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Zebrafish in Toxicology and Environmental Health

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Abstract

As manufacturing processes and development of new synthetic compounds increase to keep pace with the expanding global demand, environmental health, and the effects of toxicant exposure are emerging as critical public health concerns. Additionally, chemicals that naturally occur in the environment, such as metals, have profound effects on human and animal health. Many of these compounds are in the news: lead, arsenic, and endocrine disruptors such as bisphenol A have all been widely publicized as causing disease or damage to humans and wildlife in recent years. Despite the widespread appreciation that environmental toxins can be harmful, there is limited understanding of how many toxins cause disease. Zebrafish are at the forefront of toxicology research; this system has been widely used as a tool to detect toxins in water samples and to investigate the mechanisms of action of environmental toxins and their related diseases. The benefits of zebrafish for studying vertebrate development are equally useful for studying teratogens. Here, we review how zebrafish are being used both to detect the presence of some toxins as well as to identify how environmental exposures affect human health and disease. We focus on areas where zebrafish have been most effectively used in ecotoxicology and in environmental health, including investigation of exposures to endocrine disruptors, industrial waste byproducts, and arsenic.

1. INTRODUCTION

Rapid growth of populations and technological advancement has resulted in innumerable pollutants and environmental toxin exposure. This has generated a vital need for toxin surveillance, identification of consequences of exposure, and understanding of the biologic, chemical, and genetic mechanisms that underlie those effects (Landrigan, 2016). The field of environmental health was established as early as the 1940s, in response to the expansion of chemical manufacturing and the occurrence of contamination of the water, soil, and air caused by widespread use of chemicals in industry and consumer products (Landrigan, 2016). Since World War II, thousands of synthetic chemical compounds have been created for industrial applications and have subsequently been introduced into consumer products. Today, approximately 70,000 chemicals are in commercial use in the United States, and 3300 of these are high production volume compounds, with annual production or importation volumes in excess of one million pounds. In addition to the risks posed by the expanding repertoire of manufactured toxins, naturally occurring chemicals, such as metals can also cause harm, as was recently brought to focus by the lead contamination of the

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drinking water in Flint, Michigan (Bellinger, 2016; Tong, Baghurst, McMichael, Sawyer, & Mudge, 1996).

Environmental toxins profoundly affect fish and wildlife. In particular, water pollution has damaged fish populations by affecting reproductive health, lifespan, and embryonic and larval development. This has a major effect on aquatic ecosystems and on the industries that depend on them. Humans are exposed to environmental toxicants through fine particulate matter in the air, endocrine-disrupting chemicals (EDCs) found in food packaging, household items and personal care products, and naturally occurring compounds such as metals. Human exposure to environmental chemicals is associated with both acute toxicity and long-term consequences (Landrigan et al., 2016), which include congenital abnormalities (Swan et al., 2005), chronic diseases (Argos et al., 2010; Mazumder, 2005), cognitive disabilities (Jacobson, Muckle, Ayotte, Dewailly, & Jacobson, 2015; Muñoz-Quezada et al., 2013; Tong et al., 1996), cancer (Liu & Wu, 2010; Selikoff & Hammond, 1968; Wang, Cheng, & Zhang, 2014), and death (Argos et al., 2010). The field of environmental health is expanding to meet the demands of surveillance and prevention of consequences of environmental toxin exposure on both wildlife and human health.

There are many unanswered questions in the field of environmental health (Henn, Coull, & Wright, 2014; Landrigan, Suk, & Amler, 1999), and a surge in research effort is required to answer these. Among the most pressing are: What are the effects of low dose, cumulative exposures, and exposures to multiple toxicants? What are the developmental processes that are altered by toxicant exposure and how are these processes affected? What are the latent effects of early life exposure? Are these effects apparent in subsequent generations? Can we develop surveillance technologies to limit exposure? How can therapeutic interventions be designed and administered to reverse the effects of exposure? The barriers to addressing these questions in human populations are both practical and logistical. In terms of low-dose and cumulative exposures, the appropriate biomarkers and the ideal tissue specimens for analysis have not been identified for every toxicant or combination of toxicants. Understanding the latent and transgenerational effects of exposure is difficult in humans due out long lifespans and relatively small number of offspring. In addition, the interaction between environmental toxicants and social “exposures” including chronic stress, exposure to violence, and nutrient scarcity are only beginning to be understood. There is a critical need for in vivo animal models to study the short- and long-term effects of environmental toxins.

Zebrafish are a valuable tool for Environmental Health researchers as evidenced by a rapidly expanding body of research using zebrafish. A PubMed search using the terms “zebrafish environmental health” reveals that the use of zebrafish in this field has been steadily increasing over the past few decades (Fig. 1). In this chapter, we will highlight the unique advantages of using zebrafish embryos, larvae, and adults to address pressing issues in Environmental Health, including contaminant detection, environmental monitoring, toxicity/teratogenicity testing, and investigations into mechanisms of action and disease phenotypes associated with exposure to chemical compounds. In a field that rapidly changes with evolving technology and manufacturing worldwide, zebrafish can offer real-time in vivo studies to address potential hazards to human health that result from naturally occurring

compounds and commercial use of new synthetics or byproducts of their production, and can improve our limited understanding of the specific effects of environmental exposures.

2. HISTORICAL PERSPECTIVE: TOXICOLOGY AND GOVERNMENT EFFORTS FOR ENVIRONMENTAL REGULATION

In 1976, the United States Congress passed the Toxic Substances Control Act, which granted the Environmental Protection Agency (EPA) the authority to require testing and reporting, and set restrictions on the manufacture and use of chemicals and mixtures (Toxic Substances Control Act, 1976). Since the passage of this law, science, and technology have made rapid progress necessitating further legislative intervention to protect the public from the health effects of chemical exposures (Birnbaum, 2010). In response, the National Toxicology Program was established in 1978 as an interagency program by the Department of Health, Education, and Welfare, which is known today as the Department of Health and Human Services, to address public, scientific, and governmental concerns that human diseases and disabilities are linked to chemical exposures (Xie, Holmgren, Andrews, & Wolfe, 2016). In 1980, as part of the Comprehensive Environmental Response, Compensation and Liability Act, more commonly referred to as the “Superfund Act,” Congress formed the Agency for Toxic Substances and Disease Registry (ATSDR). ATSDR is a science-based public health agency created to study the health effects of hazardous substances in the environment and to work with communities to keep them safe from hazardous waste. To address this mission, ATSDR collects data and conducts studies in addition to using the best available scientific data to make recommendations to the EPA and other agencies to prevent and stop exposures to ensure the health of communities. Studies in zebrafish have provided critical information about the health effects of a majority of the 250+ substances on this list. Based on the strength of published data and the size of potentially exposed population, the ATSDR publishes a biannual Priority List of Hazardous Substances to select those substances that should be the subject of toxicological profiles.

With the dissolution of the National Children’s Study 2014, the NIH reappropriated funds to create the Children’s Health Exposure Analysis Resource (CHEAR) to focus on how children’s health is shaped by the environment. Through CHEAR, researchers with NIH-funded child cohorts can apply to have biological samples analyzed for chemicals, metabolites, and biomarkers of exposure. In 2016, President Obama signed the Frank R. Lautenberg Chemical Safety of the 21st Century Act into law requiring that the EPA perform testing of chemicals currently in use, set new risk-based safety standards, require protection for vulnerable populations, and increase public transparency for chemical information. The use of zebrafish has and will continue to be a tool to provide the EPA with invaluable information regarding the short-term toxicity and long-term health effects of toxicant exposure to human health and its utility has been highlighted in a special issue of the journal *Zebrafish* that was dedicated to the use of this model in toxicology research (Gamse & Gorelick, 2016).

