Reproductive BioMedicine Online (2016) **33**, 690–702

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MALE REPRODUCTION AND FERTILITY
REVIEW

DNA methyltransferases exhibit dynamic expression during spermatogenesis

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Abstract DNA methylation is one of the epigenetic marks and plays critically important functions during spermatogenesis in mammals. DNA methylation is catalysed by DNA methyltransferase (DNMT) enzymes, which are responsible for the addition of a methyl group to the fifth carbon atom of the cytosine residues within cytosine-phosphate-guanine (CpG) and non-CpG dinucleotide sites. Structurally and functionally five different DNMT enzymes have been identified in mammals, including DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. These enzymes mainly play roles in two DNA methylation processes: maintenance and *de novo*. While DNMT1 is primarily responsible for maintenance methylation via transferring methyl groups to the hemi-methylated DNA strands following DNA replication, both DNMT3A and DNMT3B are capable of methylating unmodified cytosine residues, known as *de novo* methylation. However, DNMT3L indirectly participates in *de novo* methylation, and DNMT2 carries out methylation of the cytosine 38 in the anticodon loop of aspartic acid transfer RNA. To date, many studies have been performed to determine spatial and temporal expression levels and functional features of the DNMT in the male germ cells. This review article comprehensively discusses dynamic expression of the DNMT during spermatogenesis and their relationship with male infertility development in the light of existing investigations.

KEYWORDS: DNA methylation, DNA methyltransferase, epigenetics, male infertility, spermatogenesis

Introduction

DNA methylation is one of the epigenetic marks and plays critical roles in transcriptional activation or repression of development-related genes, genomic imprinting and X-chromosome inactivation (Jones and Liang, 2009; Kaneda et al., 2004). Mammals have two copies of every gene, and both maternal and paternal genes have the same function so only one of the parental alleles must be expressed. Control of this expression is known as genomic imprinting (Barlow and

http://dx.doi.org/10.1016/j.rbmo.2016.08.022

Bartolomei, 2014). In this process, DNA methylation plays an important role in the repression or activation of imprinting genes such as *H19* (Bartolomei et al., 1993) and *IGF2* (Stoger et al., 1993). In X-chromosome inactivation, a widespread DNA methylation at cytosine-phosphate-guanine (CpG) sites occurs to inactivate one of the X-chromosomes in females (Heard et al., 1997), and DNA methylation provides maintenance of the inactivation during the lifespan of female cells (Hansen et al., 1996).

In DNA methylation, a methyl group is added to the fifth carbon atom of the cytosine residues using S-adenosyl-l-methionine (SAM) as a methyl donor (Chen and Li, 2004; Portela and Esteller, 2010). DNA methylation generally occurs in CpG dinucleotide sites, but rarely appears in non-CpG sites such as cytosine-phosphate-thymine (CpT), cytosine-phosphate-adenine (CpA) and cytosine-phosphate-cytosine (CpC) (Chen and Li, 2004; Reik and Dean, 2001) except for CpA islands which are highly methylated in embryonic stem cells (Ramsahoye et al., 2000). To date, two different DNA methylation mechanisms have been identified: *de novo* and maintenance. While maintenance methylation primarily functions in maintaining the previously methylated genomic sites during DNA replication, the *de novo* methylation process creates new marks on DNA. Moreover, *de novo* methylation plays crucial roles in re-establishing genomic imprints following global DNA demethylation in germ cells (Law and Jacobsen, 2010). In both mechanisms, DNA methylation is catalysed by specific DNA methyltransferase (DNMT) enzymes. To date, five structurally distinct DNMT have been characterized: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (Bestor, 2000).

In general, the DNMT encompass three different structural regions: N-terminal regulatory domain, C-terminal catalytic domain and a central linker region (**Figure 1**). The N-terminal regulatory domain is particularly implicated in determining subcellular localization of the DNMT and in allocating unmethylated DNA strands from hemi-methylated ones. The C-terminal catalytic domain consists of 10 different characteristic motifs, and six of them (I, IV, VI, VIII, IX and X) are evolutionally conserved among mammals. They have specific functions: while the motif I consists of an S-adenosyl-L-methionine (AdoMet) binding site, the motif IV can associate with the substrate cytosine at the active site. The motif VI is composed of glutamyl residues serving as a donor, the motif IX confers stability to the substrate-binding site and the motif X creates an AdoMet binding site; however, the function of motif VIII remains unclear. The third region of the DNMT, the central linker domain, involves repeated Lysine-Glycine (GK)

