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Fishing for Fetal Alcohol Spectrum Disorders: Zebrafish as a Model for Ethanol Teratogenesis

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Abstract

Fetal Alcohol Spectrum Disorders (FASD) describes a wide array of ethanol-induced developmental defects, including craniofacial dysmorphology and cognitive impairments. It affects ~1 in 100 children born in the United States each year. Due to the pleiotropic effects of ethanol, animal models have proven critical in characterizing the mechanisms of ethanol teratogenesis. In this review, we focus on the utility of zebrafish in characterizing ethanol-induced developmental defects. A growing number of laboratories have focused on using zebrafish to examine ethanol-induced defects in craniofacial, cardiac, ocular, and neural development, as well as cognitive and behavioral impairments. Growing evidence supports that genetic predisposition plays a role in these ethanol-induced defects, yet little is understood about these gene–ethanol interactions. With a high degree of genetic amenability, zebrafish is at the forefront of identifying and characterizing the gene–ethanol interactions that underlie FASD. Because of the conservation of gene function between zebrafish and humans, these studies will directly translate to studies of candidate genes in human populations and allow for better diagnosis and treatment of FASD.

Fetal Alcohol Spectrum Disorders: A Debilitating Disease for Patients and Society

Ethanol (alcohol, ethyl alcohol) is the most commonly consumed and abused drug. In the 1700s, when the mass distillation of alcohol made large quantities of potent alcohol readily available to the masses, alcohol abuse became rampant in areas. At this time, some noted that the offspring of women who consumed large amounts of distilled alcohol were often small and feeble with a higher rate of infant mortality.[1] Yet, two centuries would pass before the detrimental effects of alcohol on embryonic and fetal development were appreciated.

The first documented clinical description of ethanol teratogenesis in humans came in 1968, when a French physician noted a set of abnormal features in children who were exposed to alcohol before birth.[2] Five years later, the terminology Fetal Alcohol Syndrome (FAS) was coined to describe a characteristic set of birth defects in children prenatally exposed to alcohol.[3] In addition to verifying maternal alcohol consumption during pregnancy, individuals must meet three criteria: growth retardation, craniofacial malformations, and central nervous system (CNS) abnormalities to be diagnosed with FAS.[4] While FAS is the most severe outcome of prenatal alcohol exposure, it has become clear that it is not the only outcome.

Since 1973, it has been found that prenatal ethanol exposure causes wide-ranging deficits with similarly wide-ranging terminology. Newer terms include partial FAS, Alcohol-Related Birth Defects, and Alcohol-Related Neurodevelopment Disorders.[4] These terms differ based on the type of deficits involved (physical abnormalities versus CNS abnormalities) and exemplify the variation that can result from prenatal ethanol exposure.[5] The terminology Fetal Alcohol Spectrum Disorders (FASD) has been coined to define any defect caused by embryonic or prenatal alcohol exposure.[1,6–9]

Due to the high prevalence of drinking, FASD is a widespread disease in many ''Westernized'' nations.[8] In the United States alone, amounts of ethanol consumption during pregnancy vary greatly. For example, Flak *et al.*[10] found that from 1991 to 2005, 12% of pregnant women reported consuming at least a single drink of ethanol over the course of the previous 30 days. A study by Ethen *et al.*[11] stated that during a similar period, 1997–2002, more than 25% of pregnant women reported drinking alcohol. This is likely an underestimate because more than 50% of women of childbearing age in the United States consume ethanol and approximately half of pregnancies are unplanned[10] Based on these estimates, it is possible that 1 in 100 births in the United States could result in an FASD diagnosis.

More recently, data gathered from South Africa, Italy, and the United States suggest FASD prevalence rates to range between 2% and 5%.[8] These rates may still be underestimates since pediatricians fail to frequently recognize FASD. Achieving reliable rates of FASD will require a detailed understanding of the many defects that can be caused by embryonic alcohol exposure.

