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Expressional Profiling of Molecules Associated with Epigenetic Methylation-Related Fertility in the Rat Testis during Postnatal Period

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ABSTRACT

The male reproduction is precisely controlled by a number of intrinsic and extrinsic factors. These factors usually involve in expressional regulation of various molecules influencing on sperm production in the testis. A number of ways are employed to control the transcription of specific genes, including epigenetic modifications of DNA and histone molecules. DNA methylation of CpG dinucleotides is a commonly used regulatory mechanism for testicular genes associated with the fertility. Previous studies have demonstrated the infertility induced by improper DNA methylation of these genes. In the present research, we attempted to determine transcriptional expression of some of these genes in the rat testis at different postnatal ages using real-time PCR analysis. These genes include neurotrophin 3 (*Ntf3*), insulin-like growth factor II (*Igf2*), JmjC-domain-containing histone demethylase 2A 1 (*Jhm2da*), paired box 8 transcription factor (*Pax8*), small nuclear ribonucleoprotein polypeptide N (*Snrpn*), and 5,10-methylenetetrahydrofolate reductase (*Mthfr*). The expression levels of *Ntf3, Igf2,* and Snrpn genes were the highest at the neonatal age, followed by transient decreases at the prepubertal age. Expression of *Jhm2da* and *Mthfr* genes were continuously increased from the neonate to 1 year of age. The levels of *Pax8* mRNA at the early ages were higher than those at the later ages of postnatal development. These findings suggest that expression of some fertility-associated testicular genes in the rat during postnatal period could be differentially regulated by the control of the degree of DNA methylation.
(**Key words :** Testis, DNA methylation, Fertility, Gene expression, Postnatal development)

INTRODUCTION

The genome is defined as the total content of genetic information possessed by an organism. The epigenome is consisted of the modifications of DNA and histone structure influencing gene expression. The epigenomic modifications include DNA methylation, posttranslational histone modification, and chromatin remodeling (Rajender et al., 2011). Extensive researches have demonstrated relationships between gene expression and epigenomic modifications. For example, hypermethylation of DNA is associated with suppression of gene expression, while hypomethylation of DNA is correlated with genetic expression (Biermann and Steger, 2007). In addition, histone acetylation is closely related with an increase of gene transcription (Berger, 2002). Thus, these findings suggest that any types of epigenomic modifications could influence on gene expression, activation or suppression of gene expression. Of these epigenomic modifications, the degree of methylation of DNA and histone is precisely controlled by actions of methyltransferases (Biermann and Steger, 2007). Therefore, it is possible that changes of degree of epigenomic modifications during the development could induce the differential expression of specific gene within a tissue.

Most of male infertility could be described by clinically diagnosed causes, but no explanation for about 15% of infertile cases has been provided. One of possible contribution to male infertility is aberrant DNA methylation. In general, compared to somatic tissues, testicular DNA has highly hypermethylated loci (Oakes et al., 2007). An increasing number of recent researches have shown the presence of a strong relationship between improper DNA methylation and poor semen parameters and thus male infertility. For example, abnormal DNA hypermethylation of neurotrophin 3 (*Ntf3*) and paired box 8 transcription factor (*Pax8*) are related with a decreased sperm concentration and motility and an increase of atypical sperm morphology (Houshdaran et al., 2007). DNA hypermethylation of 5,10-methylenetetrahydrofolate reductase (*Mthfr*) is found in non-obstructive azoospermia male (Khazamipur et al., 2009; Chan

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et al., 2010). In addition, an unusual hypermethylation of small nuclear ribonucleoprotein polypeptide N (*Snrpn*) alters sperm DNA methylation patterns and is associated with poor semen parameters (Hammoud et al., 2010). Moreover, the undermethylation of insulin-like growth factor II (*Igf2*) leads into low sperm concentration in male (Poplinski et al., 2010). Interestingly, a loss of JmjC-domain-containing histone demethylase 2A (*Jhm2da*) results in improper DNA packaging and thus possibly infertility (Okada et al., 2007). Together, these biological data reveal that abnormal methylation of certain genes could influence the male fertility.