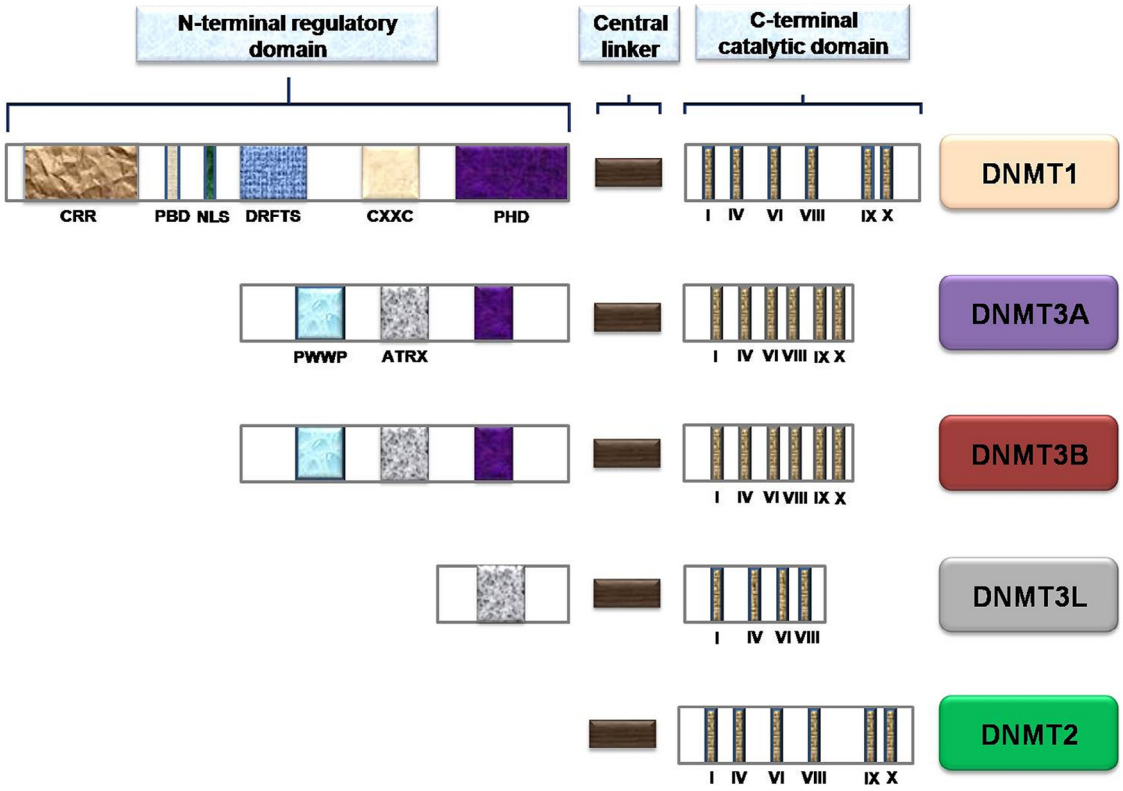

Figure 1 The general structure of mammalian DNA methyltransferase (DNMT). They include three main parts: N-terminal regulatory domain, central linker region and C-terminal catalytic domain. The N-terminal regulatory domain consists of subdomains such as charge rich-region (CRR), proliferating cell nuclear antigen-binding (PBD), nuclear localization signal (NLS), cytosine-rich zinc finger DNA-binding (ATRX), polybromo homology (PHD), and tetra peptide chromatin binding (PWWP). DNMT1 has an exclusive domain, cysteine rich region (CXXC), which enables it to strongly bind to the unmethylated DNA sequences at CpG sites. The C-terminal catalytic domain consists of ten different characteristic motifs, and six of them (I, IV, VI, VIII, IX and X) are evolutionally conserved in mammals. The third region of the DNMT, the central linker domain, involves repeated Lysine-Glycine (GK) dipeptides and provides a connection between the N- and C-terminal domains. CpG = cytosine-phosphate-guanine; DRFTS = DNA replication foci targeting sequence.

Table 1 The main basic functions and knockout phenotypes of the *DNMT* genes.

Protein	*Basic function*	*Knockout phenotype*
DNMT1	Maintenance methylation	Embryonic lethality, loss of imprinting, demethylation of the genome
DNMT3A	*De novo* methylation	Embryonic lethality, loss of imprinting in both male and female germ cells, spermatogenic arrest at spermatogonial stage and azoospermia
DNMT3B	*De novo* methylation	Embryonic lethality between E13.5 and E16.5 and ICF syndrome
DNMT3L	No catalytic activity, regulation of DNMT3A and DNMT3B activity	Abnormal maternal imprinting, male infertility, and azoospermia
DNMT2	Methylation of cytosine 38 in the anticodon loop of aspartic acid transfer RNA	Viable, fertile, normal genomic methylation and disruption of RNA methyltransferase activity

DNMT = DNA methyltransferase.

dipeptides and provides a connection between the N- and C-terminal domains (Turek-Plewa and Jagodzinski, 2005) (**Figure 1**).

Particular functions of the DNMTs

The most extensively studied DNMT type, DNMT1, is responsible for maintaining previously established DNA methylation marks after DNA replication, and it has a high affinity to hemi-methylated DNA strands (Cedar and Bergman, 2012; Pradhan et al., 1999). DNMT1 methylates the hemi-methylated DNA strands and ensures maintenance of the DNA methylation patterns during mitotic cell divisions (Cedar and Bergman, 2012). Interestingly, it has been revealed that DNMT1 partially contributes to the *de novo* methylation process independently from the *de novo* methyltransferases, DNMT3A and DNMT3B (Lorincz et al., 2002). To date, three functional DNMT1 isoforms have been identified: the pachytene spermatocyte form of DNMT1 (DNMT1p), the oocyte-specific form of DNMT1 (DNMT1o), and the somatic form of DNMT1 (DNMT1s) (Ko et al., 2005). Since all isoforms participate in the DNA methylation process, loss of *Dnmt1* (*Dnmt1*$^{-/-}$) causes embryonic lethality, lack of imprinting and extensive demethylation of the genome (Lei et al., 1996; Li et al., 1992) (**Table 1**).

The DNMT3A and DNMT3B enzymes exclusively function in *de novo* methylation (Okano et al., 1998a). DNMT3B is additionally specialized for methylation of the CpG dinucleotides at the repeated DNA sequences present in the pericentric satellite parts of eukaryotic chromosomes (Kato et al., 2007; Okano et al., 1999). While *DNMT3A* has only two RNA isoforms, *DNMT3B* contains more than twenty RNA isoforms (Ostler et al., 2007). *Dnmt3a* and *Dnmt3b* knockout mice models exhibit embryonic lethality (Kaneda et al., 2004; Okano et al., 1999), genetic immunodeficiency, centromeric instability and facial anomalies syndrome (ICF) in humans (Weemaes et al., 2013) (**Table 1**).

Another member of the DNMT family, DNMT3L, does not have any methyltransferase catalytic activity (Chedin et al., 2002) because it lacks catalytic motifs in its structure (Turek-Plewa and Jagodzinski, 2005). However, it interacts with DNMT3A and DNMT3B to stimulate their activity (Neri et al., 2013). It is suggested that DNMT3L plays an important role in the establishment of maternal genomic imprints during oogenesis (Bourc'his et al., 2001). Consistently, knockout of the *Dnmt3L* gene results in abnormal maternal imprinting and male infertility (Bourc'his and Bestor, 2004) (**Table 1**).

On the other hand, DNMT2 is able to methylate the cytosine 38 in the anticodon loop of aspartic acid transfer RNA instead of transferring methyl groups to the cytosine residues in the genomic DNA (Goll et al., 2006). Also, DNMT2 shows structural and functional differences when compared with the other DNMTs. For example, it does not include N-terminal domain, and therefore cannot contribute to *de novo* or maintenance methylation process (Turek-Plewa and Jagodzinski, 2005). Consistent with its basic mission, *Dnmt2* knockout mice (Dnmt2$^{-/-}$) exhibit disruption in the RNA methyltransferase activity (Goll et al., 2006; Okano et al., 1998b) (**Table 1**).

Expression of DNMTs during testicular development

In most multicellular organisms, male and female germ cells are the origin of development of an organism, and they provide inheritance of genetic and epigenetic information across the generations. Primordial germ cells (PGCs) derive from a subset of cells in the epiblast layer at embryonic day (E)6.5–E7.5 in mouse (Matsui and Mochizuki, 2014) (**Figure 2**). Then, they begin to migrate from the epiblast to the hindgut at E7.5–E9 and reach the genital ridge at E9.5–E11.5 in mouse (Saitou and Yamaji, 2012) (**Figure 2**). The PGCs colonize in the primitive gonad under the control of *c-kit* and its ligand interaction. Importantly, they undergo many mitotic divisions during migration and colonization (Richardson and Lehmann, 2010). In humans, the primitive gonad at an undifferentiated state until 7 weeks of embryonic improvement develops into male gonad hereafter, and while the medullar region transforms into testis, the cortical area gradually regresses. Notably, the sex determining region Y (*SRY*) gene located on the short arm of the Y chromosome regulates the testes determining factor (TDF) providing testicular development (Harley and Goodfellow, 1994).

