REPRODUCTION
REVIEW

MicroRNAs and DNA methylation as epigenetic regulators of mitosis, meiosis and spermiogenesis

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Abstract

Spermatogenesis is composed of three distinctive phases, which include self-renewal of spermatogonia via mitosis, spermatocytes undergoing meiosis I/II and post-meiotic development of haploid spermatids via spermiogenesis. Spermatogenesis also involves condensation of chromatin in the spermatid head before transformation of spermatids to spermatozoa. Epigenetic regulation refers to changes of heritably cellular and physiological traits not caused by modifications in the DNA sequences of the chromatin such as mutations. Major advances have been made in the epigenetic regulation of spermatogenesis. In this review, we address the roles and mechanisms of epigenetic regulators, with a focus on the role of microRNAs and DNA methylation during mitosis, meiosis and spermiogenesis. We also highlight issues that deserve attention for further investigation on the epigenetic regulation of spermatogenesis. More importantly, a thorough understanding of the epigenetic regulation in spermatogenesis will provide insightful information into the etiology of some unexplained infertility, offering new approaches for the treatment of male infertility.

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Introduction

Epigenetic regulation refers to the inheritable changes of phenotype or gene expression caused by mechanisms other than modifications in the underlying DNA sequences (Egger *et al*. 2004). Epigenetic regulators can control the expression of genes at the transcriptional and/or post-transcriptional levels, and epigenetic disorders may lead to human diseases, including mental retardation and cancer. Formation of functional spermatozoa is a complex process which involves self-renewal of spermatogonia by mitosis, meiosis of spermatocytes to form haploid spermatids, and transformation of spermatids to spermatozoa via spermiogenesis. These orchestrated processes involve unique transcriptional regulation and comprehensive chromosome remodeling. To keep the totipotence of zygote, epigenetic markers of male germ cells must be reset. Consequently, male germ cells are particularly vulnerable to epigenetic defects. Thus, epigenetic abnormality could be a potential cause of male infertility. The epigenetic reprogramming includes primordial germ cell (PGC) specification, spermatogonial stem cell (SSC) renewal, chromosome remodeling in the meiosis and histone transition in sperm maturation.

There are several major epigenetic regulators, including small non-coding RNAs and DNA methylation. Notably, Human Genome Program indicates that genes encoding proteins comprise only about 2% of human genomics, whereas the remainder consists of non-coding nucleic acid. MicroRNA (miRNA) is a novel class of endogenous small RNA molecules (~18–22 nucleotides in length) that can negatively control their targeting genes. MiRNAs are initially transcribed by RNA endogenous polymerase as primary miRNAs and eventually incorporated into the miRNA-induced silencing complex (miRISC) under a series of processing steps (Bartel 2004). It has been demonstrated that miRNAs are involved in the mitotic, meiotic and post-meiotic phases of spermatogenesis by inhibiting the expression of target genes (Tang *et al*. 2007, Hayashi *et al*. 2008). DNA methylation refers to the process in

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which a methyl group is added to the cytosine nucleotide with the typical location in the symmetric CG contexts (Chen & Li 2004), and it is related to genomic imprinting, transposon repression and X-chromosome inactivation in normal development. DNA methylation defects are associated with infertility in humans. With the application of 5-aza-2′-deoxycytidine, a demethylation agent, aberrant male germ cell development has been detected in males with reduced fertility (Kelly *et al*. 2003). Therefore, a thorough understanding of epigenetic regulation in spermatogenesis is of great significance for the therapy of male infertility and for developing new approaches for male contraception. Based on recent advances in epigenetic regulation, we discuss herein the roles of miRNAs and DNA methylation in regulating mitosis, meiosis and spermiogenesis.

MicroRNA and spermatogenesis

MiRNAs are small non-coding regulatory RNA molecules that regulate gene expression either by targeting mRNA for degradation or by translation inhibition, as we illustrated in Fig. 1. In general, mature miRNA is processed through at least three sequential steps: i) the pre-miRNA (~70 nucleotides) is generated by the primary transcripts (pri-miRNA) through RNase III member Drosha and its cofactors Pasha/DGCR8 (Lee *et al*. 2003, Han *et al*. 2004); ii) the pre-miRNA is transported from the nucleus to the cytoplasm by Exportin 5 (Lund *et al*. 2004); and iii) the mature miRNAs are generated by Dicer cleavage (Hutvagner *et al*. 2001).