3. ENVIRONMENTAL AND DEVELOPMENTAL TOXICOLOGY

Zebrafish are commonly used to model human diseases using genetic modifications; applications including studies of heart, kidney, liver, hematopoietic, immune, and other systems detailed other chapters in this volume. They can similarly be used to model the health effects of environmental exposures to better understand the etiologies and mechanisms of environment-related disease in humans. The concern is growing over the persistence of chemical compounds in the environment as well as the acute and long-term health effects of exposure to environmental toxicants and contaminants and zebrafish provide an ideal model to study these effects. Chemicals can simply be added to the embryo medium and the developing and transparent zebrafish can be assessed for lethality and developmental abnormalities from fertilization through larval stages. Although juvenile and adult zebrafish are not transparent, the generation of the unpigmented Casper mutant line can be crossed to transgenic fluorescent reporters to aid observation and imaging of organ systems in older zebrafish (White et al., 2008).

The ability to observe effects of toxins in vivo allows for direct assessment of toxicity, as well as measurements of absorption, distribution, metabolism, and elimination. This can be extended for use in screening for treatments that can mitigate toxic effects in live animals as well. Zebrafish express a full range of *cytochrome P450* (*cyp*) genes required for xenobiotic metabolism and biotransformation (Goldstone et al., 2010). In the zebrafish genome assembly (GRCz10), a total of 86 *cyp* genes were identified (Saad et al., 2016) with many of the metabolic characteristics of the related human enzymes, demonstrating a strong evolutionary relationship with those found in humans. However, there remains a significant lack of information about the specific mechanisms of zebrafish xenobiotic Cyp activity.

Zebrafish have been used to study the compounds ranging from naturally occurring metals and metalloids, to synthetic components of consumer products, pesticides, and byproducts of industrial processing and waste incineration. In this chapter, we will present the zebrafish tools that have been employed for the detection of these toxicants and how zebrafish research is contributing to the understanding of the effects of these compounds on the environment and on human health.

4. ZEBRAFISH: TESTING THE WATERS FOR TOXICANTS

In 1982, George Streisinger, the founder of the zebrafish field (Streisinger, Walker, Dower, Knauber, & Singer, 1981), proposed the use of zebrafish as a vertebrate model to study the frequency of mutations in response to environmental carcinogens (Streisinger, 1983). In the following three decades, zebrafish have been used to identify teratogens, to uncover mechanisms of action of common toxicants, and to understand the tissue specificity of toxicant impact on vertebrates (Gamse & Gorelick, 2016). Zebrafish provides a unique, in vivo, medium-throughput system to expand cell culture assays to a whole vertebrate model, but are less expensive than rodents. The benefit of the large population size of zebrafish offspring is a major benefit, as studies in zebrafish allow for the rapid assessment of compound toxicity and the ability to study molecular mechanisms underlying developmental and health outcomes associated with toxicant exposure across a population of live

vertebrates. In addition, large numbers of offspring enable longitudinal studies that can be done on a population scale of the developmental effects of environmental exposures at a relatively less cost than longitudinal rodent studies. Zebrafish are also easily amenable to drug discovery screens as sentinels of environmental contamination, for toxicity testing, and for investigations into the mechanisms of action of pharmaceuticals and toxicants. The zebrafish model provides the opportunity to combine the power of rapid toxicology screens with the ability to study the association of exposures with long-term outcomes in a vertebrate, making zebrafish an invaluable complementary system for research in Environmental Health.

4.1 Transgenic Zebrafish as Surveillance Tools

For nearly two decades, zebrafish have been used for biomonitoring. A major advantage of using zebrafish for this work is that the embryos and larvae are transparent and generating transgenic animals is relatively easy. This has allowed the development of transgenic lines where a fluorescent protein or other measureable readout becomes activated in the presence of contaminants or environmental stressors provide a system to assess the level of response and the tissue specificity of the response (Carvan, Dalton, Stuart, & Nebert, 2000; Gorelick, Iwanowicz, Hung, Blazer, & Halpern, 2014; Lee, Green, & Tyler, 2015). In the earliest efforts, investigators developed transgenic zebrafish lines in which expression of the luciferase or green fluorescent protein (*GFP*) gene is driven by pollutant response elements that report on the presence of aromatic hydrocarbons, electrophiles/oxidants, metals, estrogenic compounds, or retinoids (Carvan et al., 2000). Transgenic lines have been used not only to detect the presence of toxins, but can facilitate investigations into the molecular mechanisms underlying pathology associated with environmental exposures. The use of transgenic reporter zebrafish lines to measure exposure to heavy metals, organic chemicals, endocrine disruptors, and electrophilic agents has been expertly reviewed elsewhere (Lee et al., 2015) and are outlined in Table 1. We describe how such tools are used to both detect the presence of contaminants and to understand their physiological impact.

4.2 Biosensors of Environmental Contaminants

Zebrafish have been used as sentinels to identify the effects of public water supplies. An early study examined the teratogenic effects of sediment and ground water in the Netherlands in zebrafish combined with a biochemical assay in tissue culture cells and found that the zebrafish teratogen assay was equally as sensitive in identifying the presence of toxic contaminants (Murk et al., 1996). In more recent work, several researchers have generated transgenic reporter lines in which a promoter drives expression of a fluorescent protein or other reporter that is regulated by exposure to a toxin (Table 1). In this section, we will highlight specific transgenic lines that have been generated and used to not only identify classes of chemical contaminants in experimental settings, but with the potential to lend insights into toxicant-induced stress responses.

4.2.1 Aromatic Hydrocarbons and the Aryl Hydrocarbon Receptor (Ahr)—The aryl hydrocarbon receptor (Ahr) is a cytosolic receptor that is expressed in various tissues during development and adulthood, and signaling through this receptor has been studied in multiple developmental processes in rodents and zebrafish (Schneider, Branam, & Peterson,

2014). The Ahr is activated in response to synthetic and natural aromatic (aryl) hydrocarbons and functions as a transcription factor to bind to the dioxin-responsive element (DRE) to induce the expression of genes including those encoding the CYP enzymes, which are involved in xenobiotic metabolism. A DRE-containing fragment of the *cyp1a1* gene, which is regulated by Ahr, was used to drive expression of a nuclear-localized GFP (*Tg(cyp1a:nls-gfp)*) and this shows activation in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Kim et al., 2013). However, more recently, the *Tg(cyp1a:gfp)* transgenic line was generated using the medaka *cyp1a* promoter, and this transgenic zebrafish provides a more sensitive biosensor for Ahr activity (Xu et al., 2015). Use of these systems has identified the kidney, liver, and gut as target tissues for TCDD and have also been shown to respond to other dioxin-like chemicals and polyaromatic hydrocarbons (Xu et al., 2015).

4.2.2 Metals—The discovery of elevated lead levels in the drinking water of Flint, Michigan in 2016 has renewed efforts to mitigate the toxic effects of human exposure to metals. In addition to lead, many other metals including copper, platinum, cadmium, and zinc have severe toxic effects on humans and animals. Zebrafish have been used extensively to study the consequences of metal exposure, and a unique transgenic animal has been developed to detect the presence of metals in water. The *Tg(mt:egfp)* transgenic zebrafish expresses enhanced green fluorescent protein (EGFP) under the transcriptional control of the metal-responsive metallothionein promoter. This line can be used as a reporter for aquatic zinc and cadmium (Liu, Yan, et al., 2016). Recent data had shown that 10 days postfertilization (dpf) zebrafish larvae did not show significant developmental abnormalities even when exposed to levels of heavy metals that exceeded current regulatory limits by 10- or 70-fold for zinc and cadmium, respectively; however, transgene activity was detected following 24 h of exposure to zinc at the current regulatory limit and cadmium at twice the current regulatory limit. Use of the *Tg(mt:egfp)* zebrafish line provides an advance in the field as it provides a more robust readout for the presence of elevated levels of heavy metals (Liu, Yan, et al., 2016). This is useful, as exposure to a number of different metals is associated with neurodevelopmental deficits. However, further work is needed to refine these tools to respond to additional environmentally relevant metals at a broader range of concentrations.