Spermatogenesis is a complex process started at the early stage of fetal development, and includes three main stages: mitosis, meiosis and spermiogenesis (**Figure 2**). The gonocytes

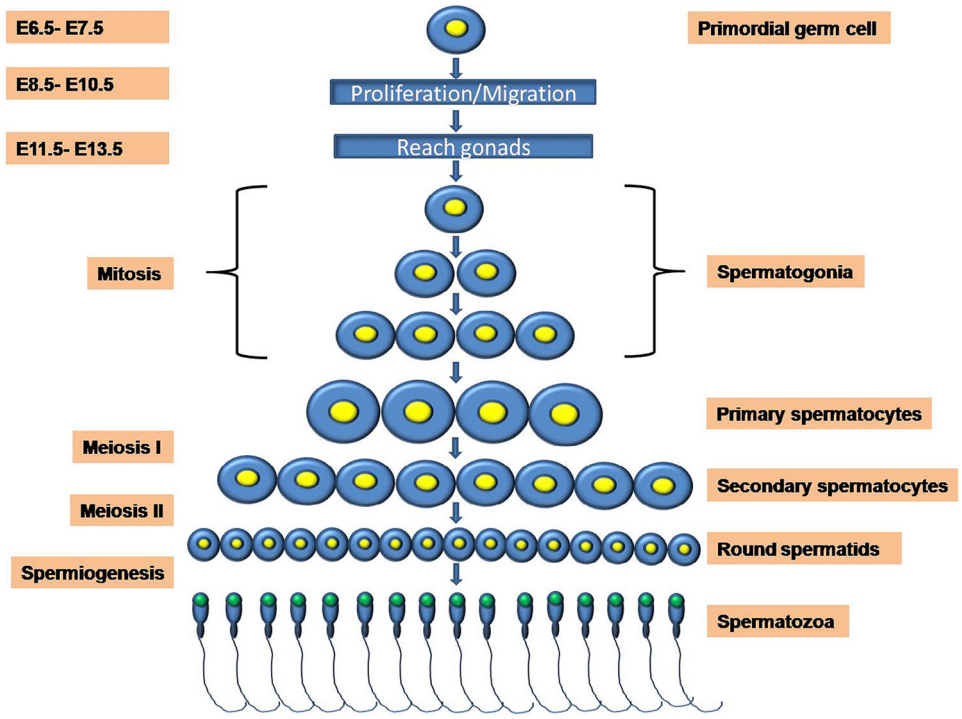

Figure 2 A schematic representation of male germ cell development from primordial germ cells (PGCs) to spermatozoa in mammals. PGC first appear at embryonic day (E)6.5–7.5 in mice, and during their migration toward the genital ridges they undergo proliferation at E8.5–10.5. After the PGC reach the primitive gonads at E11.5–13.5, they continue to proliferate, and finally differentiate into spermatogonial cells. The spermatogonial cells increase their numbers by consecutive mitotic divisions and transform into primary spermatocytes, which undergo the first round of meiotic division. The secondary spermatocytes formed by primary spermatocytes undergo the second round of meiotic division so that round spermatids are created. The round spermatids differentiate into spermatozoa at the end of process called spermiogenesis.

differentiate from PGC at E13.5 in mice. As already known, the gonocytes (fetal and neonatal germ cells) enter the primordial testis, in which they reach the base membrane of developing seminiferous tubules and then differentiate into type A spermatogonia at day six postpartum. There are two types of type A spermatogonia: type A dark spermatogonia (Ad), type A pale spermatogonia (Ap). The type Ad cells possess dark nuclei and replicate indefinitely to supply spermatogonia for spermatogenesis. The type Ap cells with pale nuclei undergo mitotic division to produce type B spermatogonia. In the spermatogonial stage, type B spermatogonia derived from a subset of type A pale spermatogonia could be identified on day eight postpartum (Bellve et al., 1977). The type B spermatogonia are the progenitor cells that undergo many mitotic divisions and gradually migrate towards the seminiferous lumen. Then, type B spermatogonia differentiate into primary spermatocytes once the organism reaches sexual maturity. The primary spermatocytes undergo one round of meiosis to form secondary spermatocytes, which undergo the second round of meiosis to form round spermatids in the meiotic stage. In the spermiogenesis stage, the round spermatids undergo important structural and nuclear changes to create mature spermatozoa (Chocu et al., 2012; Wang and Xu, 2015).

In PGC, DNA methylation marks in the parental genome are gradually erased as a result of global DNA demethylation (Popp et al., 2010), and these marks are re-established during early male germ cell development (Davis et al., 1999; Lees-Murdock et al., 2003). The re-establishment of DNA methylation is largely carried out by the specific DNA methyltransferases, DNMT3A, DNMT3B and DNMT3L (Kato et al., 2007). It is important to note that global DNA demethylation also happens during early embryonic development independent from that which occurs in the PGC (Mayer et al., 2000; Oswald et al., 2000). Additionally, it has been shown that male and female pronuclei exhibit distinct demethylation patterns after fertilization (Santos et al., 2005). Note that the dynamic expression of the DNMT in oocytes at different development stages and in the early embryos is comprehensively discussed in a recently published review article (Uysal et al., 2015).

The re-establishment of DNA methylation in the male germ cells is carried out in prospermatogonia or gonocytes (Saitou et al., 2012). Interestingly, a high rate of DNA methylation is attained in the mitotically arrested gonocytes during prenatal development. The major methylation mechanisms, *de novo* and maintenance methylation work to create new methylation marks on DNA and to maintain previously established methylation marks in mitotically active spermatogonia and primary spermatocytes at prophase I, respectively, whereas maintenance methylation occurs only in male germ cells undergoing mitotic divisions (Oakes et al., 2007). It is important to note that general DNA methylation levels in the male germ cells are at lower profiles than in the somatic cells (Urdinguio et al., 2015). In a detailed work, Abe et al. (2011) revealed that DNA methylation profiles are at lower levels in the male germ cells than those of somatic cells between E11.5

Table 2 Relative expression levels of the *DNMT* genes in the spermatogenetic cells in mice and humans.