Differential expression of genes in the testis during postnatal development has been well recognized from numerous researches, including our previous study (Seo et al., 2011). Even though transcripts of some genes in the testis are remained in relatively constant levels during the entire postnatal period, transcriptional expression of other genes are fluctuated at different developmental times. In addition, it is suggested that such variation of gene expression in the testis during postnatal period would relate with differentiation of testicular somatic cells and germ cells (Dong et al., 2007). Moreover, a growing body of recent evidence has demonstrated the importance of epigenetic regulation on gene expression during mammalian spermatogenesis (Maclean and Wilkinson, 2005). As described previously, the degree of DNA methylation influences on transcription of genes, and patterns of DNA methylation are changed during developmental stages (del Mazo et al., 1994). Thus, it is reasonable to consider that expression of certain genes which DNA methylation is closely related with male fertility would be variable at different postnatal ages.

The present research was chiefly focused to determine mRNA expressional patterns of male fertility-associated molecules in the testis during postnatal development. The molecules known to have a relationship between DNA methylation and fertility were chosen for the present study. The real-time PCR analysis was employed to obtain relative mRNA abundance at each postnatal age.

MATERIALS AND METHODS

1. Animals and tissue collection

Neonatal male rats at 1 week of age were obtained from pregnant Sprague Dawley female rats purchased from Samtako (Osan, S. Korea). Pubertal male rats at 45 days of age were also acquired from Samtako. Food and drinking water were given *ad libitum* during entire experimental period. Other rats at 5 months, 1 year, and 2 years of ages were kindly provided by Aging Tissue Bank (Department of Pharmacology, Pusan National University). Five to six rats for each age group were used for the present study, except 3 rats for 1 and 2 years of age groups.

Once experimental animals become a proper age, they were anesthetized by CO_2 stunning. The male reproductive tract was collected, and the testis was rapidly separated from the other reproductive parts in cold-PBS. The testis was further dissected out into small pieces, quickly frozen in liquid nitrogen, and then stored in −80℃ until used for total RNA isolation.

2. Isolation of total RNA from the testis and generation of complementary DNA (cDNA)

A piece of the frozen testis was homogenized in easy-Blue total RNA extract solution (iNtRON Biotech, Sungnam, S. Korea) by using a polytron homogenizer (Fisher Scientific, Pittsburgh, USA). Total RNA was collected by phenol-chloroform extraction method, and the RNA pellet was resuspended and stored in RNA storage buffer (Ambion, Austin, USA). The RNA sample was directly utilized as a template for reverse transcription (RT) reaction or stored in −80℃ for use later. The quality and quantity of total RNA sample were determined by agarose gel electrophoresis and an UV spectrophotometer (Eppendorf, New York, USA), respectively.

The RT reaction was carried out as described in the instruction of ImProm-II™ reverse transcription system (Promega, Madison, USA). One microgram of total RNA was used for each RT reaction. Messenger RNA was separated from other types of RNA using oligo-dT primer. A mixture of total RNA, oligo-dT, dNTPs, reverse transcriptase, and buffer was sequentially exposed to following steps; at 25℃ for 5 min, 42℃ for 1 hr, and 70℃ for 15 min. The final volume of the mixture was 20 μl. Complementary DNA generated from RT reaction was directly served as a template for following real-time PCR analysis.

3. Performance of real-time polymerase chain reaction (real-time PCR) analysis

The oligonucleotide primers for real-time PCR analysis

Table 1. Primer information and PCR condition for real-time PCR

Gene	Primer sequence (5' → 3')	T_m (°C)	PCR product size (bps)
Mthfr	(F) TTGACATCTGTGTGGCAGGT (R) GTTTTACAAGCTGCCGAAGG	61.5	243
Pax8	(F) GATGCCTCACAACTCGATCA (R) TACCTGCCAAGGATCTTGCT	57	195
Ntf3	(F) TGTAAAGAAGCCAGGCCAGT (R) ACAAGGCACACACACAGGAA	57	169
Jhm2da	(F) GACATGATGCCTTCCAGGTT (R) GGTCCCATATTTCCGATCCT	53	183
Igf2	(F) GTGTCCCCAGATTTGCAGTT (R) GGACTGAGTTGGGGCAAATA	60	247
Snrpn	(F) ATTGAGGTCCAGGTCAAACG (R) TTCCACCACCTTGAAGTTCC	60	185
Ppia	(F) GGCAAATGCTGGACCAAACAC (R) TTAGAGTTGTCCACAGTCGGAGATG	59	196