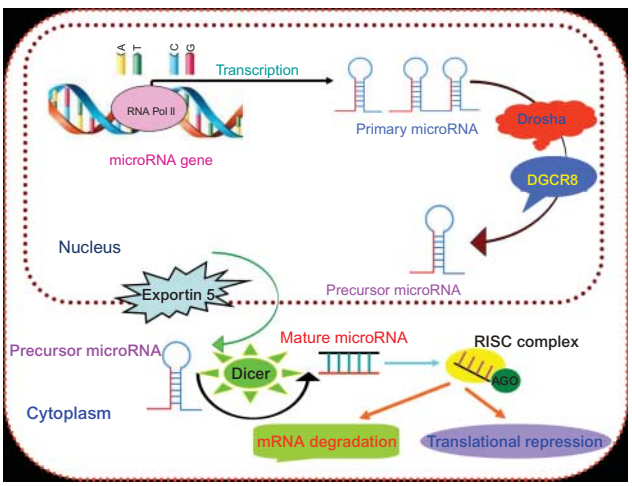

Figure 1 Schematic diagram shows miRNA biogenesis. MiRNA genes are transcribed as the primary capped and polyadenylated precursors of miRNA (pri-miRNAs) by RNA polymerase II in the nucleus, and the pri-miRNAs are further cleaved by Drosha and DGCR8. The processing of the pri-miRNAs by RNase III enzyme Drosha along with cofactor DGCR8 gives rise to the stem-loop pre-miRNA that is exported by Exportin 5 from the nucleus to the cytoplasm. In the cytoplasm, the RNase III enzyme Dicer catalyzes the pre-miRNA to form mature miRNAs. The mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) that guides the 3′-UTR of the target gene. The association of the miRNA-RISC results in the silencing of the target gene by mRNA degradation or translational repression.

MiRNA interacts with the targeting genes through specific base-pairing with the key domain of the mature miRNA, particularly at bases 2–8 of the 5′-end known as the 'seed' region. The mRNA is cleaved under the condition of perfect base-pairing between miRNAs and miRNA regulatory elements (MRE) in the 3′UTR of targeting mRNAs, whereas imperfect interaction between the miRNA and MRE leads to translational repression. The first discovered miRNA, namely, lin-4, acts as a post-transcriptional regulator (Lee *et al*. 1993, Wightman *et al*. 1993). Since then, a number of studies have shown that miRNAs are involved in various kinds of biological processes, including development, virus defense, hematopoiesis, organ formation, cell proliferation and apoptosis. Growing evidence has indicated that miRNAs play critical roles in regulating male germ cell development and are essential for epigenetic regulation of the mitosis, meiosis and spermiogenesis (Hayashi *et al*. 2008, Maatouk *et al*. 2008, Huszar & Payne 2013).

The roles of miRNAs in SSC self-renewal and differentiation

SSCs are located in the basal compartment of the seminiferous epithelium, and they play important roles in maintaining normal spermatogenesis and the transmission of genetic information to next generations. To date, studies on SSCs have been reported in other aspects, such as SSC transplantation, long-term culture of SSCs and transgenic animals (Brinster & Avarbock 1994, Khaira *et al*. 2005, Sadri-Ardekani *et al*. 2009, Kanatsu-Shinohara & Shinohara 2013). Significant advances have been made in the field of SSC biology, including gene regulation, signaling pathways and epigenetic regulators. Interestingly, a series of studies have demonstrated that miRNAs play essential roles in conferring mouse SSC fate determinations.

