51.
- [9] Westerfield, M. *The Zebrafish Book*. Univ. of Oregon Press, Eugene, OR. **1995**.
- [10] Shin, J.T.; Fishman, M.C.; From zebrafish to human: modular medical models. *Annu. Rev. Genomics Hum. Genet.* **2002**, *3*: 311-340.
- [11] Stainier, D.Y.R.; Lee, R.K.; Fishman, M.C. Cardiovascular development in the zebrafish. *Development* **1993**, *119*: 31-40.
- [12] Zon, L.I. Developmental Biology of hematopoiesis. *Blood* **1995**, *86*: 2876-2891.
- [13] Driever, W.; Fishman, M.C. The zebrafish: heritable disorders in transparent embryos. *J. Clin. Invest.* **1996**, *97*: 1788-1794.
- [14] Galloway, J.L.; Zon, L.I. Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. *Curr. Top. Dev. Biol.* **2003**, *53*: 139-158.
- [15] Barbazuk, W.B.; Kork, I.; Kadavi, C.; Heyen, J.; Tate, S.; Wun, E.; Bedell, J.A.; McPherson, J.D.; Johnson, S.L. The syntenic relationship of the zebrafish and human genomes. *Genome Res.* **2000**, *10*: 1351-1358.
- [16] Postlethwait, J.H.; Woods, I.G.; Ngo-Hazelett, P.; Yan, Y.L.; Kelly, P.D.; Chu, F.; Huang, H.; Hill-Force, A.; Talbot, W.S. Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res.* **2000**, *10*: 1890-1902.
- [17] Garrity, D.H.; Childs, S.; Fishman, M.C. The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* **2002**, *129*: 4635-4645.
- [18] Vogel, G. Zebrafish earns its stripes in genetic screens. *Science* **2000**, *288*: 1160-1161.
- [19] Karlovich, C.; John, R.; Ramirez, L.; Stainier, D.; Meyers, R. Characterization of the Huntington's disease (HD) gene homologue in the zebrafish *Danio rerio*. *Gene* **1998**, *14*: 117-125.
- [20] Leimer, U.; Lun, K.; Romig, H.; Walter, J.; Grunberg, J.; Brand, M.; Haass, C. Zebrafish (*Danio rerio*) presenilin promotes aberrant amyloid beta-peptide production and requires a critical aspartate residue for its function in amyloidogenesis. *Biochem.* **1999**, *38*: 13602-13609.
- [21] Leroy, E.; Boyer, R.; Auburger, G.; Leube, B.; Ulm, G.; Mezey, E.; Harta, G.; Brownstein, M.J.; Jonnalagada, S.; Chernova, T.; Dehejia, A.; Lavedan, C.; Gasser, T.; Steinbach, P.J.; Wilkinson, K.D.; Polymeropoulos, M.H. The ubiquitin pathway in Parkinson's disease. *Nature* **1998**, *395*: 451-452.
- [22] Son, O-L.; Kim, H-T.; Ji, M-H.; Yoo, K-W.; Rhee, M.; Kim, C-. Cloning and expression analysis of a Parkinson's disease gene, uch-L1, and its promoter in zebrafish. *Biomedical and Biophysical Research Communications* **2003**, *312*: 601-607.
- [23] Rasooly, R.S.; Heneken, D.; Freeman, N.; Tompkins, L.; Badman, D.; Briggs, J.; Hewitt, A.T. National Institutes of Health Trans-NIH Zebrafish Coordinating Committee. Genetic and genomic tools for zebrafish research: the NIH zebrafish initiative. *Dev Dyb* **2003**, *228*: 490-496.
- [24] Lo, J.; Lee, S.; Xu, M.; Liu, F.; Ruan, H.; Eun, A.; He, Y.; Ma, W.; Wang, W.; Wen, Z.; Peng, J. 15,000 Unique zebrafish EST clusters and their future use in microarray for profiling gene expression patterns during embryogenesis. *Genome Res.* **2003**, *13*: 455-466.
- [25] Ton, C.; Stamatiou, D.; Dzau, V.J.; Liew, C.-C. Construction of a zebrafish cDNA microarray: gene expression profiling of the zebrafish during development. *Biochemical and Biophysical Research Communications* **2002**, *296*: 1134-1142.