Species	*PGC*	*SA*	*SB*	*S*	*PL*	*L/Z*	*PP*	*P*	*PS*	*SC*	*RS*	*ES*	*Parameter*	*Reference*
Mouse	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	DNMT1	Sakai et al., 2001
	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	*Dnmt1*	La Salle et al., 2004
	++	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	*Dnmt3a*	La Salle et al., 2004
	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	*Dnmt3b*	La Salle et al., 2004
	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	*Dnmt3L*	La Salle et al., 2004
	NA	+++++	+++	NA	++	++++	+++	++	NA	NA	+++	+	*Dnmt1*	La Salle et al., 2006
	NA	+++++	++++	NA	+++	++++	+++	++	NA	NA	+++	+	*Dnmt3a*	La Salle et al., 2006
	NA	+++++	++	NA	++	++++	+++	++	NA	NA	+++	+	*Dnmt3b*	La Salle et al., 2006
Human	NA	NA	NA	+	NA	NA	NA	ND	NA	NA	+	NA	DNMT1	Omisanjo et al., 2007
	NA	NA	NA	+++	NA	NA	NA	NA	++++	++	+	ND	*DNMT1*	Marques et al., 2011
	NA	NA	NA	+++	NA	NA	NA	NA	+++	+	++	ND	*DNMT3A*	Marques et al., 2011
	NA	NA	NA	+	NA	NA	NA	NA	++++	ND	++	ND	*DNMT3B*	Marques et al., 2011
	NA	NA	NA	ND	NA	NA	NA	NA	ND	ND	ND	ND	*DNMT3L*	Marques et al., 2011

Note that + and +++++ are used to show the lowest and the highest expression levels, respectively. We observed that protein and mRNA expression levels of the *DNMT1*, *DNMT3A*, *DNMT3B* and *DNMT3L* genes exhibit differences in the spermatogenic cells during spermatogenesis in mice and humans.
DNMT = DNA methyltransferases ES = elongated spermatid; L/Z = leptotene/zygotene spermatocyte; NA = not analysed; ND = not detected; P = pachytene spermatocyte; PGC = primordial germ cell; PL = preleptotene spermatocyte; PP = pre-pachytene spermatocyte; PS = primary spermatocyte; RS = round spermatid; S = spermatogonia; SA = type A spermatogonia; SB = type B spermatogonia; SC = secondary spermatocyte.

and 15.5 of early development. However, the methylation status of male germ cells increases at E17.5, in which methylation marks begin to be largely established in mice (Abe et al., 2011).

In many studies, spatial and temporal expression and subcellular localizations of the DNMTs are examined in greater detail. These studies are evaluated and discussed in the remaining part of the article. DNMT1 protein expression was characterized in the genital ridge of the primitive mouse gonad at E11.5 (La Salle et al., 2004). DNMT1 was localized both nuclear and cytoplasmic, and it also surrounded the mitotic chromosomes at the genital ridge of E11.5. In male germ cells, *Dnmt1* mRNA is also highly expressed in the gonocytes of testicular tissue at 12.5–14.5 days post-coitus (dpc) of early development, but its expression is surprisingly down-regulated at 16.5–18.5 dpc in mice (Sakai et al., 2001) (**Table 2**). It is important to note that the genomic imprinting establishment of the maternally expressed *H19* (Imprinted Maternally Expressed Transcript) gene in gonocytes is achieved at around 16.5 dpc (Tada et al., 1998; Ueda et al., 2000), and genome-wide DNA methylation is given rise at 16.5–18.5 dpc (Coffigny et al., 1999) in contrast to the remarkably decreased *Dnmt1* gene expression at these time points (Sakai et al., 2001). Most likely, not only DNMT1 but also other DNMT such as DNMT3A and DNMT3A seem to be responsible for establishing DNA methylation patterns during early development. DNMT1 particularly plays a role in the maintenance of the methylation process; therefore, the *Dnmt1* gene is highly expressed in the proliferating cells (Szyf et al., 1991). Consistently, Sakai et al. (2001) found that *Dnmt1* shows an expressional correlation with the cell proliferation marker, proliferating cell nuclear antigen (PCNA) in testicular cells (Sakai et al., 2001) complying with the presence of a physical interaction of DNMT1 with PCNA (Chuang et al., 1997). In addition to the presence of DNMT1 expression in embryonic testis tissues, it is also expressed in the postnatal mouse testes, at higher levels than that of prenatal ones (La Salle et al., 2004). In contrast, both DNMT3A and DNMT3L protein expression were found to be at very high levels between E13.5 and 18.5, suggesting that these enzymes are important for prenatal testis development and responsible for the establishment of the DNA methylation patterns in male germ line cells (La Salle et al., 2004) (**Table 2**).

Expression of DNMTs in germ-line cells during spermatogenesis

Dnmt1 expression

In mice, DNMT1 protein expression has been detected in the types A and B spermatogonia, which is consistent in that DNMT1 is highly expressed in proliferating cells (Watanabe et al., 2004). The *Dnmt1* is transcribed more highly in the type A spermatogonia, but it exhibits lower expression levels in the type B spermatogonia and preleptotene spermatocytes, increases toward leptotene/zygotene spermatocytes, decreases in the pachytene spermatocytes again and finally increases in the round spermatids in mice (La Salle and Trasler, 2006) (**Figure 3**). Of note, *Dnmt1* exhibits undetectable levels in the mixture of residual bodies and elongated spermatids (La Salle and Trasler, 2006). This study revealed that *Dnmt1* gene expression shows remarkable fluctuations in the spermatogenic cells from spermatogonia to elongated spermatids during spermatogenesis.

In humans, *DNMT1* mRNA is expressed in the spermatogonia, pachytene spermatocytes and round spermatids as is observed in the mouse spermatogenic cells; however, its protein expression is not detected in the human pachytene spermatocytes (Omisanjo et al., 2007) (**Table 2**). Nevertheless, Marques et al. (2011) observed DNMT1 protein expression in the human pachytene spermatocytes (Marques et al., 2011). The differences between former and latter studies may arise from using different techniques in measuring the *DNMT1* gene

