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Epigenetic effects of environmental chemicals: insights from zebrafish

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Abstract

Zebrafish have been extensively used for studying vertebrate development and modeling human diseases such as cancer. In the last two decades, they have also emerged as an important model for developmental toxicology research and, more recently, for studying the developmental origins of health and disease (DOHaD). It is widely recognized that epigenetic mechanisms mediate the persistent effects of exposure to chemicals during sensitive windows of development. There is considerable interest in understanding the epigenetic mechanisms associated with DOHaD using zebrafish as a model system. This review summarizes our current knowledge on the effects of environmental chemicals on DNA methylation, histone modifications and noncoding RNAs in the context of DOHaD, and suggest some key considerations in designing experiments for characterizating the mechanisms of action.

Keywords

DNA methylation; noncoding RNAs; DNMTs; zebrafish; non-mammalian model

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Conflict of Interest

I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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1. Introduction

There is growing evidence from epidemiological and experimental studies that exposure to environmental stressors during critical windows of susceptibility can have long-term consequences [1,2]. Examples of association between exposure to environmental stressors during critical periods of fetal development and increased risk for cardiovascular diseases, obesity and neurological disorders are well documented [3,4]. This is a growing field of research and is collectively termed as the developmental origins of health and disease (DOHaD) [5,6]. The DOHaD hypothesis postulates that early life stressors can cause developmental reprogramming, inducing long-term changes in normal development and physiology [6]. Several studies have demonstrated that developmental exposure to environmental chemicals can cause long-term changes in physiology and behavior of the adults [7]. Some of these effects are shown to be inherited by subsequent generations. The mechanisms involved in developmental reprogramming by toxicants are not thoroughly understood; however, effects on epigenetic landscape during cellular and tissue differentiation are considered to be a potential mechanism of toxicant action [8]. Epigenetic modifications are defined as persistent changes in gene expression that occur without a change in the nucleotide sequence.

In the past two decades, there has been intense research on the impacts of environmental chemicals on various epigenetic factors using a variety of model systems [9,10]. The majority of the studies were conducted using mammalian models and to a lesser extent in non-mammalian models [11–13]. *Agouti* mouse is one of the well known model systems used to study epigenetic mechanisms of toxicant action [14]. Even though research in mammalian models can be easily translated to humans, conducting *in vivo* studies on a rapidly growing list of chemicals is time consuming and not cost-effective. In addition, studying the mechanisms of developmental reprogramming in embryos during *in utero* development is difficult. Hence, it is increasingly recognized that alternate vertebrate model systems could provide unique advantages in accelerating research in screening toxicants as well as understanding the mechanisms of action. One such model is zebrafish (*Danio rerio*), an established model in toxicology [15], developmental biology and human disease research [16]. More recently, it has been widely used as a model system for DOHaD studies and for understanding the underlying genetic and epigenetic mechanisms of action [17]. This review summarizes our current knowledge on the epigenetic effects of toxicants using zebrafish as a model organism and highlights the challenges and opportunities zebrafish offers for investigating the epigenetic mechanisms of action. Studies conducted so far have mostly focused on the impact of toxicants on the epigenetic machinery and very little is known about the mechanisms by which toxicants alter the epigenetic patterns. As zebrafish are increasingly used as an alternative model for DOHaD studies, this review summarizes the important factors to consider while conducting studies to characterize the epigenetic basis of DOHaD effects.

Zebrafish as a model for DOHaD and epigenetic toxicology

Zebrafish have become an attractive model for DOHaD and transgenerational studies because of high fecundity, short generation time (embryo to adult in 3–4 months), external

fertilization and development, and easy maintenance and breeding [16](Figure 1). In contrast to murine models where embryonic development occurs *in utero*, in zebrafish it occurs externally. This enables exposure of embryos to stressors immediately after fertilization (2–4 cell stage), in the absence of any maternal influence. Transparent zebrafish embryos allow visualization of any developmental abnormalities associated with exposure. Zebrafish are highly fecund and each female can lay hundreds of eggs at a time. This makes it possible to have relatively high sample size for each experimental condition. There are a number of larval and adult behavioral assays developed to assess the later life effects of developmental exposure to toxicants [18]. Compared to rodent models, rearing and maintenance costs for zebrafish are inexpensive. This is an important consideration for DOHaD and transgenerational studies, because costs associated with raising multiple animals from each treatment condition over a long time period, sometimes over multiple generations, can be expensive. Furthermore, in mammals transgenerational transmission of a phenotype requires assessment of the F_3 generation for embryonic exposure because primordial germ cells of the F_2 generation are exposed in pregnant dams [19]. In contrast, due to external development in zebrafish, studies in the F_2 generation are considered to be transgenerational [17].

