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Advancements in zebrafish applications for 21st century toxicology

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Abstract

The zebrafish model is the only available high-throughput vertebrate assessment system, and it is uniquely suited for studies of *in vivo* cell biology. A sequenced and annotated genome has revealed a large degree of evolutionary conservation in comparison to the human genome. Due to our shared evolutionary history, the anatomical and physiological features of fish are highly homologous to humans, which facilitates studies relevant to human health. In addition, zebrafish provide a very unique vertebrate data stream that allows researchers to anchor hypotheses at the biochemical, genetic, and cellular levels to observations at the structural, functional, and behavioral level in a high-throughput format. In this review, we will draw heavily from toxicological studies to highlight advances in zebrafish high-throughput systems. Breakthroughs in transgenic/reporter lines and methods for genetic manipulation, such as the CRISPR-Cas9 system, will be comprised of reports across diverse disciplines.

Keywords

Zebrafish; Toxicology; CRISPR; High-throughput; Transgenic/Reporter

1. Introduction

Technological advancements in molecular and genetic biotechnology have resulted in a shift in the field of toxicology from traditional observations of apical endpoints, to characterizations of the early molecular responses to chemical perturbations (NRC, 2007; Villeneuve et al., 2014). This shift in focus facilitates a deeper understanding of the associations between phenotypic and genotypic data. The unique attributes of the zebrafish model combine to produce a data stream that is rich in molecular, biochemical, functional, and behavioral processes (Figure 1). Fundamental resources, such as an annotated and

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Conflict of interest

The authors declare that there are no conflicts of interest.

sequenced genome and an expansive array of tools for genetic manipulation, continue to facilitate the extensive use of the zebrafish model across disciplines. In terms of publication, zebrafish are one of the fastest growing model organisms. While we will draw heavily from toxicological studies, the purpose of this review is to highlight the recent advancements in zebrafish research, focusing on the characteristics that are advantageous in the model, including advances in high-throughput systems, transgenic/reporter lines, and methods for genetic manipulation.

The model was founded largely on its amenability to large-scale forward genetic approaches since zebrafish embryos develop externally, and those studies required the ability to make detailed morphological observations on large populations of developing embryos. The first large-scale forward genetic screens in zebrafish provided the foundation for the discovery of a slew of novel genes and pathways fundamental to vertebrate development (Driever et al., 1996; Haffter et al., 1996). In the field of toxicology and pharmacology, zebrafish embryos can be used as biosensors to rapidly test the bioactivity and toxicity of a large number of chemicals (Noyes et al., 2015; Padilla et al., 2012; Truong et al., 2014). Subsequently, we can assess whether the chemical compounds have the potential to perturb processes in the human body, which may lead to adverse health effects or amelioration of disease phenotypes.

The zebrafish model represents a viable alternative to the mammalian models currently used in toxicity testing and other biological research. Zebrafish are easier and less expensive to house and care for than popular rodent models. The ability to more accurately reproduce the natural social conditions of a vertebrate model reduces housing stress and its impact on experimental outcomes (Balcombe et al., 2004). Zebrafish are also one of the most genetically tractable vertebrate models since they can be easily injected with gene modifying constructs and absorb chemical mutagens through their water. Many of the advantages of zebrafish accrue from the embryonic and early larval stages. Zebrafish embryos are transparent and develop externally, allowing the use of noninvasive imaging techniques to track the impact of genetic manipulation or chemical treatment. Noninvasive procedures minimize animal suffering and reduce the stress levels of the animal, which should lead to more accurate and reproducible data sets (Balcombe et al., 2004). Zebrafish embryos also develop very rapidly compared to mammalian models, potentially reducing the time needed to complete experimentation. Table 1 highlights the temporal differences in developmental life stages between human, rat, and zebrafish (Carlson, 2013; Kimmel et al., 1995; O'Rahilly, 1973; Witschi, 1962). The heart, liver, brain, pancreas, and other organs are developed by 5 days post fertilization (dpf) (Kimmel et al., 1995). Nutrients are provided by a yolk sac for the first 7 dpf, and embryos for chemical screening are routinely housed in 96-well microtiter plates; thus, some *in vitro* assays, such as those that measure the oxygen consumption rate as an indicator of mitochondrial respiration, can be applied to zebrafish embryos (Knecht et al., 2013). Zebrafish also have a shorter life cycle and much larger capacity for generating offspring than rodents, which ensures a steady supply of animals for research purposes. A typical rodent mating pair produces only 5–10 offspring per mating event, in comparison to the 200–300 embryos produced by zebrafish. Due to their small size and higher fecundity, zebrafish assays can have larger sample sizes and achieve greater statistical power than mammalian studies, which means biologically meaningful responses

are more likely to be detected. The capacity to investigate complex and rare biological processes is also enhanced.

2. Relevance of the zebrafish to human health

A key issue with zebrafish, or any translational model, is identifying which biological interactions and responses are functionally conserved between the model and humans. Empirically, we know that embryonic developmental stages reflect broader phylogenetic similarity than adult stages; therefore, zebrafish embryogenesis is the most likely stage to recapitulate human biological interactions and responses. Most of the transcriptome is highly active during development with many genes expressed in conserved patterns across phyla with growing evidence that most of the overarching signal transduction pathways are also conserved. (Irie and Kuratani, 2011; Kalinka et al., 2010). Furthermore, because of our shared evolutionary history, many of the anatomical and physiological features of fish are highly homologous to humans (Eimon and Rubinstein, 2009; Nishimura et al., 2015; Pickart and Klee, 2014). For example, zebrafish and mammalian brains share many features, which include well-conserved neurotransmitter structures and systems, such as GABA, glutamate, dopamine, serotonin, noradrenaline, histamine, and acetylcholine (Panula et al., 2006; Schmidt et al., 2013). Data have also shown that the expression of genes involved in controlling neurotransmitters in zebrafish are similar to those in mammalian rodent models (Mueller et al., 2006). Additionally, zebrafish and mammalian brains share broad anatomical features, such as the presence of the cerebellum, telencephalon, diencephalon, spinal cord, and enteric-autonomic nervous systems (Guo, 2004; Mueller et al., 2008; Schmidt et al., 2013; Wullimann and Mueller, 2004)

