

REVIEW

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# Environmental epigenetics in zebrafish

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## Abstract

It is widely accepted that the epigenome can act as the link between environmental cues, both external and internal, to the organism and phenotype by converting the environmental stimuli to phenotypic responses through changes in gene transcription outcomes. Environmental stress endured by individual organisms can also enforce epigenetic variations in offspring that had never experienced it directly, which is termed transgenerational inheritance. To date, research in the environmental epigenetics discipline has used a wide range of both model and non-model organisms to elucidate the various epigenetic mechanisms underlying the adaptive response to environmental stimuli. In this review, we discuss the advantages of the zebrafish model for studying how environmental toxicant exposures affect the regulation of epigenetic processes, especially DNA methylation, which is the best-studied epigenetic mechanism. We include several very recent studies describing the state-of-the-art knowledge on this topic in zebrafish, together with key concepts in the function of DNA methylation during vertebrate embryogenesis.

**Keywords:** Environmental epigenetics, Zebrafish, DNA methylation, Methylome, Histone modifications, Embryogenesis, Toxicant, Transgenerational inheritance

## Background

Research in the field of environmental epigenetics focuses on how gene regulatory mechanisms operate on chromatin, in the absence of changes in the genome sequence, during adaptive responses to external stimuli [1–3]. The main epigenetic mechanisms include DNA methylation, histone post-translational modifications, and replacement of canonical histones by specialized histone variants, nucleosome density, three-dimensional chromatin organization, noncoding RNAs, and transcription factor regulatory networks [4–11]. Close interlinking among all these mechanisms establishes the so-called epigenotype displayed by a given cell/organism within a given environment. The dynamic nature of such a finely tuned epigenetic equilibrium implies that the epigenotype fluctuates rather rapidly in response to external stimuli, potentially allowing gradual adaption of genome transcriptional outputs and phenotype variation [12, 13]. On the other hand, especially in the case of the germline

and stem cells, some particular epigenetic patterns may persist in the chromatin across generations, constituting the basis for long-term adaption [14, 15].

A growing body of evidence shows that there are critical time windows during embryogenesis and primordial germ cells specification in which the epigenome is extremely sensitive to environmental cues, which can therefore modify the epigenetic information both within developing individuals and across generations [16, 17].

Because of the variability in reproductive and developmental processes, and their response to environmental stress, a wide variety of model and non-model organisms have been employed to study both the individual and transgenerational inheritance of epigenetic information [18–22].

The focal point of this review is the use of zebrafish to evaluate DNA CpG methylation, which is the best-studied epigenetic mechanism among those that can covalently modify DNA. It consists in the enzymatic transfer of a methyl group from the *S*-adenosyl-methionine donor to the 5th carbon position of a cytosine pyrimidine ring [23]. Accumulation of 5-methyl cytosines mostly occurs in the so-called CpG islands, which are genomic regions with densely clustered CG dinucleotides. Control of gene expression can be affected by context-dependent changes

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in CpG methylation. In particular, while CpG methylation at promoters generally causes stable transcriptional gene silencing [4], high levels of CpG methylation within the gene body are associated with highly expressed genes [24]. CpG methylation also plays fundamental roles in genomic imprinting [25] and X-chromosome inactivation [26].

Various organisms contain cytosine methylation in CpA, CpT, and CpC dinucleotides, which are collectively referred to as non-CpG methylation [27–29]. The overwhelming majority of non-CpG methylated sites, primarily CpA dinucleotides, are enriched in brain tissue and pluripotent cells compared to other differentiated cell types [24, 28, 29]. However, the functional significance of this occurrence is poorly understood in the vertebrate genome, and it will not be covered in this review.

DNA methylation patterns are primarily imposed by de novo DNA methyltransferases (Dnmts), and then, they are semi-conservatively transferred onto the newly synthesized DNA strand after each cell division by a maintenance Dnmt [23, 30, 31]. Reversion of DNA methylation, especially during embryogenesis, is thought to be obtained by a passive replication-dependent mechanism involving the inhibition of Dnmts [32–34]. Alternatively, a multistep process embracing both ten-eleven translocation (Tet) proteins and the DNA repair machinery mediates active demethylation [34–37]. Due to the combination of all of these events, DNA methylation patterns are highly dynamic throughout embryonic development, particularly during epigenetic reprogramming, in which the bulk of paternal and maternal epigenetic asymmetries become harmonized into the zygotic genome [38–42].