4.3 Zebrafish Transgenics Shine Light on the Mechanisms of Toxicant-Related Disease

Cellular stress is a central and conserved response to toxin exposure. Many pollutants, including metals, pesticides, and oxidative agents are known or suggested to induce endoplasmic reticulum (ER) stress (Chen, Melchior, & Guo, 2014; Kitamura, 2013). Induction of ER stress contributes to a variety of human diseases including neurodegenerative diseases, metabolic dysfunction, inflammatory diseases, and cancer, the risks for which may be compounded by underlying toxicant exposure (Wang & Kaufman, 2016). Oxidative stress is one cause of ER stress, and the metabolism of many toxic compounds, including pesticides and metals, results in the generation of reactive oxygen species. As of yet, there have been few animal models in which to investigate the consequences of toxicant exposure and metabolism, and zebrafish represent a significant advance in this field.

4.3.1 Cellular Stress Reporters—CHOP (also called DDIT3) is a transcription factor that is strongly induced and translocated to the nucleus in response to some types of ER stress (Harding, Zhang, & Ron, 1999; Palam, Baird, & Wek, 2011). The transgenic zebrafish line *huORFZ* contains a GFP transgene under the control of the upstream open reading frame of the human *CHOP* cDNA (Lee et al., 2011). There are several important features of the *huORFZ* model that make it ideal for first-line pollution monitoring: (1) exposure to different chemical stressors results in distinct patterns of GFP expression, indicating the cell types and organ systems that respond to a given toxicant; (2) the system is responsive to several pollutants at the range of concentrations enforced by current World Health Organization guidelines; and (3) GFP expression decreased following the exposure period, which suggests that expression is a direct result of the physiological response upon toxicant exposure. Lee et al. (2015) have demonstrated that this transgenic line can be used to detect the presence of environmental contaminants, including heavy metals and EDCs (Lee et al., 2014). The response of this transgenic line is not limited to a particular stressor and can be applied to a range of chemicals or toxicants that induce ER stress.

The heat shock response is a cellular strategy used to protect the cell, by the induction of a number of protein chaperones, which prevents the aggregation of unfolded and misfolded proteins that accumulate due to stress. In addition to heat stress, this survival-promoting response can be induced by aging, protein-folding diseases, and exposure to toxic chemicals (Scheff Jeremy, Stallings Jonathan, Reifman, & Rakesh, 2015). The heatshock promoter driving GFP *TgBAC(hspb11:GFP)* has been used as a surrogate marker to identify the tissue-specific effects of pesticides (Shahid et al., 2016). This recent study highlights how different cell types are impacted by exposure to the same stressor. Zebrafish embryos were exposed to a number of different pesticides from 9 to 48 hours postfertilization (hpf) and examined for induction of the *TgBAC(hspb11:GFP)* transgene and muscle integrity. This transgene largely is activated in the muscle and notochord of embryos exposed to pesticides, however, the magnitude of the response varied: azinphosmethyl had a moderate effect on induction of the *hspb11* transgene and also only modestly affected muscle integrity, whereas, galanthamine caused severe disruption of muscle integrity and strongly activated the *hspb11* promoter (Shahid et al., 2016). Interestingly, the transgene remained active in muscle tissue up to 48 h after the pesticides were removed, indicating the long-lasting effects of toxin exposure on these cells.

4.3.2 Reporters of Endocrine Activity—Activation of estrogen receptors (ERs) is important for developmental processes and sexually dimorphic behaviors. In addition to estradiol and environmental estrogens, several synthetic or exogenous compounds are known to interfere with hormone signaling and have endocrine-disrupting activity (EDCs). One of the main challenges of assessing the effects of EDC is that these compounds are typically functional at very low concentrations and exhibit nonlinear dose responses (Vandenberg et al., 2012). Because of this, the Endocrine Society recommends a “no-threshold” approach to risk assessment for EDC (Zoeller et al., 2012). Zebrafish transgenic reporters thus provide a unique system in which to detect endocrine activity in the absence of gross morphological abnormalities.

Two common zebrafish transgenic reporters that are used for the detection of estrogen receptor signaling are the *Tg(5xERE:GFP)* (Gorelick & Halpern, 2011; Gorelick et al., 2016) and *Tg(cyp19a1b:GFP)* (Cano-Nicolau et al., 2016; Sonavane et al., 2016) zebrafish lines. These lines respond to a range of estrogenic compounds at different doses. For instance, 17α-ethynylestradiol (EE) and diethylstilbestrol (DES) induce GFP expression at the pM to nM range, whereas bisphenol A (BPA) does not induce fluorescence at exposures below the μM range (Cano-Nicolau et al., 2016; Gorelick & Halpern, 2011). In addition, reporters of endocrine activity have been used to detect environmental contamination in water samples (Gorelick et al., 2014; Sonavane et al., 2016) with similar sensitivity to the established bioluminescent yeast assay (Gorelick et al., 2014). Both of these transgenic lines have been used to detect estrogens in samples collected using the Polar Organic Chemical Integrative Sampler, which concentrates estrogens from environmental water samples. Samples extracted from the membranes are diluted in embryo water, at concentrations higher than that found directly at sampling sites, for exposure and assessment of reporter activity. Due to the nature of the sampling method, these studies determine the estrogenic effects of environmental mixtures. Although use of these transgenic reporters for detection of environmental estrogens has not yet resulted in policy change, these studies highlight the use of zebrafish not only for the detection of estrogenic activity at a single point, but reveal their use in determining variations in contamination levels over time.

While these and other reporters have been highly effective in providing both a practical tool for water quality surveillance and for studying the mechanism of toxin-mediated damage, one major limitation is that by using fluorescent proteins such as GFP, which are slow to mature and have a long half-life, these reporters cannot capture the dynamic response to toxins. A second limitation is that the detection of fluorescent reporters in high-throughput automated imaging systems may be hindered by suboptimal embryo positioning (or nonuniform transgene expression across the zebrafish), such that the brightest parts of some embryos are not imaged accurately. New approaches to surmount these challenges are currently being developed and will enhance the utility of zebrafish transgenics to uncover mechanisms underlying environmental toxicant exposures.

5. TRANSCRIPTIONAL PROFILING TO IDENTIFY CONTAMINANTS

Integration of “-omic” technologies into environmental toxicology has been occurring at a rapid pace as new advances in image processing and data analysis make the use of these applications more feasible. Connectivity mapping is a data-driven approach combining transcriptomics and machine learning technology, and has previously been used to link disease and drug-induced phenotypes on the basis of differential gene expression patterns (Lamb et al., 2006). This approach has been applied to assess exposure and toxicity to chemical groups based on mechanism(s) of action and transcriptomic changes. Software packages have been developed to allow users to compare gene expression profiles under their treatment or exposure conditions to those archived in publicly available databases (Sandmann, Kummerfeld, Gentleman, & Bourgon, 2014). Wang et al. (2016) published the first use of connectivity mapping in environmental health using zebrafish. By mining publicly available microarray datasets, the group compared the transcriptional responses to a range of chemical exposures and doses in different organs from zebrafish and fathead

minnow. Mapping is more successful within species and among those samples run on the same platform. As the cost of mRNA sequencing technologies reduces, whole genome data will be available for an increasing number of model systems under different experimental conditions. Connectivity mapping also offers researchers the opportunity to generate hypotheses about the mechanisms of action for environmental pollutants or toxins for which mechanistic pathways were previously unknown.

