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Modulation of DNA methylation machineries in Japanese rice fish (*Oryzias latipes*) embryogenesis by ethanol and 5-azacytidine

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ABSTRACT

As a sequel of our investigations on the impact of epigenome in inducing fetal alcohol spectrum disorder (FASD) phenotypes in Japanese rice fish, we have investigated on several DNA methylation machinery genes including DNA methyl transferase 3ba (*dnmt3ba*) and methyl binding proteins (MBPs), namely, *mbd1b*, *mbd3a*, *mbd3b*, and *mecp2* at the transcription level. Studies were made during normal development, from 0 day post fertilization (dpf) to hatching, and also exposing the fertilized eggs to ethanol or a DNMT inhibitor, 5-azacytidine (5-azaC). We observed that during development, all these genes followed distinct expression patterns, generally high mRNA copies in early phases (0–1 dpf) and significantly low mRNA copies prior to or after hatching. Ethanol (100–500 mM, 0–2 dpf) was unable to alter any of these mRNAs in 2 dpf; additional four day (2–6 dpf) maintenance of these embryos in ethanol-free environment, on 6 dpf, was also unable to establish any significant difference in these mRNA levels in comparison with the corresponding controls. However, continuous exposure of fertilized eggs in 300 mM ethanol, 0–6 dpf, showed significantly high mRNA copies only in MBPs (*mbd1b*, *mbd3a*, *mbd3b*, *mecp2*). 5-azaC (2 mM) on 2 dpf was able to enhance only *mbd3b* mRNA. Removal of 5-azaC and maintenance of these embryos in clean medium, 2–6 dpf, showed significantly enhanced *mbd3b* and *mecp2* mRNAs compared to corresponding controls on 6 dpf. Our studies showed that in Japanese rice fish embryogenesis both ethanol and 5-azaC have the potential to specifically modulate the developmental rhythm of DNA methylation machineries.

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1. Introduction

DNA methylation is considered as a key contributor to normal development (Messerschmidt et al., 2014). Two families of proteins, termed DNA methyltransferases (DNMTs) and methyl-CpG-binding proteins (MBPs), are associated with DNA methylation. DNMT proteins catalyzed the addition of the methyl group from S-adenosyl methionine (SAM) to the 5 positions of cytosine found in CpG dinucleotides (reviewed by Hermann et al., 2004) and MBPs can specifically recognize DNA and bind once it is methylated (Li et al., 2015). The Dnmt families in mammals are comprised of Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3l. Among them, Dnmt1 is known as maintenance methyltransferase, and Dnmt3 families (Dnmt3a and Dnmt3b) are considered as de novo methyltransferases (Smith and Meissner, 2013). Dnmt2 harbors only the catalytic domain of a Dnmt and involved in RNA cytosine methylation (Schaefer and Lyko, 2010). Dnmt3l lacks the catalytic subunit but plays a key role in allowing DNA methylation during the maturation of germ cells (Tang et al., 2009). MBPs belong to three different families of proteins: namely the MBD family (proteins containing a methyl-CpG-binding domain or MBD), the Kaiso and Kaiso-like proteins, and the SRA domain proteins (Fournier et al., 2011). In mammals, four MBD proteins, MeCP2, Mbd1, Mbd2, and Mbd4, bind methylated DNA. Moreover, three other proteins, namely, Mbd3, Mbd5 and Mbd6 are members of this family but do not bind methylated DNA (Hendrich and Tweedie, 2003; Laget et al., 2010). The role of DNA methylation and Dnmts in mammalian development has been comprehensively studied and described. MBD proteins are generally thought to govern normal embryogenesis by a range of mechanisms which are still unknown (Ruddock-D'Cruz et al., 2008; Bogdanvic and Veenstra, 2009). In mice, Mecp2 depletion exhibits a phenotype markedly similar to the individuals with the symptoms of Rett syndrome, a neurological disorder in humans caused by MECP2 mutation (Amir et al., 1999; Guy et al., 2001).