### The zebrafish model

Zebrafish (*Danio rerio*) are small tropical freshwater fish native to the inland water bodies of the Himalayan region [43]. These vertebrate organisms are small, the adults being about 2–3 cm in length, and can be maintained relatively cheaply in laboratory. They have a short life cycle and generation time, as well as high fecundity, single female individuals being capable of generating hundreds of eggs per week [44]. Altogether, these features allow rapidity and high statistical power for downstream experimental procedures.

The directly developing embryos grow outside the maternal fish, elaborating the general body plan and organ systems within 48 h from fertilization [45]. Furthermore, zebrafish embryos appear quite translucent to microscopic observation, facilitating noninvasive live imaging of morphogenetic processes at a single-cell level in the context of the whole organism [46, 47].

Importantly, zebrafish embryos are relatively permeable to water-soluble molecules, being ideally suited for

drug discovery and monitoring of pollutants [48]. The typical experimental strategy consists of large-scale pharmacological/toxicological screenings based on exposure to chemical compounds followed by high-throughput molecular studies. As discussed below, the zebrafish has recently been proved to be a premier model to explore changes in the epigenetic state, especially DNA methylation, following exposure to several environmental stressors.

It is worth mentioning that it is estimated that the zebrafish genome has approximately 70% homology to human genes [49] and that ~99% of embryonic-essential fish genes are homologs in human embryonic development [50]. Last but not least, the main epigenetic mechanisms and events, especially those occurring during germ cell programming, are common to zebrafish and mice [51, 52]. Undoubtedly, these aspects are of fundamental importance in allowing researchers to extrapolate results to other vertebrates, including humans.

### DNA methylation in zebrafish

DNA methylation machinery and mechanisms in zebrafish are generally conserved with those of mammals [53–56], with the significant exception that fish do not require imprinting of genes or sex chromosomes for viability [30, 57]. This feature provides a simplified system for exploring methylome dynamics in response to environmental challenges during the so-called epigenetic reprogramming process, which includes the establishment of DNA methylation patterns during vertebrate embryogenesis [58].

Measurements of overall DNA methylation at different developmental time points during zebrafish development revealed that over 80% of CpGs are methylated, and modest gain from this high baseline occurs as the embryo progresses toward gastrulation [51, 52, 59–62]. Interestingly, the paternal DNA methylation pattern is maintained throughout early embryogenesis, while the hypomethylated maternal DNA is reprogrammed to a pattern similar to that of the sperm [51, 52]. Moreover, the overall DNA methylation level in zebrafish is higher than those of endothermic animals, probably due to a lower deamination rate of methylated cytosine to thymine [53, 63].

Zebrafish possess multiple *dnmt* genes representing the homologs of mammalian maintenance *dnmt1* [64], and de novo *dnmt3a* (*dnmt3a1* and 2) and *dnmt3b* (*dnmt3b1*, 2, 3, and 4), which arose following the genome duplication event characterizing the teleost fish lineage, as well as tandem gene duplications [65, 66]. Zebrafish also contain the three Tet family proteins shared in vertebrates, but fish embryos do not express them during early development [67].

Nonetheless, a recent study has shown that a massive wave of Tet-dependent DNA demethylation begins at about 24 h post-fertilization, temporally encompassing the so-called phylotypic stage [68], which is the period in which developing embryos of species in the same phylum display maximal similarity. Strikingly, such an epigenome reconfiguration is evolutionary conserved across zebrafish, *Xenopus*, and mouse [68], suggesting that DNA demethylation could be a preeminent epigenetic mechanism accounting for co-regulation of key developmental genes in multiple vertebrate species. Accordingly, the chromatin contexts exhibiting DNA hypomethylation contain thousands of enhancers embedded into gene regulatory networks controlling body plan and organ formation [68].