Mthfr : 5,10-methylenetetrahydrofolate reductase; *Pax8* : Paired box 8 transcription factor; *Ntf3* : Neurotrophin 3; *Jhm2da* : JmjC-domain-containing histone demethylase 2A; *Igf2* : Insulin-like growth factor II ; *Snrpn* : small nuclear ribonucleoprotein polypeptide N; *Ppia* : peptidylprolyl isomerase A (cyclophilin A).

were designed by using Primer 3 software (http://www.bioneer.co.kr/cgi-bin/primer/primer3.cgi: Whitehead Institute/MIT Center for Genomes Research, USA). The primers utilized in the present study are shown in Table 1.

To carry out real-time PCR, 1 μl of cDNA, 10 pmol of primer set, and 10 μl of master mixture (Finnzymes, Espoo, Finland) were mixed, and dH_2O was added into the mixture to make a final volume of 20 μl. The PCR procedure was employed with an initial denaturation at 95℃ for 5 min, followed by cycles of denaturation at 95℃ for 30 sec, annealing at Tm for 30 sec, and extension at 72℃ for 30 sec. The final extension at 72℃ for 10 min was additionally included to make sure all PCR products in double-stranded form. Agarose gel fractionation was used to check the sizes of PCR products. For real-time PCR analysis, cyclophilin A (*Ppia*) was included as an internal PCR control.

4. Data presentation and analysis

A mean and a standard error for each experimental group were calculated from results of 4-5 repeated RT reaction and real-time PCR analyses. The expressional levels of molecules examined in the present study were normalized by those of *Ppia* and represented as relative ratios between target molecule and *Ppia*. Statistical comparison among experimental groups for each molecule was made by one-way ANOVA, followed by a post-hoc analysis, Duncan's test. If *P* value was lower than 0.05 level, results were considered significant.

RESULTS

1. Expressional Patterns of Neurotrophin 3 (*Ntf3*) and Insulin-Like Growth Factor II (*Igf2*) in the Testis during Postnatal Development

The pattern of *Ntf3* expression in the rat testis during postnatal development is shown in Fig. 1A. The highest level of *Ntf3* transcript was found at 1 week of age, followed by a transient decrease of *Ntf3* mRNA level at 45 days of age (Fig. 1A). Such minimal level of *Ntf3* mRNA was remained throughout the rest of postnatal period (Fig. 1A). Expressional pattern of *Igf2* in the rat testis during postnatal time was very similar with that of *Ntf3* (Fig. 1B). The mRNA level of *Igf2* was the highest at 1 week of age and significantly decreased at 45 days of age (Fig. 1B). After

then, the amount of *Igf2* transcript was not significantly changed until 2 years of age (Fig. 1B).

2. Comparison of JmjC-Domain-Containing Histone Demethylase 2A 1 (*Jhm2da*) and Paired Box 8 Transcription Factor (*Pax8*) mRNA Levels in the Testis at Different Postnatal Ages

Fig. 2 is showing expressional patterns of *Jhm2da* and *Pax8* genes in the rat testis at different postnatal ages. The lowest level of *Jhm2da* mRNA was detected at 1 week of age, and a significant increase of *Jhm2da* mRNA level was found at 45 days of age (Fig. 2A). A further increase of *Jhm2da* transcript level was observed at 5 month and 1 year of ages, followed by a drastic decrease of *Jhm2da* mRNA level at 2 years of age (Fig. 2A). The transcriptional expression of *Pax8* gene was significantly increased at 45 days of age, compared with that at 1 week of age (Fig. 2B). However, the mRNA level of *Pax8* was drastically decreased at 5 months of age, followed by a significant increase at 1 year of age (Fig. 2B). There was no statistical difference on the level of *Pax8* transcript between 1 and 2 years of ages (Fig. 2B).