- [26] Stickney, H.L.; Schmutz, J.; Woods, I.G.; Holtzer, C.C.; Dickson, M.C.; Kelly, P.D.; Myers, R.M.; Talbot, W.S. Rapid mapping of zebrafish mutations with SNPs and oligonucleotide microarrays. *Genome Res.* **2002**, *12*: 1929-1934.
- [27] Geisler, R. et al. A radiation hybrid map of the zebrafish genome. *Nature Genetics* **1999**, *23*: 86-89.
- [28] Kwok, C.; Critcher, R.; Schmitt, K. Construction and characterization of zebrafish whole genome radiation hybrids. *Methods Cell Biol.* **1999**, *60*: 287-302.
- [29] Hukriede, N.A.; Joly, L.; Tsang, M.; Miles, J.; Tellis, P.; Epstein, J.A.; Barbazuk, W.B.; Li, F.N.; Paw, B.; Postlethwait, J.H.; Hudson, T.J.; Zon, L.I.; McPherson, J.D.; Chevrette, M.; Dawid, I.B.; Johnson, S.L.; Ekker, M. Radiation hybrid mapping of the zebrafish genome. *PNAS* **1999**, *96*: 9745-9750.
- [30] Kelly, P.D.; Chu, F.; Woods, I.G.; Ngo-Hazelett, P.; Cardozo, T.; Huang, H.; Kimm, F.; Liao, L.; Yan, Y.L.; Zhou, Y.; Johnson, S.L.; Abagyan, R.; Schier, A.F.; Postlethwait, J.H.; Talbot, W.S. Genetic linkage mapping of zebrafish genes and ESTs. *Genome Research* **2000**, *10*: 558-567.
- [31] Knapik, E.W.; Goodman, A.; Ekker, M.; Chevrette, M.; Delgado, J.; Neuhauss, S.; Shimoda, N.; Driever, W.; Fishman, M.C.; Jacob, H.J. A microsatellite genetic linkage map for zebrafish. *Nature Genetics* **1998**, *18*: 338-343.

- [32] Shimoda, N.; Knapik, E.W.; Ziniti, J.; Sim, C.; Yamada, E.; Kaplan, S.; Jackson, D.; de Sauvage, F.; Jacob, H.; Fishman, M.C. Zebrafish genetic map with 2000 microsatellite markers. *Genomics* **1999**, *58*: 219-232.
- [33] Gates, M.A.; Kim, L.; Egan, E.S.; Cardozo, T.; Sirotkin, H.I.; Dougan, S.T.; Lashkari, D.; Abagyan, R.; Schier, A.F.; Talbot, W.S. A genetic linkage map for zebrafish: comparative analysis and localization of genes and expressed sequences. *Genome Research* **1999**, *9*: 334-47
- [34] Postlethwait et al. Vertebrate genome evolution and the zebrafish gene map. *Nature Genetics* **1998**, *18*: 345-349.
- [35] Axelrod, H.R.; Emmens, C.W.; Sculthorpe, D.; Winkler, V.W.; Pronek, N. Exotic Tropical Fishes. TFH Publications, Inc. Jersey City, NJ. **1971**.
- [36] Eaton, R.C.; Farley, R.D. Spawning cycle and egg production of Zebrafish, *Brachydanio rerio*, in the laboratory. *Copeia* **1974**, *1*: 195-204.
- [37] Jayram, K.C. The freshwater fishes of India, Pakistan, Bangladesh, Burma, and Sri Lanka- A handbook. Zoological Survey of India. Calcutta. **1981**, 475 pp. + 13 plates.
- [38] Sterba, G. The Aquarium Encyclopedia. The MIT Press. Cambridge, Massachusetts. **1983**, 605 pp.
- [39] Hamilton, F. An account of the fishes found in the river Ganges and its branches. Edinburgh & London. Fishes Ganges i-vii + 1-405 **1822**, (first observation).
- [40] Talwar, P.K.; Jhingran, A.G. Inland fishes of India and adjacent countries. Vol. I. Oxford and IBH Publishing Co. PVT Ltd. New Delhi **1991**.