Many toxins specifically affect the liver, as this is the primary site of xenobiotic metabolism in vertebrates. Pathologies ranging from necrosis and fatty liver, to steatohepatitis and liver cancer have been found to result from occupational and environmental exposure to chemicals and toxicants (Al-Eryani et al., 2015; Wahlang et al., 2013). Toxin-specific hepatic responses have been identified in zebrafish using gene expression analysis on a variety of platforms to determine the hepatic response to a range of toxins, including arsenic, acetaminophen, and ethanol (Xu, Lam, Shen, & Gong, 2013; Zhang, Li, & Gong, 2014). A well-defined genome, easy access to target organs, and conserved responses to toxins make zebrafish amenable to emerging genomic, proteomic, and metabolomics approaches to better understand the molecular changes caused by toxins.

6. HIGH-THROUGHPUT SCREENING FOR TOXICITY STUDIES

A major goal for toxicology studies is to be able to screen many compounds in a short amount of time and with accuracy in predicting human toxicity. In 2007, the EPA began the ToxCast program to screen chemicals in order to develop protocols that would lead to improved human toxicity prediction (Dix et al., 2007). The pilot study, ToxCast Phase I, included 310 compounds (mostly pesticides) that were screened in a large number of medium- and high-throughput screening assays (Judson et al., 2010). That same year, the National Research Council published a report titled “Toxicity testing in the 21st Century: A Vision and Strategy” (National Research Council, 2007), which prompted rapid expansion of ToxCast, and in Phase II of the ToxCast program, the chemical library was expanded to 1878 compounds for which testing concluded in 2013 (Richard et al., 2016). ToxCast Phase I and II library compounds have been tested in model organisms including *C. elegans* and zebrafish (Boyd et al., 2016; Padilla et al., 2012; Sipes, Padilla, & Knudsen, 2011). Phase III of the ToxCast program contains greater than 3800 unique chemicals and compounds under evaluation (Richard et al., 2016).

Toxicology in the 21st Century (Tox21) program is a collaboration between the NIEHS National Toxicology Program, the EPA, and the National Center for Advancing Translational Science to test more than 10,000 environmental chemicals and drugs to elucidate their toxicity in biochemical and cell-based assays (Collins, Gray, & Bucher, 2008). The FDA’s ToxCast joined the collaboration in 2010 and are now jointly referred to as the “ToxCast chemical library” (Richard et al., 2016). The European community has also responded with the EU *R*egistration, *E*valuation, *A*uthorization and restriction of *CH*emical substances (REACH) legislation, requiring the collection of toxicity data for chemicals that are produced or marketed in quantities in excess of one ton per year (Selderslaghs, Blust, & Witters, 2012). Several research centers in Europe now use zebrafish as the central animal

model for toxicology studies and centralized groups have issued a white paper calling for increased resources for using zebrafish for toxicology research (www.eufishbiomed.kit.edu).

Limitations to these approaches are that xenobiotic metabolism cannot be studied in vitro, determining active in vivo doses and blood concentrations from in vitro studies is not possible, understanding the effects of chronic exposure is impossible in vitro, and knowing whether or when a given genetic or signaling perturbation would result in a phenotypic change in an animal is difficult to ascertain (Tice, Austin, Kavlock, & Bucher, 2013).

Zebrafish are being used as a first-pass screen to identify chemicals with the highest likelihood of posing risk to humans and require further testing (Dix et al., 2007). Researchers at the EPA used the zebrafish developmental assay to add information to the toxicity assay database of the ToxCast Phase I library (Padilla et al., 2012). In this first large-scale screen of the effects of environmental contaminants zebrafish, embryos were exposed from 6 hpf to 5 dpf to a single dose and then a concentration range from 1 nM to 80 μM. Survival and morphological defects were assessed at 6 dpf. This was expanded in a subsequent study that analyzed the effects of several hundred chemicals from the ToxCast Phase II library on 18 different endpoints in zebrafish larvae at 5 dpf (Truong et al., 2014). More recently, 1060 compounds from the ToxCast I and II chemical libraries have been tested in a phenotype-based screen in zebrafish to predict teratogenic effects. This study showed that hypoactivity at 24 hpf in exposed zebrafish embryos is associated with an increased risk of 17 specific developmental abnormalities as assessed 5 dpf larvae (Reif et al., 2016). Interestingly, this study also identified a group of chemical compounds that caused the same degree of hypoactivity at 24 hpf, with no corresponding morphologic defect at 5 dpf, indicating that this protocol may prevent false negatives. Efforts to build databases and develop assays to predict human toxicity have capitalized on the use of zebrafish as a quick, medium throughput in vivo system to accurately predict human toxicity.

In 2009, an international group of pharmaceutical companies formed a consortium to develop a zebrafish development assay that could correctly classify a set of 10 teratogenic and 10 nonteratogenic compounds (Gustafson et al., 2012). The results of these toxicity tests were compared to mammalian data, and found to have an overall concordance of 60–70%. In a second phase of this consortium project, 38 proprietary pharmaceutical compounds were tested by two independent laboratories, and 79% of the classifications were the same between the laboratories, although the laboratories differed in their concordance with in vivo data (Ball et al., 2014). The Dechorionated Zebrafish Embryo Developmental toxicity assay was developed to identify the no-adverse-effect-level (NOAEL) and the concentration resulting in 25% lethality (LC_{25}) for a training set of 31 compounds (Brannen, Panzica-Kelly, Danberry, & Augustine-Rauch, 2010). This approach yielded 87% concordance with published in vitro teratogenicity data (Brannen et al., 2010). Improvements to this assay, including enzymatic removal of the chorion, repeating the assay with a distinct set of text compounds, and using various zebrafish strains, have been attempted to make a direct comparison between the chorion-on data published by the pharmaceutical company consortium and the chorion-off data to determine whether the presence of the chorion affected the sensitivity and specificity of the zebrafish embryo assay (Ball et al., 2014;

Brannen et al., 2010; Gustafson et al., 2012; Panzica-Kelly, Zhang, & Augustine-Rauch, 2015).

New advances in the morphological assessment of toxicant-exposed zebrafish larvae allow for the determination of the effects of test compounds on developmental endpoints. Computational approaches, including the Cellomics® ArrayScan® V^{TI} high-content image analysis platform reduce the time required for analysis and reduce variability between experiments while providing an image that can be kept for permanent record or reevaluated manually (Deal et al., 2016). Bright field image analysis will identify a large number of phenotypes that may be undetectable using other methods. The use of zebrafish offers a valuable tool for high-throughput screening of compounds with demonstrated accuracy in predicting human toxicity. Further, development of these technologies and platforms will be important for identifying target organs and generating hypotheses about mechanisms of action for chemicals for which no biological data are available.

7. ASSESSING HEALTH IMPACTS OF ENVIRONMENTAL EXPOSURES USING ZEBRAFISH

The ability to assess tissues for toxin accumulation and its associated phenotypes can lead to valuable insights into disease processes and enable therapeutic compound screening. While transgenic reporter lines can monitor differentiation of distinct cell lineages and detect the induction of signaling pathways, much of the data acquisition is limited to low- and medium-throughput applications due to the time required for screening individual zebrafish embryos and larvae by fluorescence microscopy.

7.1 Automated Reporter Quantification In Vivo (ARQiv)

Automated reporter quantification in vivo (ARQiv) is a high-throughput screening platform that uses a microplate reader to detect changes in the intensity of transgenic fluorescent reporters in live zebrafish embryos and larvae over time (Walker et al., 2012). Recently, ARQiv technology has been applied to test FDA-approved drugs and their ability to increase the number of insulin-producing pancreatic β cells in a transgenic reporter zebrafish line (*Tg(ins:PhiYFP-2a-nsfB; sst2:tagRFP)lmc01*), demonstrating the feasibility of this approach for both quantification of cell number and fluorescent reporter intensity (Wang et al., 2015). An increase of as little as 10 β cells in the developing pancreas was detected, highlighting the ability of this technique to identify small changes in the development of this important organ.