3. Evaluation of Transcript Levels of Small Nuclear Ribonucleoprotein Polypeptide N (*Snrpn*) and 5, 10-Methylenetetrahydrofolate Reductase (*Mthfr*) in the Testis during Postnatal Period

The highest expression of *Snrpn* gene in the rat testis

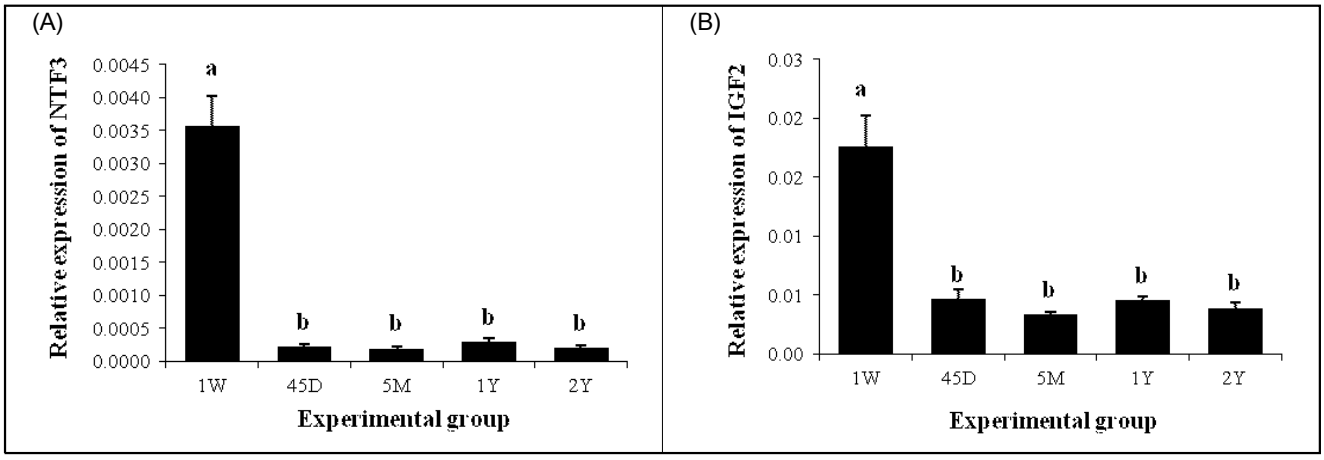

Fig. 1. Expression patterns of *Ntf3* and *Igf2* in the rat testis during postnatal period. Graphs are showing the relative expressional levels of *Ntf3* (A) and *Igf2* (B). Different letters in the graphs indicate statically significant at $p < 0.05$. d: day, m: month, and y: year.

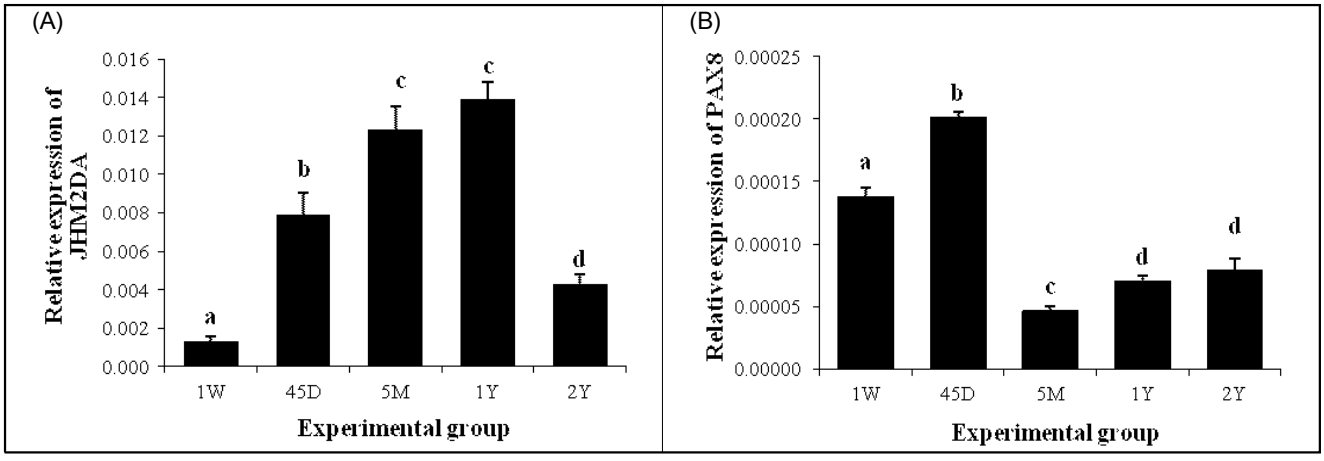

Fig. 2. Expression patterns of *Jhm2da* and *Pax8* in the rat testis during postnatal period. Graphs are showing the relative expressional levels of *Jhm2da* (A) and *Pax8* (B). Different letters in the graphs indicate statically significant at $p<0.05$. d: day, m: month, and y: year.

