1 Modulation of DNA Methylation Machineries in Japanese Rice Fish (*Oryzias latipes*)
2 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 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 days (2-6 dpf) maintenance of these embryos in ethanol-free environment, on 6 dpf, were 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 has the potential to specifically modulate the developmental rhythm of DNA methylation machineries

Key words: DNA methylation, DNA methyl transferase, Methyl binding protein, Japanese rice fish embryogenesis, ethanol, 5-azacytidine.

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 methyl group from S-adenosyl methionine (SAM) to the 5 position 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 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., 2012). 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; Bogdanovic and Veenstra, 2009). In mice, Mecp2 depletion exhibit a phenotype markedly similar to the individuals with the symptoms of Rett syndrome, a neurological disorder in human caused by MECP2 mutation (Amir et al., 1999; Guy et al., 2001).

Although research in DNA methylation has been concentrated predominantly on mice and human, studies in other species, especially in zebrafish, are emerging rapidly (Williams et al., 20014). Several aspects of zebrafish methylation are very similar to mammals (Mackay 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, 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, 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 genome of both zebrafish and Japanese rice fish, that makes the studies more complicated. But the addition of duplicate genes also provide 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 been 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 pattern 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 modulate 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 MgSO₄, and 0.3 mM CaCl₂, with required amount of NaHCO₃ 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 MgSO₄, 0.36 mM CaCl₂ with required amount of NaHCO₃ 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 (10X4.5 cm) in 150-200 mL hatching solution (50-100 embryos/bowl) with 50% static renewal of the media every day. Viable 0- (Iwamatsu stage 9-10), 1- (Iwamatsu stage 17-18), 2- (Iwamatsu stage 23-25), 3- (Iwamatsu stage 27-28), 4- (Iwamatsu stage 29-30), and 6-dpf (Iwamatsu stage 34-38) embryos and hatchlings (within 24h 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 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 stage 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 6dpf (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 (Thermo Scientific, Wilmington, DE) and the quality of the purified RNA was evaluated on 1% agarose gel electrophoresis containing 0.1% ethidium bromide. Purified RNA was reverse transcribed to cDNA by iScript supermix (BioRad Laboratories, Hercules, CA), in a 20 µl final volume following manufacturers protocol; iScript supermix uses a combination of oligo-dT and random hexamers for priming cDNA synthesis. Gene-specific primers (IDT, Coralville, IA) were designed (Table 1), and annealing temperatures were optimized by gradient PCR. The nucleotide sequences of the amplicons are also verified with the sequences reported in GenBank. All the amplicons showed 100% sequence identity with the sequences reported in GenBank. The standards used in RT-qPCR analysis were prepared for each set of target genes by PCR, using cDNAs prepared either from normal hatchling or 0 dpf embryos (Iwamatsu stages 9-10) as template and the same sense and antisense primer sets (Table1) were used during RT-qPCR analysis for each target gene (Wu et al., 2010). The amplified standards were purified by agarose gel (1%) electrophoresis containing 0.1% ethidium bromide, eluted, and quantified by Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA). The standards once prepared were aliquoted out into separate tubes and stored at -80 °C until use. During RT-qPCR analysis (Wu et al., 2010), the respective standard was serially diluted to the desired concentrations ($\sim 10^2$ - 10^8 copy number/µL) with nuclease-free water and run parallel with the samples. For each target gene 1µL of cDNA or standards in duplicate in a total reaction volume of 20 µL (10 µL iQ™ SYBR®

Green super mix from BioRad laboratory, 50pM each of forward and reverse primers of the target gene, 1 µL cDNA or standards, and the volume was adjusted to 20 µL by nuclease-free water) was amplified in a thermal cycler (DNA Engine Opticon2, BioRad) (Wu et al., 2010). The quantification cycle or Cq was set at the point manually with the best R² value which ranged from 0.70-0.99 for standard curves. The Cq was applied to all wells for consistent analysis of standards and individual samples. Standards were run each time in every set of RT-qPCR analysis. The copy numbers of RNA in each tube were determined by using a software program (MJ Opticon Monitor Analysis Software, version 3.1, Bio-Rad laboratories, Hercules, CA). The data were expressed as copy number of target gene mRNA/ng of total RNA (Wu et al., 2010).