- [41] Menon, A.G.K.; Check list - fresh water fishes of India. *Rec. Zool. Surv. India, Misc. Publ.; Occas. Pap.* **1999**, *175*: 366.
- [42] Courtenay, W.R. Jr.; Hensley, D.A.; Taylor, J.N.; McCann, J.A. Distribution of exotic fishes in the continental United States. Pages 41-77 in W.R. Courtenay, Jr.; and J.R. Stauffer, Jr. Distribution, Biology and Management of Exotic Fishes. John Hopkins University Press. Baltimore **1984**.
- [43] Courtenay, W.R. Jr, Jennings, D.P.; Williams, J.D. Appendix 2. Exotic Fishes. Pages 97-107 in C.R. Robins, R.M. Bailey, C.E. Bond, J.R. Brooker, E.A. Lachner, R.N. Lea, and W.B. Scott. Common and Scientific Names of Fishes from the United States and Canada. American Fisheries Society Special Publication 20. Bethesda, Maryland. **1991**.
- [44] Courtenay, W.R. Jr, Sahlman, H.F.; Miley, W.F. II, Herrema, D.J. Exotic fishes in fresh and brackish waters of Florida. *Biological Conservation* **1974**, *6*: 292-302.
- [45] Courtenay, W.R. Jr.; Stauffer, J.R. Jr. The introduced fish problem and the aquarium fish industry. *Journal of the World Aquaculture Society* **1990**, *21*: 145-159.
- [46] Welcomme, R.L. International introductions of inland aquatic species. *FAO Fish. Tech. Pap.* **1988**, *294*: 318.
- [47] Wright, D.; Rimmer, L.B.; Pritchard, V.L.; Krause, J.; Butlin, R.K. Inter and intra-population variation in shoaling and boldness in the zebrafish (*Danio rerio*). *Naturwissenschaften*. **2003**, *90*: 374-377.
- [48] Poor, E.A.; Dziewczynski, T.; Rowland, W. Social interactions among zebrafish (*Danio rerio*) shoaling and inspection behavior. *REU Abstracts from Animal Behavior Bulletin* **2003**, *5*: 7-7.
- [49] Gerhard, G.S.; Kauffman, E.J.; Wang, X.; Stewart, R.; Moore, J.L.; Kasales, C.J.; Demidenko, E.; Cheng, K.C. Life spans and senescent phenotypes in two strains of zebrafish (*Danio rerio*). *Experimental Gerontology* **2002**, *37*: 1055-1068.
- [50] Roubenoff, R.; Hughes, V.A. Sarcopenia: current concepts. *J. Gerontol. A. Biol. Sci. Med. Sci* **2000**, *55*: M716-M724.
- [51] Johnson, S.L.; Midson, C.N.; Ballinger, E.W.; Postlethwait, J.H. Identification of RAPD primers that reveal extensive polymorphisms between laboratory strains of zebrafish. *Genomics*. **1994**, *19*: 152-156.
- [52] Nechiporuk, A.; Finney, J.E.; Keating, M.T.; Johnson, S.L. Assessment of polymorphism in zebrafish mapping strains. *Genome Res.* **1999**, *9*: 1231-1238.
- [53] McCune, A.R.; Fuller, R.C.; Aquilina, A.A.; Dawley, R.M.; Fodool, J.M.; Houle, D.; Travis, J.; Kondrashov, A.S. A low genomic number of recessive lethals in natural populations of bluefin killifish and zebrafish. *Science* **2002**, *296*: 2398-2401.
- [54] McKusick, V.A. Genomics: structural and functional studies of genomes. *Genomics* **1997**, *45*: 244-249.
- [55] Kaiser, J. Environment institute lays plans for gene hunt. *Science* **1997**, *278*: 569-570.
- [56] Guengerich, F.P. The environmental genome project: functional analysis of polymorphisms. *Environ.* **1989**, *106*: 365-368.
- [57] Brown, P.O.; Hartwell, L. Genomics and human disease- variations on variation. *Nat. Genet.* **1998**, *18*: 91-93.