### Impact of environmental compounds on DNA methylation during zebrafish embryogenesis

Some recent studies have primarily focused on global DNA methylation changes and phenotypic alterations triggered by environmental pollutants during epigenetic reprogramming of zebrafish embryos. An overview of the studies examined in this review is shown in Table 1.

One of these studies reported that the overall DNA methylation level of developing embryos continuously exposed to benzo[*a*]pyrene, a well-known carcinogen and epigenetic modifier [69–71], was about half of that of control untreated embryos [62]. Consistently, significant loss of methylation in the promoter region, as well as concomitant increase in mRNA transcription, was specifically detected for the *vasa* gene [62]. Because *vasa* is required for differentiation and migration of primordial germ cells [72–74], the authors claimed that irregular epigenetic modulation of *vasa* gene expression could in turn arouse reproductive toxicity.

In a more recent study, the established relationship between fetal androgen exposure and reproductive defects in animal models [75, 76] inspired exploration of global DNA methylation in ovaries of adult zebrafish antecedently exposed to testosterone or dihydrotestosterone during embryogenesis [77]. Interestingly, the authors observed a biphasic dose response in the methylome of androgenized zebrafish, with an inverse relationship between global methylation status and androgen dose exposure. This finding is in accordance with evidence reported by similar studies in other organisms [78, 79],

**Table 1 Overview of studies examining the epigenetic effects in zebrafish embryos exposed to several compounds**

Compound	Epigenetic effect	References
Benzo[ <i>a</i> ]pyrene	Global and gene-specific hypomethylation Upregulation of <i>dnmt3b2</i> Downregulation of <i>dnmt1</i> and <i>dnmt3a2</i> Stimulation of Gmmt activity	[62, 99]
Androgens	Global hypomethylation	[77]
Arsenic	Differential spatiality-specific global methylation	[84]
Estrogens	Gene-specific hypomethylation: <i>vasa</i>	[85]
Nickel, cadmium	Gene-specific hypermethylation: <i>vasa</i>	[85]
Bisphenol-A	Gene-specific alterations of DNA methylation Downregulation of <i>dnmt1</i> , <i>dnmt3b3</i> , <i>dnmt3b4</i> <sup>a</sup>	[85, 122, 123]
Perfluorooctanoic acid	Gene-specific alterations of DNA methylation	[85]
S-(+) fipronil	Global and gene-specific hypermethylation	[90]
TCDD	Gene-specific hypomethylation: <i>cfos</i> Gene-Specific hypermethylation: <i>ahrra</i> Upregulation of <i>dnmt1</i> and <i>dnmt3b2</i> Downregulation of <i>dnmt3a1</i> , <i>dnmt3b1</i> and <i>dnmt3b4</i>	[85, 92, 93]
Lead	Overall DNA hypomethylation Inhibition of Dnmt1 activity Downregulation of <i>dnmt3b1</i> and <i>dnmt3b3</i>	[98]
Heat stress/copper	Upregulation of <i>dnmt3</i> genes	[103]
Methylmercury	Differential methylation of noncoding DNA	[92]
MEHP, 5-azacytidine	Upregulation of <i>dnmt1</i> , <i>dnmt3b1</i> and <i>dnmt3b2</i> Downregulation of <i>dnmt3a1</i> and <i>dnmt3a2</i> Overall hypomethylation <sup>b</sup>	[109]
Ethanol	Upregulation of specific miRNAs	[111]
Perfluorooctane sulfonate	Differential alterations of miRNAs abundance	[113]
DZNep	Gene-specific depletion of H3K27me3 and H3K9me3	[114]

<sup>a</sup> Transgenerational effect; <sup>b</sup> in F0 liver of female fish

and it has been explained by downregulation of androgen receptors at higher exposure levels or adaptive responses through complex signaling pathways.