In the past several years, a number of miRNA expression profiles have been identified in the murine testes by miRNA microarrays, RT-PCR or small RNA sequencing technology (Hayashi *et al*. 2008, Buchold *et al*. 2010, Jung *et al*. 2010). The expression profiling of interesting miRNAs in the testis has been outlined in Table 1, and their potential roles in regulating SSC self-renewal and differentiation are illustrated in Fig. 2. In total, 141 miRNAs have been identified in mouse testis, of which 29 are novel by small RNA sequencing method (Ro *et al*. 2007). These miRNAs may be involved in mouse spermatogenesis. It has been reported that miR-21 is functionally important in regulating SSC ($Thy1^+$) population by working with ETV5, a critical transcription factor for maintaining the self-renewal of SSCs (Niu *et al*. 2011). Meanwhile, miR-34c, miR-182, miR-183 and miR-146a have been shown to be preferentially expressed in the SSC ($Thy1^+$)-enriched population

Table 1 Expression and roles of miRNAs in mammalian testis.

microRNA	Expression and roles	References
miR-17–92 and miR-290–295 clusters	miR-17–92 and miR-290–295 clusters are highly expression in spermatogonia	Hayashi *et al.* (2008)
miR-141, -200a, -200c and -323	Expression of miR-141, -200a, -200c and -323 is reduced during PGC development	Hayashi *et al.* (2008)
let-7a, d, e, f and g	Expression of let-7a, d, e, f and g is increased during PGC development	Hayashi *et al.* (2008)
miR-34c, -182, -183 and -146a	miR-34c, -182, -183, and -146a are preferentially expressed in Thy1$^+$-enriched population	Niu *et al.* (2011)
miR-21	miR-21 is important in maintaining mouse Thy1$^+$ population	Niu *et al.* (2011)
miR-20 and miR-106a	miR-20 and miR-106a regulate mouse SSC renewal by targeting STAT3 and Ccnd1	He *et al.* (2013)
miR-135a	miR-135a contributes to the maintenance of mouse SSC by regulating FoxO1	Moritoki *et al.* (2014)
miR-221 and 222	miR-221 and 222 play an important role in the maintenance of the undifferentiated state of mouse spermatogonia	Yang *et al.* (2013)
miR-17–92 cluster and miR- 106b–25	miR-17-92 cluster and miR-106b-25 are involved in the regulation of mouse spermatogonial differentiation	Tong *et al.* (2012)
Mirlet7 family	let-7 family miRNAs play a role in retinoic acid-induced spermatogonial differentiation	Tong *et al.* (2011)
miR-146	miR-146 regulates spermatogonial differentiation by retinoic acid	Huszar & Payne (2013)
miR-449/, miR-34b/c	miR-449 and miR-34b/c are located in mouse spermatocytes and spermatids	Bouhallier *et al.* (2010), Bao *et al.* (2012) and Wu *et al.* (2014)
miR-34a, -34b and -34c	miR-34a, -34b and -34c are expressed in bovine sperm	Tscherner *et al.* (2014)
miR-34c	miR-34c is highly expressed in mouse pachytene spermatocytes and round spermatids	Liang *et al.* (2012)
miR-469	miR-469 expression is associated with sperm development	Dai *et al.* (2011)
miR-122	miR-122 expression is involved in sperm development	Yu *et al.* (2005)

(Niu *et al.* 2011), reflecting potential roles of these miRNAs in mediating self-renewal and maintenance of mouse SSCs. On the other hand, miR-34c has been demonstrated to be involved in the differentiation of mouse SSCs via targeting Nanos2 (Yu *et al.* 2014). Taken together, miR-34 seems to be associated with both division and differentiation of SSCs through targeting different genes. Moreover, miR-135a has been shown to retain the undifferentiated state of SSCs via FoxO1 (Moritoki *et al.* 2014). Impairing the function of X chromosome-clustered miR-221/222 in mouse undifferentiated spermatogonia causes a transition from KIT$^-$ to KIT$^+$ state and the loss of stem cell ability to repopulate spermatogonia (Yang *et al.* 2013). This study suggests that miR-221/222 might play an important role in the maintenance of the undifferentiated state of mammalian spermatogonia. In addition, the expression of miR-17–92 (Mirc1) and miR-106b–25 (Mirc3) clusters has been shown to be significantly down-regulated by retinoic acid induction (Tong *et al.* 2012). During the development of male germ cells, miR-17–92 and miR-290–295 cluster are highly expressed in mouse spermatogonia (Hayashi *et al.* 2008). Collectively, these findings suggest that miR-17–92 clusters play potential roles in maintaining the stemness of SSCs. Consistent with these observations, we have recently demonstrated that miR-20 and miR-106a are required for the proliferation and survival of mouse SSCs through targeting Stat3 and Ccnd1 (He *et al.* 2013).