- [58] Olden, K.; Wilson, S.H. Environmental health and genomics: visions and implications. *Nat. Rev. Genet* **2000**, *1*: 149-153.
- [59] Laale, H.W. Ethanol induced notochord and spinal chord duplications in the embryo of the zebrafish, *Brachydanio rerio*. *J. Exp. Zool.* **1971**, *177*: 51-64.
- [60] Lele, Z.; Krone, P.H. The zebrafish as a model system in developmental, toxicological, and transgenic research. *Biotechnology Advances* **1996**, *14*: 57-72.
- [61] Vitozzi, L.; DeAngelis, G. A critical review of comparative acute toxicity data on freshwater fish. *Aquat. Toxicol.* **1991**, *19*: 167-204.
- [62] Ensenbach, U.; Nagel, R. Toxicity of complex chemical mixtures: acute and long-term effects on different life stages of zebrafish (*Brachydanio rerio*). *Ecotoxicol. Env. Saf.* **1995**, *30*: 151-157.
- [63] Ribeyre, F.; Amiard-Triquet, C.; Boudou, A.; Amiard, J-C. Experimental study of interactions between five trace elements-Cu, Ag, Se, Zn, Hg,- toward their bioaccumulation by fish (*Brachydanio rerio*) from the direct route. *Ecotoxicol. Env. Saf.* **1995**, *32*: 1-11.
- [64] Ruoppa, M.; Nakari, T. The effects of pulp and paper industry Tervakovski oy wastewaters on the fertilized eggs and alevins of zebrafish and on the physiology of rainbow trout. *Water Sci. Technol.* **1988**, *20*: 201-202.
- [65] Aardema, M.J.; MacGregor, J.T. Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies. *Mutation Research* **2002**, *499*: 13-25.
- [66] Simmons, P.T.; Portier, C.J. Toxicogenomics: the new frontier in risk analysis. *Carcinogenesis* **2002**, *23*: 903-905.
- [67] Farr, S, Dunn, R.T. 2nd. Consise review: gene expression applied to toxicology. *Toxicol. Sci* **1999**, *50*: 1-9.
- [68] Olden, K.; Guthrie, J. Genomics: implications for toxicology. *Mutation Research* **2001**, *473*: 3-10.
- [69] Hoyt, P.R.; Doktycz, M.J.; Beattie, K.L.; Greeley, Jr M.S. DNA microarrays detect 4-nonylphenol-induced alterations in gene expression during zebrafish early development. *Ecotoxicology* **2003**, *12*: 469-474.
- [70] Shrader, E.A.; Henry, T.R.; Greeley, Jr M.S.; Bradley, B.P. Proteomics in zebrafish exposed to endocrine disrupting chemicals. *Ecotoxicology* **2003**, *12*: 485-488.
- [71] Marchant, G.E. Toxicogenomics and toxic torts. *Trends in Biotechnology* **2002**, *20*: 329-332.
- [72] Stern, H.M.; Zon, L.I. Cancer genetics and drug discovery in the zebrafish. *Nat. Rev. Cancer* **2003**, *3*: 533-539.
- [73] Darland, T.; Dowling, J.E. Behavioral screening for cocaine sensitivity in mutagenized zebrafish. *Proc. Natl. Acad. Sci. USA* **2001**, *98*: 11691-11696.
- [74] Peterson, R.T.; Link, B.A.; Dowling, J.E.; Schreiber, S.L. Small molecules developmental screens reveal the logic and timing of vertebrate development. *Proc. Natl. Acad. Sci. USA* **2000**, *97*: 12965-12969.
- [75] Stuart, G.W.; McMurray, J.V.; Westerfield, M. Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* **1988**, *103*: 403-412.
- [76] Muller, F.; Lele, Z.; Varadi, L.; Menczel, L.; Orban, L. Efficient transient expression systems based on square-pulse electroporation and *in vivo* luciferase assay of fertilized fish eggs. *FEBS Lett.* **1993**, *324*: 27-32.
- [77] Zelenin, A.V.; Alimov, A.; Barmintzev, V.A.; Bniunov, A.O.; Zelenina, I.A.; Krasnov, A.E.; Kolesnikov, V.A. The delivery of foreign genes into fertilized fish eggs using high velocity microprojectiles. *FEBS Lett.* **1991**, *287*: 118-120.