In addition to these advantages, zebrafish are also ideal for studying the epigenetic mechanisms of action. The availability of numerous transgenic fish strains enables characterization of cell and tissue-specific effects. Zebrafish are also amenable for genetic manipulation, and targeted gene-editing with CRISPR-Cas9 is widely used [20]. The availability of genomic resources [21] and the sequencing methods needed to conduct transcriptomic and epigenomic profiling have garnered enormous attention in the use of zebrafish as a model species in DOHaD studies. In the past few years, there have been several studies characterizing the developmental profiles of DNA methylation [22,23], histone modifications [24] and noncoding RNAs [25–27] providing base line information on the dynamics of epigenetic regulation during embryogenesis. Several studies have demonstrated the long-term effects of developmental exposure to toxicants. Most of these studies have reported later-life effects and in some cases intergenerational or transgenerational effects (Table 1). However, studies aimed at understanding the mechanisms behind DOHaD and multigenerational studies are still in their infancy.

Despite the unique advantages zebrafish offer, there are some distinct differences between zebrafish and mammals in epigenetic programming. Mammals undergo two rounds of reprogramming of DNA methylation, first at the time of fertilization in the zygote, and then in primordial germ cells (PGCs). In zebrafish the second wave of reprogramming has not yet been demonstrated. In addition, the methylomes of sperm and oocytes are significantly different and the paternal genome is resistant to demethylation in zebrafish [22,23]. Furthermore, zebrafish do not have genomic imprinting making them unsuitable for studying parent-of-origin effects.

DNA methylation

Similar to mammals, DNA methylation is one of the most well studied epigenetic modifications in zebrafish. Methylation of cytosine residues in CpG islands are generally

considered to cause stable silencing of gene expression. Recently, Long et al; [28] empirically demonstrated that CpG islands in gene promoters are conserved among all vertebrates, including zebrafish. DNA methyltransferases (DNMTs) are responsible for the addition of the methyl groups on CpG dinucleotides. Zebrafish possess orthologs of both maintenance and *de novo* DNMTs [29]. Maintenance DNA methyltransferase, *dnmtl*, ensures inheritance of methylation patterns during cell division by preferentially methylating hemimethylated CG dinucleotides, whereas *de novo* methyltransferases are involved in establishing new methylation patterns. In contrast to two *de novo* DNMTs (DNMT3A and DNMT3B) in mammals, zebrafish possess multiple homologs. These include two DNMT3A (*dnmt3aa* and *dnmt3ab*) and four DNMT3B (*dnmt3ba, 3bb1, 3bb2* and *3bb3*) genes[30]. In general, due to genome duplication in the fish lineage, there are two or more orthologues of many mammalian genes. This often leads to subfunction partitioning among the duplicated genes, providing a unique opportunity for obtaining new mechanistic insights into the multiple functions of a single human gene [31]. For example, DNMT3A knockout mice die postnatally at 4–8 weeks and DNMT3B knockouts die embryonically at 14.5 days making it difficult to study their roles beyond early embryonic development [32]. In contrast, we observed that *dnmt3aa* and *dnmt3ab* (DNMT3A homologs) knockout zebrafish generated using TALEN technology develop normally, making it possible to study their roles beyond development. Previous studies using morpholino oligonucleotide knock down approach and TALEN knockouts have demonstrated that proper expression of DNMT3 genes is critical for cellular and tissue differentiation such as hematopoiesis [33]. The results from these studies suggest that toxicants affecting the expression of DNMTs could have long-term consequences by altering DNA methylation. Several studies have investigated the effect of environmental chemicals on DNMT gene expression in zebrafish exposed during early development (Table 2). These studies have shown that altered DNMT gene expression patterns are developmental stage-specific, but whether these changes alter genome wide DNA methylation remains to be determined. So far most of these studies have used gene-specific DNA methylation profiling targeting early developmental genes or those targeting specific pathways and determined alterations to DNA methylation at specific time points [11,34]. Even though these results demonstrate the fact that altered DNMT expression could affect DNA methylation patterns, the prevalence of these changes genomewide are just beginning to be understood. In addition, the persistence of DNA methylation changes observed after developmental exposure remains to be determined. With the availability of high throughput bisulfite sequencing methods, it is feasible to investigate genomewide changes in differential methylation and persistence of these effects over developmental time post-exposure. One recent study demonstrated genomewide transgenerational changes in DNA methylation following developmental exposure to mono (2-ethylhexyl) phthalate in zebrafish [35]. Similar studies needs to be conducted with other toxicants to determine the role of DNA methylation in toxicant-induced phenotypes.