SSC differentiation is an essential step in spermatogenesis, which has been found to be modulated by miRNAs. The expression of miR-141, miR-200a, miR-200c and miR-323 are down-regulated during male germ cell development, whereas the levels of let-7 family (let-7a, d, e, f and g) are up-regulated (Hayashi *et al.* 2008), indicating distinct roles of these miRNA in male germ cell differentiation. This speculation has been verified by the findings that the members of miRNA let-7 family (e.g., let-7a/b/c/d/e) regulate mouse SSC differentiation (Tong *et al.* 2011, 2012). MiR-146 is involved in mouse

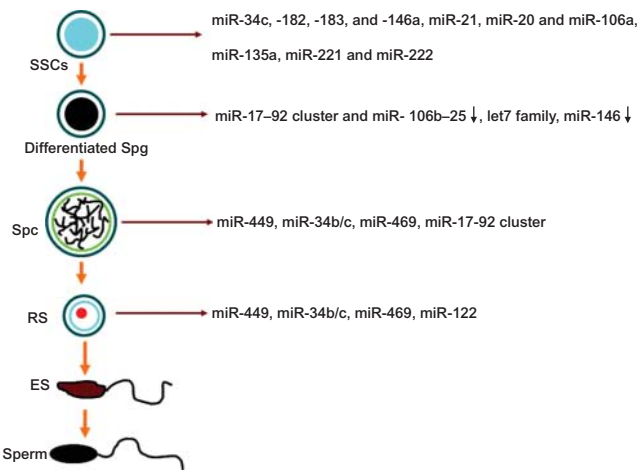

Figure 2 The expression patterns of miRNAs in various types of cells in mammalian testis. MiR-17-92 and miR-290–295 cluster are highly expressed in mouse spermatogonia, and numerous miRNAs (e.g. miR-34c, -182, -183 and -146a, miR-21, miR-20, miR-106a, miR-135a, miR-221, and miR-222) have been shown to regulate SSC self-renewal. In contrast, miR-17–92 cluster, miR-106b–25, let-7 family and miR-146 are involved in the regulation of mouse spermatogonial differentiation. MiR-449, miR-34b/c and miR-469 are located in mouse spermatocytes and spermatids, while miR-34a, -34b, -34c and miR-122 are associated with sperm development. SSCs, spermatogonial stem cells; Spc, spermatocytes; RS, round spermatids; ES, elongating spermatids.

SSC differentiation induced by retinoic acid signaling, because its expression is diminished by ~180-fold in differentiating spermatogonia vs undifferentiated spermatogonia by targeting mediator complex subunit 1, a coregulator of retinoid receptors (RARs and RXRs) (Huszar & Payne 2013). In addition to regulating the differentiation, Mir-17-92 (Mirc1) cluster also protects meiotic cells from apoptosis by down-regulating E2F1 translation (Novotny *et al*. 2007). Although numerous miRNAs have been found in modulating the self-renewal and differentiation of SSCs in rodents, there are no reports in the literature supporting miRNA function in human SSCs due to difficulties in obtaining human testis tissues in particular human SSCs. We have recently compared the global miRNA profiles among human spermatogonia, pachytene spermatocytes and round spermatids, illustrating that 110 miRNAs are differentially expressed between human spermatogonia and pachytene spermatocytes (Liu *et al*. 2015), suggesting that these miRNAs are involved in mitosis and meiosis.