- [78] Szelei, J.; Varadi, L.; Muller, F.; Erdelyi, F.; Orban, L.; Horvath, L.; Duda, E. Liposome-mediated gene transfer in fish embryos. *Transgenic Res.* **1994**, *3*: 116-119.
- [79] Muller, F.; Ivics, Z.; Erdelyi, F.; Papp, T.; Varadi, L.; Horvath, L.; Maclean, N.; Orban, L. Introducing foreign genes into fish eggs with electroporated sperm as a carrier. *Mol. Mar. Biol. Biotechnol.* **1992**, *1*: 276-281.
- [80] Khoo, H-W.; Ang, L-H.; Lim, H-B.; Wong, K-Y. Sperm cells as vectors for introducing foreign genes into zebrafish. *Aquaculture* **1992**, *107*: 1-19.

- [81] Nebert, D.W.; Stuart, G.W.; Solis, W.A.; Carvan, M.J. 3rd. Use of reporter genes and vertebrate DNA motifs in transgenic zebrafish as sentinels for assessing aquatic pollution. *Environ. Health Perspect.* **2002**, *110*: A14.
- [82] Perz-Edwards, A.; Hardison, N.L.; Linney, E. Retinoic acid-mediated gene expression in transgenic reporter zebrafish. *Developmental Biology* **2001**, *229*: 89-101.
- [83] Yan, C.H.; Chan, K.M. Characterization of zebrafish metallothionein gene promoter in a zebrafish caudal fin cell-line, SJD.1. *Mar. Environ. Res.* **2002**, *54*: 335-339.
- [84] Blechinger, S.R.; Warren, J.T. Jr.; Kuwada, J.Y.; Krone, P.H. Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line. *Environ. Health Perspect* **2002**, *110*: 1041-1046.
- [85] Gong, Z.; Wan, H.; Tay, T.L.; Wang, H.; Chen, M.; Yan, T. Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. *Biochem. Biophys. Res. Commun.* **2003**, *308*: 58-63.
- [86] Briggs, J.P. The zebrafish: a new model organism for integrative physiology. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.* **2002**, *282*: R3-R9.
- [87] Schwerte, T.; Fritsche, R. Understanding cardiovascular physiology in zebrafish and *Xenopus* larvae: the use of microtechniques. *Comparative Biochemistry and Physiology Part A* **2003**, *135*: 131-145.
- [88] Ho, S.Y.; Pack, M.; Farber, S.A. Analysis of small molecule metabolism in zebrafish. *Methods Enzymol.* **2003**, *364*: 408-426.
- [89] Barrionuevo, W.R.; Burggren, W.W. O₂ consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O₂. *Am. J. Physiol* **1999**, *276*: R505-R513.
- [90] Bagatto, B.; Pelster, B.; Burggren, W.W. Growth and metabolism of larval zebrafish: effects of swim training. *The Journal of Experimental Biology* **2001**, *204*: 4335-4343.
- [91] Jacob, E.; Drexel, M.; Schwerte, T.; Pelster, B. Influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *Am. J. Physiol. Regul. Integr. Comp. Physiol* **2002**, *283*: R911-R917.
- [92] Pelster, B.; Sanger, A.M.; Siegele, M.; Schwerte, T. Influence of swim training on cardiac activity, tissue capillarization, and mitochondrial density in muscle tissue of zebrafish larvae. *Am. J. Physiol. Regul. Integr. Comp. Physiol* **2003**, *285*: R339-R347.
- [93] Fritsche, R.; Schwerte, T.; Pelster, B. **2000**, Nitric oxide and vascular reactivity in developing zebrafish, *Danio rerio*. *Am. J. Physiol. Regulatory Integrative Comp. Physiol* **279**: R2200-R2207.
- [94] Hsieh, DJ-Y.; Liao, C-F. Zebrafish M₂ muscarinic acetylcholine receptor: cloning, pharmacological characterization, expression patterns and roles in embryonic bradycardia. *British Journal of Pharmacology* **2002**, *137*: 782-792.