Non-coding RNAs

The importance of non-protein coding RNAs (ncRNAs) in the regulation of various biological processes in eukaryotes is well documented. The number of known ncRNAs continues to grow and the most widely known regulatory ncRNAs include microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-associated RNAs (piRNAs) and long

noncoding RNAs (lncRNAs). Zebrafish is widely used as a model to study developmental roles of ncRNAs and to determine the mechanisms of regulation of gene expression [26,27,36]. MiRNAs are the most widely studied group in developmental toxicology and a variety of environmental chemicals are shown to affect their expression. However, most of these studies in zebrafish have determined the expression patterns after acute developmental exposure to toxicants; very few studies have characterized the role of individual miRNAs in toxicant-induced phenotypic changes later in life [37–39]. As epigenetic changes are considered to mediate transgenerational effects, it is essential to determine persistent changes that are transmitted to subsequent generations. Recently, a few studies in mice have demonstrated that sperm miRNAs are involved in the transgenerational transmission of stress-induced phenotypes[40,41]. One recent study reported that prenatal exposure of mice to vinclozolin caused an upregulation of miRNA-23b and let-7 in the primordial germ cells of developing embryos [42]. These changes were shown to persist in three subsequent generations. Similar mechanistic studies with other chemicals are needed in order to conclusively demonstrate that miRNAs are a mediator of transgenerational effects. Another group of ncRNAs that are expressed in germ cells and could potentially play a role in transgenerational effects are piRNAs [43]. They originate from the intergenic repetitive elements in the genome and associate with the PIWI subfamily members of the Argonaute family of proteins [43]. Their role is to repress transposable elements in the germline and maintain genomic integrity [44]. The biogenesis of piRNAs in various model systems [45,46], including zebrafish is well known [47–49], but there are no studies on the effects of toxicants on piRNA expression. Recent studies in *D. virilis* and *C. elegans* have demonstrated that piRNA-mediated stable long-term gene silencing as a potential mechanism of transgenerational effects [50,51]. However, the hypothesis that ncRNAs act as a mediator of DOHaD and multigenerational effects needs further experimentation.

Histone modifications

Histone modifications play an important role in the regulation of chromatin structure. There is a growing list of these modifications and they exert their effects either by directly influencing the overall chromatin structure or by regulating the binding of effector molecules [52]. For example, methylation of histones can activate (e.g., histone H3 lysine4 trimethylation; H3K4me3) or repress (e.g., histone H3 lysine 27 trimethylation; H3K27me3) gene expression. These histone modifications can alter the chromatin accessibility, thereby generating binding sites for RNA polymerase II or other epigenetic modifiers such as DNMTs and presumably fine-tuning the regulation of gene expression [52]. Similar to the effects on DNA methylation, environmental chemicals that alter the availability of methyl donors can disrupt histone modifications. In addition, histone and DNA demethylases are also shown to impact histone modifications [53]. For example, histone demethylases belonging to Jumonji family are affected by exposure to metals. This is hypothesized to be due to the displacement of iron (Fe), an essential cofactor in the catalytic activity of DNA and histone demethylases by heavy metals [53]. There is some evidence suggesting that altered histone modifications can have persistent effects on gene expression [54,55]. Similar studies are lacking in zebrafish despite considerable progress in characterizing the role of histone modifications during early development and in disease states.