Although transgenic reporters are routinely used to visualize the effects of drugs and chemical compounds on developing organ systems (Lam et al., 2011; Ma et al., 2015), this technology can also be applied to environmental exposures using the same tissue- and signaling pathway-specific transgenic reporter lines to assess toxin-induced effects on the development of cell types and organ structures. One major limitation is that it only provides quantification of reporter levels without corresponding images to allow for the analysis of morphological changes associated with changes in reporter activity (Wang et al., 2015). Data

generated using ARQiv will need to be coupled with that from other imaging techniques to obtain full understanding about the phenotypic effects of a particular exposure.

7.2 Laser Ablation-Inductively Coupled Plasma-Mass Spectroscopy (LA-ICP-MS)

Laser ablation-inductively coupled plasma-mass spectroscopy (LA-ICP-MS) can be used to provide spatial information about element distribution in biological samples (Hare, Austin, & Doble, 2012). This technique can be used in calcified tissue (teeth) and soft tissue (placenta) (Arora et al., 2014; Niedzwiecki et al., 2016). We are currently optimizing the use of LA-ICP-MS to determine tissue accumulation and organ-specific distribution of elements in whole zebrafish larvae (data not shown). The technique is able to detect compounds at concentrations below parts-per-million and has spatial resolution capacity at the micrometer range allowing for detailed analysis of tissue; however, quantification of trace elements within tissue samples using LA-ICP-MS analysis is not yet reliable due to properties of the ablation process (Hare et al., 2012).

7.3 Automated Assessment of Behavior and Morphologic Phenotypes

Complex developmental effects associated with exposures can be studied in zebrafish using behavioral profiling (Rihel et al., 2010) and phenotype-driven screens (Gallardo et al., 2015). Behavioral profiling is most useful for modeling effects on brain activity and has recently been used to identify phenotypic suppressors of autism in a zebrafish genetic model of hyperactivity (Hoffman Ellen et al., 2016). Similar efforts could be used to identify environmental modifiers of genes associated with autism spectrum and other neurological disorders. Phenotype-driven chemical screening has been used to identify compounds that altered the collective migration of fluorescently marked cells (Gallardo et al., 2015).

Automation of image capture and phenotype analysis will improve the ability of researchers to screen larger libraries of compounds over wider concentration ranges, while limiting bias in the assays (Deal et al., 2016; Jeanray et al., 2015; Mikut et al., 2013). Optimized techniques for embryo immobilization will enable imaging the developing zebrafish larvae using state of the art techniques including light sheet fluorescence microscopy (Höckendorf, Thumberger, Wittbrodt, 2012; Kaufmann, Mickoleit, Weber, & Huisken, 2012). Zebrafish can facilitate analysis of developmental and structural changes over time, but require development of advanced video capabilities. Recently, an open source application for the video analysis of movement of larval zebrafish has been created for academic use (Cario, Farrell, Milanese, & Burton, 2011). The rapid advances in imaging and computational technologies to identify the morphologic consequences of toxicant exposure in zebrafish, put this model at the forefront of the field with the potential to advance the identification of teratogenic and tissue-specific effects of toxins.

8. LONG-TERM AND TRANSGENERATIONAL EFFECTS OF TOXIN EXPOSURES

Exposure to environmental toxicants during development can have both acute consequences to the embryo, leading to congenital anomalies and poor birth outcomes, as well as long-term health consequences throughout the life of an individual. In addition, exposure to low

doses of environmental contaminants can have latent health effects that are not apparent for years, even after the cessation of exposure. The fetal origins hypothesis, also called Barker hypothesis, was first described by David Barker in 1986 following the observation that poor infant nutrition was associated with poor cardiovascular outcomes among men in England and Wales (Barker & Osmond, 1986). Adverse health effects in this cohort are thought to be the result of altered developmental programming or physiological changes that make an individual susceptible to disease.

A well-known examples of this phenomenon is the causal association between the development of vaginal clear cell adenocarcinoma in women who were exposed to diethylstilbestrol in utero (Hatch, Palmer, Titus-Ernstoff, et al., 1998; Herbst, Ulfelder, & Poskanzer, 1971). This hypothesis has recently been tested in zebrafish to study the latent effects of embryonic exposure to atrazine, an herbicide and suspected endocrine disruptor (Wirbisky et al., 2015, 2016), and TCDD, a persistent environmental pollutant (Baker, Peterson, & Heideman, 2013). Both male and female adult zebrafish that developed from embyros exposed to atrazine, a widely used herbicide, demonstrate altered expression of genes related to neuroendocrine function (Wirbisky et al., 2016, 2015). In another study, early life exposure to low doses of TCDD during the embryonic period caused few malformations in the fish during the exposure period; more profound were the transgenerational effects of early exposure: the offspring of adults which developed from embryos exposed to TCDD showed morphological abnormalities, including skeletal defects, and reduced reproductive success (Baker et al., 2013). Zebrafish that were exposed to TCDD during the sex determination period (3–7 weeks postfertilization) displayed skeletal anomalies in adulthood and mismatches between secondary sex characteristics and the sex of the gonads as determined by histological analysis (Baker et al., 2013). These studies exemplify the power of the zebrafish system to feasibly demonstrate early, late, and transgenerational effects of toxin exposure.

9. PATHWAYS AND MECHANISMS OF TOXICANT-INDUCED DISEASE

Zebrafish can provide a powerful tool to investigate the mechanisms of action of environmental pollutants and its related diseases, and can be used to test therapeutic candidates or intervention measures to mitigate the effects of environmental contaminants, with the goal of translation to human disease. Here, we highlight examples of mechanistic insights generated from zebrafish models of exposure, including inorganic arsenic, BPA and TCDD (Fig. 2).

9.1 Arsenic

Inorganic arsenic is a naturally occurring element that epidemiological studies have linked with multiple adverse health outcomes (Vahter, 2008). Arsenic is classified as a human carcinogen by the International Agency for Research on Cancer and long-term exposure is associated with increased risk of several cancers, including bladder, kidney, liver, and skin cancer (IARC, 2004; Wang et al., 2014). Data from the first longitudinal study of people chronically exposed to inorganic arsenic through drinking water has found that the latency for health effects can be decades (Ahsan et al., 2006; Argos et al., 2010). Studying the

underlying mechanisms of arsenic toxicity in zebrafish can provide much needed information of how arsenic causes disease in humans.

Although arsenic is one of the most common metalloid contaminants of drinking water, and can be found at high levels in common foods such as rice and apple juice (Davis et al., 2012; Sauvé, 2014), the precise mechanism of arsenic toxicity is relatively unknown. Work in tissue culture cells and rodent models has identified oxidative stress, biotransformation and methylation, and ER stress as potential mechanisms of arsenic-induced toxicity (Gamble et al., 2005; Hughes, 2002; Vahter, 2002), although studies linking aberrant cellular processes to specific arsenic-induced disease phenotypes are lacking. Work in zebrafish is shedding light on this field.

Zebrafish express aquaglyceroporins and the trivalent arsenic specific methyltransferase (*zas3mt*), enzymes required for the uptake and metabolism of inorganic arsenic, respectively (Hamdi et al., 2009, 2012). Early studies of zebrafish models of inorganic arsenic exposure provided descriptive analysis of the effects of arsenic on the gross morphological development of zebrafish embryos and larvae, demonstrating acute toxicity and cardiovascular defects (Li et al., 2009; Seok et al., 2007). While information about the rate of arsenic metabolism by zebrafish in vivo and the production of specific metabolites are still emerging, it is clear that rodent models do not always accurately reflect the effects of human arsenic exposure. For instance, studies in rodents showed increased excretion and slower but more extensive methylation of arsenic when compared to humans so that ingested arsenic remains in the rodent blood stream for prolonged periods (Hallauer et al., 2016; States et al., 2011).