Among the most common and well-researched defects associated with FASD are facial and neural defects. As noted

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earlier, facial and neural defects are required for a diagnosis of FAS. In addition, maternal alcohol consumption is a risk factor for orofacial clefting.[12] Numerous structural defects of the brain are found in children with FASD. This includes changes to the brain shape and size, with individuals with FAS showing a reduction in total size.[9] In addition, prenatal ethanol exposure has also been shown to reduce the size of the cerebellum and to cause structural changes to the corpus collosum.[9,13]

Furthermore, prenatal alcohol exposure is also the leading cause of preventable intellectual disability,[7] impairing both cognition and behavior.[9] Individuals with FASD suffer from learning and memory impairments.[14] Individuals with FASD also frequently fail to consider the consequences of their actions, are unresponsive to social cues, or lack the appropriate initiative to form and maintain reciprocal friendships.[15,16] The inability to attain appropriate social skills can lead to a lack of social relationships, employment problems, trouble with the law, inappropriate sexual behavior, suicide, and depression.[17,18]

Anxiety, low self-esteem and relationship, and economic hardships are all also associated with craniofacial malformations adding to the burden of prenatal ethanol exposure.[19] Thus, FASD is one of, if not the most, prevalent birth defects, leaving individuals with lifelong disabilities. Our understanding of the causes of the defects associated with FASD will require intensive research in both humans and animal model organisms.

Models of FASD: Return of the Fish

Despite the inklings in the 1700s, it was not until this past century that alcohol was clearly demonstrated to be a teratogen. Perhaps the first evidence of this comes from a fish model in 1910, where ethanol was shown to disrupt development of several organ systems, including the brain, face, and eyes of *Fundulus heteroclitus*.[20] Despite this initial work, aquatic models of FASD have lagged behind mammalian and avian models in the latter half of the century. Much has been learned from these other model systems and is reviewed elsewhere.[21–24] For the purpose of this review, we focus primarily on the contributions of fish systems, particularly zebrafish, to our understanding of FASD.

The zebrafish became a major model organism in the early 1980s, as a small group of researchers, led notably by George Streisinger and Charles Kimmel, sought a model organism in which to analyze genetics and morphogenesis during vertebrate development (Reviewed by Grunwald and Eisen[25]). Zebrafish was chosen, in part, due to their external fertilization and high fecundity, as well as the optic clarity of the embryos. Since then, many tools have been generated for transgenesis and genetic manipulation in zebrafish. As a disease of development, these very same characteristics and tools make zebrafish an excellent model of FASD.

One of the challenges of establishing zebrafish as a model system for FASD was determining physiologically relevant dosages of ethanol. As detailed below, dosage regimens have varied widely, from 0.12% to 10%, and those studies that attempted to quantify tissue levels of ethanol also varied widely in conclusion. Recently, a consensus has been reached by groups of individual researchers using differing techniques. Using enzyme-based assays, two separate laboratories have reported the tissue levels of ethanol to be ~31%–35% of the concentration of ethanol in the media.[26,27] Using an Analox assay, a separate group approximated the tissue levels to be 35% of the media levels.[28] Our group used Headspace Gas Chromatography and found tissue levels to be ~24%–37%, depending upon the age of the embryo, with older embryos having lower tissue levels.[29] One possible reason for the early disagreement in tissue levels of ethanol may be the very rapid rate at which ethanol levels equilibrate,[28–31] and therefore, extended or multiple washes would result in an underestimation of the ethanol concentration. Collectively, these studies indicate that the use of ethanol concentrations at or above 2% in the media is likely to result in suprapharmacological levels of ethanol.

Other potential variables that have been studied do not appear to have a substantial effect on tissue levels of ethanol. Genetic background (AB, Tu, or *fli1:EGFP*) did not appear to have a substantial impact on the tissue levels of ethanol.[29] Specifically, consistent but nonsignificant effects were observed with AB appearing to accumulate ethanol to a lower extent than the other backgrounds.[29] Similarly, the chorion does not appear to be a major barrier to ethanol.[28,29,32]

However, the developing embryo can show varying ethanol sensitivities based on genetic backgrounds (Ekkwill, AB, or Tu).[33] Interestingly, the tissues most affected by ethanol exposure varied among the different backgrounds with Tu having the lowest survival rate, but being the most resistant to craniofacial malformations. Work in different adult zebrafish backgrounds (wild-type AB, wild-type long fin, wild-type short fin, or Leopard danio) demonstrated that the backgrounds exhibited different sensitivities to ethanol, while tissue levels of ethanol were consistent across the backgrounds.[34–37] In addition, the outbred 5D background was also sensitive to ethanol and this could be phenocopied by knockdown of two different microRNAs.[38] Thus, it appears that the wide range of genetic and transgenic resources available for zebrafish researchers will be readily and easily applicable to studies of FASD.