- [95] Rees, B.B.; Sudradjat, F.A.; Love, J.W. Acclimation to hypoxia increases survival time of zebrafish, *Danio rerio*, during lethal hypoxia. *Journal of Experimental Zoology* **2001**, *289*: 266-272.
- [96] Ton, C.; Stamatou, D.; Liew, C-C. Gene expression profiling of zebrafish exposed to hypoxia during development. *Physiol. Genomics* **2003**, *13*: 97-106.
- [97] Nasevicius, A.; Larson, J.; Ekker, S.C. Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. *Yeast* **2000**, *17*: 294-301.
- [98] Denko, N.C.; Fontana, L.A.; Hudson, K.M.; Sutphin, P.D.; Raychaudhuri, S.; Altman, R.; Giaccia, A.J. Investigating hypoxic tumor physiology through gene expression patterns. *Oncogene* **2003**, *22*: 5907-5914.
- [99] Val, A.L. Oxygen transfer in fish: morphological and molecular adjustments. *Braz. J. Med. Biol. Res.* **1995**, *28*: 1119-1127.
- [100] Wang, G.L.; Semenza, G.L. Molecular basis of hypoxia induced erythropoietin expression. *Curr. Opin. Hematol* **1996**, *3*: 156-162.
- [101] Pelster, B.; Burggren, W.W. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of the zebrafish (*Danio rerio*). *Circ. Res.* **1996**, *79*: 358-362.
- [102] Padilla, P.A.; Roth, M.B. Oxygen deprivation causes suspended animation in the zebrafish embryo. *Proc. Natl. Acad. Sci. USA* **2001**, *98*: 7331-7335.
- [103] Warren, K.S.; Fishman, M.C. Physiological genomics: mutant screens in zebrafish. *Am. J. Physiological Heart Circ. Physiol* **1998**, *275*: H1-H7.
- [104] Talbot, W.S.; Hopkins, N. Zebrafish mutations and functional analysis of the vertebrate genome. *Genes Dev* **14**: **2000**, 755-762.
- [105] Warren, K.S.; Baker, K.; Fishman, M.C. The slow mo mutation reduces pacemaker current and heart rate in adult zebrafish. *Am. J. Physiol. Heart Circ. Physiol* **2001**, *281*: H1711-H1719.
- [106] Snyder, M.J.; Mulder, E.P. Environmental endocrine disruption in decapod crustacean larvae: hormone titers, cytochrome P450, and stress protein responses to heptachlor exposure. *Aquat. Toxicol.* **2001**, *55*: 177-190.
- [107] Liu, J.; Kadiiska, M.B.; Liu, Y.; Lu, T.; Qu, W.; Waalkes, M.P. Stress-related gene expression in mice treated with inorganic arsenicals. *Toxicol. Sci* **2001**, *61*: 314-320.
- [108] Lyons-Alcantara, M.; Mooney, R.; Lyng, F.; Cottell, D.; Mothersill, C. The effects of cadmium exposure on the cytology and function of primary cultures from rainbow trout. *Cell. Biochem. Funct.* **1998**, *16*: 1-13.
- [109] Bartosiewicz, M.; Penn, S.; Buckpitt, A. Application of gene arrays in environmental toxicology: fingerprints of gene regulation associated with cadmium chloride, benzo(a)pyrene, and trichloroethylene. *Environ. Health Perspect* **2001**, *109*: 71-74.
- [110] Ryan, J.A.; Hightower, L.E. Stress proteins as molecular biomarkers for environmental toxicology. *EXS* **1996**, *77*: 411-424.
- [111] Whitesell, L.; Bagatell, R.; Falsey, R. The stress response: implications for the clinical development of hsp90 inhibitors. *Curr. Cancer Drug Targets* **2003**, *3*: 349-358.
- [112] Yenari, M.A. Heat shock proteins and neuroprotection. *Adv. Exp. Med. Biol.* **2002**, *513*: 281-299.
- [113] Krone, P.H.; Sass, J.B.; Lele, Z. Heat shock protein gene expression during embryonic development of the zebrafish. *Cell Mol. Life Sci* **1997**, *53*: 122-129.