Zebrafish embryos treated with inorganic arsenic have multiple defects, and some studies implicated downregulation of Dvr1, a factor involved in mesoderm induction and the establishment of left-right asymmetry (Li et al., 2012). These processes are essential for proper cardiac morphogenesis. Interestingly, depletion of Dvr1 using morpholino knockdown led to heart defects that were similar to those seen upon exposure to 2 mM inorganic arsenic from 4 to 48 hpf (Li et al., 2012). Overexpression of human GDF1, a homolog of Dvr1, led to a reduction in the number of zebrafish that displayed morphological defects upon arsenic exposure (Li et al., 2012). Further studies from the same group showed folic acid prevented arsenic-induced toxicity in zebrafish by reducing generation of reactive oxygen species and rescuing the decrease in Dvr1 expression (Ma et al., 2015). However, the protective effect of folic acid diminished after 48 hpf (Ma et al., 2015), indicating that other mechanisms may underlie later developmental anomalies caused by arsenic exposure.

Transcriptomic and metabolomic approaches in zebrafish have also been used to gain insight into the molecular mechanisms of arsenic toxicity, particularly in the adult liver (Lam et al., 2006; Li et al., 2016; Xu et al., 2013; Yang et al., 2007). The effect of acute arsenic exposure and changes of gene expression patterns over time in the adult liver was first examined using microarray, with gene expression changes occurring as early as 8 h of exposure (Lam et al., 2006). Differentially expressed genes were grouped into categories to examine the adaptive response of the zebrafish liver to arsenic exposure, and revealed that arsenic-induced liver injury is the result of DNA and protein damage and oxidative stress resulting from the

metabolism of inorganic arsenic. Gene ontology and pathway analysis of RNA-SAGE data were applied and used to identify a panel of biomarker genes to predict arsenic toxicity (Xu et al., 2013). Network analysis identified *nr2f2*, *jun*, *k-ras*, and *apoE* as four central factors that were upregulated in zebrafish liver following arsenic exposure. Each of these factors is implicated in pathways that can contribute to arsenic-induced liver disease: Jun and Kras are known oncoproteins, Nr2f2 regulates many genes involved in oxidative stress, and drug metabolism and ApoE is required for lipoprotein synthesis. Interestingly, arsenic exposure has been shown to accelerate the formation of atherosclerosis in *ApoE* deficient mice, highlighting the conservation of pathways affected by arsenic exposure in zebrafish and mammalian models (States et al., 2012; States, Srivastava, Sen, & D'Souza, 2007).

Chronic exposure of zebrafish to environmentally relevant concentrations revealed retention of arsenic in the eye, skin, and liver of 6-month-old fish and resulted in increased heart rate during larval stages and neurologic defects (Hallauer et al., 2016). Progeny of arsenic-exposed fish had reduced biomass at 3 months of age relative to the progeny of their unexposed siblings (Hallauer et al., 2016). Zebrafish studies have recapitulated the effects of arsenic on the cardiovascular system (Hallauer et al., 2016; Li et al., 2012) and have shown alterations in liver metabolism and liver function (Lam et al., 2006; Li et al., 2016; Xu et al., 2013). Our research is focused on using zebrafish to understand the mechanisms that underlie arsenic-induced liver disease in human populations (Mazumder, 2005; Santra, Das Gupta, De, Roy, & Guha Mazumder, 1999). Metabolic changes in the liver of adult zebrafish after acute arsenic exposure was investigated using gas chromatography coupled with mass spectroscopy (Li et al., 2016), identifying 34 potential metabolite markers of arsenic exposure. Additionally, histological examination of the livers of arsenic-exposed zebrafish showed cellular changes and accumulation of lipid droplets, liver function tests showed little alteration (Li et al., 2016), suggesting that metabolic changes may be a sensitive method to detect alterations in liver function induced by arsenic. Although the use of zebrafish to study arsenic toxicity is relatively recent, this model system has provided important insights into both the acute (Li et al., 2016; Xu et al., 2013) and chronic effects of arsenic exposure (Hallauer et al., 2016). Zebrafish studies of the effects of arsenic exposure will provide insight into the mechanisms of these physiological consequences and will also allow for the examination of transgenerational effects more feasibly than with rodent models.

9.2 Bisphenol A

BPA is one of the most common endocrine-disrupting environmental contaminants. It is a high production volume chemical and is present in many consumer and industrial products such as plastics. It is also present in the environment as a result of manufacturing processes and leaching from the products in which it is used. BPA is defined as an endocrine disruptor because of its ability to elicit both proestrogenic and antiestrogenic effects by binding to estrogen receptors ERα and ERβ and altering transcription in tissue- and context-specific manners (Santangeli et al., 2016). BPA binds to the zebrafish estrogen-related receptor gamma (ERRγ) in vivo (Tohmé et al., 2014). BPA has been reported to have adverse effects on reproductive health, early development, and contributes to obesity (Rochester, 2013). Both mammalian and zebrafish exposure studies have revealed related phenotypes.

Environmentally relevant doses of BPA were found to inhibit oocyte maturation by binding to the membrane estrogen receptor, Gper, and activating Egfr/Mapk3/1 signaling, which prevents resumption of meiosis (Fitzgerald, Peyton, Dong, & Thomas, 2015). Interestingly, this pathway is independent of signaling through the estrogen receptor (Fitzgerald et al., 2015). BPA is suggested to act through epigenetic mechanisms through histone modification and alteration of DNA methylation (Faulk et al., 2016; Kundakovic & Champagne, 2011; Santangeli et al., 2016), and zebrafish studies have provided key mechanistic insights. The effect of BPA on histone methylation patterns and DNA methylation has recently been shown in adult zebrafish (Laing et al., 2016; Santangeli et al., 2016). Global DNA methylation has also recently been shown to be reduced in the ovaries and testes of adult zebrafish exposed to 15 μg/L BPA for 7 days (Liu, Zhang, et al., 2016) and 1 mg/L BPA for 15 days (Laing et al., 2016). Adult female zebrafish exposed to environmentally relevant concentrations of BPA, from 5 to 20 μg/L, displayed nonmonotonic effects in that the lowest dose tested led to a complete block in ovulation, accompanied by more significant reduction in gene expression of the estrogen receptors *esr1* and *esr2a*, and induction of apoptosis markers *caspase3* and *p53* (Santangeli et al., 2016). Expression of the DNA methyltransferases *dnmt1* and *dnmt3* were upregulated in the ovaries of female zebrafish exposed to 5 μg/L BPA. Some of these gene expression changes were associated with changes in the levels of H3K4me3 and H3K27me3 levels (Santangeli et al., 2016). In contrast, adult male and female zebrafish exposed to higher doses of BPA (up to 1 mg/L) were shown to have reduced expression of *dnmt1* in the liver and ovaries when exposed to 10 μg/L, 100 μg/L, and 1 mg/L BPA, while no significant differences in expression level were observed in the testes of male zebrafish at the same exposure concentrations (Laing et al., 2016). Interestingly, the DNA methylation patterns were not strictly correlated with changes in gene expression. For instance, while no change in *dnmt1* expression was observed in the testes, analysis of 11 CpG sites in the *dnmt1* promoter revealed significant increases in some of the sites in this tissue. In the ovary, where the most consistent changes in *dnmt1* expression were observed, no significant changes in site-specific DNA methylation in the *dnmt1* promoter were found (Laing et al., 2016).

In addition to studying BPA-induced defects in reproduction, zebrafish have also been used to understand the neurotoxic effects of BPA (Cano-Nicolau et al., 2016; Kinch, Ibhazehiebo, Jeong, Habibi, & Kurrasch, 2015; Saili et al., 2012). In the zebrafish brain, BPA can activate gene expression through the canonical estrogen receptor signaling pathway (Chung, Genco, Megrelis, & Ruderman, 2011). Zebrafish exposed to BPA for a narrow (8–58 hpf) or longer (8–120 hpf) window were examined for effects on behavior. Zebrafish larvae (5 dpf) that were exposed to low dose BPA from 8 to 58 hpf demonstrated hyperactivity, and adult zebrafish that were exposed to low dose BPA from 8 to 120 hpf had behavioral and learning deficits, including larval hyperactivity and reduced ability to choose the correct arm of a T-maze to avoid an electric shock, compared to unexposed controls (Saili et al., 2012).