Zebrafish Models of FASD

FASD is highly variable and extensive research has gone into understanding the many abnormalities in ethanol teratogenesis. Some of the more varied defects in FASD include cardiac and eye defects. Moreover, the embryo appears most sensitive during gastrulation when the progenitors of the CNS and the face are being generated. Therefore, disrupting embryonic development during these times can lead to the cardinal features of FAS, growth retardation, facial dysmorphologies, and CNS abnormalities.[4] With or without these structural defects, individuals with FASD also have behavioral and/or cognitive impairments. Zebrafish have modeled these varied and cardinal phenotypes associated with FASD.

Cardiac and eye defects are some of the most widely reported defects in ethanol-treated zebrafish. For instance, 0.5% ethanol can cause significant increases in the occurrence of cardiac edema, reduction in heart volume, and reduced ventricular thickness.[39] Slightly higher doses of 100 or 150 mM (0.6%–0.9%) can disrupt cardiac precursor migration, heart looping, and cardiac gene expression.[40] The zebrafish inner ear is disrupted in embryos treated with 2% ethanol.[41] Using the lateral line as a model of hair cells, Uribe

et al.[42] demonstrated a reduction in sensory hair cells in embryos treated with 1% ethanol. Eye diameter and visual acuity are reduced in embryos treated with 1.5% ethanol.[43,44] A separate study suggested that the eye diameter was sensitive to even 0.4% ethanol.[45] The difference between these findings is unclear, but may relate to genetic background as Dlugos and Rabin[45] used an outbred background and Bilotta *et al.*[43,44] used an unspecified background.

The earliest use of zebrafish in FASD research focused on structural defects caused by ethanol. In modeling the similarities of holoprosencephaly and some children with FASD, Blader and Strähle[32] discovered that treatment with 2.4% ethanol over a short time window, from dome to 30% epiboly, resulted in cyclopic phenotypes similar to those observed in *wnt11* mutants, in which Wnt/PCP signaling is disrupted. The phenotype observed strongly suggested that ethanol was disrupting migration of the prechordal plate mesoderm during gastrulation. Likewise, treatment with 3% ethanol at similar times caused a split body axis, often associated with cyclopia, and these defects associated with altered gastrulation cell movements.[26]

Recent findings support the conclusion that ethanol alters gastrulation cell movements. Genetic background plays a significant role in the susceptibility to ethanol-induced cyclopia, with the EK background displaying cyclopia in even 1% ethanol.[46] The Wnt/PCP pathway is critically involved in gastrulation movements and genetically interacts with 1% ethanol to produce cyclopia in *vangl2* mutants,[47] which also have disrupted Wnt/PCP signaling. In addition, in the highly ethanol-sensitive TL strain, 0.6% ethanol has been shown to alter the movement of cells during gastrulation.[40] These zebrafish findings are consistent with characterizations in Xenopus showing that ethanol exposure disrupts gastrulation.[48]

These ethanol-induced defects in gastrulation can result in general growth retardation in zebrafish. Loucks and Ahlgren[49] demonstrated that zebrafish exposed to 1% or 1.5% ethanol had small, but significant reductions to the overall size of the embryo. The effect of ethanol was more substantial as higher concentrations of ethanol were tested[50] and Ali *et al.*[51] found that a 1-h pulse of 10% ethanol resulted in smaller larvae with numerous structural defects. However, given the kinetics and partitioning of ethanol, discussed earlier, this treatment is likely to be far outside the physiological norms in humans.

Different morphometric analyses have found that an ethanol concentration of 1.5% or less results in changes to specific facial measurements, presumably due to elevated levels of cell death in the neural crest progenitors of the facial skeleton.[28,52] However, in our analyses discussed below, we have found that 1% ethanol merely sensitizes embryos to craniofacial defects,[53] differences that may be due to genetic background.

Due to the large number of transgenic backgrounds available, new insights are being gained into how ethanol disrupts neural development in zebrafish. Exposure to 1% ethanol reduces the number of elavl3-positive neural progenitors[54] and 100 mM (0.6%) increases the branching of secondary motor neurons and decreased the diameter of Mauthner axons.[55] In addition, Zhang *et al.*[56] reported that 0.5% ethanol exposure leads to disruption of the mid-hindbrain boundary. These and other structural brain abnormalities have been found to have an adverse relationship with cognitive function and behavior in the clinical population.[13,57] More recently, the zebrafish has been shown to model some of the behavioral and cognitive deficits of FASD.