Another pertinent example refers to exposure of zebrafish embryos to arsenic, an environmental contaminant known to have adverse effects on human health by causing a series of cancers and cardiovascular and neurological diseases [80–83]. Consistent with this, when used at a concentration of 2.0 mM, sodium arsenite inflicted severe malformations of neural and cardiac structures in developing zebrafish and provoked substantial changes in the genomic DNA methylation pattern throughout the embryonic body [84]. By means of fluorescent immunostaining of 5-methylcytidine, the authors determined that, when compared to control unperturbed embryos, arsenic-treated embryos displayed abnormal hypomethylation in the trunk and tail at early developmental stages. This trend was overturned during the remaining phases of development and aberrant hypermethylation was detected across the whole embryo body, especially in the tail [84]. Notably, this information highlights the versatility of the zebrafish model for inspecting changes in the overall DNA methylation pattern among distinct spatial sectors of a whole organism.

Paradoxically, however, the global DNA methylation level could not be an informative epigenetic marker, being the epigenetic effects driven by site-specific changes that may be obscured on a global scale, as highlighted by several studies. Among these, Bouwmeester et al. [85] performed a systematic screening and exposing of fish embryos to subtoxic concentrations of a range of environmentally relevant xenobiotics of known epigenetic effects, which potentially play a role in developmental origins of adult diseases. The authors found that the bulk genomic methylation level did not vary in embryos exposed to any of the test compounds. Nevertheless, pyrosequencing analysis of methylation in the promoter of selected informative target genes displayed significant differences between control and exposed embryos [85]. For instance, the estrogenic compounds diethylstilbestrol and 17 $\alpha$ -ethynylestradiol induced reproducible hypomethylation in the CpG island of the germline-specific marker *vasa*, while the metals Ni and Cd both induced hypermethylation in the same genomic region. It is worth mentioning that a subset of the tested compounds, which includes bisphenol-A and perfluorooctanoic acid, specifically affected site-specific DNA methylation at concentrations unable to inflict overt adverse phenotypes. Altogether, these findings not only reaffirm the applicability of the zebrafish embryo as a valuable screening model for epigenetic modifications after xenobiotic exposure, but also suggest that in these assays opposed locus-specific methylation changes could balance each other,

not being reproduced on the global genome-scale methylation level.

Identification of changes in gene-specific methylation represents a fundamental issue in the emerging field of enantioselective environmental epigenetics. In this connection, several pollutants contain a chiral structure consisting of enantiomers that, despite having identical physical–chemical properties, selectively impinge on biological mechanisms [86–89]. To date, only a single report has shed new light on the toxicity of chiral compounds from the perspective of enantioselective epigenetic regulation in a developing organism, and zebrafish was the model successfully used [90]. In this study, the authors focused on the modification of DNA methylation induced by the R(–) and S(+) enantiomers of fipronil, a *m*-phenylpyrazole insecticide [91]. They found that the S(+) fipronil exerted significantly greater developmental toxicity compared to the R(–) enantiomer, resulting in a massive increase in both global and gene-specific DNA methylation [90]. In this analysis, no fewer than 22 molecular pathways each containing more than five hypermethylated genes were identified by the KEGG database, and seven of these pathways were strictly associated with pivotal developmental processes [90]. As expected, five out of seven randomly selected genes containing hypermethylated promoters were confirmed to be transcriptionally downregulated to a greater extent by S(+) fipronil, rather than R(–) fipronil, exposure.

### Environmental effects mediated by Dnmts

A few recent studies have suggested that pollutant exposure could induce alteration in DNA methylation patterns by disturbing *dnmt* gene expression during zebrafish embryogenesis. Among these, a couple of reports described the impact of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on DNA methylation of embryos, larvae, and adult zebrafish [92, 93]. TCDD is a halogenated polycyclic hydrocarbon acting as a ligand for the aryl hydrocarbon receptor (AHR) transcription factor, which plays a role in mediating the toxic developmental effects of TCDD in zebrafish. Indeed, binding of TCDD allows nuclear translocation and recruitment of AHR to xenobiotic response elements in the promoter regions of a variety of target genes [94–97].

In accordance with findings from other groups [85], both the studies mentioned concordantly highlighted that, although TCDD did not affect the overall amount of 5-methylcytosine during development, specific methylation of the CpG islands in the promoter of AHR target genes was either unchanged or differentially affected. For instance, hypomethylation was observed in 11 out of 22 CG dinucleotides within the *cfos* promoter, while 14 out of 34 CG sites were hypermethylated in the *ahrra* promoter [93].