2.3. Statistics:

All data (Figures 1-4) were analyzed by using one way ANOVA followed by either post-hoc Tukey's multiple comparison test (Figures 1,2, and 4) or unpaired "t" test (Figure 3). Data were expressed as mean ±SEM of 4 or more observations and $p < 0.05$ was considered as significant.

3. Results:

3.1. Expression of *dnmt3ba* and MBDs were found to be organ specific in adult fish.

By applying RT-qPCR techniques and gene-specific primers (Table 1), we have analyzed the expression of *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNAs in the brain of both male and female reproductively active adult fish and in the liver, and ovary of egg-laying female fish (Table 2). Our data indicated that these organs expressed all these mRNAs in adult stages, however, *dnmt3ba* mRNA in liver of female fish is found to be undetectable (Table 2). Moreover, expression of these mRNAs are comparatively less in liver than brain and ovary if the data are expressed as mRNA copy/ ng RNA. Among the investigated MBDs, *mbd1b* mRNA copies was found to be highest and *mbd3a* mRNA copies was found to be at the lowest level in male brain. In female brain, the distribution pattern of *mbd* mRNA copies are more or less identical with male, however, *mbd3b* mRNA was at the highest level and *mbd3a* was remained to be in the lowest level. In case of ovary, the expression of *mbd1b*, *mbd3a*, and *mbd3b* were remained almost at the same level and *mecp2* was found to be at the minimum level compared to other MBDs. Although the distribution of *mbd* mRNAs in liver showed significantly lowest values than other two organs (brain and ovary), *mbd1b* was found to be highest and *mecp2* was found to be at the lowest level in this organ (liver) (Table 2).

3.2. Developmental regulation of *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNAs in Japanese rice fish embryogenesis.

The mRNA expression pattern of *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b*, and *mecp2* were investigated in Japanese rice fish embryogenesis starting from 0 dpf (Iwamatsu stage 9-10) to hatching (Iwamatsu stage 40) by RT-qPCR using gene-specific primers (Table 1). During development, the expression of these mRNAs maintained a rhythm, in general high mRNA copies in early phases of development (0-1 dpf) and then gradual reduction as the embryos approached hatching (Figure 1). However, critical examination of the expression patterns of these mRNAs during development indicate that these genes followed three distinct patterns of expression; *dnmt3ba*, *mbd3b*, and *mecp2* mRNAs were found to be at the highest levels in the embryos of 1 dpf and gradually reduced until hatching (Figures 1.1, 1.4 and 1.5); *mbd1b* mRNA was at the maximal level in 0 dpf embryos and then reduced gradually until hatching (Figure 1.2). However, *mbd3a* showed two peak levels of expression, the first one is on 1dpf and the other one is on 4 dpf embryos, and minimal level is in hatchlings (Figures 1.3). Therefore, it appears from the data that the mRNA levels of all these genes are reached maximal levels in the early phases of development (0-1 dpf) and reduced to minimal level during hatching or prior to hatching.

3.3. Effects of ethanol on expression of *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNAs in Japanese rice fish embryogenesis

The effects of ethanol on mRNA expression of these methylation machinery genes were investigated in two ways. First, 0 dpf embryos (Iwamatsu stages 9-10) were exposed to five different concentrations of ethanol (100, 200, 300, 400, and 500 mM) including controls (no ethanol) for first 48 h of development in tightly capped tubes and then ethanol was removed from the culture and 50% of the survived embryos (both from control and ethanol-treated group) were used for RNA extraction. Rest were maintained in hatching solution in a 48-well tissue culture plate without ethanol for another 4 days (2-6 dpf) and used for RNA extraction and analyses on 6 dpf (Figures 2.1-2.5). In the second approach, 0 dpf embryos (Iwamatsu stages 9-10) were exposed to 300 mM of ethanol until 6 dpf of development in tightly capped tubes and the survived embryos on 6 dpf were used for RNA extraction and mRNA analyses (Figures 3.1-3.5).