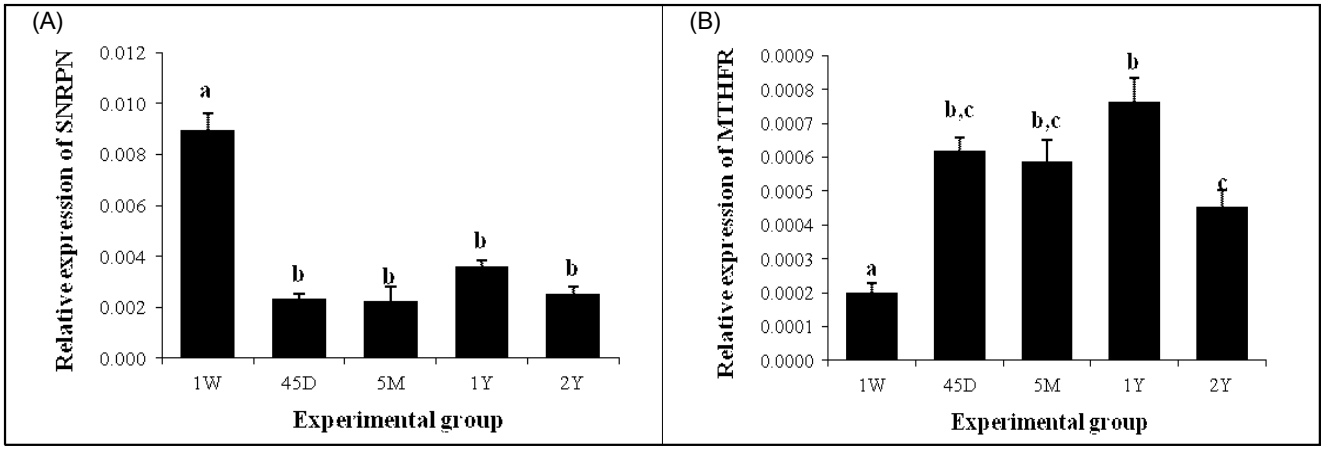

Fig. 3. Expression patterns of *Snrpn* and *Mthfr* in the rat testis during postnatal period. Graphs are showing the relative expressional levels of *Snrpn* (A) and *Mthfr* (B). Different letters in the graphs indicate statically significant at $p<0.05$. d: day, m: month, and y: year.

during postnatal period was detected at 1 week of age (Fig. 3A). Then, a significant reduction of *Snrpn* mRNA amount in the testis was followed at 45 days of age, and the minimal level of *Snrpn* mRNA was remained throughout the rest of postnatal period (Fig. 3A). The expression of *Mthfr* gene was found in the rat testis during entire postnatal period (Fig. 3B). A significant increase of *Mthfr* mRNA level was detected at 45 days of age, compared with that at 1 week of age (Fig. 3B). The amount of *Mthfr* transcript was not significantly changed until 1 year of age (Fig. 3B). However, compared with that at 1 year of age, a significant drop of *Mthfr* mRNA level was observed at 2 years of age (Fig. 3B).

DISCUSSION

There is no doubt that proper expressional regulation of male fertility-related factors is necessary for production of functional spermatozoa in the testis. Expression of some of these molecules is dependent upon epigenetic modification, especially DNA methylation. The present study has demonstrated differential expression of some of these male fertility-associated molecules during postnatal period. Expressional patterns of *Ntf3, Igf2,* and *Snrpn* were quiet similar, and the highest mRNA levels of these molecules were detected at 1 week of age. The expression of *Jhm2da* and *Mthfr* transcripts were significantly increased at adult ages, followed by transient decreases at an old age. The levels of *Pax8* mRNA at pre-pubertal ages were higher than those at post-pubertal ages.