Using transgenic *Tg(cyp19a1b:GFP)* reporter fish, a reporter of estrogen signaling, zebrafish larvae exposed to BPA on 4 or 7 dpf resulted in activation of the estrogen-specific marker which likely occurred through activation of ERα (Cano-Nicolau et al., 2016). Reporter expression was localized to specific brain regions including the posterior telencephalon, preoptic area, and caudal hypothalamus. In the zebrafish brain, it has also recently been

shown that exposure of developing zebrafish to low doses BPA caused precocious neurogenesis in the hypothalamus which resulted in hyperactivity and brain changes (Kinch et al., 2015). Together, these data show that zebrafish are capable of demonstrating not only the molecular and cellular responses to the endocrine disruptor BPA, but also provide evidence of the pathological effects of BPA exposure.

Most recently, zebrafish have been used to study the toxic effects of BPA and the products of its degradation (Makarova, Siudem, Zawada, & Kurkowiak, 2016). The degradation products of BPA were found to have lower binding affinity for both human and zebrafish estrogen receptors than BPA itself but one degradation product, 4-isopopylphenol, was predicted to have a higher binding affinity for the human ERRγ and slightly lower affinity for zebrafish ERRγA. 4-Isopopylphenol has the ability to permeate biological membranes similar to BPA, but appears to be more toxic as it caused acute lethality to zebrafish embryos while the same dose of BPA did not. These zebrafish studies emphasize that degradation products of environmental contaminants can be more toxic than their parent compounds, and that toxicity testing of intermediates may be warranted (Gamse & Gorelick, 2016).

Zebrafish have provided useful insights into the effects of BPA on the developing brain and reproductive organs. These systems are most the most likely to be affected by environmental exposures to BPA and similar compounds that rely heavily on estrogen signaling (Patisaul & Adewale, 2009; Saili et al., 2012). Studies of BPA toxicity in zebrafish have highlighted the nonmonotonic effects of this common environmental contaminant and other EDCs (Santangeli et al., 2016; Vandenberg et al., 2012).

9.3 TCDD

TCDD is one of the most widely studied environmental contaminants in zebrafish (Carney, Prasch, Heideman, & Peterson, 2006). This chemical is a polychlorinated dibenzo-*p*-dioxin, an anthropogenic, lipophilic persistent environmental contaminant, commonly found in air and soil as the result of solid waste incineration and industrial processing. Human occupational and environmental exposure may be associated with a wide range of chronic diseases, including cancer, diabetes, endometriosis, cardiovascular disease, reduced testosterone, and thyroid hormone levels (White & Birnbaum, 2009). The effects of aromatic hydrocarbon exposure on the health of wild fish populations are more difficult to assess; however, lake trout populations in regions with high levels of aromatic hydrocarbon contamination have been unable to sustain their numbers (King-Heiden et al., 2012). Zebrafish have been proposed as a model to understand not only the health effects of human exposure to aromatic hydrocarbons and dioxin-like compounds, but also to predict the effects of contamination on wild fish populations. A comprehensive review of the contributions of the zebrafish model to our understanding of the molecular mechanisms of TCDD reproductive and developmental toxicity has been published elsewhere (King-Heiden et al., 2012).

Zebrafish have been used to study TCDD-induced endocrine disruption and reproductive toxicity (Baker et al., 2013; Heiden et al., 2008), cardiovascular toxicity (Antkiewicz, Burns, Carney, Peterson, & Heideman, 2005; Goldstone & Stegeman, 2006), and skeletal abnormalities (Baker et al., 2013; Burns, Peterson, & Heideman, 2015; Henry, Spitsbergen,

Hornung, Abnet, & Peterson, 1997; Teraoka et al., 2006). Developmental malformations in zebrafish embryos and larvae exposed to TCDD are prevented by depletion of Ahr2 (Prasch et al., 2003), indicating that metabolism is required for TCDD toxicity. *Cyp1a* transgenic reporter zebrafish have been used to investigate the mechanisms of TCDD toxicity and to identify target organs for the effects of TCDD (Kim et al., 2013; Mattingly, McLachlan, & Toscano, 2001; Xu et al., 2015); however, pathways independent of Cyp1a1 also contribute to the developmental toxicity of TCDD as knockdown of zebrafish *cyp1a* does not prevent TCDD-induced phenotypes (Carney, Peterson, & Heideman, 2004). Here, we compile some of the most recent studies of zebrafish exposed to TCDD.

TCDD has been shown to affect both ovarian function and follicle maturation (Baker, Peterson, & Heideman, 2014; Heiden et al., 2008). Studies in numerous fish species have demonstrated many impairments in female reproduction that are caused by TCDD (King-Heiden et al., 2012). A microarray analysis of the zebrafish adult ovary examined the transcriptional changes that precede the physiologic dysfunction following exposure to a TCDD dose curve (Heiden et al., 2008). Exposure to TCDD resulted in downregulation of genes involved in estradiol synthesis and follicle maturation, as well as genes encoding structural proteins Krt4 and Lgals3l (Heiden et al., 2008). While this study found that gene expression changes were not dose dependent and that a majority of the differentially expressed transcripts were unknown or poorly characterized, ~40% of the differentially expressed probes contained both putative aryl hydrocarbon-response elements and estrogen response elements.

Cardiac toxicity is one of the most obvious end points of zebrafish exposure to TCDD (King-Heiden et al., 2012). Gene expression analysis over a time course was performed to understand the molecular pathways that are altered in response to TCDD exposure (Carney, Chen, et al., 2006). Within 1 h of exposure, a cluster of 42 genes involved in xenobiotic metabolism, proliferation, contractility, and regulation of heart development were induced (Carney, Chen, et al., 2006). In addition a “cell cycle gene cluster” was downregulated in zebrafish exposed to TCDD and negative regulators of cell cycle progression were upregulated, indicating that reduced cardiomyocyte number may underlie TCDD-induced cardiac toxicity (Carney, Chen, et al., 2006). Increased and ectopic expression of *Bmp4* and *Notch1b* transcripts in the region of nascent cardiac valve formation were found to be responsible for TCDD-induced failure of heart valve formation in the zebrafish (Mehta, Peterson, & Heideman, 2008). Failure to restrict these transcripts, as determined by in situ hybridization, was associated with loss of endothelial cell pattern in the region where this morphogenic process should occur. This study highlights one of the most significant advantages to using the zebrafish system, in that alterations in stereotypical developmental processes can yield insight into the cellular and molecular mechanisms underlying toxicity.

Skeletal malformation is another predominant developmental defect associated with TCDD exposure in fish and rodent species (Baker et al., 2014; Birnbaum, Harris, Stocking, Clark, & Morrissey, 1989; Henry et al., 1997; King-Heiden et al., 2012). Craniofacial malformations in TCDD-exposed zebrafish are dependent on Ahr2/Arnt1 signaling (Prasch, Tanguay, Mehta, Heideman, & Peterson, 2006; Prasch et al., 2003). A transgenic reporter *Tg(sox9b:EGFP)*, which marks perichondrial endoderm in the developing jaw, was used to

demonstrate that craniofacial abnormalities in TCDD-exposed zebrafish larvae resulted from reductions in chondrocyte size and number and decreases in ossification of the jaw (Burns et al., 2015). In addition to being a marker of craniofacial and jaw development in the zebrafish, *sox9b* is required for this process. Heterozygous *sox9b* mutant zebrafish are more susceptible to TCDD-induced craniofacial malformations, and overexpression of *sox9b* in TCDD-treated zebrafish mitigated the effects of the toxicant on jaw development (Xiong, Peterson, & Heideman, 2008). Scoliosis is frequently observed in adult fish following exposure to TCDD during the embryonic or larval periods (Baker et al., 2013, 2014).