- [114] Sass, J.B.; Martin, C.C.; Krone, P.H. Restricted expression of the zebrafish *hsp90a* gene in slow and fast muscle fiber lineages. *Int. J. Dev. Biol* **1999**, *43*: 835-838.
- [115] Lele, Z.; Hartson, S.D.; Martin, C.C.; Whitesell, L.; Matts, R.L.; Krone, P.H. Disruption of zebrafish somite development by pharmacologic inhibition of Hsp90. *Dev. Biol* **1999**, *210*: 56-70.
- [116] Halloran, M.C.; Sato-Maeda, M.; Warren, J.T.; Su, F.; Lele, Z.; Krone, P.H.; Kuwada, P.H.; Shoji, W. Laser-induced gene expression in specific cells of transgenic zebrafish. *Development* **2000**, *127*: 1953-1960.
- [117] Shoji, W.; Isogai, S.; Sato-Maeda, M.; Obinata, M.; Kuwada, J.Y. Semaphorin3a1 regulates angioblast migration and vascular development in zebrafish embryos. *Development* **2003**, *130*: 3227-3236.
- [118] Martin, C.C.; Tang, P.; Barnardo, G.; Krone, P.H. Expression of the chaperonin 10 gene during zebrafish development. *Cell Stress Chaperones* **2001**, *6*: 38-43.
- [119] Martin, C.C.; Tsang, C.H.; Beiko, R.G.; Krone, P.H. Expression and genomic organization of the zebrafish chaperonin gene complex. *Genome* **2002**, *45*: 804-811.
- [120] Martin, C.C.; Laforest, L.; Akimenko, M-A.; Ekker, M. Role for DNA methylation in gastrulation and somite patterning. *Dev. Biol.* **1999**, *206*: 189-205.
- [121] Yamakoshi, K.; Shimoda, N. De novo DNA methylation at the CpG island of the zebrafish no tail gene. *Genesis* **2003**, *37*: 195-202.
- [122] Dong, W.; Teroka, H.; Kondo, S.; Hiraga, T. 2,3,7,8-tetrachlorodibenzo-p-dioxin induces apoptosis in the dorsal midbrain of zebrafish embryos by activation of arylhydrocarbon receptor. *Neurosci. Lett.* **2001**, *303*: 169-172.
- [123] Dong, W.; Teraoka, H.; Yamazaki, K.; Imani, S.; Imagawa, T.; Stegeman, J.J.; Peterson, R.E.; Hiraga, T. 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. *Toxicol. Sci.* **2002**, *69*: 191-201.
- [124] Nagel, R. DarT: the embryo test with the zebrafish *Danio rerio* - a general model in ecotoxicology and toxicology. *ALTEX* **2002**, *19*: 38-48.
- [125] Van de Peer, Y.; Taylor, J.S.; Joseph, J.; Meyer, A. Wanda: a database of duplicated fish genes. *Nucleic Acids Res.* **2002**, *30*: 109-112.
- [126] Jozefowicz, C.; McClintock, J.; Price, V. The fates of zebrafish Hox gene duplicates. *J. Struct. Funct. Genomics* **2003**, *3*: 185-194.
- [127] Farber, S.A.; Pack, M.; Ho, S.Y.; Johnson, I.D.; Wagner, D.S.; Dosch, R.; Mullins, M.C.; Hendrickson, H.S.; Hendrickson, E.K.;

- Halpern, M.E. Genetic analysis of digestive physiology using fluorescent phospholipid reporters. *Science* **2001**, 292: 1385-1388.
- [128] Isogai, S.; Lawson, N.D.; Torrealday, S.; Horiguchi, M.; Weinstein, B.M. Angiogenic network formation in the developing vertebrate trunk. *Development* **2003**, 130: 5281-5290.
- [129] Lawson, N.D.; Weinstein, M.B. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* **2002**, 15: 307-318.
- [130] Pelster, B. Developmental plasticity in the cardiovascular system of fish, with special reference to the zebrafish. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **2002**, 3: 547-553.
- [131] Patton, E.E.; Zon, L.I. The art and design of genetic screens: zebrafish. *Nat. Rev. Genet.* **2001**, 2: 956-966.