Interestingly, these alterations have been supposed to be dependent upon TCDD-induced deregulation of *dnmt* gene expression. Indeed, TCDD exposure during early embryogenesis provoked developmental stage-specific upregulation of *dnmt1* and *dnmt3b2*, coupled to downregulation of *dnmt3a1*, *dnmt3b1*, and *dnmt3b4* [93]. The specificity of these effects is further supported by the observation that expression of *dnmt3a2* and *dnmt3b3* was not affected by TCDD treatment [93]. These findings strongly suggest that TCDD could impact both establishment and maintenance of DNA methylation patterns of genomic loci not necessarily restricted to AHR targets.

Another relevant study, focusing on the epigenetic effect evoked by lead (Pb), also advocated a direct relationship between changes in Dnmt activity/expression and the DNA methylation level of zebrafish embryos [98]. The authors first determined that Pb exposure modulates the activity of the maintenance Dnmt enzyme via non-competitive inhibition, *in vitro*. They also described the alteration of expression patterns of the *de novo* Dnmt enzymes during development of zebrafish Pb-exposed embryos, which in turn displayed overall DNA hypomethylation [98].

By contrast, divergent findings come from other studies indicating that gene expression and activity of the various Dnmts are not affected by exposure to environmental pollutants. For example, treatment of developing zebrafish embryos with benzo[*a*]pyrene, a potent DNA-hypomethylating compound, did not alter either transcriptional or enzymatic activity of Dnmts [62]. However, it should be emphasized that the authors measured the global activities from all the Dnmt isozymes in nuclear extracts derived from benzo[*a*]pyrene-exposed embryos, so that potential compensative changes among the activity of individual Dnmts cannot be excluded. Indeed, a more recent study confirmed that the mRNA abundance of the various *dnmts* was differentially altered in benzo[*a*]pyrene-treated zebrafish embryos at 24 h post-fertilization [99]. In particular, while the transcript levels of *dnmt3b2* were elevated, those of *dnmt1* and *dnmt3a2* were significantly reduced, and those of *dnmta1* and *dnmtb1* were not affected [99].

Beyond this, Fang et al. [62] also noted that benzo[*a*]pyrene exposure substantially stimulated activity, but not gene transcription, of the glycine *N*-methyltransferase (Gnmt) enzyme. Gnmt is probably the most important enzyme regulating the metabolic transmethylation flux in animal organisms, where it catalyzes the transfer of a methyl group from *S*-adenosyl-methionine (SAM) to glycine-forming *S*-adenosyl-homocysteine [100]. Interestingly, there is a functional relationship between Gnmt expression and DNA methylation, mediated by SAM concentrations [101, 102]. Based on this, an increase in

Gnmt activity, in the absence of changes in Dnmt activity, could account for the decreased SAM amount, which in turn could explain the loss of global DNA methylation in benzo[*a*]pyrene-exposed embryos.

Zebrafish, like other aquatic organisms, are likely exposed to multiple environmental stressors, which could impose additive effects on the epigenomic landscape. Following this consideration, Dorts et al. [103] reported that the combination of heat stress and copper exposure provokes synergistic adverse developmental effects upregulating the expression of all the *dnmt3* genes without apparent changes in the global DNA methylation level. Once again, this finding does not necessarily mean that DNA methylation modifications did not occur. Therefore, although the authors did not determine site-specific DNA methylation, a potential effect on the establishment of DNA methylation patterns in the promoter of selected genes cannot be excluded.

### **Transgenerational inheritance of DNA methylation by environmental compounds**

Although environmental stressors acting on somatic cells can potentially influence the epigenetic program of the individual developing organism exposed, epigenetic alterations can be propagated to subsequent generations through the germline, even in the absence of further stressor exposures [104]. With so far very few though intriguing studies, zebrafish is also emerging as a useful model for studying long-term transgenerational effects of environmental factors on both epigenetic and phenotypic variations.