Zebrafish research into the pathways and molecular mechanisms underlying TCDD toxicity have provided information about outcomes relevant to human populations, most notably cardiac and reproductive defects. TCDD is also the most commonly studied environmental toxicant with regard to transgenerational effects, as discussed in Section 8 (Baker, King-Heiden, Peterson, & Heideman, 2014; Baker et al., 2014).

10. LIMITATIONS TO THE ZEBRAFISH MODEL SYSTEM

While zebrafish will allow researchers to answer many questions that are limited by the realities of epidemiological researchers, there are several limitations to this model. For instance, the physiological differences between zebrafish and mammals mean that disease outcomes such as asthma or placental defects are not observable in zebrafish. However, although not all disease-related phenotypes can be identified in zebrafish, many of the developmental and signaling pathways leading to these diseases are conserved between zebrafish and humans (Padilla et al., 2012).

A second consideration is that in zebrafish, some exposures may not be equivalent to the experience of human populations. In most studies, the toxicant is added directly to the water, recapitulating a dermal exposure during the early stages of zebrafish development when the embryos are not swallowing water in order to breathe. However, many toxicants are introduced into the human body via oral exposure through contaminated drinking water or food, and, as such exposure is intermittent and affects involves the gastrointestinal system. This may lead to substantial differences in the absorption, tissue distribution, metabolism, and excretion depending on the uptake and biotransformation pathways based on the route of exposure. Metabolic differences between zebrafish and mammals may also be affected by differences in the expression patterns of xenobiotic metabolism enzymes and incompletely conserved enzyme functions (Saad et al., 2016), which may also contribute to the differences in dosing required to elicit phenotypes. Urinary biomarkers of exposure and metabolism are also unavailable from zebrafish.

Studies have demonstrated that gender can play an influential role in response to toxin exposure. For example, arsenic exposure in humans leads to changes in DNA methylation in isolated cord blood cells that are different in males and females (Pilsner et al., 2012), and endocrine disruptors have been shown to have different neurobehavioral effects in boys and girls (Evans et al., 2014; Roen et al., 2015). While zebrafish have no discernible sex chromosomes and do not become sexually dimorphic until 3 weeks postfertilization (Sola &

Gornung, 2001; Tong, Hsu, & Chung, 2010), toxicant exposure during this window can influence sex characteristics as seen with early TCDD exposure (Fig. 2) (Baker et al., 2013).

11. CONCLUSIONS

As manufacturing processes and development of new synthetic compounds proceed in order to keep pace with the growing world economy, environmental health, and the effects of toxicant exposure are emerging as critical areas of research. The main benefit to using zebrafish in toxicology and environmental health studies is that their unique combination of developmental features provides a system with the benefits of both in vitro and in vivo schemes. Combining the large scale of embryo production with rapid development allows for short-term assessment of toxicity in a whole animal system. In addition, the relative ease and comparatively low cost of raising large numbers of individuals allows for unprecedented investigation into latent effects and adverse outcomes in response to early life exposure to environmental contaminants. Many of the genetic, molecular, and cellular processes are conserved between zebrafish and mammals, allowing close applicability to human exposure and disease. As such, studies using zebrafish have uncovered important insights into the effects of environmental contaminants on normal development in a live vertebrate system.

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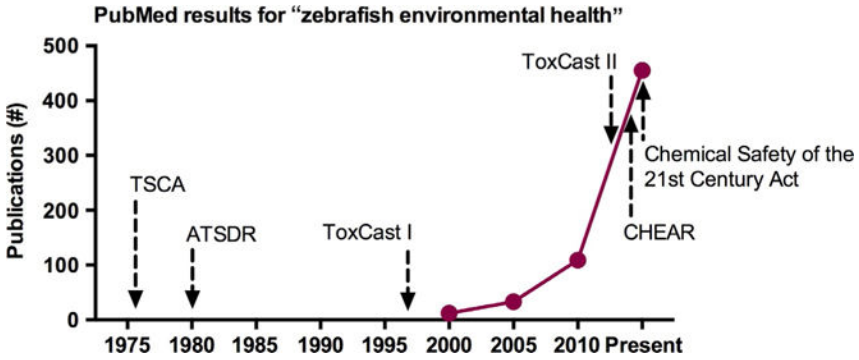

Fig. 1.

Increasing use of zebrafish in environmental health studies. The use of zebrafish has steadily increased during the past three decades. *Line graph* represents the number of Pubmed articles categorized under "zebrafish environmental health." *Dashed arrows* indicate government programs and legislation focused on toxicology and environmental health from 1975 to present.

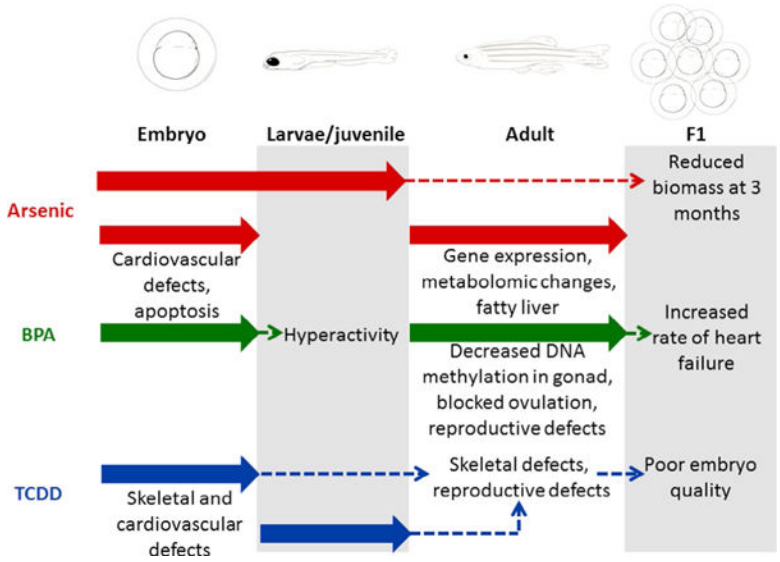

Fig. 2.

Using zebrafish to model the health effects of toxicant exposure. Zebrafish exposed to a range of water-soluble environmentally relevant contaminants during different stages of the life cycle display a wide range of outcomes, from reduced reproductive success to skeletal and neurodevelopmental defects. *BPA*, bisphenol A; *TCDD*, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Illustrations by Christopher Smith. Copyright Mount Sinai Health System 2016. Images used with permission.

Table 1

Transgenic Zebrafish Lines for Reporting Toxicant Exposure

Transgenic Line	Reporter for	Toxicants Tested	References
Tg(cyp1a:nls-gfp)	Cytochrome p450 Cyp1a	Aromatic hydrocarbons, dioxin-like compounds	Kim et al. (2013)
Tg(cyp1a:gfp)	Cytochrome p450 Cyp1a	Aromatic hydrocarbons, dioxin-like compounds	Xu et al. (2015)
Tg(mt:egfp)	Metallothionein	Heavy metals	Liu, Yan, Wang, Wu, and Xu (2016)
Tg(huORFZ:gfp)	Human CHOP	Heavy metals, endocrine disruptors	Lee et al. (2014)
TgBAC (hspb11:GFP)	Small heat shock protein hsbp11	Pesticides	Shahid et al. (2016)
Tg(5xERE: GFP)	Estrogen receptor activity	Estradiol, xenoestrogens, environmental water samples	Gorelick et al. (2014), Gorelick and Halpern (2011), and Gorelick, Pinto, Hao, and Bondesson (2016)
Tg(cyp19a1b: GFP)	Cytochrome p450 cyp19a1b, estrogen receptor activity	BPA, environmental water samples	Cano-Nicolau et al. (2016) and Sonavane et al. (2016)