Our data indicate that in the first approach, all mRNAs of DNA methylation machineries investigated in this study (*dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2*) were remained unaltered both on 2 (immediately after ethanol removal) and 6 dpf embryos when compared with the corresponding controls. However, in second approach, when the embryos were exposed to 300 mM ethanol 0-6 dpf (continuous exposure), except *dnmt3ba* mRNA (Figure 3.1), all other *mbd* mRNAs (*mbd1b*, *mbd3a*, *mbd3b*, and *mecp2*) were found to be remained in significantly enhanced level in comparison with the corresponding 6 dpf controls (Figures 3.2, 3.3, 3.4 and 3.5).

From our developmental studies, we have observed that these mRNAs were found to be down-regulated in 6 dpf embryos when compared with the embryos of early phases (0-1 dpf) of development (Figure 1). To establish the influence of ethanol on modulating developmental expression patterns of these DNA methylation machinery genes, we have compared all mRNA data observed in 2 dpf embryos with the mRNA data observed on 6 dpf embryos with regard to different ethanol concentrations. Our data indicated that compared to 2dpf embryos *dnmt3ba* mRNA on 6 dpf showed a general tendency of reduction; however, unable to establish significant difference except 100 mM group (2 dpf vs. 6dpf; $p<0.05$) (Figure 2.1). For *mbd1b*, the mRNA level of 400 mM of embryos on 6 dpf established significant enhancement with 2 dpf. Other ethanol groups (100, 200, 300 and 500 mM) as well as control group were also unable to establish any significant difference. In case of *mbd3a*, *mbd3b*, and *mecp2*, comparison of the data between two age groups (2 dpf and 6 dpf) either in ethanol-treated conditions or controls, no significant differences were established.

3.4. Effect of 5-azaC on *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNA levels in Japanese rice fish embryogenesis.

The effects of DNMT inhibitor 5-azaC on the expression of mRNAs of DNA methylation machinery genes are also investigated. The embryos were exposed to 2 mM 5-azaC 0-2 dpf and then either used for RNA extraction or maintained in clean hatching solution without 5-azaC for another 4 days (2-6 dpf) and used for RNA extraction and analyses. It was observed that mRNA expression pattern of *dnmt3ba*, *mbd1b*, *mbd3a* and *mecp2* were remained unaltered after 2 dpf of continuous exposure to 2 mM 5-azaC (Figures 4.1, 4.2, 4.3 and 4.5), however, *mbd3b* mRNA was found to be enhanced significantly when compared with corresponding control (Figure 4.4). The embryos on 6 dpf showed significant increase in *mbd3b*, and *mecp2* mRNAs when compared with the corresponding controls on 6 dpf (Figures 4.4, and 4.5), while *dnmt3ba*, *mbd1b*, and *mbd3a* mRNAs, in this condition, remained at the same level as in control embryos in 6 dpf (Figures 4.1,4.2, and 4.3). Age-related comparison (between 2 dpf and 6 dpf embryos) showed that *dnmt3ba* mRNA was reduced significantly both in control and 5-azaC-treated embryos on 6 dpf in comparison to 2 dpf embryos (either control 2 dpf vs control 6 dpf, $p<0.05$ or 2 dpf 5-azaC vs 6 dpf 5-azaC, $p<0.05$). However, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* showed inconsistency. In case of *mbd1b*, *mbd3a*, *mbd3b* down regulation were observed in 6 dpf control embryos only when the comparison were made with 2 dpf embryos exposed to 5-azaC (Figures 4.2, 4.3, and 4.4). However 6 dpf embryos showed significant enhancement in *mecp2* mRNA when compared with corresponding 2 dpf control embryos or the embryos exposed to 5-azaC (Figure 4.5).

4. Discussion.

DNA methylation is an epigenetic mechanism consisting in the addition of a methyl group covalently bound to the 5-carbon of cytosine of a CpG dinucleotide. In animals, DNA methylation is mediated by Dnmts, which are evolutionarily conserved enzymes. The addition of methyl groups to the DNA regulates gene expression in two ways, either by interfering directly with the binding of proteins that interact with DNA elements (Iguchi-Ariga and Schaffner, 1989; Comb and Goodman, 1990; Bell and Felsenfeld, 2000), or indirectly, through recruitment of MBPs which contain a methyl-CpG-binding domain (Jaenisch and Bird, 2003; Fatemi and Wade, 2006). Most MBPs specifically recognize and bind to methylated DNA, and can associate with histone deacetylases (HDACs) and other chromatin remodeling proteins, resulting transcriptional repression (Wade, 2001).