Recent advances in ‘omics’ technologies have greatly increased the utility of the zebrafish model and have allowed cross-species comparisons at the molecular level. The zebrafish genome has been sequenced and annotated, which allows investigations at the biochemical, genetic, and cellular levels to be anchored to observations at the structural, functional, and behavioral level. A comparison of the human reference genome to the zebrafish genome reveals approximately 70% of human genes and about 82% of potential human disease related genes have at least one obvious zebrafish ortholog (Howe et al., 2013b). Zebrafish also have several additional orthologs for 15% of human genes due to an ancient genome duplication event (Howe et al., 2013b). The high degree of conservation between zebrafish and humans coupled with advancements in genome editing technologies have greatly expanded the number of zebrafish models of human disease, including cardiovascular disorders (Staudt and Stainier, 2012; Staudt et al., 2014), neurological and psychiatric disorders (Mahmood et al., 2013; Zdebik et al., 2013; Ziv et al., 2013), cancer (reviewed in Yen et al., 2014), and many more (Phillips and Westerfield, 2014; Pickart and Klee, 2014). A valuable resource that will facilitate cross-species comparison at the molecular level is The Zebrafish GenomeWiki (http://genome.igib.res.in/twiki/), which uses a crowd sourcing method to systematically curate biological annotations data (Singh et al., 2014). Thus far, 600 genes, 52,896 transcripts, and 4,150 proteins have been annotated. An additional resource is the Zebrafish Mutation Project (http://www.sanger.ac.uk/resources/zebrafish/zmp/). The Sanger Institute recently launched its Zebrafish Mutation Project with the goal of creating a knockout allele in every protein coding gene in the zebrafish genome, followed by

phenotypic analysis. (Kettleborough et al., 2013). Each mutant allele identified as producing any morphological abnormality will have its transcriptome analyzed. The mutant lines are currently available from the Zebrafish International Resource Center (https://zebrafish.org). For a more comprehensive list of zebrafish genomic resources, the reader is referred to (Varshney et al., 2015b). These types of resources are key in promoting a broader understanding of vertebrate gene function and building a mechanistic understanding of pathways involved in chemical toxicity and disease etiology.

In order to understand or predict the toxic potential of a compound, whether it be an environmental toxicant or a potential therapeutic drug, knowledge of absorption, distribution, metabolism, and elimination of the compound is required. One of the advantages of using zebrafish is that chemicals are easily delivered via addition to the water, analogous to adding chemicals to cell culture medium, and typically only requiring a 100 μL total volume during development. Thus, very little test compound is needed when compared to mammalian dosing studies.

Zebrafish embryos are protected by a chorion until 2 or 3 dpf. The chorion is an acellular envelope containing pores that are approximately 0.5 μm in diameter and 2 μm spacing (Lee et al., 2007), with still poorly characterized chemico-permeability. To eliminate a potential confounding factor, the chorion can be manually or enzymatically removed without negatively impacting development. The main route of exposure is dermal until around 3 dpf when the mouth is opened (Wallace and Pack, 2003). Both dermal and enteral routes exist from 3 to 14 dpf. Zebrafish also develop a blood brain barrier at 3 dpf, which is reported to be similar to those found in mammals (Jeong et al., 2008; Xie et al., 2010). Furthermore, the blood brain barrier chemical penetration or exclusion profile has been shown to be the same between zebrafish and mammals (Fleming et al., 2013). Zebrafish larvae also possess a high conservation of essential metabolizing enzymes, such as the Phase I cytochrome P450 enzymes (Goldstone et al., 2010). The kidney is formed and functioning by 2 dpf (Drummond, 2003) and the liver becomes vascularized and functional by 3 dpf (Field et al., 2003). Zebrafish larvae can excrete various chemicals through both the kidney and liver by 3 dpf (Matz et al., 2007). For a more in depth analysis of the pharmaco/toxicodynamics of zebrafish see (Nishimura et al., 2015).

Mammalian toxicity studies remain the gold standard for predictive modeling of chemical risk to humans; however, these studies are poorly suited for early stage toxicity screening because they are expensive, time-consuming, and require substantial amounts of oft times precious test compound. Cell-based assays are amenable to high-throughput screening, but are limited in their ability to accurately model multicellular processes and recapitulate the metabolism of a whole animal. The zebrafish is increasingly used as a vertebrate model for *in vivo* drug discovery and chemical risk assessment because it combines the scale and throughput of *in vitro* systems with the physiological complexity of vertebrate whole animal research (Ali et al., 2011; Baraban et al., 2013; Bruni et al., 2014). Numerous studies have been conducted to evaluate the predictive capabilities of zebrafish assays by examining large libraries of compounds with known toxicity profiles in mammals including cardiotoxicity (Arnaout et al., 2007; Chico et al., 2008; Park et al., 2013), seizure liability (Genschow et al., 2002; Koseki et al., 2014), and otic toxicity (Chiu et al., 2008). The results from these

studies ranged from 63–100% predictivity, suggesting that the underlying molecular mechanisms are conserved between zebrafish and humans (reviewed in Eimon and Rubinstein, 2009; He et al., 2014; Hung et al., 2012)).