Although research in DNA methylation has been concentrated predominantly on mice and humans, studies in other species, especially in zebrafish, are emerging rapidly (Williams et al., 2014). Several aspects of zebrafish methylation are very similar to mammals (Mackay

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et al., 2007; Anderson et al., 2009; Smith et al., 2011). For instance, the dynamic changes in methylation seen during the course of mammalian embryonic development have been demonstrated in zebrafish embryos (Mackay et al., 2007). Until now eight different *dnmt* genes are reported in zebrafish. Like mammals they have only one dnmt1, and one dnmt2, while other six dnmts are most similar to the mammalian Dnmt3 (Kamstra et al., 2014). In addition, zebrafish lacks dnmt3l protein (Smith et al., 2011). During initial phases of development all zebrafish dnmt mRNA levels were declined and increased again after zygotic genome activation (Martin et al, 1999; Smith et al., 2011). Like mammals, interactions of dnmts with other proteins were also observed in zebrafish embryogenesis (Kamstra et al., 2014). Moreover, a zebrafish genome also contains MBPs and characterization of these proteins are already initiated (Hendrich and Tweedie, 2003; Coverdale et al., 2004; Albalat, 2008; Pietri et al., 2013; Shimoda et al., 2014). Zebrafish *mecp2* showed 43.7% amino acid identity with human, expressed in multiple organs and enriched in the nervous system (Coverdale et al., 2004; Gao et al., 2015). Moreover, mecp2-null zebrafish are viable and fertile (Pietri et al., 2013). The coding region required for methyl-CpG binding domain is lacking in zebrafish mbd4 (Shimoda et al., 2014).

DNA methylation is not universal to all animals; however, in those that have occurred, it appears to be essential for proper development (Suzuki and Bird, 2008). The preferred models in methylation studies are mammals; but one difficulty associated with mammalian models is the least accessibility of the embryos when many of these methylation-associated changes are occurring. In other vertebrate models such as fish, particularly zebrafish (*Danio rerio*) and Japanese rice fish, external fertilization, optically clear embryos, large progeny, and rapid ontogeny, can provide significant potential for investigations on DNA methylation. However, due to genome duplication events that have occurred during the evolution of teleost (Howe et al., 2013), multiple DNA methylation paralog genes relative to mammals are found in the genome of both zebrafish and Japanese rice fish, that makes the studies more complicated. But the addition of duplicate genes also provides an opportunity to examine DNA methylation mechanisms in a unique genetic context. For example, in contrast to mammals, the presence of multiple dnmt3 paralogs and the lack of dnmt3l in zebrafish genome enable us to separate their roles in development and imprinting (Smith et al., 2011).

Japanese rice fish is a small fish, easy-to-maintain in the laboratory, and is used as a useful animal model to study human disease. We have successfully used this fish in inducing fetal alcohol spectrum disorder (FASD) phenotypes after exposing the fertilized eggs to physiologically relevant concentrations of ethanol. We propose that despite genetic mechanisms epigenetic mechanisms are also involved in inducing FASD phenotypes in this fish (reviewed by Haron et al., 2012). In a previous study, we have demonstrated that dnmt enzyme mRNAs (*dnmt1*, *dnmt3aa*, and *dnmt3bb.1*) are expressed during Japanese rice fish embryogenesis and their expression patterns are modulated by both ethanol and 5-azacytidine (a known inhibitor of DNMT enzyme activity). Moreover, 5-azaC, like ethanol, is also able to induce FASD-like developmental features in the neurocranial cartilages of Japanese rice fish (Dasmahapatra and Khan, 2015). We hypothesize that both ethanol and 5-azaC may share a common pathway which is effective in transforming developing embryos into FASD phenotypes. In this communication, we have extended our investigations to *dnmt3ba*, and several MBPs such as *mbd1b*, *mbd3a*, *mbd3b*, and *mecp2* at the transcription level. Our data indicate that like DNMTs the expression of MBPs in Japanese rice fish embryogenesis reached the highest level during early stages of development followed by gradual reduction as the embryos approached hatching. Both ethanol and 5-azaC were unable to modulate the expression pattern of *dnmt3ba* mRNA; however, specifically modulated the expression of MBP genes at the transcriptional level.