Functions of miRNAs in meiosis and spermiogenesis

Meiosis of spermatocytes and spermiogenesis of spermatids are two indispensable phases of spermatogenesis through which functional male gametes are generated. These intricate processes must be tightly regulated, and any mistake in these processes leads to the abnormality of spermatogenesis. Some unexplained male infertility may be the result of meiotic arrest in spermatocytes and/or defects of spermiogenesis in post-meiotic spermatids. Notably, miRNAs likely play a role in regulating gene expression throughout these developmental stages. Drosha is required for the biogenesis of miRNAs in spermatogenesis, and conditional knockout of Drosha in male germ cells leads to a significant decrease of miRNAs, reduces testis weight and a severe disruption in both meiotic and haploid phases of spermatogenesis (Wu *et al*. 2012). Meanwhile, Dicer is essential for the biogenesis of both miRNAs and siRNAs in spermatogenesis. After conditional knockout of miRNA maturation-related key enzyme Dicer in testes, the number of mouse SSCs is decreased and their differentiation is also blocked. In addition, loss of Dicer1 results in male infertility in mice (Maatouk *et al*. 2008), illustrating an essential role of miRNAs in spermatogenesis. Sertoli cell-specific deletion of Dicer, a central component of the RNAi machinery, severely impairs Sertoli cell competence, leading to male infertility due to the absence of mature spermatozoa and testicular degeneration (Papaioannou *et al*. 2009), reflecting an important role of the Dicer for male germ cell development. Germ cell-specific deletion of Dicer1 leads to overexpression of genes for meiotic sex chromosome inactivation (MSCI), an increase in spermatocyte apoptosis and defects in chromatin organization and nuclear shaping of elongating spermatids (Korhonen *et al*. 2011, Romero *et al*. 2011, Greenlee *et al*. 2012, Zimmermann *et al*. 2014), suggesting that Dicer1 is required for the meiotic and haploid phases of spermatogenesis. In addition to Dicer, DGCR8 has been shown to be indispensable for the biogenesis of miRNAs but not endo-siRNAs, and similar symptom occurs in the conditional DGCR8-knockout mice, although the phenotype is less severe compared to the Dicer1-knockout mice (Zimmermann *et al*. 2014). Moreover, the knockout study of canonical enzymes in the miRNA biogenesis process reveals that miRNAs are of great importance in meiosis and spermiogenesis phases of spermatogenesis since spermatogonial differentiation appears to be unaffected by canonical enzyme deficiency. These findings suggest that miRNAs necessary to confer spermatogonial differentiation may be synthesized by non-canonical enzymes, and the underlying mechanism remains to be elucidated.

It has recently been found that miR-449 is predominantly expressed in mouse testes and it is mainly located in spermatocytes and spermatids (Bao *et al*. 2012, Wu *et al*. 2014). Interestingly, miR-34b and miR-34c have been found to resemble the 'seed' sequence of miR-449. Coincidentally, miR-34b and miR-34c exhibit a similar effect to that of miR-449 during the development of male germ cells and spermatogenesis (Bouhallier *et al*. 2010, Bao *et al*. 2012, Wu *et al*. 2014). Individual deficiency in miR-34b/c or miR-449 appears to have no obvious effect; however, simultaneous inactivation of miR-34b/34c and miR-449 leads to mouse oligoasthenoteratozoospermia (Wu *et al*. 2014), implicating that double or triple knockout approach of miRNAs is needed to obtain the phenotype for certain miRNAs. It has been demonstrated that all the members of miR-34 family (i.e. miR-34a, -34b and -34c) are found in bovine sperm (Tscherner *et al*. 2014). Additionally, miR-34c seems to be important for the later steps of spermatogenesis because it is highly expressed in mouse pachytene spermatocytes and round spermatids in murine testis by targeting the activating transcription factor 1 (ATF1; Liang *et al*. 2012). Moreover, the induction of ATF1 in spermatocytes of the pachytene stage is essential for maintaining cell viability and mouse early development (Bleckmann *et al*. 2002, Persengiev & Green 2003). These findings illustrate that the members of miR-34 family are vital for spermatogenesis. The expression of transition protein (TP) and protamine (Prm) proteins are required for spermatid elongation and spermiogenesis. MiR-469 has been found to be essential for the timely translation at later stages of spermiogenesis by inhibiting of TP2 and Prm2 transcripts (Dai *et al*. 2011). Another miRNA, namely miR-122, has been shown to reduce the expression of TP2 at transcription level by mRNA cleavage (Yu *et al*. 2005). These studies illustrate that miR-469 and miR-122 are associated with spermiogenesis. MiR-18 is abundantly expressed in the testis and it displays cell type-specific expression, with the highest intensity in spermatocytes. Meanwhile, miR-18 directly targets heat