3. High-throughput screening is practical in a vertebrate model

Translation, cost efficiency, and transparency of the developing zebrafish have established it as the go-to vertebrate for high-throughput screening (HTS). The design and implementation of screening formats that target an expanding number of pathways and endpoints (e.g. teratogenicity, endocrine disruption, cardiotoxicity, etc.) has accelerated immensely in recent years. Zebrafish screens are now used to test an array of different environmental toxicants, pharmaceutical agents, and chemical libraries across a range of life stages, transgenic and mutant lines, test concentrations, and exposure durations (Rennekamp et al. 2015). Zebrafish are also unaffected by up to 1% dimethyl sulfoxide vehicle, and this tolerance to the most common pharmaceutical research vehicle makes it possible to test essentially any small molecule structures or hydrophobic chemicals that resist aqueous dissociation (Hallare et al. 2006). Thus, zebrafish HTS assays become highly relevant in early research and design screens where novel molecules with unknown properties and differing structural attributes and moieties may be extremely limited in quantity, and due to custom synthesis or extraction, may be very costly.

Some of the recent advances in the development of rapid chemical toxicity screens with zebrafish embryos have been developed in the United States as part of the Environmental Protection Agency's National Center for Computational Toxicology Toxicity Forecaster, or ToxCast, program (Dix et al., 2007; Kavlock et al., 2012; Padilla et al., 2012; Truong et al., 2014). These high-content screening platforms are generally consistent in that they use a multi-well plate format to test chemical effects on embryonic development by assessing deformities, mortality, and chemical structures across concentration ranges (Figure 2). For instance, Padilla et al. (2012) implemented embryonic zebrafish screens of developmental toxicity for about 300 chemicals (mostly pesticides) identified in the Phase I ToxCast chemical library. Survival and overt malformations were evaluated in larvae at 144 hours post fertilization (hpf). A subsequent study in our lab by Truong et al. (2014a) employed an enhanced approach to rapidly screen over 1,000 chemicals, which included all the Phase I ToxCast chemicals plus several hundred chemicals in the Phase II ToxCast library. While the dose ranges tested were generally similar for the two studies, several differences are noted.

Padilla et al. (2012) exposed embryos with intact chorions under static renewal conditions for 5 days with evaluations on day 6. The enhanced HTS platform developed and implemented by Truong et al. (2014) included mechanized dechorionation of embryos followed by static non-renewal exposures and more comprehensive phenotypic evaluations on days 1 and 5. The automated nature of the HTS platform and data handling employed in our laboratory also allowed for larger sample sizes (n = 32 embryos/concentration). Padilla et al. scored 6 malformation endpoints based on severity, whereas we opted to use binary scoring of 22 endpoints as either present or absent. Finally, Truong et al. computed lowest effect levels while Padilla and colleagues calculated a half-maximal activity concentration based on their toxicity scoring index. Despite these differences in study design, 75% of the

ToxCast Phase I chemicals scored as toxic in the Truong study were also scored as toxic in the Padilla study, suggesting reasonably good concordance across the two studies. Thus, it appears that for chemicals with expected bioactivity, such as those in the Phase I ToxCast library, the more limited phenotypic screening by Padilla et al. still detected chemical-induced deformity. Important questions remain for compounds with unknown toxicity. For instance, Truong et al. identified early notochord deformities associated with thiocarbamate pesticide exposure in developing zebrafish, which might be missed in a more limited phenotypic screen. Breadth versus depth or throughput versus false negatives – this is the fulcrum between high-throughput and moderate-throughput.

Zebrafish assays have been accepted by the Food and Drug Administration for toxicity and safety assessments for investigative new drug approval (He et al., 2014). In an effort to identify novel therapeutics for treatment in humans, many high-throughput chemical screens using the zebrafish model have been aimed at modulating ototoxicity (Esterberg et al., 2013), seizures (Baraban et al., 2013; Rahn et al., 2014), and cancer (Lee et al., 2007; Nguyen et al., 2012). Work from Leonard Zon and colleagues at Harvard Medical School provides an excellent proof of concept on how to translate high-throughput zebrafish screens from the tank to the bedside. Currently, umbilical cord blood transplants are the only option for patients who are unable to find a suitable marrow donor to replenish their hematopoietic stem cells (HSCs) after chemotherapy or bone marrow transplant (Broxmeyer et al., 1989). Zon's laboratory sought a chemical that would promote HSC proliferation and eliminate the need for a second umbilical cord transplant. In 2007, they conducted a chemical genetic screen exposing zebrafish larvae to a library of small molecules (2,500 chemicals) in order to identify compounds that modulate HSC formation and homeostasis (North et al., 2007). The screen used whole mount *in situ* hybridization to identify chemicals capable of inducing the HSC genes *runx1* and *c-myb* in the dorsal aorta of larval zebrafish. The screen identified 16,16-dimethyl prostaglandin E2 (PGE2), which is the first small molecule discovered capable of amplifying a stem cell population within an organ. Subsequent experiments using PGE2-treated cord blood transplantation in immunodeficient irradiated mice provided the preclinical data to support an FDA-approved Phase I clinical trial (Goessling et al., 2011). The Phase I trial has been completed and suggests PGE2 enhances cord blood engraftment, while a Phase II clinical trial is ongoing. The time from the tank to the bedside was 36 months (Zon, 2014). The work by Zon and colleagues demonstrates how the zebrafish model can be used in an initial screen to rapidly identify potential human therapeutic targets to shorten the process of translational research.

4. Advances in the high-throughput pipeline

One of the more rapidly advancing areas of HTS testing with embryonic zebrafish involves chemical impacts on neurobehavior. Embryonic photomotor responses (PMRs) have been used to screen large chemical libraries, including approximately 14,000 neuroactive drugs (Kokel et al., 2010). This method was recently optimized by our lab for use in chemical toxicity screening (Noyes et al., 2015; Reif et al., 2015). The basic approach is a 50 second assay in a 96-well plate using approximately 24 hpf embryos that were chemically exposed starting at approximately 6 hpf. The PMR is a burst of tail flexions in response to a short pulse of intense visible light in an otherwise dark (infrared-lighted) assay chamber. This

assay queries the function of hindbrain photoreceptors and associated developing motor neurons and muscles, and produces a very predictable and reproducible tail flexion readout (Kokel et al., 2013). Deviations from the normal pattern, due to chemical exposure, are thus easily detected by a shifted response pattern. Kokel et al. found that different structural and functional classes of neuroactive chemicals clustered and elicited specific and reproducible embryonic PMRs (Kokel et al., 2010). Chemical psychostimulants and anxiolytics increased and decreased motor activity, respectively, throughout the assay regardless of light. Dopamine agonists lengthened PMR latency periods, while serotonin reuptake inhibitors showed brief but robust responses to light in the refractory period.