In one of these studies, adult zebrafish females were fed with a diet enriched in either TCDD, methylmercury (MeHg), or 5-aza-2'-deoxycytidine, and offspring from two subsequent generations was assessed for changes in DNA methylation [92]. Surprisingly, the authors observed weak evidence of alteration in the methylome of the F2 individuals, concluding that the compounds mentioned did not cause transgenerational effects in zebrafish. However, at least two technical flaws in the experimental strategy employed could have accidentally distorted the interpretation of their results. First, it should be noted that the exposure window did not include epigenetic reprogramming of DNA methylation occurring during early embryogenesis, which is critical for transgenerational effects. In addition, only female individuals were exposed to the above-mentioned compounds, probably based on the evidence of a previous study by other authors hypothesizing the exclusion of potential effects on the male germline [52]. In particular, these authors explored the DNA methylation dynamics of fifteen selected genes in maternal haploid parthenogenic embryos, which do not have paternal genome

contribution. Strikingly, they found that the mentioned genes underwent reprogramming timely to an extent that was indistinguishable from control embryos derived from normal mating [52]. This finding suggests that the maternal genome/transcriptome/proteome in the embryo is sufficient for instructing DNA methylation reprogramming. Although intriguing, this conclusion suffers from two main weaknesses, viz. the extremely exiguous set of genes explored and the absence of data about the DNA methylation dynamics of noncoding loci. Following this line of reasoning, most paternal transgenerational effects could be potentially conveyed through the noncoding genome fraction.

Some support to this theory has been lent by observations from a very recent study highlighting the influence of developmental exposure to MeHg on the inheritance of phenotypic malformations in correlation with epimutations consisting in reproducible patterns of differential DNA methylation [105]. In particular, the authors noted that fertilized eggs of the F0 generation exposed to MeHg until 24 h post-fertilization show hyperactivity, visual deficits, and altered retinal electrophysiology [105]. Strikingly, although these fish, as well as their offspring of the F1 and F2 generations, were reared without additional exposures to MeHg for their entire life cycle, the F2 individuals displayed exactly the same phenotypic defects mentioned above. Compared to unexposed controls, the sperm DNA isolated from the F2 fish ancestrally exposed to MeHg did contain a highly reproducible set of differentially methylated regions. Intriguingly, although a number of these regions map within the promoter of genes that may correlate with the behavioral phenotypes observed, the vast majority of differentially methylated sites did not have gene associations [105]. Such a captivating finding could suggest that these regions of noncoding genome are probably involved in the regulation of gene expression by either *cis*-regulatory mechanisms or production of noncoding RNA.

In a coeval study performed by a distinct group, the authors assessed the transgenerational effects of two distinct compounds, the well-known Dnmt1 inhibitor 5-azacytidine [106, 107] and the plasticizer derivative mono(2-ethylhexyl)phthalate (MEHP), which is ubiquitously present in the environment [108]. It is worth mentioning that in this study only fertilized eggs of the F0 generation were exposed once in a lifetime, until 6 days post-fertilization, to 5-azacytidine or MEHP at concentrations unable to elicit detectable adverse effects on development [109]. Despite this, both compounds altered *dnmt* gene expression and DNA methylation level to a different extent in the directly exposed individuals. Comparative genome-wide analysis of the DNA methylation patterns of the offspring of these fish and unperturbed

controls at the F0, F1, and F2 generations indicated that methylation changes provoked by ancestral exposure to the compounds mentioned are persistent across generations. Even in this case, in perfect agreement with the finding described above, differential methylation was frequently found outside gene bodies and promoters, being enriched at distal noncoding regions that could have relevant regulatory roles. This interesting hypothesis is further supported by the evolutionary conservation of these genomic regions across vertebrate organisms, including humans [110].

### Modification of additional epigenetic profiles by environmental compounds

As summarized throughout this review, the vast majority of the environmental epigenetic studies in zebrafish interrogated DNA methylation. However, as outlined by studies using other organisms, additional epigenetic factors are equally important for sensing of environmental stressors. To date, limited studies in zebrafish have highlighted the variation in epigenetic marks, such as miRNAs and histone post-translational modifications, following exposure to toxicants or pollutants. For example, a recent study indicated that the teratogenic effects of sublethal concentrations of ethanol on zebrafish embryogenesis are mediated by a major increase in the abundance of a specific subset of miRNAs, which the authors proposed to be a signature for ethanol-induced toxicity in vertebrates [111].