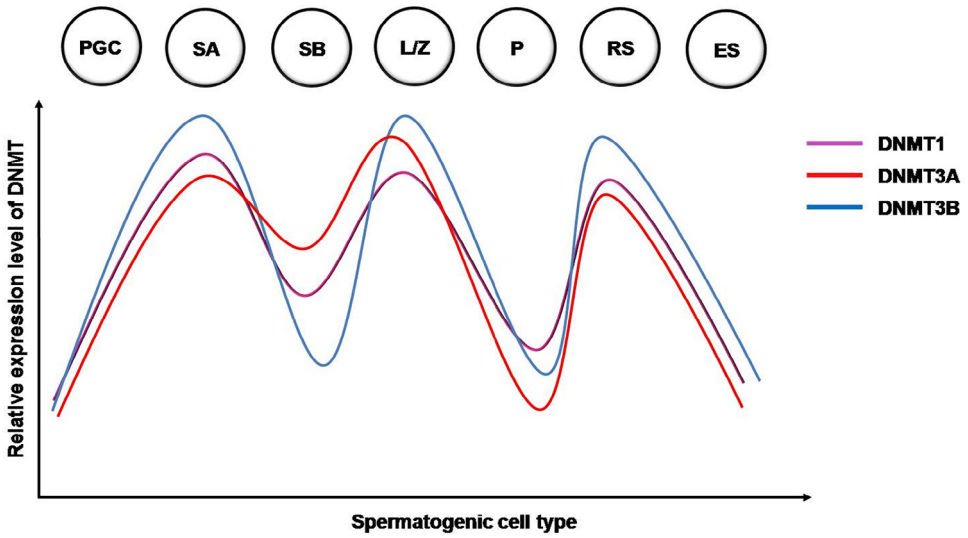

Figure 3 Expression levels of DNA methyltransferases (DNMTs) in male germ cells during spermatogenesis. The DNMT1, DNMT3A and DNMT3B exhibit almost similar expression patterns though there are small fluctuations in their relative expression. The colours pink, red and blue represent the DNMT1, DNMT3A and DNMT3B genes expression, respectively. ES = elongated spermatid; L/Z = leptotene/zygotene spermatocyte; P = pachytene spermatocyte; PGC = primordial germ cell; RS = round spermatid; SA = type A spermatogonia; SB = type B spermatogonia.

expression and in isolating spermatogenic cells. Marques et al. (2011) also revealed that the *DNMT1* mRNA expression gradually decreases from spermatogonia, primary spermatocytes and secondary spermatocytes to round spermatids in humans (Marques et al., 2011). By contrast, there was no *DNMT1* expression in the elongated spermatids (Marques et al., 2011) (**Table 2**). DNMT1 protein is localized in both nucleus and cytoplasm of type A spermatogonia, zygotene and pachytene spermatocytes, but it shuttles to nucleus and exhibits nuclear localization in the leptotene spermatocytes, secondary spermatocytes and late spermatids, and it resides in the cytoplasm of round spermatids (Marques et al., 2011) (**Figure 4A**). *DNMT1* is at detectable levels in the ejaculated sperm cells, in which DNMT1 is surprisingly located in the equatorial region of these cells (Marques et al., 2011) (**Figure 4B**). Similarly, DNMT1 protein is localized in the nuclei of spermatogonial cells and in the cytoplasm of round spermatids in a previous work (Omisanjo et al., 2007). DNMT1p, one of the DNMT1 isoforms, is exclusively expressed in the pachytene spermatocytes at mRNA levels, but not at protein level in mice (Mertineit et al., 1998; Trasler et al., 1992). Since histone-protamine exchange occurs during spermiogenesis in the round/elongating spermatids to provide strict packaging of nuclear genome, there is no need of making DNA methylation at this stage of spermatogenesis. Therefore, the subcellular distribution and expression pattern of DNMT1 protein may be closely related to necessitating DNA methylation marks during spermatogenesis. Consistent with this prediction, infertile patients diagnosed as round spermatid maturation arrest based on pathological analysis did not show any *DNMT1* mRNA or protein expression (Omisanjo et al., 2007).

Dnmt3a and *Dnmt3b* expression

As is known, DNMT3A and DNMT3B enzymes exclusively function in the *de novo* methylation process (Okano et al., 1999) so that they contribute to regulating spermatogenic process, and establishing either maternal or paternal imprint (Kaneda et al., 2004). In mice, DNMT3A protein expression is determined in type B spermatogonia and pre-leptotene spermatocytes at only tubuler stages IV–VII (Watanabe et al., 2004). The *Dnmt3a* mRNA expression is at high levels in the type A spermatogonia, is slightly reduced in the type B spermatogonia, and further decreased in preleptotene and pachytene spermatocytes, but there is no *Dnmt3a* expression observed in the round and elongating spermatids (La Salle and Trasler, 2006) (**Figure 3**). On the other hand, *Dnmt3a2* is expressed at higher levels than that of *Dnmt3a* in the type A spermatogonia when compared with other spermatogenic cells, and *Dnmt3a2* is at detectable levels in the round and elongating spermatids (La Salle and Trasler, 2006). The presence of different expression patterns of the *Dnmt3a* isoforms suggests that they may compensate each other's function to establish and sustain the DNA methylation marks during spermatogenesis. Unlike *DNMT1 and DNMT3A*, there was no *DNMT3B* mRNA expression in the ejaculated sperm cells, while their protein expression has been observed (Marques et al., 2011).

DNMT3A and *DNMT3B* genes are expressed in human spermatogenic cells including type A spermatogonia, primary spermatocytes, secondary spermatocytes and round spermatids, but at lower levels than that of *DNMT1* (Marques et al., 2011). Notably, *DNMT3A* exhibits high levels of expression in the ejaculated sperm cells (Marques et al., 2011). *DNMT3A* mRNA expression has been identified in the spermatogonia, primary spermatocytes, secondary spermatocytes and round spermatids, but it was at low levels in the secondary spermatocytes (Marques et al., 2011) (**Table 2**). DNMT3A is intensively located in the cytoplasm of most spermatogenetic cell types; however, it is found in the nuclei of pachytene spermatocytes (**Figure 4A**) and secondary spermatocytes (Marques et al., 2011). In the ejaculated spermatozoa, DNMT3A protein interestingly resides in the mid-piece region (Marques et al.,