The power of this very quick and simple assay to predict more classical signs of chemical hazard was most recently demonstrated by Reif et al. using the Phase I and II ToxCast chemical libraries (Reif et al. 2015). Abnormal responses in the embryonic PMR at 24 hpf were highly predictive of teratogenicity in larvae at 5 dpf. We used the embryonic PMR and a slightly more complex larval PMR assay to test more than 40 flame retardant chemicals with variable structural attributes and largely unknown toxicities (Noyes et al. 2015). By integrating the results of both life stage PMRs with morphometric endpoints, we were able to greatly expand our capacity to detect and dissect structure-bioactivity relationships among diverse flame retardants. The two approaches employed in our lab involved implementing a hierarchical cluster analysis and principal component analysis to evaluate interactions and differences in bioactivity across the morphological and behavioral platforms to discern chemical classes and structural features that confer increased bioactivity. Consistent with observations by Reif et al. for the ToxCast dataset, the presence or lack of PMR effects in 24-hpf embryos exposed to flame retardant chemicals was predictive of mortality and teratogenicity detected later in larvae at 5 dpf. The 24-hpf PMR assay here predicted the presence or absence of morphological defects for approximately 80% of the flame retardants examined morphologically at 5 dpf. Moreover, when combined with PMR testing of larvae at 5 dpf, the concordance increased in that the presence or absence of 24-hpf and 5-dpf PMR effects predicted teratogenicity and survival for 93% of the flame retardants tested.

Other behavioral screening methods have been applied to take advantage of these earliest movements in embryonic zebrafish. For instance, chlorpyrifos insecticide and other well-known developmental neurotoxicants have been used as training sets to guide and validate embryonic zebrafish spontaneous tail contractions for use in developmental neurotoxicity screening (Selderslaghs et al., 2012; Selderslaghs et al., 2010). In addition, Raftery et al. (2014) recently used a 384-well plate format and exposed transgenic embryonic zebrafish (*fli1:egfp*) from 5–25 hpf to 16 chemicals from the ToxCast Phase I library (Raftery et al., 2014). This study employed eGFP stably expressed in the vascular epithelium of this transgenic line to measure spontaneous tail contractions, similar to the endpoint targeted by Selderslaghs et al., as an early indicator of developmental neurotoxicity. While the advances being made in these types of neurobehavioral screening platforms are compelling, additional work is needed to further characterize the specificity of the embryonic PMR mechanism (e.g. altered patterns of stimulation of non-ocular photoreceptors) to neurodevelopmental toxicity.

5. Transgenic reporter lines

The zebrafish model has the capacity for *in vivo* multi-reporter imaging options, which makes this model uniquely suited for *in vivo* cell biology (Figure 3). The use of zebrafish transgenic reporters was traditionally restricted to early life stages; however, lines like *Casper*, a double mutant lacking all melanocytes and iridophores, in both embryogenesis and adulthood, allows *in vivo* imaging of internal organs in adult zebrafish (White et al., 2008). Many reporter strains have already been developed that are driven by restricted promoter expression patterns that can provide readouts for cell/tissue specificity, major signaling pathways, and many other cellular processes. A comprehensive list is curated and maintained by the Zebrafish Model Organism Database (ZFIN; Howe et al., 2013a).

Molecular markers and reporters can be leveraged to achieve more detailed and reliable phenotypic screening. Zebrafish transgenic reporters have been used in mid- to large-scale screens to identify compounds that produce changes in angiogenesis (Kitambi et al., 2009; Tran et al., 2007), stem cell specification (Goessling et al., 2009; Trompouki and Zon, 2010), heart rate (Burns et al., 2005; Milan et al., 2006), and cell metabolism (Walker et al., 2012). Transgenic zebrafish reporters have also been used in fate mapping (Dougherty et al., 2012; Wang et al., 2011) and transplantation (Li et al., 2011; Taylor and Zon, 2009) experiments to aid in morphogenic studies of vertebrate development and cancer progression. Zebrafish reporters can act as *in vivo* biochemical sensors via small-molecule signaling to measure physiological functions, such as indicators of cardiac function (Hou et al., 2014) and wound healing (Niethammer et al., 2009). Hou et al. created a transgenic line (*CaViar*) to study the development of the heart *in vivo* using a genetically encoded dual function calcium and voltage reporter under the control of the heart-specific *cmlc2* promoter. They also developed a high-sensitivity spinning disc confocal microscope and associated software to capture three-dimensional optical mapping of membrane voltage and calcium ions in the developing heart.

Cellular responses are intimately linked with the physiological, mechanical, and molecular signals provided by their surrounding environment. None of these parameters can currently be recapitulated in cell based assays. *In vivo* live imaging of zebrafish transgenic reporters has the ability to enhance our understanding of cell behavior by allowing researchers to monitor cells in their natural environment. For example, the processes controlling the maintenance and recruitment of adult neural stem cells (aNSCs) in the vertebrate brain is not completely understood, and advancements have been hampered by technical limitations. The behavior of aNSCs in mammals remains unclear, particularly related to whether NSCs are depleted with age or undergo self-renewal, which has important implications for recovery from neurodegenerative disease, brain injuries, and repeated traumas. The pallial germinal zone of the zebrafish brain is relevant to mammals as it includes structural and functional properties that resemble mouse aNSCs (Dirian et al., 2014). Two recent reports have used a transgenic zebrafish line to capture live images of aNSCs in their endogenous niche (Barbosa et al., 2015; Dray et al., 2015). Barbosa *et. al.* used the labeled aNSC transgenic line, *Tg(gfap:GFP)mi2001*, to better understand the molecular and cellular processes governing aNSCs in zebrafish brains. For one month, repetitive imaging was used to track individual stem cells in an intact or injured adult zebrafish telencephalon *in vivo.* The results

indicated that after injury, neuronal progenitors are generated by symmetric divisions that deplete the stem cell population as progenitors are recruited to the site of injury. Advancements in the imaging capabilities of neural activity in freely moving zebrafish have also been developed (Feierstein et al., 2015; Fosque et al., 2015; Prevedel et al., 2014).