shock factor 2, which is a key transcription factor essential for influencing embryogenesis and gametogenesis (Bjork *et al*. 2010).

Collectively, miRNAs play essential roles in regulating each step of male germ cell development, including mitosis, meiosis and spermiogenesis in rodents. Nevertheless, it remains to be defined whether miRNAs are required for spermatogenesis in humans. Recently, we have identified 559 miRNAs distinctively expressed by human spermatogonia, pachytene spermatocytes and round spermatids (Liu *et al*. 2015). Using miRNA microarray, we have identified 144 miRNAs that are significantly up-regulated while 29 miRNAs are down-regulated in pachytene spermatocytes vs round spermatids (Liu *et al*. 2015), reflecting essential roles of these miRNAs in mediating spermiogenesis. A number of novel binding targets of the differentially expressed miRNAs have been identified using various bioinformatics software and verified by real time PCR (Liu *et al*. 2015). Significantly, our ability to unveil the global distinct miRNA signatures and binding targets of human spermatogonia, pachytene spermatocytes and round spermatids could provide novel small RNA regulatory mechanisms mediating three stages of human spermatogenesis and offers new targets for treatment of male infertility.

DNA methylation and spermatogenesis

DNA methylation, by definition, is an epigenetic modification that DNA methyltransferase transfers a methyl unit from the *S*-adenosyl-L-methionine to the 5th position of cytosine residues in nucleotides (Chen & Li 2004, Portela & Esteller 2010). DNA methylation occurs in the cytosine-phosphate-guanine dinucleotides (CpGs) context, although it is also located in few non-CpG sites; however, the function of DNA methylation situated in non-CpG sites remains unclear (Jones 2012). Dinucleotides with high frequency of CpG palindromes are called CpG islands (CGI). The CpG island is defined as the DNA region whose G & C content should be equal to or $>55\%$ of the base pairs, and the ratio of observed-to-expected CpGs is over 0.65 (Takai & Jones 2002). Normally, CpG islands located in transcription start sites (TSS) are unmethylated in developmental and housekeeping genes, and the active TSS is marked by the trimethylation of histone H3 lysine 4 (H3K4me3) (Thomson *et al*. 2010, Messerschmidt *et al*. 2014). The CGI promoter can be repressed by various molecular strategies. On one hand, CGI promoter undergoes *de novo* methylation and it can be kept in methylated state by maintaining methyltranferase activity. This mechanism is stable and lasts for a long period, and it is responsible for X chromosome inactivation and genomic imprinting (Jones & Liang 2009). On the other hand, CGI promoter can be repressed by a large protein complex through binding to the target genes so that the chromatin is enclosed (Boyer *et al*. 2006). Its target gene is marked with repressive and active H3K4me3, which is called the bivalent modification pattern (Bernstein *et al*. 2006, Pan *et al*. 2007). This strategy is unstable and the silenced genes can be reactivated under certain circumstances (Cedar & Bergman 2009). The CpG islands located in gene bodies are marked by H3K9me3 and they bind to MeCP2 which is associated with histone deacetylation and chromatin condensation to repress gene expression (Nguyen *et al*. 2001). In contrast to CpG islands located in the TSS, methylation of CpG islands located in gene bodies is able to activate genes (Jones 1999).