In a similar study, microarray analysis was applied to assess the differential variation of a panel of miRNAs following exposure of zebrafish embryos to perfluorooctane sulfonate, a widely distributed environmentally organic compound, which has been found to cause developmental toxicity [112, 113]. Being the predicted targets of these miRNAs involved in a broad spectrum of developmental, cellular, and metabolic processes, this preliminary study could address the epigenetic explanation of toxicity induced by the compound mentioned.

An additional noteworthy study evaluated the genome-wide occupancy of H3K27 and H3K9 histone trimethylation following exposure of developing zebrafish embryos to 3-deazaneplanocin-A (DZNep), an anti-cancer drug that unselectively inhibits EZH2 histone methyltransferase of the polycomb repressive complex 2 responsible for H3K27 methylation [114, 115]. Interestingly, DZNep exposure provoked a dose-dependent depletion and alteration in distribution of H3K27me3 and H3K9me3 from a substantial number of gene promoters. These epigenetic variations were associated with severe neuronal and cranial malformations in the exposed fish, although they unexpectedly did not result in significant changes in gene expression levels. An explanation for this

paradoxical observation could be that, as noted by the authors, DZNep does not prevent de novo acquisition of histone lysine methylation [114].

### **Toward a deeper understanding of mode(s) of epigenetic inheritance**

Presently, experimental clues suggesting that epigenetic marks acquired by the germline are perpetuated to fish of subsequent generations remain quite limited. As described in the previous sections, DNA methylation actually represents the best-characterized epigenetic factor to be involved in transmission of epigenetic information. The paradigm of epigenetic inheritance is certainly the genomic imprinting that mediates paternal or maternal allelic transmission of specific DNA methylation patterns [116]. An auxiliary example has been provided by studies on the tonguefish *Cynoglossus semilaevis*. This teleost fish employs a primary mechanism of sex determination based on chromosome inheritance, whereas female and male individuals bear either a ZW or ZZ chromosome configuration, respectively [117, 118]. The complex mechanism responsible for male sex determination relies on a gene regulatory network triggered by the Z-linked *dmrt1* gene, which is repressed and heavily methylated in the promoter region during gonadal differentiation of female individuals [119].

Interestingly, a fraction of ZW females is spontaneously sex-reversed into phenotypic males, referred to as pseudomales, which can mate with normal females to produce viable offspring [119]. More importantly, the extent of sex reversal responds to changes in environmental temperature, and it is inherited by the subsequent generation reared in normal conditions [119]. Consistently, the sex-reversed pseudomales (as well as normal males) show high gonadal *dmrt1* expression coupled to extremely low methylation levels of the *dmrt1* promoter [120]. Although the cause–effect relationship between differential DNA methylation and sex reversal remains to be clarified, this study clearly highlights that DNA methylation plays a fundamental role in transgenerational epigenetic inheritance in tonguefish.

Similar DNA methylation-based mechanisms probably regulate transgenerational epigenetic inheritance also in zebrafish, which has been postulated to have female dominant (ZW/ZZ) sex determination system [121]. Moreover, the genomic distribution of CpG islands and the percentage of 5-methylcytosine are both generally conserved between tonguefish and zebrafish [120].