Zebrafish reporter strains allow the evaluation of cellular and physiological functions during developmental life stages that are not typically accessible in most animal models. Improvements in imaging technologies and software allow us to track the activity of individual cells over long periods of time or reconstruct high resolution images of the *in vivo* three-dimensional shape of internal organs (Feierstein et al., 2015; Mickoleit et al., 2014). Zebrafish reporter strains will provide an opportunity to develop more comprehensive explanations of chemical toxicity mechanisms and chemical structure-bioactivity relationships in vertebrates. The unique ability to monitor diverse sets of cellular and physiological readouts in a living vertebrate organism is historically unprecedented and will facilitate a deeper understanding of many biological processes.

6. Modulation of gene expression

Knocking out a gene and altering the expression levels of a gene represent two different experimental questions; although, they may produce the same phenotype. One of the main advantages of using a transient gene modulating tool is the speed in which hypotheses can be tested. In comparison, generating a zebrafish mutant line can take upwards of a year. Morpholino oligonucleotides (MO) are the most frequently used antisense knockdown tool within the zebrafish community. MOs have been used in large-scale screens (Eckfeldt et al., 2005; Pickart et al., 2006) and to investigate candidate gene functions (Mathew et al., 2009; Porazinski et al., 2015). MOs are ~25-mer nucleic acid bases that are linked to morpholine rings with a neutrally charged phosphorodiamidate backbone, which has been hypothesized, but not experimentally validated, to inhibit electrostatic MO-protein interactions limiting toxicity and degradation via nucleases (Summerton, 2007). The two types of MO applications in zebrafish are splice blocking (Morcos, 2007) and translation blocking (Summerton, 1999). Photoactivatable MOs have been created, allowing spatial and temporal control of gene expression (Shestopalov et al., 2007). The Zebrafish Model Organism Database (Sprague et al., 2008) and Morpholino Database (http://www.morpholinodatabase.org; Knowlton et al., 2008) are two MO database resources that collect published MO sequences and the combined collection of MOs from several large scale screens, respectively. A published list of recommendations to ensure the reliable application of MOs in zebrafish is also available (Eisen and Smith, 2008).

Many researchers are currently reassessing the use of MOs to knockdown gene expression after several reports indicate a poor correlation between MO-induced and knockout (KO) phenotypes in zebrafish (Aranguren et al., 2011; Chapman et al., 2013; Kok et al., 2015; Su et al., 2014; Swift et al., 2014). Kok et al. recently conducted a reverse genetic screen using site specific nucleases targeting 20 genes that had published MO-induced overt phenotypes (morphants) and 50% of the KOs failed to reproduce the morphant phenotype. Additionally, they compared 98 published morphant phenotypes to the corresponding KO phenotypes in the Sanger Zebrafish Mutation Project and ~80% of the morphant phenotypes were not

observed in the KOs (Kok et al., 2015). A majority of the KOs investigated appeared normal, which is consistent with the low rates of overt phenotypes detected in a characterization of close to 1,000 KO zebrafish lines (Kettleborough et al., 2013), which may imply the zebrafish genome operates under a high degree of redundancy and/or may indicate the presence of genetic compensatory networks. The authors suggest that the most essential guideline to evaluate a morphant phenotype is by comparison to an established KO line. This recommendation is based on the assumption that knocking down a gene should produce the same phenotype as knocking out the gene, which does not take into account mechanisms that compensate for gene dosage. Rossi et al. investigated the reasons underlying the differences in phenotypes produced by morphants and KOs in zebrafish by comparing *egfl7* KO and morphant proteomes and transcriptomes (Rossi et al., 2015). *egfl7* morphants display severe vascular defects, while the KO appears normal. The study identified a set of proteins and transcripts upregulated in the KOs, but not in the morphants. The upregulated gene list included a set of extracellular matrix genes known to rescue *egfl7* morphants, suggesting deleterious mutations in *egfl7* may be inducing a compensatory network, which is not activated after transcriptional or translational knockdown. The MO controversy is unlikely to go away, since off-target effects cannot easily be distinguished from target-specific effects.

MOs are not the only gene knockdown tool in the zebrafish tool kit. RNA interference (RNAi) is an antisense process where RNAs act post-transcriptionally to inhibit gene expression via the RNA-induced silencing complex (Bartel, 2009). RNAi has had limited use in the zebrafish model. Injection of long dsRNA was shown to lead to global mRNA knockdown via induction of the interferon response (Zhao et al., 2001). Injection of small interfering RNA raised concerns regarding the non-specific effects produced by the dysregulation of the endogenous microRNA pathway, which is responsible for degrading maternal transcripts during embryonic development (Oates et al., 2000; Zhao et al., 2008). Recent approaches that used tissue specific expression of short hairpin RNA (De Rienzo et al., 2012) and RNAi triggered by convergent transcription to induce heterochromatin formation (Andrews et al., 2014) have had more success, demonstrating efficient and specific targeting of zebrafish genes achieving 70% and 92% gene silencing, respectively; however, RNAi using convergent transcription did not induce repression for all the targeted genes. The earlier studies that produced RNAi-mediated non-specific phenotypes injected dsRNA at concentrations ranging from 40 to 250 pg, while the more recent approaches injected 11.25 to 30 pg of a DNA construct that restricts expression of the RNAs to inside the nucleus. The global injection of dsRNA into the embryo may have been responsible for the non-specific effects reported in earlier publications and is not an inherent incompatibility of RNAi methods in the zebrafish model. Additionally, RNAi using *mir-155* and *mir-218* backbones has been used to generate heritable gene knockdowns in zebrafish, creating hypomorphic states that facilitate the study of gene function and human diseases in which the expression level of a gene is critical (Giacomotto et al., 2015). The data suggest that for certain target loci, RNAi is a valuable tool that can be used in the zebrafish model system to control gene expression in a spatial and temporal manner.