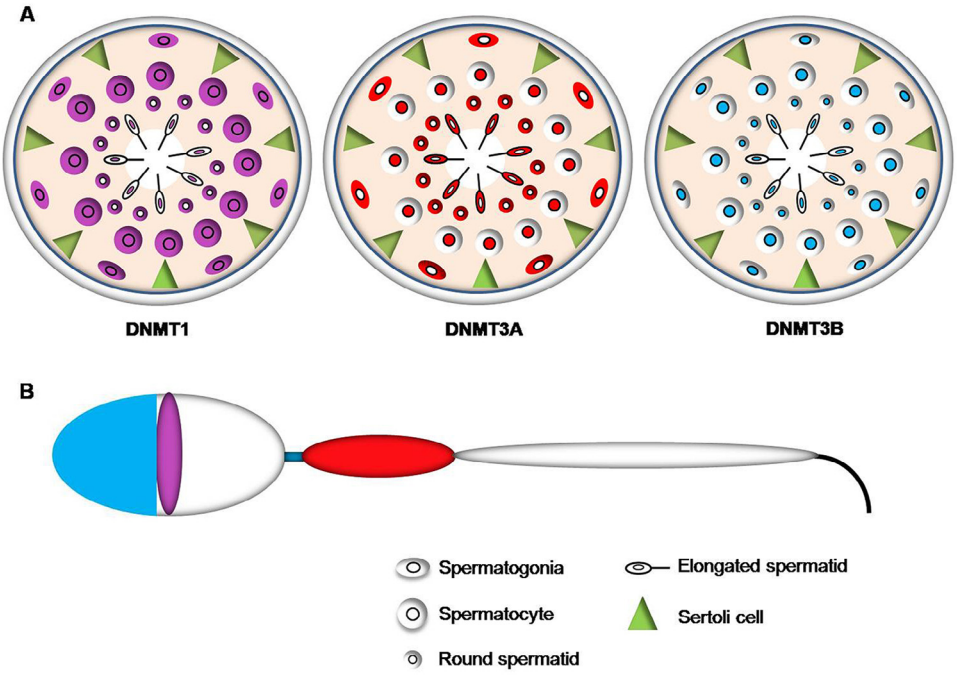

Figure 4 Cellular and subcellular localizations of the DNA methyltransferases (DNMTs) in spermatogenic cells. Nuclear or cytoplasmic localization of the DNMT1 (A), DNMT3A (B) and DNMT3B (C) are generated based on the studies in this field. Furthermore, we have drawn a sperm cell on which localization of the same DNMTs are shown (D). DNMT1, DNMT3A and DNMT3B exhibit different subcellular distributions in the spermatogenic cells. The colours purple, red and blue represent the DNMT1, DNMT3A and DNMT3B proteins, respectively.

2011) (**Figure 4B**). In parallel with these findings, *Dnmt3a* conditional knockout mouse models manifest spermatogenic arrest at the spermatogonial stage (Kaneda et al., 2004) (**Table 1**).

In mice, DNMT3B is expressed in type A spermatogonia at seminiferous tubule stages IV–VII (Watanabe et al., 2004). Interestingly, both DNMT3A and DNMT3B proteins were not defined in the same spermatogonial cell types (Watanabe et al., 2004). *Dnmt3b* mRNA is more highly expressed in the type A spermatogonia, but it is significantly decreased in the type B spermatogonia, preleptotene and pachytene spermatocytes, and increased in the leptotene spermatocytes and round spermatids in mice (La Salle and Trasler, 2006) (**Figure 3**). On the other hand, it is important to note that the elongating spermatids have *Dnmt3b* expression at very low levels (La Salle and Trasler, 2006). Of note, the remaining spermatogenic cell types show almost the same expression patterns for the *Dnmt3a* gene as is observed for the *Dnmt3b* gene (La Salle and Trasler, 2006). Based on these studies outlined above, *Dnmt3a* and *Dnmt3b* gene expression are downregulated at two developmental time points during spermatogenesis: differentiation of spermatogonial cells into spermatocytes and pachytene spermatocyte stages. This may result from requiring high levels of transcriptional activity for the testis-specific genes, which are necessary during later periods of spermatogenesis. The *Dnmt3a* null mice die around 3 weeks of age, but conditional knockout of the *Dnmt3a* in male germ cells leads to spermatogenic defects and loss of methylation at paternally imprinted loci in the spermatogonial cells, whereas the *Dnmt3b* conditional knockout mice exhibit no phenotype (Kaneda et al., 2004; Okano et al., 1999). On the other hand, Yaman and Grandjean (2006) reported that the absence of DNMT3A protein causes meiotic delay and impaired *H19* imprint establishment, suggesting that this gene seems to be important in meiotic progression (Yaman and Grandjean, 2006).

In humans, *DNMT3B* has been found to be transcribed in the spermatogonia, primary spermatocytes and round spermatids; however, it was not detected in the secondary spermatocytes (Marques et al., 2011). DNMT3B protein was predominantly localized in the nuclei of spermatogenic cells tested in this study (Marques et al., 2011) (**Figure 4A**). In the ejaculated sperm cells, DNMT3B was detected in the anterior half of the sperm head (Marques et al., 2011) (**Figure 4B**).

Dnmt3L expression

Basically, DNMT3L contributes indirectly to establishing maternal imprints and regulating spermatogenesis process (Bourc'his et al., 2001) via participating *de novo* methylation although it does not have any catalytic activity (Aapola et al., 2000). In the *Dnmt3L* knockout (*Dnmt3L*$^{-/-}$) mice models, although preleptotene spermatocytes and Sertoli cells are present, the pachytene spermatocytes onward are not detected in the testicular tissue (Hata et al., 2006). Consistent with the impaired *Dnmt3L* expression in the *Dnmt3L*$^{-/-}$ mice, there was a remarkably reduced expression of gonad specific and sex-chromosome-associated genes in the mouse testes (Hata et al., 2006). As is known, DNMT3L includes ATRX (ADD) domain, which interacts with the N-terminus of histone H3 protein (Vlachogiannis et al., 2015). Binding of DNMT3L to the histone H3 through ATRX domain is necessary for

Table 3 The relationship between male infertility and altered DNA methylation and DNMT expression.

Category of male infertility	*Cause*	*Reference*
Spermatogenesis impairment	Abnormal DNA methylation and DNMT1 expression	Hartmann et al., 2006; Takashima et al., 2009
Reduced sperm count	DNA hypomethylation in the germ cells	Kobayashi et al., 2007; Raman et al., 1995
Oligospermia	Abnormal DNA methylation in the imprinting genes, changed *DNMT3A* expression, polymorphism in *DNMT1* gene	Cheng et al., 2014; Filipponi et al., 2009; Marques et al., 2008
Azoospermia	Aberrant DNA methylation in the testicular cells, lack of either *Dnmt3L* or Dnmt3a	Bourc'his et al., 2004; Kaneda et al., 2004; Marques et al., 2010; Minor et al., 2011
Obstructive azoospermia	Reduced DNA methylation at the *H19* gene	Minor et al., 2011
Unexplained infertility	Abnormal DNA methylation patterns in the sperm cells	Urdinguio et al., 2015

DNMT = DNA methyltransferase.

recognizing the target CpG or non-CpG DNA strands to be methylated during spermatogenesis (Vlachogiannis et al., 2015). Consistently, *Dnmt3L*-deficient male mice exhibit azoospermic phenotype due to blockage in meiosis I at the leptotene-zygotene transition (Vlachogiannis et al., 2015). Also, *Dnmt3L*-deficiency causes a decreased expression of the *Dazl*, *Sox3*, *CD9*, *Ngn3* genes in the spermatogonial stem cells (Vlachogiannis et al., 2015). Furthermore, it has been suggested that a reduced DNMT3L protein expression leads to upregulation of the premeiotic stage-specific gene, *Stra8* (Vanhoutteghem et al., 2014). Based on the important functions of the DNMT3L, the loss of this gene results in spermatogenetic defects as is seen in the *Dnmt3a* knockout mice (Sakai et al., 2004; Takashima et al., 2009).