Embryonic ethanol exposure impairs social behavior in adult zebrafish.[58–62] Zebrafish form social groups called shoals.[63] Research has demonstrated that a single 2-h exposure to 1% ethanol (v/v) at 24 hours postfertilization (hpf) can impair an individual's response to a virtual shoal and between groups of live zebrafish.[58,59,61,62] These social impairments are present in adult fish ranging in age from 4 to 24 months.[58,59,61,62] Parker *et al.*[60] examined the effect a 0.12% developmental dose of ethanol (48 to 216 hpf—9 days) had on individual and group shoaling behavior in 4-month-old zebrafish. They found both the individual's response to a social cue (five live zebrafish) and group shoaling were impaired by embryonic ethanol exposure in adult zebrafish.[60] The social impairments of FASD are not the only CNS impairments zebrafish can be used to model.

Individuals with FASD are known to have deficits in learning and memory[14] and zebrafish have been shown to recapitulate some of these deficits.[52,64,65] Associative learning in adult zebrafish has been shown to be impaired by a 2-h ethanol exposure starting at 16 hpf.[64] Carvan *et al.*[52] found adult zebrafish that were exposed from 0 to 24 hpf to 0.06% and 0.18% ethanol took significantly more trial to predict the location of a food reward. However, Bailey *et al.*[66] found that fish exposed to either 1% or 3% ethanol from either 8 to 10 hpf or 24 to 27 hpf did not have impaired spatial discrimination. Taken together, these results demonstrate that not only can zebrafish be used to model the learning impairments of FASD but also to show the variability of embryonic ethanol exposure.

Embryonic ethanol exposure increases maladaptive behaviors in zebrafish.[60,66,67] Parker *et al.*[60] reported that exposure to 0.12% ethanol from 48 to 216 hpf caused adult zebrafish to spend significantly more time at the bottom of the tank compared to untreated controls. This behavior is interpreted as elevated anxiety. However, spending too much time at the top of the water column can also be interpreted as a maladaptive behavior.[66,68] Exposure to 3% ethanol between 24 to 27 hpf leads to a significant time spent in the top of the tank, suggesting the acquisition of maladaptive behaviors.[66] The exposure regimens across these studies differ substantially, suggesting that the timing, duration and length of exposure may contribute to the variability in behavioral outcomes.

FASD affects individuals from childhood and throughout adulthood.[15] More recently, the effects of embryonic ethanol exposure on larval behavior have been examined. Zebrafish larvae demonstrate a rapid escape response/startle response known as a C-start.[69] Shan *et al.*[55] exposed embryos from 5.25 to 10.75 hpf to ethanol concentration of 0%, 0.006%, 0.3%, and 0.6%, and measured C-starts among other criteria in 2-day-old larvae. They found that ethanol-treated fish showed an increase in abnormal C-starts and tail speed. Relatively low doses of ethanol have been shown to induce hyperactivity in larval and adult zebrafish, while higher doses have been shown to reduce activity.[70,71] Thus, ethanol concentration is an important variable in the effect of ethanol on locomotion.

Insights into the Mechanisms of Ethanol Teratogenicity from Zebrafish

Ethanol is a small molecule that will likely have many pleiotropic effects. Thus, there is unlikely to be a single

mechanism by which ethanol teratogenicity proceeds. Data from zebrafish and other model organisms support this, with several mechanisms that appear conserved across species being demonstrated. Ethanol-induced neural crest cell apoptosis is a cause of facial defects in FASD. In both chicken and zebrafish, Flentke *et al.* have shown that this ethanol-induced death is calcium and CaMKII dependent.[28,72] Collectively, these findings suggest that the mechanisms of facial deformity in FASD are conserved across vertebrates.