Recent advancements in our ability to modulate gene expression in a temporal and spatial manner has greatly expanded the versatility of the zebrafish model. Zebrafish researchers

can now use a repertoire of tools to rapidly assess the function of a gene or in the case of RNAi and CRISPRi (discussed in section 7), the relatively unexplored endogenous functions of non-coding elements such as promoters, enhancers, silencers, or insulators. The method of choice will depend on the specific question being asked, and using a combination of the methods available will provide confidence in differentiating specific versus non-specific effects.

7. Advances in precision genome editing

This section will provide a brief overview on the implementation of precision genome editing technologies in the zebrafish model and discuss their current and potential future use in toxicology studies. The development of techniques that produce targeted genome edits is revolutionizing the study of gene function and has the potential to unravel the molecular mechanisms involved in toxic pathways at a remarkable pace. The initial venture into precision genome editing relied on human engineered nucleases called zinc finger nucleases (Kim et al., 1996) and transcription-activator-like effector nucleases (Christian et al., 2010), both of which create targeted double strand breaks (DSBs) in the genome, followed by repair via nonhomologous end joining, which often creates codon reading frameshifts leading to impaired protein functions; however, if a donor DNA template is provided, the DSB can be repaired via homology-dependent repair to create precise alterations in the genome (Symington and Gautier, 2011). These techniques were successfully applied in zebrafish, but their use was constrained by their limited multiplexing capabilities and the considerable amount of time and cost required in designing the nucleases (Cade et al., 2012; Doyon et al., 2008). Fortunately, nature has provided us with a template to create a simpler and more efficient genome editing tool. The scientific community has co-opted the clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 system, which relies on a single guide RNA (sgRNA) and the Cas9 nuclease to generate targeted DSBs next to specific recognition sites called protospacer adjacent motifs, followed by repair via nonhomologous end joining or homology-dependent repair mechanisms. This revolutionary method is simple, economical, and advancements are being reported at a rapid rate. For example, Cas9 nucleases with altered protospacer adjacent motif specificities have been engineered and confirmed to generate somatic mutations in zebrafish, thus expanding the number of targetable loci within the zebrafish genome (Kleinstiver et al., 2015). Importantly, the CRISPR-Cas9 system was recently shown to be six-times more efficient at generating germline mutations in zebrafish when compared to zinc finger and transcription-activator-like effector nucleases (Varshney et al., 2015a). There are a plethora of great reviews on the application and ethical considerations of the rapidly developing CRISPR system (Baltimore et al., 2015; Ledford, 2015; Mali et al., 2013; Sander and Joung, 2014).

The CRISPR-Cas9 system in zebrafish has been used to generate gene KOs (Hruscha et al., 2013; Hwang et al., 2013; Jao et al., 2013), tissue specific gene disruptions (Ablain et al., 2015), and single nucleotide substitutions (Hwang et al., 2013), as well as introduce exogenous DNA at specific target sites (Auer et al., 2014; Hisano et al., 2015; Kimura et al., 2014; Li et al., 2015). A zebrafish codon optimized Cas9 has been developed (Jao et al., 2013) and has reported an approximate 35% increase in mutagenesis efficiency (Liu et al., 2014). Two resources supporting efficient sgRNA design include CRISPRz (Varshney et al.,

2015c) and CRISPRscan (Moreno-Mateos et al., 2015), which are websites that provide experimentally determined mutation efficiencies of sgRNAs or predictions of highly efficient sgRNAs, respectively.

One of the more revolutionary aspects of CRISPR technology, in terms of biological research, is the ability to rapidly identify causal genes responsible for producing or ameliorating various phenotypes. The first high-throughput CRISPR-Cas9 phenotyping screen, which targeted 162 loci (83 genes) in the zebrafish genome, reported a 99% success rate in generating somatic mutations with an average germline transmission rate of 28% (Varshney et al., 2015a). The Moen's lab from the Fred Hutchinson Cancer Research Center recently published a reverse genetic screen examining 48 zebrafish loci and identified two new genes involved in electrical synapse formation (Shah et al., 2015). The somatic mutation efficiency was high enough to induce an observable phenotype in the injected animal, which reduced the experimentation time to 3 weeks. The speed of discovery is unprecedented and would not be attainable in a mammalian system. These studies used multiplexing of multiple loci to demonstrate the feasibility and power of mid- to large-scale rapid screens using the CRISPR-Cas9 system in the zebrafish model. A major concern using any genome editing tool is off-target effects; however, reports suggest that the frequency of CRISPR off-target effects in zebrafish are low (0–4%) with little risk of confounding the phenotypic analysis of mutations when segregation of unlinked mutations and the low occurrence of predicted off-target sites are also considered (Hruscha et al., 2013; Li et al., 2015; Varshney et al., 2015a).

In addition to generating gene KOs, the CRISPR system has enhanced our ability to generate knockin animals, which is a versatile tool for all biological research. Previous methods, such as Tol2-mediated transgenesis, have successfully generated hundreds of zebrafish lines, which have aided in understanding mechanisms of development and gene regulation, as well as characterizing functional regulatory elements (Abe et al., 2011; Fisher et al., 2006; Kawakami, 2007). The CRISPR-mediated knockin represents an advancement in knockin methods due to its increased control over the copy number of insertional events and the site of integration into the genome. Both the number of copies and location of insertion into the genome have been shown to effect the pattern and levels of gene expression (Giraldo and Montoliu, 2001; Moro et al., 2013).