While studying the mechanisms of FASD targeting Japanese rice fish epigenome, we have previously shown that like mammals, DNMTs are operative in this species during development and *dnmt* mRNA transcripts (*dnmt1*, *dnmt3aa*, and *dnmt3bb.1*) were reached the maximal level in early phases of embryogenesis. Moreover, the expressions of *dnmt* transcripts are modulated by ethanol and 5-azaC (Dasmahapatra and Khan, 2015). In this communication, we have extended our investigations to other members of DNA methylation machineries, such as *dnmt3ba*, and several MBDs like *mbd1b*, *mbd3a*, *mbd3b*, and *mecp2* at the transcription level. In addition to development, we have also studied the expression of these genes in brain of adult fish (both male and female) and in the liver and ovary of female fish (egg laying) at the transcription level. Our data indicated that all these mRNA transcripts are expressed in brain (both male and female) and ovary of reproductively active fish. However, compared to brain and ovary, the MBD mRNA levels are significantly low in liver and *dnmt3ba* mRNA is undetectable/below the detection limit in this organ. These data showed that the expression of DNA methylation machinery genes, especially *dnmt3ba*, are organ-specific. In zebrafish there are eight different *dnmt* genes and three of them (*dnmt3*, *dnmt4* and *dnmt7*) are mammalian *dnmt3b* orthologs (Shimoda et al., 2005; Kamstra et al., 2014; Seritrakul and Gross, 2014); among *dnmt3*, *dnmt3ab* mRNA expressed in brain, retinae, pharyngeal arches, swim bladder, and intestine, but not in liver and pancreas of 72 hpf embryos (Takayama et al., 2014). The lack of expression of *dnmt3ba* mRNA transcript in liver of Japanese rice fish adult and *dnmt3ab* in zebrafish (72 hpf) liver may indicate a functional identity between these two genes. Moreover, expression of *mecp2* mRNA is also reported in zebrafish embryogenesis and in adult brain (Coverdale et al., 2004) which indicated significant importance of methylation machinery genes during embryogenesis as well in adult brain function of Japanese rice fish.

Our studies also showed that during Japanese rice fish embryogenesis, *dnmt3ba* maintained a developmental rhythm like other *dnmts* (*dnmt1*, *dnmt3a*, and *dnmt3bb.1*) as we have observed in our previous studies (Dasmahapatra and Khan, 2015); higher mRNA copies in early phases of development (highest mRNA copies in 0-1 dpf embryos) and then gradual reduction until hatching (lowest mRNA copies in 6 dpf embryos and hatchlings) (Figure 1). Ethanol (100-500 mM, 0-2 dpf) by short-term exposure (0-2 dpf) is unable to alter *dnmt3ba* mRNA level in both in 2 and 6 dpf age groups in comparison with the corresponding controls (Figure 2.1). Continuous exposure of the fertilized eggs to 300 mM ethanol (0-6 dpf) is also unable to alter *dnmt3ba* mRNA transcript in 6 dpf embryos (Figure 3.1). Finally, 5-azaC (2 mM) either on 2 dpf or 6 dpf is also unable to alter *dnmt3ba* mRNA transcript in these embryos. From these observations we suggest that the developmental rhythm of *dnmt3ba* mRNA is found to be very stable; because exposure of the embryos to ethanol whether short-term (0-2 dpf 100-500 mM and then maintained in clean hatching solution 2-6 dpf) or continuous (0-6 dpf in 300 mM ethanol) or to 5-azaC (either on 2 or 6 dpf) were unable to modulate *dnmt3ba* mRNA expression pattern in these embryos compared to corresponding controls (Figure 2.1 and 3.1). Moreover, present data on developmental expression analysis of *dnmt3ba* mRNA is also unable to establish significant difference between the embryos of 2 dpf and 6 dpf even though the mRNA expression maintained a trend of downregulation (Figure 1.1). However, in 5-azaC experiment, both control and 5-azaC treated embryos on 2 dpf showed significantly higher level of *dnmt3ba* mRNA than 6 dpf embryos (2 dpf control v/s 6 dpf control or 2 dpf 5-azaC v/s 6 dpf 5-azaC; $p < 0.05$) (Figure 4.1) which supports our hypothesis of down regulation of *dnmt3ba* mRNA as the embryos approached towards hatching. Our previous studies on *dnmt3bb.1* mRNA, which seems to be a paralog of *dnmt3ba*, is also maintained identical developmental pattern as followed by *dnmt3ba* mRNA observed in the present study. Moreover, ethanol (300 mM, 0-2 dpf and then 2-4 dpf no ethanol) treatment was also unable to alter the developmental pattern of *dnmt3bb.1* mRNA. However, 5-azaC (2 mM, 0-2 dpf and then no 5-azaC, 2-6 dpf) was able to prevent the down regulation of *dnmt3bb.1* mRNA during Japanese rice development (Dasmahapatra and Khan 2015). Therefore, the current studies in *dnmt3ba* and previous studies with *dnmt3bb.1* showed identity in developmental expression pattern and ethanol sensitivity but differ in response to DNMT inhibitor.