Using genomewide approaches, recent studies have documented the patterns of histone modifications during zygotic genome activation (ZGA), a period of development characterized by major remodeling of chromatin [56]. These results suggest that the ZGA is accompanied by major changes in the patterns of zygotic histone methylation. Based on the evidence that environmental chemical exposures during ZGA affect gene expression patterns, it is conceivable that some of these changes are associated with altered histone modifications. In the past few years there has been significant progress in the development of genomewide profiling methods for analyzing histone modifications in zebrafish [56]. Using these approaches to investigate the effect of environmental chemicals on histone modifications during early embryonic development and their persistence in later life stages is an exciting avenue of research. Furthermore, there is considerable crosstalk between different epigenetic factors. Conducting integrative analysis of gene expression, DNA methylation and histone modifications will be extremely useful to capture the influence of different epigenetic factors on gene expression and the phenotypes.

Considerations for conducting DOHaD studies

Zebrafish have become a popular vertebrate model for studying the long-term implications of exposure to toxicants during sensitive windows of development. Several studies have demonstrated morphological and behavioral phenotypes, but the experiments characterizing the underlying mechanisms are still lacking. Recently, Yamada and Chong [57] described strategies while designing experiments to investigate epigenetic mechanisms of action in DOHaD studies. Some of the factors to consider include the dynamic nature of the epigenome, the relationship between the epigenetic changes and gene expression patterns, cell and tissue-specific differences in the epigenome and the influence of the genome on the epigenetic landscape.

Similar to gene expression patterns, epigenetic changes are dynamic and differ with age. Studies in humans and rodent models have demonstrated that global DNA methylation declines with age [58]. As DOHaD studies investigate the effects a long time after the exposure, it is possible that the environmental factors during the rearing period can influence epigenetic changes. So far most DOHaD studies have investigated epigenetic changes at one time point, often weeks or months after the initial exposure. In order to determine the stable and persistent nature of epigenetic changes, future studies should consider measuring epigenetic changes at multiple time points.

It is also becoming increasingly clear from genomewide profiling studies that the relationship between gene expression and methylation patterns can be ambiguous. Recent studies using genomewide profiling of DNA methylation have shown that the inverse relationship between gene expression and DNA methylation in the promoter regions is not always true [59]. In addition, DNA methylation changes are seen in the intergenic regions far away from any known genes and the functional significance of these changes is yet to be determined.

Most of the DOHaD studies in zebrafish have used gene-specific methods such as quantitative PCR and bisulfite conversion PCR or pyrosequencing for measuring gene expression and DNA methylation, respectively. As the sequencing costs continue to

decrease, it is cost-effective to use RNAseq and RRBS (Reduced Representation Bisulfite Sequencing) methods to assess genomewide changes and identify the potential mechanisms. In addition to DNA methylation, other chromatin modifiers such as histones are important players in the regulation of gene expression. Furthermore, the overall chromatin structure is regulated at multiple levels. Determining the nucleosome positioning is a useful approach to initially identify the open and closed chromatin states before conducting targeted analysis of DNA methylation or specific histone modification. Recent development of methods such as Assay for Transposase Accessible Chromatin sequencing (ATAC-seq) have made it less labor intensive to identify regions of open and closed chromatin states, in comparison to Micrococcal Nuclease digestion followed by sequencing (MNase-seq) or DNase hypersensitivity assays [60].

Another important consideration in DOHaD studies is the tissue-specific analysis of epigenetic effects of toxicants. It is well established that despite having the same genome, each cell and tissue type have unique epigenomic patterns. Hence, the effects of toxicant exposure on epigenetic machinery may not be uniform across all tissues and cell types. For example, prenatal bisphenol A exposure in BALB/c mice caused hypermethylation in *estrogen receptor 1* gene in the prefrontal cortex, but not in the hypothalamus in male offspring [61]. Therefore, it is important to consider investigating the tissue-specific differences in epigenetic patterns. This may not be possible while working with samples containing heterogenous cell types, such as developing embryos because it is difficult to assign the cellular origin to the sequencing data. However, this problem can be overcome by generating transgenic strains expressing fluorescent proteins in any specific cell type and conducting FACS prior to sequencing. This is particularly feasible in zebrafish where tissue-specific transgenic strains are readily available and methods for FACS are established.

Finally, the most important and poorly understood factor is the role of genetic variation on epigenetic responses. There are widespread associations between single nucleotide polymorphisms (SNPs), the most common source of genetic variation, and DNA methylation in humans [62], but the consequences are poorly understood. It is recently demonstrated that SNPs at CpG sites can impact transcription factor binding [63]. The other well known cause of genomic variation is polymorphisms in the genes involved in the regulation of epigenetic machinery. The effect of genomic variation can be minimized by using inbred strains in DOHaD studies. Even though zebrafish has been used as a laboratory model organism for several decades, the degree of genetic variation among different strains is still quite high [64]. The development of inbred zebrafish lines is necessary in order to reduce confounding effects of genetic variability not only on the epigenome but also on other physiological traits [65].