Additionally, the use of the endogenous regulatory elements overcomes other obstacles, such as a limited knowledge of both proximal and distal regulatory sequences. Several zebrafish labs have generated transgenic animal models using CRISPR-Cas9, where the gene of interest is fused to a peptide linker and florescent reporter to drive multicistronic expression, which allows the endogenous promoter to drive the expression of the target gene and reporter in an approximate 1:1 ratio without interfering with the resulting structure of the protein (Auer et al., 2014; Hisano et al., 2015; Li et al., 2015). Transgenic zebrafish reporters that use enhancer trapping, a method that co-opts the transcriptional regulators of a nearby promoter element to drive expression of the fluorescent reporter, have also been developed (Kimura et al., 2014). Additionally, the CRISPR-Cas9 system has been successfully modified to function as a synthetic transcriptional regulator to either repress (CRISPRi) or activate (CRISPRa) gene expression levels using a catalytically inactive Cas9

(dCas9) nuclease fused to a transcriptional activator ($dCas9^{VP64}$) or repressor domain ($dCas9^{KRAB}$) using an *in vitro* cell culture model (Gilbert et al., 2013; Kiani et al., 2014; Sander and Joung, 2014). A novel CRISPRa method employs epigenome editing using dCas9 fused to the catalytic core of the human histone acetyltransferase p300 ($dCas9^{p300\ CORE}$), which is strongly associated with active promoters and enhancers, has also been developed and was shown to induce higher levels of transcriptional activation when compared to $dCas9^{VP64}$ in HEK293T cells (Hilton et al., 2015). A recent publication using CRISPRi targeting two genes (*fgf8a* and *foxi1*) in zebrafish, reported that multiple sgRNAs are needed to induce gene knockdown since a single sgRNA was insufficient to alter gene expression; however, pooling those same sgRNAs resulted in a 40–70% reduction in the relative mRNA expression levels of the targeted genes (Long et al., 2015). It is unclear if these results will hold true for other zebrafish target loci.

To the best of our knowledge, no report has been published that uses the CRISPR-Cas9 system in zebrafish toxicity screens. The coupling of the CRISPR-Cas9 system with zebrafish screens has the potential to provide unprecedented mechanistic insight in all fields of modern biology because mutational analysis is a primary tool in understanding how a genotype correlates with a phenotype. Furthermore, integrating the CRISPR system with tools that have already been validated in zebrafish, such as the Cre-Lox system (Hans et al., 2011), the Gal4/UAS system (Scott, 2009), and doxycycline or tamoxifen responsive promoters (Hans et al., 2009), will greatly expand our ability to generate conditional KOs. In the context of toxicology, the various CRISPR applications can be used to rapidly identify functional regulatory elements, genes, and pathways that confer resistance or susceptibility to chemicals. Additionally, epistasis experiments could also be conducted to ‘place’ genes in their proper order in toxicity or developmental pathways. Ultimately, the CRISPR system applied to the zebrafish model will provide greater predictive power for adverse health effects in humans since molecular information is generally critical in determining interspecies differences to toxicological responses and in translation of model organism data to humans.

8. Conclusion

The zebrafish model provides a very unique vertebrate data stream that allows researchers to anchor hypotheses at the biochemical, genetic, and cellular levels to observations at the structural, functional and behavioral level in a high-throughput format. As the tools with which we perturb and then monitor biological, physiological, and behavioral processes advance, so will our understanding of said processes. In short, the zebrafish model is bringing us closer to closing the elusive genotype-phenotype gap.

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Acronyms

CRISPR	Clustered, Regularly Interspaced, Short Palindromic Repeats
Dpf	Days Post Fertilization
DSB	Double Strand Break
Hpf	Hours Post Fertilization
HSC	Hematopoietic Stem Cell
HTS	High-Throughput Screen
KO	Knockout
MO	Morpholino Oligonucleotide
NSC	Neural Stem Cell
PGE2	16,16-dimethyl prostaglandin E2
PMR	Photomotor Response
RNAi	RNA interference
sgRNA	Single Guide RNA

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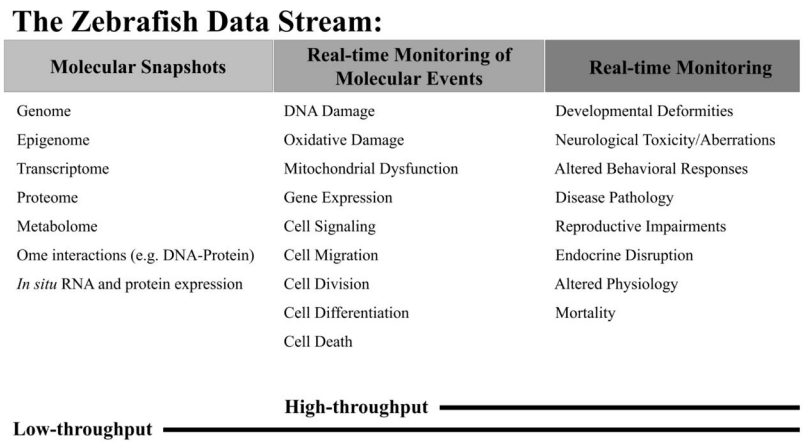