There are two patterns of DNA methylation, namely, the maintenance methylation and the *de novo* methylation (Holliday & Pugh 1975). A number of DNA methyltransferases, including DNMT1, DNMT1o, DNMT3a, DNMT3b and DNMT3L, are involved in these two mechanisms. DNMT1 is responsible for maintenance methylation activity and it has a high affinity for hemi-methylated DNA (Pradhan *et al*. 1999). It is composed of C-terminal methyltransferase domain and N-terminal regulation domain (Song *et al*. 2012). N-terminal domain interacts with proliferating cell nuclear antigen (PCNA; Chuang *et al*. 1997) and UHRF1, which facilitates DNMT1 binding to the replication fork (Bostick *et al*. 2007, Hashimoto *et al*. 2008). DNMT1 recognizes the hemi-methylated DNA and methylates the newly generated strand so that the DNA methylation patterns can be maintained (Cedar & Bergman 2012). DNMT3a and DNMT3b are responsible for the *de novo* methylation (Okano *et al*. 1998). After the wave of demethylation in PGCs, the imprinted genes and transposon methylation patterns can be reestablished through the *de novo* methylation (Law & Jacobsen 2010). The *de novo* methylation mechanism is interpreted by both histone modification and piRNA pathways (Ooi *et al*. 2007). As an example, H3K4 methylation may be involved in *de novo* methylation. DNMT3L lacking the methyltransferase activity (Chedin *et al*. 2002) can recruit DNMT3a2 to the unmethylated H3K4 tail site (Jia *et al*. 2007), whereas this activity is inhibited by the methylation of the H3K4 tails (Ooi *et al*. 2007). Consequently, only the unmethylated H3K4 leads to the *de novo* methylation. Additionally, piRNA pathways may also explicate *de novo* methylation, since piRNA is related to the transposon silencing and the silenced transposons are methylated through *de novo* methylation (Aravin *et al*. 2007). PiRNA can interact with PIWI proteins, including MILI and MIWI. Notably, male germ cells with MILI and MIWI2 deficiency assume DNA methylation defect in retrotransposons regulation regions in the phase of *de novo* methylation (Kuramochi-Miyagawa *et al*. 2008). Meanwhile, piRNA–PIWI complex could indirectly recruit the *de novo* methyltransferase through interacting with chromatin modifiers (Aravin & Bourc'his 2008). Together, these findings indicate that the

de novo methylation regulates the development of male germ cells.

There are also two different modes for demethylation, namely the passive and active demethylation. Aberrant maintenance of methylation pathways in the replication may decipher the passive demethylation which is replication-dependent (Mertineit *et al.* 1998, Kagiwada *et al.* 2013). Meanwhile, the active demethylation is likely an enzymatic process. First of all, 5mC is hydroxylated by ten–eleven translocation enzyme to become 5hmC and further to 5-formylcytosine (5fC) or 5-carboxycytosine (5caC) (He *et al.* 2011, Ito *et al.* 2011). Secondly, the modified 5mC can be recognized by the activation-induced deaminase (Aid) or apolipoprotein B mRNA-editing enzyme catalytic polypeptide 1 (Apobec1) and converted to thymidine, which results in T:G mismatch. After the removal of the T:G mismatch by the glycosylase thymine–DNA glycosylase and methyl-CpG binding domain protein 4, the site can be repaired by a base pair excision repair mechanism to regenerate the unmethylated cytosine (Rai *et al.* 2008, Cortellino *et al.* 2011).

Bimodal pattern of DNA methylation in male germ cells

As illustrated in Fig. 3, DNA methylation in male germ cell specification and maturation displays a bimodal pattern. PGC, the origin of male germ cells, derives from epiblast at E6.5–E7.5. Once stimulated by BMP4, PGCs migrate from the epiblast to hindgut at E7.5–E9 and the genital ridge at E9.5–E11.5 (Saitou & Yamaji 2012). In the E6.5 mouse embryo, the DNA patterns in PGCs are similar to those in somatic cells in term of their fates. For instance, the pluripotent markers are repressed by DNA hypermethylation (Seisenberger *et al.* 2012). During the migration of PGCs, the epigenetic marks are widely erased (Popp *et al.* 2010). In particular, the paternal imprinted genes and transposons of PGCs are also demethylated. The re-establishment of DNA methylation patterns in male germ cells initiates from the prospermatogonia or gonocytes. Although a great proportion of DNA methylation is acquired during the prenatal mitotic arrest of the gonocytes, the *de novo* methylation and maintenance methylation occur in the mitosis of spermatogonia and meiotic prophase I, while the maintenance methylation appears only in mitosis (Santos *et al.* 2005, Oakes *et al.* 2007).