Across animal species, degradation of ethanol is a multistep enzymatic process, with each step a potential target of ethanol sensitivity. Ethanol can also be degraded by retinaldehyde dehydrogenases, which are necessary to convert retinal to retinoic acid. Retinoic acid is critical for the development of many organ systems, leading to the hypothesis that ethanol is a competitive inhibitor of retinoic acid synthesis.[73,74] However, there is disagreement regarding how ethanol may impact retinoic acid signaling. Napoli[75] showed that ethanol either upregulates or has no effect on retinoic acid signaling, depending on tissue context. This work demonstrates that retinoic acid has variable effects on ethanol teratogenesis.

Specific to zebrafish, retinoic acid supplementation restores proper facial development in fish treated with 100 mM ethanol (0.6%).[76] However, retinoic acid supplementation fails to rescue microphthalmia in ethanol-exposed zebrafish.[77] It is likely that the involvement of retinoic acid in ethanol teratogenicity is cell-type specific, as Zhang *et al.* also found retinoic acid failed to rescue eye development but did restore mid-hindbrain development.[56] The precise mechanism by which retinoic acid may be involved in ethanol teratogenesis is unknown but it may relate to cross talk with another signaling pathway, Sonic hedgehog (Shh),[56] which is an indirect target of retinoic acid signaling.

Due to the similarity in phenotype of holoprosencephaly and FAS, impairment of the Shh pathway has been postulated as a cause of FAS.[21] Loss of Shh signaling results in holoprosencephaly, which consists of neural tube defects, loss of midline craniofacial structures, and neural crest-specific cell death.[78] Work in chicken, mouse, and zebrafish has shown that ethanol exposure reduces Shh signaling leading to increased CNCC death, as well as disrupted midline and eye development.[30,79–82] These ethanol-induced phenotypes can be rescued by injection of *shh* mRNA.[50] In addition to altering retinoic acid signaling, one alternative (or additional) mechanism by which ethanol attenuates Shh is the disruption of cholesterol modification of Shh needed for proper signaling, as has been found in zebrafish.[82] Further support for an involvement of Shh signaling in ethanol teratogenesis comes from genetic analyses (as we discuss in the next section).

Identifying Gene–Ethanol Interactions: The Way of the Fish

There is strong and growing evidence for genetic risk factors for FASD. Human twin studies show that monozygotic twins are 100% concordant for FASD, while dizygotic twins are only 64% concordant.[83] Studies in mice, chick, and zebrafish show that different strains have different sensitivities to ethanol-induced defects.[33,34,84–86] However, direct analysis of the genetic loci regulating sensitivity to ethanol-induced birth defects is still in its infancy. Only a handful of gene–ethanol interactions have been identified to date. A detailed discussion of these interactions can be found elsewhere,[87,88] whereas here, we focus on findings in zebrafish.

The many genetic tools available in zebrafish have proven successful in expanding our understanding of developmental biology. While they have recently come under scrutiny,[89] well-designed experiments using morpholinos[90] can speed genetic analyses. Tilling projects, such as the Zebrafish Mutation Project, have generated mutations in approximately 60% of the predicted coding genes in zebrafish. Genome editing techniques, such as CRISPR/Cas9, making it possible to directly target nearly any gene of interest.[91] Furthermore, forward genetic screens are a mainstay of the zebrafish community, and we have begun to take advantage of this technique to identify ethanol-sensitive loci (our unpublished data). These tools are readily applicable to studies of ethanol teratogenicity and have already begun to inform our understanding of FASD.

Some of the early evidence for gene–ethanol interactions in model organisms came from studies of the Shh pathway. Using morpholino oligonucleotides against *agrin*, coding for a membrane protein that mediates Shh signaling, Zhang *et al.* found that ethanol genetically interacted with the Shh pathway during eye development.[26] Expanding on these studies, the same group has shown that *shha* interacts with ethanol disrupting GABAergic and glutamatergic neural development.[30] An interaction between ethanol and Shh pathway members has been independently identified in mouse.[92–94] Collectively, these studies provide strong evidence for an evolutionarily conserved interaction between ethanol and the Shh pathway.