We aimed to study the roles played by MBDs in FASD. As an initial attempt, we have analyzed the expression of *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNA transcripts in Japanese rice fish embryogenesis as well as in the embryos exposed to ethanol or 5-azaC. The Japanese rice fish genome databases (Ensembl/GenBank) consists several MBDs which showed sequence homology with human. However, to our knowledge, these MBDs of Japanese rice fish are never

been characterized. The founding member of the MBD family is the MECP2, which is ubiquitously expressed and highly abundant in the mammalian brain. Mutations in *MECP2* gene caused the Rett syndrome and other neurodevelopmental disorders (Amir et al., 1999; Moretti and Zoghbi, 2006). In general, members of the MBD family contain a methyl-CpG binding domain. Moreover, MBD1, MBD2 and MeCP2 also contain a transcriptional repressor domain (TRD). The DNA binding activity in Mbd3 protein has been lost in mammals, however, such activity is preserved in amphibians and fishes (Hendrich and Tweedie, 2003). Moreover, in contrast to MeCP2-null mouse model, *mecp2*-null zebrafish are viable and fertile (Pietri et al., 2013) which is unique and useful to study the mechanisms associated with Rett syndrome (Pietri et al., 2013). Therefore, more studies in MBD gene families of non-mammalian vertebrates are necessary.

Our data showed that all these MBDs, like DNMTs, are also maintained a distinct developmental rhythm/cycle during rice fish embryogenesis; high mRNA copies in early phases of development (0-1 dpf) and low copies prior to or after hatching (Figures 1.2, 1.3, 1.4 and 1.5). Although we have not analyzed *dnmt* or *mbd* proteins or *dnmt* enzyme activities in this fish, from our current mRNA data we could speculate that the mRNA expression of these two families of DNA methylation machinery genes are positively/directly correlated. When *dnmt* mRNAs reached the optimum level, to get the DNA methylation successful, the *mbd* genes are also reached the optimum level of expression. For this reason, in medaka embryogenesis, when *dnmt* mRNA levels are high as in the early phases of embryogenesis (0-1 dpf), the *mbd* mRNAs are also obtained the peak level which suggests that DNA methylation and epigenetic reprogramming that occurred during embryogenesis is a cooperative effort between these two gene families. During development, successful methylation of genes mediated by DNMTs and binding of MBDs to methylated DNA recruits histone modifiers for turning off or on of that particular gene which ultimately leads epigenetic reprogramming of the zygote genome. In zebrafish, *mecp2* was found to be expressed in fertilized eggs to 24-48 hpf embryos (Coverdale et al., 2004) and then enriched in the developing brain at 48 hpf (Gao et al., 2015) suggesting its importance in neuronal function.