In humans, DNMT3L at mRNA (**Table 2**) and protein levels have not been identified in any spermatogenetic cell types (Marques et al., 2011). However, it is reported that DNMT3L is capable of enhancing the *de novo* methylation activity of DNMT3A enzyme in humans at endogenous sequences in the genome (Chedin et al., 2002). Overall, these data suggest that DNMT3L is an important cofactor for DNMT3A activity (Kaneda et al., 2004). New studies aiming to characterize the spatial and temporal expression of DNMT3L in reproduction-related tissues are required because there may be an indirect impact of this gene on the spermatogenetic activity.

DNMTs and male infertility

Infertility is a worldwide reproductive problem that affects 13–18% of couples, who cannot conceive despite unprotected intercourse during one year, and it arises from either the male factor or the female factor or both male and female factors (Dube et al., 2008; Gnoth et al., 2005). The male factor-associated infertility comprises half of the infertile couples (Dube et al., 2008).

Azoospermia, one of the commonly encountered reasons for male infertility is briefly defined as complete absence of functional sperm in the ejaculate, and it affects 10–15% of infertile men (Hamada et al., 2013; Jarow et al., 1989). Azoospermia is generally divided into two subgroups: obstructive azoospermia resulting from obstruction of the genital duct and non-obstructive azoospermia derived from testicular failure (Wosnitzer et al., 2014). Other infertility-associated spermatogenetic defects such as reduced sperm concentration (oligospermia), defective sperm motility (asthenozoospermia) and distorted sperm morphology (teratozoospermia) are encountered in IVF clinics (Friemel et al., 2014). Oligozoospermia is characterized by low concentration of spermatozoa in ejaculated semen. According to the World Health Organization (WHO), normal ejaculated semen includes approximately 60 million sperm/ml, but in oligozoospermic men it is identified as a concentration of less than 20 million sperm/ml in ejaculate.

Because spermatogenesis is a complex process consisting of spermatogonia, meiosis and differentiation stages, DNA methylation is one of the epigenetic mechanisms playing an important role for normal spermatogenesis (Marchal et al., 2004; Rajender et al., 2011). It has been reported that abnormal DNA methylation in spermatogenic cells due to genetic failure, environmental factors and disturbed expression of the DNMTs (Massart et al., 2012; Toshimori et al., 2004) may lead to spermatogenetic impairments (Hartmann et al., 2006; Takashima et al., 2009) (**Table 3**). As a result, hypomethylation or hypermethylation of the target DNA strands might negatively influence the process of spermatogonial cell differentiation into spermatozoa, resulting in subfertility or infertility (Kobayashi et al., 2007; Raman and Narayan, 1995) (**Table 3**).

Many studies have found that there is an aberrant DNA methylation in the imprinting control region of development-related genes in the spermatozoa of oligozoospermic men (Kobayashi et al., 2007; Marques et al., 2004, 2008), which most likely results from expressional changes in the DNMTs (**Table 3**). It should be kept in mind that since abnormal DNA methylation in the spermatozoa from oligozoospermic men would transmit its genomic content to fertilizable oocytes, it may negatively affect early development and results in abnormal growth (Filipponi and Feil, 2009). Similarly, if these sperm cells from oligozoospermic men were used in intracytoplasmic sperm injection, developmental outcome would be quite poor (Kobayashi et al., 2007). However, there is no investigation designed to examine the correlation between imprinting disorders and aberrant DNA methylation offspring of oligozoospermic men.

Minor et al. (2011) reported that DNA methylation in the DMR region of the *H19* gene is significantly decreased in the testicular sperm cells from obstructive azoospermia pa-

tients compared with fertile men (Minor et al., 2011) (**Table 3**). They also revealed that a similar decrease has been identified in the men undergoing reversal vasectomy in comparison with fertile men. These data show that aberrant DNA methylation or imprinting abnormalities seem to associate with the obstruction in the genital tract and can change the testicular microenvironment, ending with spermatogenetic failure. Pacheco et al. (2011) have found that 9189 CpG sites had different DNA methylation levels in the infertile patients, which can be associated with low-motility in the sperm samples when compared with fertile individuals (Pacheco et al., 2011). In that study, an increased *DNMT3A* expression has been detected in the sperm cells having low-motility and hypomethylated CpG sites (Pacheco et al., 2011).

Aberrant DNA methylation has been defined in the definite imprinting genes such as *H19*, *GTL2* (Gene trap locus 2), *PEG1* (Paternally expressed genes 1), *LIT1* (Long qT intronic transcript 1), *ZAC* (Sterile alpha motif and leucine zipper containing kinase AZK), *PEG3* (Paternally expressed genes 3), *SNRPN* (Small nuclear ribonucleoprotein polypeptide N) in oligozoospermic patients, who have partial spermatogenesis failure (Boissonnas et al., 2010; Kobayashi et al., 2007; Marques et al., 2008). Besides, global methylation errors have been identified in the testicular cells obtained from azoospermic patients (Marques et al., 2010; Minor et al., 2011). The observed DNA methylation alterations in the imprinting genes most likely originates from changed DNMT expression, especially that of *Dnmt3L* and *Dnmt3a* gene because *Dnmt3L* and *Dnmt3a* knockout male mice models exhibit azoospermia and DNA methylation aberration (Bourc'his and Bestor, 2004; Kaneda et al., 2004) (**Table 3**). Similarly, hypomethylation of the paternally imprinted *H19* gene in the oligozoospermic men might derive from impaired *Dnmt3a* and *Dnmt3L* gene expression (Marques et al., 2010). Ferfouri et al. (2013) demonstrated that there are 212 differentially methylated CpG sites in the testicular samples between obstructive azoospermia and non-obstructive azoospermia groups (Ferfouri et al., 2013). Overall, abnormal methylation at the imprinting genes in the spermatozoa from azoospermic patients seems to be associated with obstructive azoospermia and non-obstructive azoospermia development since the imprinting genes are largely methylated in the sperm samples from the obstructive azoospermia or non-obstructive azoospermia group (Marques et al., 2010).