Conclusions and Future directions

So far most of the DOHaD research using zebrafish as a model system has been focused on identifying the latent effects of developmental exposures. Based on the emerging evidence from mammalian model systems, there is growing recognition that the latent effects of early life exposure could be due to the altered epigenome. In the last few years, there has been a sudden spurt in the number of studies characterizing developmental profiles of various

epigenetic factors in zebrafish. These studies suggest that the majority of the epigenetic machinery is highly conserved among vertebrates. In addition, the progress in the development of sequencing techniques and the availability of open source bioinformatic analysis software have made it possible to quantify genomewide epigenetic changes in zebrafish. The next steps in characterizing the epigenetic mechanisms associated with DOHaD include designing experiments that will allow us to identify the developmental basis of persistent changes, and functionally characterizing the genes using forward and/or reverse genetic approaches. Genome editing and transgenesis tools are well established and straightforward, providing unique opportunities to conduct physiological as well as functional studies in an *in vivo* model system.

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Highlights

- Epigenetic mechanisms are highly conserved among all vertebrates.
- Zebrafish is an ideal *in vivo* model for investigating epigenetic mechanisms associated with the developmental origins of adult health and disease.
- Among key considerations in conducting DOHaD studies are examination of multiple time-points and tissues, as well as integration of gene expression and methylation analyses.