Our data further indicated that ethanol in short-term exposure (0-2 dpf) is unable to modulate the expression of these MBD genes (Figures 2.2, 2.3, 2.4, 2.5) either in 2 dpf or in 6 dpf embryos. Age-related comparison (embryos of 2 dpf vs embryos of 6 dpf) of the mRNA contents of these *mbd* genes are also mostly failed to establish significant difference. However, continuous exposure of these embryos to 300 mM of ethanol (0-6 dpf) showed enhanced level of *mbd* mRNAs (*mbd1b*, *mbd3a*, *mbd3b*, and *mecp2*) (Figures 3.2-3.5). In murine embryonic fibroblasts (MEF), ethanol (200 mM, 48 h exposure) enhances MeCP2, and reduces Mbd2 and Mbd3

mRNAs while degrades MeCP2, MBD-2 and MBD-3 proteins (Mukhopadhyay et al., 2013) which indicate that MBDs play significant role in ethanol-mediated effects such as FASD. Although we are unable to explain properly the effect of short-term exposure of ethanol on mbds, from our continuous exposure data we suggest that MBD expressions would have modulated if the embryos are exposed to ethanol during the entire period of development.

Our studies also showed that 5-azaC is able to modulate the expression of mbds in a gene-specific manner (Figure 4). In 2 dpf embryos only *mbd3b* (Figure 4.4) and in 6 dpf embryos both *mbd3b* (Figure 4.4) and *mpcp2* (Figure 4.5) are in enhanced state in comparison with the corresponding 2 or 6 dpf controls. Further, age-related down regulation in 6 dpf embryos compared to 2 dpf embryos were noticed in control embryos with regard to *mbd1b*, *mbd3a* and *mbd 3b* mRNAs. However, 5-azaC treatment (0-2 dpf and then maintenance in clean hatching solution 6 dpf) is able to maintain same status of *mbd1b*, *mbd3b* and *mecp2* mRNAs in 6 dpf embryos as observed in 2 dpf. These data indicated that in early stages when the embryos were exposed to 5-azaC (or ethanol), the normal expression of these genes were disrupted/delayed probably due to cessation/retardation of development. When 5-azaC (or ethanol) is removed from the medium and the embryos were maintained in clean medium, the gene became reactivated and functional. However, more studies are needed to confirm the role played by *mbd* genes in inducing FASD phenotypes.

Taken together, our data indicated that the genes of DNA methylation machineries investigated in this study (*dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2*) are able to maintain a distinct expression pattern at the transcription level during development, high mRNA copies in early phases (0-1 dpf) and minimal expression in prior to or after hatching. Both ethanol and 5-azaC is able to disrupt the developmental rhythm of some of these genes in a specific manner and played significant role in developing FASD phenotypic features in Japanese rice fish embryos.

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Table 1: List of Primers used for amplification of *dnmt3ba* and *mbd* genes in Japanese rice fish.

gene	Sense (5'-3')	Antisense (5'-3')	Annealing temperature (° C)	Product (bp)	Ensembl number
<i>dnmt3ba</i>	gctcatcctgaaagaagccag	gggctcagcagctgagc gatg	62	315	ENSORLT00000018522
<i>mbd1b</i>	cctcgccgtaggcgaacgcac	gcaccgaggccagttctactc	52	178	ENSORLT00000017785
<i>mbd3a</i>	ctgggaaaggaagctgagcgg	gccagacgccggggttttctc	65	209	ENSORLT00000019084
<i>mbd3b</i>	gctccttgattccgcacg	cgctggctattgccgacagcagc	65	372	ENSORLT00000006113
<i>mecp2</i>	ggctggacccgcaaactgaaac	ccagtgcggtgaaatcaaagtc	65	176	ENSORLT00000024967

Table 2: Expression of *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b*, *mecp2* mRNAs in brain (male and female), liver (female) and ovary of reproductively active Japanese rice fish.

	Brain (male)	Brain (female)	Liver (female)	Ovary
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Dnmt3ba	42.38±10.01	8.92±4.68	ND	23.18±13.31
Mbd1b	3840±1410	894.4±86.05	29.79±5.61	261.3±139
Mbd3a	220.9±34.35	231.3±47.55	10.44±0.91	312.7±135.1
Mbd3b	1597±207.5	1164±212.6	1.21±0.14	496.4±274.5
Mecp2	667.4±254.6	374.4±68.23	0.87±0.16	66.59±46.82

The organs were quickly dissected out, weighed to nearest mg and homogenized in trizol for RNA extraction. The mRNA analysis was made by RT-qPCR. The data were expressed as mRNA copy/ng RNA and presented as mean ±SEM of four observations.