The expression of *Ntf3* in the testis has been determined by other previous researches (Russo et al., 1999; Müller et al., 2006). The *Ntf3* is mostly localized in germ and Sertoli cells, even though the Leydig cells in the testis also express the *Ntf3* transcript (Müller et al., 2006). The expressional level of *Ntf3* is higher at embryonic stage than at postnatal period (Russo et al., 1999; Müller et al., 2006), in agreement with our present results showing the highest expression at the earliest postnatal age. These findings together imply that *NTF3* would involve in the development of embryonic and early postnatal testis, rather than testicular development at later postnatal age. Similarly with *Ntf3*, the expression of *Igf2* in interstitial cells of human testis is higher at the early postnatal age than at pubertal age (Berensztein et al., 2008). During postnatal development, *IGF2* stimulates the expression of the steroidogenic acute regulatory protein within the Leydig cells (Colón et al., 2007). It is considered that *IFG2* would promote the differentiation of immature Leydig cells into mature steroid-producing Leydig cells during postnatal development. Thus, it is proposed that *NTF3* and *IGF2* play an important role on the regulation of proper growth and differentiation of testicular cells during the earlier stage of the development.

A recent study has revealed a functional role of JHDM2A in the testis (Okada et al., 2007). As a histone demethylase, JHDM2A controls gene expression of various molecules requiring for DNA packaging and condensation of sperm chromatin (Okada et al., 2007). Deficiency of JHDM2A results in disruption of spermatogenesis, oligozoospermia, and infertility in mice (Liu et al., 2010). Together, these

observations indicate that JHDM2A is necessary for proper chromatin condensation during spermatogenesis. Developmental change of *Jhdm2a* expression in the testis has not been examined. Our present study revealed a progressive increase of *Jhdm2a* transcript level until they reached sexual maturity, while the level of *Jhdm2a* transcript was significantly dropped at old age. Spermatogenesis in the testis becomes active near the puberty and occurs at the full capacity at adult age. Thus, it is believed that an increased function of JHDM2A for chromatin condensation would be favorably required at puberty and adulthood, rather than at the prepubertal age. Even though no report is currently available for the expression of *Pax8* in the testis, the present study has demonstrated the presence of *Pax8* mRNA in the prepubertal testis at non-neglect level. It is suggested that PAX8 is likely involved in the embryonic development of the excurrent duct system of male reproductive tract (Tong et al., 2011). Additional researches are required to determine the functional role of PAX8 in the testis during postnatal development.

The testis of adult mouse possesses a high level of MTHFR activity, compared with other organs (Kelly et al., 2005). Deficiency and/or abnormal expression of *Mthfr* gene results in disruption of spermatogenesis, a significant reduction of spermatogonia, an increased apoptotic index of germ cells, and eventually infertility (Kelly et al., 2005). Even though a functional role of *Mthfr* in the testis has not been precisely examined, it is generally believed that *Mthfr* is important for initiation and progress of spermatogenesis in the male. Indeed, it is suggested that MTHFR contributes to the maintenance of cellular methyl donor pool, an important factor for DNA methylation (Chan et al., 2010). Our present study showed a significant increase of *Mthfr* expression during the transient time from neonatal age to prepubertal age, the time at which the rate of spermatogenesis becomes greatly increased. Therefore, these observations lead into a potential role of MTHFR on the regulation of spermatogenesis in the testis. A function of SNRPN in the testis has not been suggested. However, the highest expression of *Snrpn* transcript at neonatal age implies a possible role of SNRPN in participation of differentiation of testicular cells and/or initiation of spermatogenesis. Aberrant methylation of *Snrpn* gene is associated with nonobstructive male infertility (Dasoula et al., 2007). Thus, it is reasonable to consider that SNRPN would involve in the regulation of sperm formation, including control of meiotic division and germ cell development. Detailed examination should be performed to resolve the precise role of SNRPN in the testis.

As discussed earlier, DNA methylation is controlled by an action of DNA methyltransferase (DNMT). There are 3 major types of DNMTs, which have different functions on DNA methylation and different expressional patterns during the development (Data et al., 2012). DNMT1 is usually involved in maintaining programmed methylation patterns, while other DNMTs participate in generation of a new pattern of DNA methylation. Because the testis possesses all of these DNMTs, the control of activity and expression of each DNMT and/or interaction of 3 DNMTs is important to regulate DNA methylation patterns of specific genes. Thus, future researches should be conducted to provide information about the relationship between the expression of DNMTs and functional regulation of fertility-associated molecules examined in the present study.

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