As *DNMT1* is expressed in all human spermatogenetic cell types, it seems to be an important factor for normal spermatogenesis (Marques et al., 2011). Therefore, abnormal DNMT1 expression has been defined in the testicular tissues obtained from patients having spermatogenetic impairment and the similar issue was also observed in the mouse models (Takashima et al., 2009). The abnormal DNMT1 expression may also result from the presence of any mutation or polymorphism in the *DNMT1* gene: such a study by Cheng et al. (2014), three exonic single nucleotide polymorphisms (SNP; rs16999593, rs2228612 and rs2228611) in the *DNMT1* gene have been found to be related with development of severe oligospermia (Cheng et al., 2014). Additionally, Adiga et al. (2011) evaluated the *DNMT3B* gene expression in the spermatogenic cells at different stages of spermatogenesis in the fertile men and patients with bilateral spermatogenetic arrest (Adiga et al., 2011). They documented that *DNMT3B* is differentially expressed in the preleptotene/zygotene and pachytene spermatocytes from fertile and in infertile men. However, no remarkable expression difference for this gene is characterized in the elongated spermatids. Of note, although there are expressional differences in certain spermatogenic cells for the *DNMT3B* gene, the global DNA methylation do not exhibit any predominant discrepancy (Adiga et al., 2011).

The knockout mice models of the DNMTs gene shed light on understanding the molecular background of male infertility development. *Dnmt1*$^{-/-}$ causes demethylation of the genome (Lei et al., 1996; Li et al., 1992) (**Table 1**). The *Dnmt3a* conditional knockout mouse models exhibit spermatogenic arrest at the spermatogonial stage (Kaneda et al., 2004) and also *Dnmt3a* knockout male mice have azoospermia (Bourc'his and Bestor, 2004; Kaneda et al., 2004) whereas the *Dnmt3b* conditional knockout mice shows no infertile phenotype (Kaneda et al., 2004; Okano et al., 1999) (**Table 1**). The *Dnmt3L* knockout (*Dnmt3L*$^{-/-}$) mice are viable, but female mice fail to create viable pups (Saitou et al., 2012); however, the males are infertile showing the characteristic features of azoospermia (Bourc'his et al., 2001; Hata et al., 2002) (**Table 1**). Additionally, lack of the *Dnmt3L* gene causes loss of paternal methylation imprints, especially on the *H19* locus (Bourc'his and Bestor, 2004; Kaneda et al., 2004; Webster et al., 2005). As is known, although DNMT3L does not include any catalytic activity, it plays an important role in normal spermatogenesis (Vlachogiannis et al., 2015). Hata et al. (2006) examined histological structures of the *Dnmt3L*$^{-/-}$ testes at 7 weeks after birth (Hata et al., 2006). They found that the *Dnmt3L*$^{-/-}$ testes involve no spermatids and consist of only preleptotene or leptotene spermatocytes and Sertoli cells, meaning that there is no development pachytene spermatocytes or subsequent cell types. Furthermore, the expression of sex-chromosome-linked genes and gonad-specific genes are prominently decreased in the *Dnmt3L*$^{-/-}$ testes when compared with wild type counterparts (Hata et al., 2006). In another work, disruption of the *Dnmt3a* or *Dnmt3L* gene functioning in *de novo* methylation, causes *H19* hypomethylation in the prospermatogonial cells in mice (Kato et al., 2007).

Recently, Urdinguio et al. (2015) indicated that DNA methylation levels in the sperm cells obtained from patients with unexplained infertility exhibited different methylation patterns compared with the spermatozoa from fertile men: 2752 CpG sites exhibited abnormal DNA methylation patterns (Urdinguio et al., 2015) (**Table 3**). Of the total CpG sites analysed here, 1305 CpG sites were found to be hypomethylated, whereas 1447 of them underwent hypermethylation in the infertile patients. The data suggested that the significant changes of DNA methylation may be due to the presence of sperm specific methylation regions, which show predominant differences between fertile and infertile groups (Urdinguio et al., 2015). In recently published studies, researchers found that some demographic factors such as cigarette smoking and ageing may negatively affect DNA methylation establishment during spermatogenesis. For example, cigarette smoking causes an increased DNA methylation at the promoter region of the *Pebp1* (Phosphatidylethanolamine binding protein 1) gene in mice testis, and some of the distinctly methylated genes are known to be closely associated with spermatogenesis (Xu et al., 2013) and the remaining genes function in other somatic tissues (Scoccianti et al., 2011; Wangsri et al., 2012; Word et al., 2013). In a study evaluating the effect of ageing on DNA methylation, sperm cells from old mice pos-

sessed remarkably lower global DNA methylation levels in comparison with the sperm cells from young ones (Milekic et al., 2015). In addition, environmental stress also induces alterations in DNA methylation status of mammalian cells (Elhamamsy, 2016). Environmental factors such as cadmium exposure (Virani et al., 2016) and tobacco smoking can cause DNA methylation changes (Ambatipudi et al., 2016), and maternal smoking during pregnancy leads to DNA methylation impairments in the newborns (Joubert et al., 2016). In a recent study by Radford et al. (2014), they revealed that the in-utero nutritional environment potentially affects the male germ cell methylome, which may have an association with improving metabolic diseases in offspring (Radford et al., 2014). On the other hand, in females, aging is capable of altering global DNA methylation levels and expression profile of DNMT1, DNMT3A, DNMT3B and DNMT3L in MII oocytes and preimplantation embryos (except blastocyst). The global DNA methylation and expression of the DNMT proteins were predominantly reduced in the MII oocytes and preimplantation embryos from old mice when compared with young mice (Yue et al., 2012).

Conclusion

In conclusion, DNA methylation is a major epigenetic mark, which regulates temporal and spatial expression of the testicular genes required for normal spermatogenesis process. There are two types of methylation, maintenance and *de novo* methylation, which are catalysed by DNMT1, DNMT3A and DNMT3B enzymes, respectively. The lack of DNMTs leads to embryonic lethality, abnormal imprinting genes expression, loss of global DNA methylation levels and syndromes such as immunodeficiency, centromere instability and facial anomalies based on the lost DNMT type. The expression patterns of the DNMTs at mRNA and protein levels have been generally characterized in the spermatogenetic cell types in mice and humans. However, the molecular mechanisms regulating the DNMTs in the male germ cells are not fully understood. Also, there are a limited number of studies aimed to determine the relationship between DNMTs and male infertility development. New technologies such as genome-wide analysis enable us to assess many imprinted genes, other genes and repetitive regions, in which possible alterations may lead to male infertility development. Therefore, we think that new studies are necessary related to characterizing the potential effects of the abnormal expression of the DNMTs and subsequently appearing aberrant DNA methylation on regulating the testicular genes.

Acknowledgement

The authors thank to Donald Desmond for helpful comments and corrections on this article.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 26 February 2016; refereed 22 August 2016; accepted 23 August 2016.