Legend to the Figures:

Figure 1: Expression of *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNA in Japanese rice fish embryogenesis. Total RNA was prepared from 6 to 8 pooled Japanese rice fish embryos or hatchlings, reverse transcribed and analyzed by RT-qPCR. The data were analyzed by one-way ANOVA followed by post-hoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Each bar is the mean ±SEM of six to eight observations. Bar heads with asterisks (*), pound (#), dollar (\$), and (&), and percent (%) indicate that the data are significantly ($p < 0.05$) different from 0 dpf, 1 dpf, 2 dpf, 3 dpf, or 4 dpf embryos, respectively. All these mRNAs maintained a distinct pattern of expression during Japanese rice fish embryogenesis. Fig. 1.1= *dnmt3ba*; Fig. 1.2 = *mbd1b*; Fig.1.3= *mbd3a*; Fig. 1.4= *mbd3b*; Fig. 1.5= *mecp2*.

Figure 2: Effects different ethanol concentrations on *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNA of Japanese rice fish embryos. Fig. 2.1= *dnmt3ba*; Fig. 2.2 = *mbd1b*; Fig.2.3= *mbd3a*; Fig. 2.4= *mbd3b*; Fig. 2.5= *mecp2*. Fertilize Japanese rice fish embryos (Iwamatsu stages 9-10) were exposed to different concentrations of ethanol (100-500 mM), and analyzed either on 2 dpf or maintained in clean hatching solution without ethanol (2-6 dpf) and analyzed on 6 dpf. Each bar is the mean ±SEM of four to eight observations. The data were analyzed by one-way ANOVA followed by post-hoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Bar head with dollar (\$) symbol indicated that the data on 2 dpf are significantly different from the corresponding embryos on 6 dpf.

Figure 3: Effect of ethanol (300 mM; continuous exposure, 0-6 dpf) on *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNA of Japanese rice fish embryos. Fig. 3.1= *dnmt3ba*; Fig. 3.2 =

mbd1b; Fig.3.3= *mbd3a*; Fig. 3.4= *mbd3b*; Fig. 3.5= *mecp2*. Fertilize Japanese rice fish embryos (Iwamatsu stages 9-10) were exposed to ethanol (300 mM; 0-6 dpf), and analyzed on 6 dpf. Ethanol (300 mM) was continuously present in the culture media. Each bar is the mean $\pm$ SEM of six to eight observations. The data were analyzed by one-way ANOVA followed by unpaired “t” test; $p < 0.05$ was considered as significant. Bar head with pound (#) symbol indicates that the data are significantly different from the corresponding 6 dpf controls.

Figure 4: Effect of 5-azacytidine (2 mM) on *dnmt3ba*, *mbd1b*, *mbd3a*, *mbd3b* and *mecp2* mRNA of Japanese rice fish embryos. Fig. 4.1= *dnmt3ba*; Fig. 4.2 = *mbd1b*; Fig.4.3= *mbd3a*; Fig. 4.4= *mbd3b*; Fig. 4.5= *mecp2*. Fertilize Japanese rice fish embryos (Iwamatsu stages 9-10) were exposed to 5-azacytidine (2 mM) and analyzed either on 2 dpf or maintained in clean hatching solution 2-6 dpf without 5-azaC and analyzed on 6 dpf. Each bar is the mean $\pm$ SEM of six to ten observations. The data were analyzed by one-way ANOVA followed by post-hoc Tukey’s multiple comparison test; $p < 0.05$ was considered as significant. Bar head with asterisks (*) indicates that the data are significantly different from corresponding 2 dpf controls; bar head with pound (#) symbol indicates that the data are significantly different from the corresponding 6 dpf controls, and the bar head with dollar (\$) symbol indicates that the data on 6dpf are significantly different corresponding 2 dpf embryos exposed to 5-azaC.

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