Most studies of gene–ethanol interactions have been guided by FASD phenotypes. However, the large number of readily available zebrafish mutants allows for shelf screening to identify potentially unexpected gene–ethanol interactions. Initially, we screened five mutant lines, *smoothened* (a Shh pathway member), *cyp26b1* (RA catabolizing enzyme), *smad5* (bone morphogenetic protein pathway member), *gata3* (transcription factor), and *pdgfra* (platelet-derived growth factor receptor) for gene–ethanol interactions in craniofacial development. Of these, only *pdgfra* interacted with ethanol and this interaction was highly synergistic.[53]

In zebrafish, mice, and human, loss of *pdgfra* results in cleft palate, and in model organisms, this has been shown to be due to disrupted neural crest cell migration.[95–98] When treated with ethanol, *pdgfra* mutant zebrafish have massive reductions of the entire craniofacial skeleton.[53] In addition, over two-thirds of *pdgfra* heterozygous embryos had craniofacial defects. These ethanol-induced defects proved to be due to elevated cell death. Pdgfra acts through the PI3K/mTOR pathway to regulate cell survival, proliferation, and growth,[99,100] and we found that this pathway mediates the *pdgfra*–ethanol interaction.[53]

Pdgfra function is conserved among zebrafish, mice, and human[94,96,97] and, in human, we identified single-nucleotide polymorphisms in *PDGFRA* and *PDGFRB* that significantly associated ethanol-induced changes in outer canthal width and midfacial depth, respectively.[53] This suggests that growth factor signaling as a target of ethanol teratogenesis and that studies in animal models can predict gene–ethanol interactions in human FASD.

We built upon this initial shelf screen using zebrafish mutants available from the Zebrafish International Resource

Center (ZIRC). We screened 20 mutant lines, analyzing for increases in cell death, deficits in neural development, and general growth retardation in addition to craniofacial malformations. From this screen, we identified five ethanol-sensitive mutant lines, nearly doubling the number of known gene–ethanol interactions.[47] Spanning a wide range of cellular functions, the genes include *mars*, a methionine-tRNA synthetase necessary for protein translation; cell cycle components, *hinfp* and *plk1*; *foxi1*, a transcription factor important in craniofacial development; and the cell polarity gene, *vangl2*.

Embryos lacking *mars* develop mild viscerocranial defects that are exacerbated in ethanol. In addition, the size of the eye is reduced. Ethanol treatment results in exacerbation of the microcephaly and microphthalmia phenotypes, as well as loss of the lower jaw in *hinfp* mutants. Embryos lacking *plk1* lose the entire viscerocranium as well as display defects to the neurocranium. In addition, axonal projections are reduced, and localized increase in cell death is observed. Ethanol treatment leads to severe growth retardation, complete loss of the craniofacial skeleton and axon projections, and extensive cell death throughout the embryo. Necessary for development of the posterior craniofacial elements, the hyosymplectic and ceratobranchials,[101] *foxi1*–ethanol interactions result in loss of these cartilage elements, mislocalized axon projections, and reduced ear size.

Of these genes, *vangl2* interacted most strongly with ethanol. Developmentally, *vangl2*, is necessary for convergent/extension of the body axis with loss resulting in a shortened body axis and, at low incidence, synophthalmia.[102] Ethanol exposure in mutants leads to loss of the posterior viscerocranial elements, narrowing of the palate, and severe synophthalmia, as well as disrupted axon projections. These phenotypes were also observed in a small percentage of heterozygotes. It remains to be seen if the *vangl2* or any of the other gene–ethanol interactions are conserved in humans.

Conclusions

FASD remains a significant problem in our society, impacting on the lives of millions of people. Estimates place the incidence of FASD at 10 in 1000 births, which still might be an underestimate.[103–106] Given that (1) more than 50% of childbearing women report using ethanol,[10] (2) nearly 50% of pregnancies are unplanned,[107,108] and (3) embryos are sensitive to ethanol before a woman is typically aware of pregnancy, abstinence-based approaches are unlikely to eliminate FASD. Thus, a complete understanding of the underpinnings of FASD is critical to human health.

Animal studies have been invaluable in elucidating the biochemical, molecular, and genetic events that lead to FASD.[21–23,109,110] Studies linking animal models with human patients will be vital to advancing our understanding of FASD and to identify potential therapeutic approaches to overcome its catastrophic effects. Zebrafish is particularly well suited for studying the pathology of FASD because of its high fecundity, external fertilization, embryo transparency, rapid development time, and genetic tractability. Due to the high degree of conservation of gene function across vertebrate species, studies in zebrafish should greatly accelerate our understanding of FASD.[111–113] With its future bright, zebrafish is poised to become one of the prominent model systems at the forefront of FASD research.

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