Figure 1.
Conceptual image of the zebrafish data stream

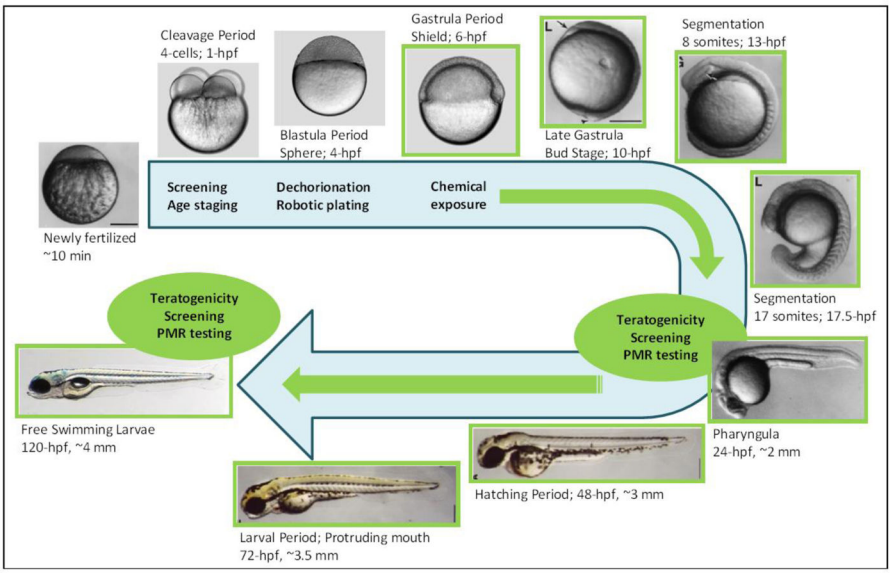

Figure 2.

Example of embryonic zebrafish high-throughput screening (HTS) platform. Embryos synchronized at a specific developmental stage are selected, screened for viability, and placed into well plates. Embryos are generally exposed to chemicals between 6–120 hours post fertilization (hpf). Morphological evaluations and behavioral assays are frequently conducted during (1) the early pharyngula stage at 24 hpf when the heart is first clearly visible in a distinct pericardial sac and body/tail flexions initiate with development of the sensory-motor system; and (2) free-swimming larvae represented by inflation of the swim bladder, largely completed developmental morphogenesis, and rapid growth (Haffter et al., 1996; Kimmel et al. 1995; Noyes et al. 2015; Truong et al. 2014).

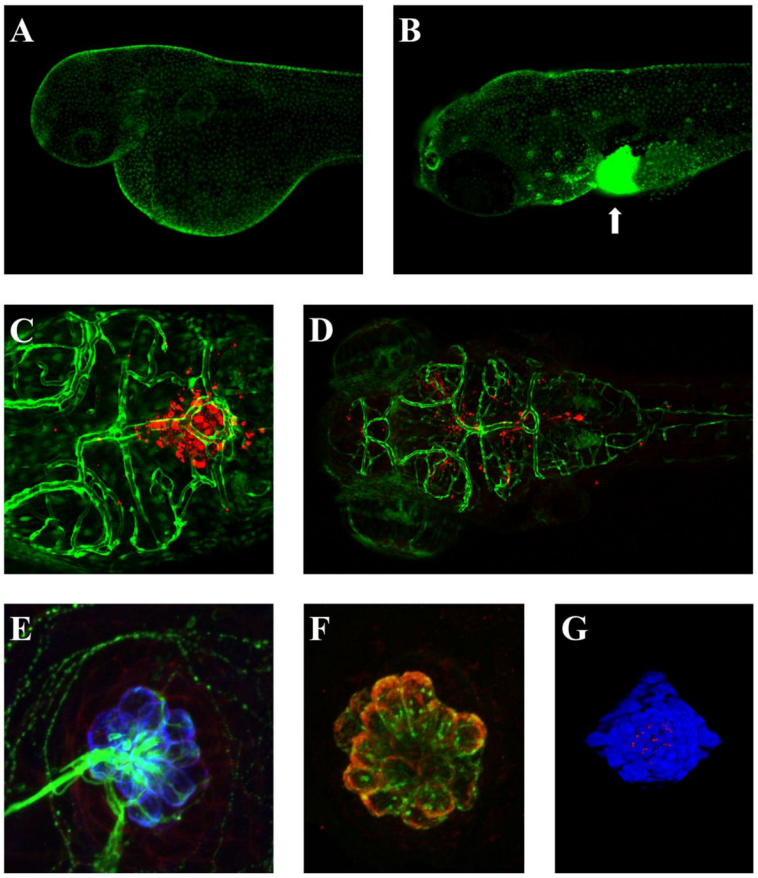

Figure 3.

Representative images from embryonic transgenic zebrafish. (**A** and **B**) The *Tg(cyp1a:nls-egfp)* line can be used as a surrogate for AHR activity to identify the target tissues of chemical exposure. Embryos were continuously exposed to a chemical starting at 6 hpf and imaged at 48 hpf (**A**) and 120 hpf (**B**), with noticeable cyp1a expression in the liver at 120 hpf (white arrow). (**C** and **D**) The *Tg(fli:gfp)* line, which expresses GFP in endothelial cells of the entire vasculature, were injected with glioblastoma cells (red) into the brain of 4 dpf larvae (**C**) and reimaged at 7 dpf (**D**) in order to capture the invasion and migration behavior of the brain cancer cells. (**E**–**G**) Immunohistochemistry was used to determine the expression pattern of various genes in the hair cells of the lateral line neuromast of 4 dpf larvae. (**E**) 2D composite image stained with antibodies targeting otoferlin (blue), acetylated tubulin (green), and maguk (red). (**F**) 2D composite image stained with antibodies targeting otoferlin (green) and vglut3, a synaptic vesicle marker (red). (**G**) 3D composite image stained with DAPI and the synaptic protein ribeye (red clusters). While images (**E**–**G**) are not from a transgenic line, the images were included to highlight the ability to capture high quality *in situ* expression patterns of genes across development, which is the function of transgenic reporter lines.

Table 1

Comparison of early developmental life-stages of human, rat, and zebrafish

Developmental Stage	Human (Day)	Rat (Day)	Zebrafish (Hour)
Blastula/Blastocyst	4–6	3–5	2–5
Implantation	8–10	6	n/a
Neural Plate Formation	17–19	9.5	10
First Somite	19–21	9–10	10–11
10 Somite Stage	22–23	10–11	14
Neural Tube Formation	22–30	9–12	18–19
First Pharyngeal Arch	22–23	10	24
Initiation of Organogenesis	21	5	10
First Heartbeat	22	10.2	24
Birth/Hatching	253	21	48–72