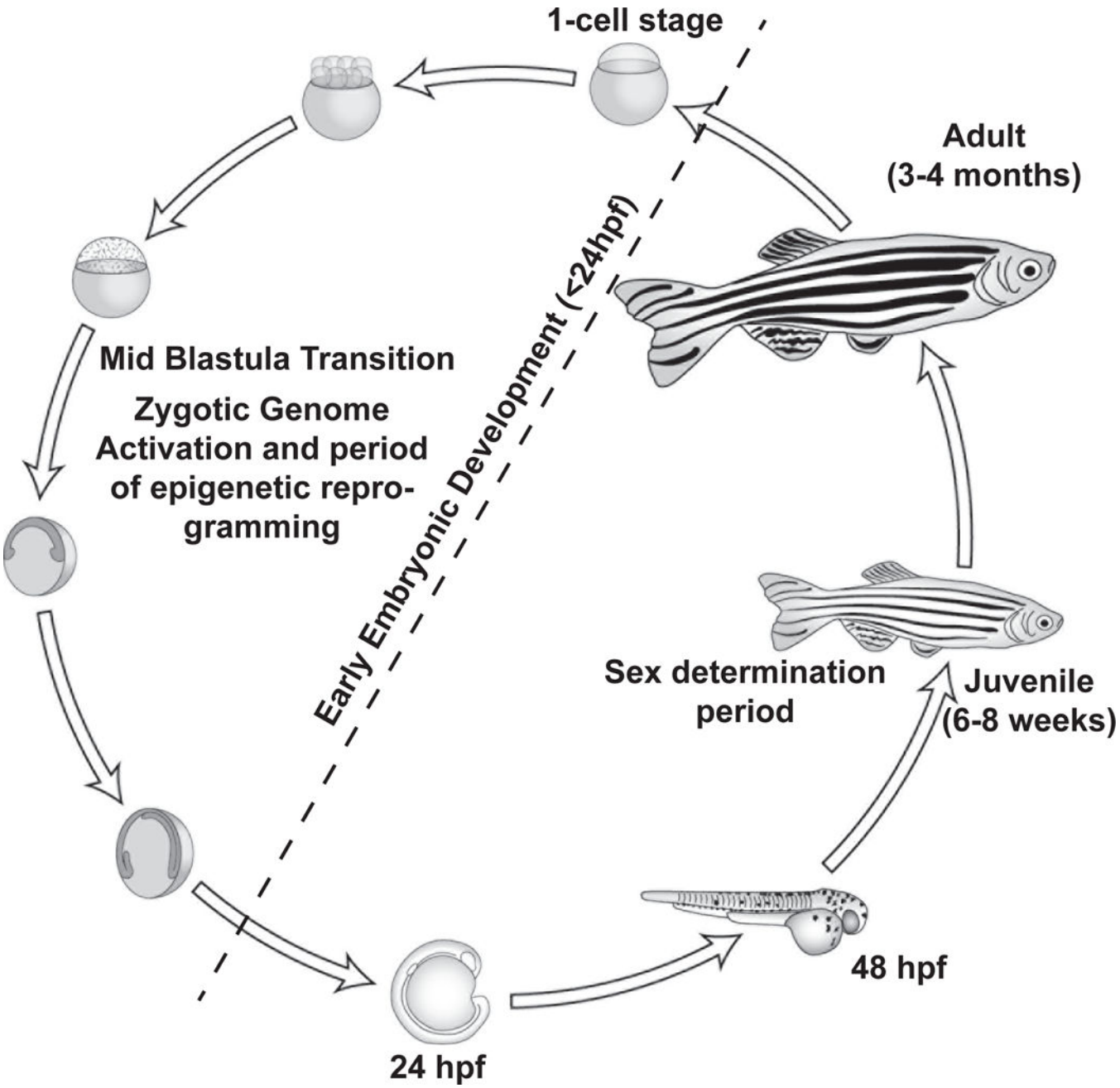

Figure 1.

Zebrafish is an ideal model for DOHaD studies. Because of its short life cycle and rapid development they are ideal for conducting long-term studies including multi- and transgenerational studies. Similar to mammals, zebrafish undergo zygotic genome activation as well as epigenetic reprogramming. Exposure to environmental chemicals during sensitive windows of development can have later life consequences.

Table 1

List of published studies investigating the long-term effects of developmental exposure to toxicants in zebrafish.

Chemical	Exposure	Effects	Reference
TCDD	Developmental and juvenile exposure	Decrease in spermatozoa and germinal epithelium thickness; Increase in spermatogonia	[66]
TCDD	Developmental and juvenile exposure	Increased female to male ratio in all three generations. Scoliosis like phenotype, reduced egg production and fertilization success in F1 and F2 generations.	[17]
TCDD	Developmental exposure	Egg production and fertilization success were reduced Increased mortality of F1 embryos, Reduced egg production and fertility	[67]
Bisphenol A	Adult exposure	Heart defects in F1 and F2 generation	[68]
PCB126	Developmental exposure	Altered adult behavior (lack of habituation) Altered gene expression in the brain	[69] [70]
PAH mixture	Chronic dietary exposure	Altered locomotory activity in F1 and F2 larvae	[71]
Testosterone and Dihydrotestosterone	Developmental and juvenile exposure	Global hypomethylation in the ovary (F0) and in F1 larvae. Altered glucose homeostasis	[72]
Atrazine	Developmental exposure	Reduction in 5-hydroxyindoleacetic acid (5-HIAA) levels and serotonin turnover. Reproductive dysfunction in adults	[71] [73,74]
Cadmium	Developmental exposure	Adults displayed anxiety like behavior in novel tank assay Altered antioxidant levels	[75]
Vinclozolin	Juvenile exposure	Shift in sex ratios towards females, and affected gonadal maturation.	[76]
PFOS	Chronic exposure	Effects on adult behavior (F0) and larval survival, morphology and behavior in F1 generation.	[77]
Organophosphate flame retardants	Developmental exposure	Impaired larval and adult behavior	[78]

Table 2

Summary of published studies in zebrafish demonstrating the effects of toxicants on epigenetic mechanisms.

Chemical	Epigenetic alteration	Developmental stage	References
Estrogen	Aromatase gene promoter DNA methylation	Adults brain and liver	[79]
TCDD	DNMT expression Gene-specific DNA methylation	Embryos	[34]
BaP	DNMT expression Gene-specific DNA methylation	Embryos	[11,80]
Depleted Uranium	Global DNA methylation	Adults	[81,82]
TDCPP	Global DNA methylation	Embryos	[83]
Lead	Global DNA methylation	Embryos	[84]
TCDD	miRA-451, 23a, 23b, 24, 27e	Embryos	[85]
Ethanol	miR-9/9*, 153c	Embryos	[86]
Ethanol	miR-153a, miR-725, miR-30d, let-7k, miR-100, miR-738, and miR-732	Embryos	[87]
Ethanol	miR-9	Embryos	[39]
Valproic acid	miR-16a, 18c, 122, 132, 457b, and 724	Embryos	[88]