2. Materials and methods

The institutional Animal Care and Use Committee (IACUC) of the University of Mississippi (UM) approved all the experimental protocols.

2.1. Animal rearing, embryo collection, and exposure to ethanol and 5-azaC

Methods of animal maintenance, collection of fertilized eggs, identification of the different developmental stages (Iwamatsu, 2004) and the culture conditions of Japanese rice fish embryos in the laboratory were described previously (Hu et al., 2009; Dasmahapatra and Khan, 2015). In brief, the orange–red varieties of adult Japanese rice fish (breeders) are maintained in Balanced Salt Solution (BSS, 17 mM NaCl, 0.4 mM KCl, 0.3 mM $MgSO_4$, and 0.3 mM $CaCl_2$, with required amount of $NaHCO_3$ to maintain the pH 7.4) in Aquatic Habitats ZF0601 Zebrafish Stand-Alone system (Aquatic Habitats, Apoka, FL). The fish were fed twice daily with TetraMin flakes and brine shrimp nauplii (Artemia). Fertilized eggs after collection and screening were maintained in hatching solution (17 mM NaCl, 0.4 mM KCl, 0.6 mM $MgSO_4$, 0.36 mM $CaCl_2$ with required amount of $NaHCO_3$ to maintain the pH 7.4 and 0.0002% methylene blue to reduce fungal infection) under a 16L:8D light cycle in a Precision High Performance Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 26 ± 1 °C. For studies on *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3* and *mecp2* mRNA expression during embryogenesis, the collected 0 dpf embryos were maintained in clear glass bowls (10 × 4.5 cm) in 150–200 mL hatching solution (50–100 embryos/bowl) with 50% static renewal of the media every day. Viable 0- (Iwamatsu stages 9–10), 1- (Iwamatsu stages 17–18), 2- (Iwamatsu stages 23–25), 3- (Iwamatsu stages 27–28), 4- (Iwamatsu stages 29–30), and 6-dpf (Iwamatsu stages 34–38) embryos and hatchlings (within 24 h of hatching) were used for RNA extraction (8 embryos or hatchlings pooled together/sample). To observe the organ-specific expression of these DNA methylation machinery genes, four reproductively active adult male and four egg laying female fish were used for the collection of brain (male and female), liver (female) and ovarian (female) tissues. To study the effects of ethanol (100–500 mM) and 5-azaC (2 mM) on *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNA expression at the message level, viable 0 dpf embryos (Iwamatsu stages 9–10) were transferred to 2 mL tubes (1 egg/tube) in 1 mL medium (hatching solution) containing either 100–500 mM of ethanol or 2 mM of 5-azaC (5-azacytidine, Sigma-Aldrich, St. Louis, MO) depending upon the nature of the experiments. The tubes were tightly capped to stop evaporative loss. Control embryos were maintained in 1 mL hatching solutions (1 egg/tube). The medium was changed every day. Some of the control and ethanol (100–500 mM) or 5-azaC (2 mM)-treated embryos after 2 days of treatment were utilized for RNA extraction. The remaining embryos (control and embryos treated with 100–500 mM of ethanol or 2 mM 5-azaC) were transferred to a 48-well plate and maintained in clean hatching solution (one embryo/well/mL medium) for another 4 days (2–6 dpf) with 50% static renewal of the media and on 6 dpf the viable embryos were used for RNA extraction. In a separate experiment, the embryos (0 dpf, Iwamatsu stages 9–10) were exposed to 300 mM ethanol from 0 to 6 dpf (continuous exposure) with change of media once every day and the viable embryos were used for RNA extraction on 6 dpf.

2.2. RNA isolation, cDNA synthesis, priming strategy, and RT-qPCR

After the required period, the viable embryos were pulled (6–8 per sample) and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA extraction (Dasmahapatra et al., 2005; Dasmahapatra and Khan, 2015). The brain of adult male and female fish and the liver and ovary of egg laying female fish were also used for RNA extraction. To remove genomic DNA from the samples, the extracted RNA was treated with nuclease-free RQ1 DNase (Promega, Madison, WI). The concentration of the purified RNA was determined in a Nano Drop