An interesting line of questioning to pursue in the future would be to correlate the inheritance of environmentally altered DNA methylation patterns with changes in the expression of the gene toolkit responsible for DNA methylation and demethylation. So far, very scarce and

confusing information is available on this point. For example, Olsvik et al. reported that the F2 offspring of F0 adult female zebrafish exposed to MeHg has only modest effects on both DNA methylation and *dnmts* expression, even though a number of site-specific methylation changes were detected in the F1 fish [92]. These data are difficult to interpret because the experimental design conceived by the authors (breeding of MeHg-treated F0 female with non-exposed F0 male fish) precluded examination of the paternal chromatin role in the transmission of DNA methylation patterns from one generation to the next. A pertinent study in this trajectory reported the transgenerational inheritance of heart disorders in the F2 offspring derived from F0 male adult fish exposed to bisphenol-A [122]. The aberrant phenotypes were consistently associated with downregulation of several genes involved in cardiac embryo development [122]. Unfortunately, although this finding suggests that the epigenetic landscape of these genes have probably changed, the authors did not address DNA methylation at their promoters. Indirect complementary observations come from a distinct study highlighting that chronic exposure to bisphenol-A, at concentrations that do not produce any obvious malformations, alters the expression of *dnmt1*, *dnmt3b3*, *dnmt3b4* genes across two generations of fish [123]. Future systematic analysis should uncover the specific contribution for each of these genes to transgenerational epigenetic inheritance.

Beyond the DNA methylation machinery, a series of compelling evidence also suggested that retention of prepatterned histone modifications in sperm chromatin could have instructive roles for the developmental program. Unlike the mammalian male gametes, the mature zebrafish sperm chromatin lacks protamine, transition proteins, and testis-specific histone variants [124]. Nonetheless, chromatin compaction is entrusted to hypoacetylated nucleosomal histones and higher amounts of linker histone compared to somatic cells [124]. Notably, coincidence of several permissive and repressive histone modifications has been found in blocks of multivalent sperm chromatin containing developmental genes with regulatory functions, constituting a mark predictive for their embryonic expression [124, 125]. Relevant to this idea, the histone modifications mentioned are not erased at fertilization, persisting in the early developing embryo [125]. Altogether, these findings strongly support a model of transgenerational epigenetic inheritance along the paternal lineage in zebrafish.

On the other hand, this model apparently clashes with earlier antithetic observations, indicating that histone modification patterns are initially not associated with the chromatin of the early developing zebrafish embryo, emerging following zygotic genome activation

[126]. Such a negative result could be explained by the insufficient sensitivity of the detection assay used by the authors. Indeed, early embryonic stages are technically challenging to examine due to the low level of modified histones. In addition, it could be speculated that the overall amount of histone modifications is partially erased or diluted or replaced by other epigenetic marks in the embryo before the onset of zygotic genome activation.

More recently, a number of attractive studies in mice suggested regulatory roles for further epigenetic factors, such as noncoding RNA and three-dimensional chromatin architecture, in epigenetic transgenerational inheritance [127–129]. Although similar studies have not yet been accomplished in zebrafish, it could be syllogistically inferred that the multidimensional coordination of distinct epigenetic processes likely governs the environmentally induced epigenetic transgenerational inheritance phenomenon.

## Conclusions

In this review, we have reported and discussed recent evidence that strongly supports the idea that the zebrafish can be a valuable animal model for exploring both individual and transgenerational epigenetic variations induced by a wide variety of environmental stimuli. So far, experimental investigation has focused mostly on DNA methylation due to the functional link between epigenetic (re)programming and DNA methylation. Future studies are required to adequately elucidate the roles played by additional epigenetic processes involving histone modifications, noncoding RNA, and chromatin structure. Clearly, more research on this field using zebrafish is warranted, in order to fully understand the impact of the environment on the epigenome, and in turn the phenotype, of vertebrate organisms.

## Abbreviations

AHR: aryl hydrocarbon receptor; Dnmt: DNA methyltransferase; DZNep: 3-deazaneplanocin-A; Gnm1: glycine N-methyltransferase; H3K9me3: histone H3 lysine 9 trimethylation; H3K27me3: histone H3 lysine 27 trimethylation; KEGG: Kyoto Encyclopedia of Genes and Genomes; MeHg: methylmercury; MEHP: mono(2-ethylhexyl)phthalate; miRNA: microRNA; SAM: S-adenosyl-methionine; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Tet: ten-eleven translocation protein.

## Authors' contributions

VC was a major contributor in reviewing the literature, conceiving, and writing the manuscript. GS participated in writing during the final stage of manuscript preparation. Both authors read and approved the final manuscript.

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