The global erasure of the DNA methylation also occurs during early embryonic development (Mayer *et al.* 2000, Oswald *et al.* 2000). The outcomes and gene expression patterns of demethylation may differ between early embryogenesis and PGCs. First, the demethylation during the early embryogenesis results in totipotency, whereas the demethylation of the PGCs derived from the epiblast leads to pluripotency. Secondly, the demethylation manner during the early embryogenesis is similar to the process of demethylation in PGCs with the exception of the imprinted genes, which facilitate the expression of parent-specific genes in embryos. Finally, it has been demonstrated that the sperm and oocyte genomes have different demethylation modes during embryogenesis, and active DNA demethylation appears in parental genome compared to the replication-dependent demethylation in maternal genome (Santos *et al.* 2005).

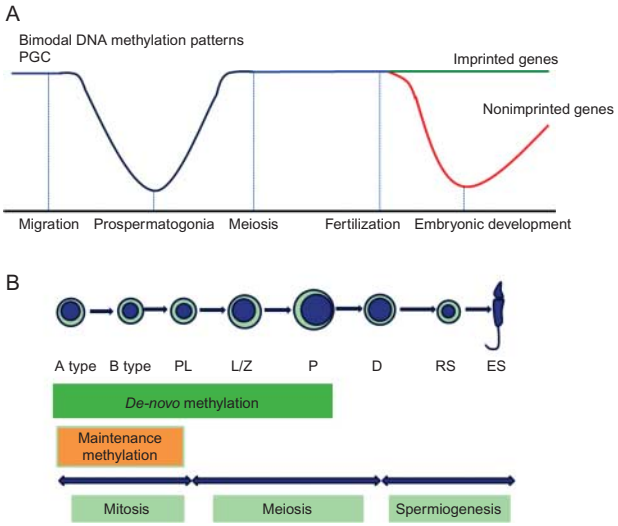

Figure 3 Schematic diagram reveals the expression of DNA methylation profiles in mammalian spermatogenesis. (A) Bimodal DNA methylation patterns in male germ cell development. PGCs are derived from epiblast at E6.5 and they migrate to the genital ridge. During the migration of PGCs, the epigenetic marks are widely erased. After the erasure of the DNA methylation markers, reestablishment of the male germ cell DNA patterns initiates from prospermatogonia to entering meiosis. After fertilization, DNA patterns are broadly erased by active demethylation activity, whereas the imprinted genes are maintained by DNMT1o activity. (B) The *de-novo* methylation and maintenance methylation activity initiates from type A spermatogonia to meiosis. A type, type A spermatogonia; B type, type B spermatogonia; PL, preleptotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; RS, round spermatids; ES, elongating spermatids.

DNA methylation and SSC division and differentiation

To maintain the stem cell pool, SSCs either remain in a quiescent state or undergo self-renewal. DNA methyltransferase 3-like (DNMT3L) is involved in SSC quiescence. DNMT3L positively regulates the stability of promyelocytic leukemia zinc finger (PLZF) in THY1$^+$ SSCs through down-regulating CDK2 expression, which may degrade PLZF through the ubiquitin pathway (Liao *et al.* 2014). The distal CpG island of Cdk2 promoter has been shown to be hypomethylated in Dnmt3l-knockout SSCs compared to the wide-type cells (Liao *et al.* 2014). On the other hand, it has been reported that the expression of DNMT3a2 and DNMT3b is undetectable in PLZF-positive and KIT-negative SSCs (Shirakawa *et al.* 2013). Thus, there are distinctive roles for different

members of DNMT3 subfamily in regulating mitotic self-renewal of mouse SSCs.

Besides undergoing self-renewal, SSCs also give rise to differentiated spermatogonia. DNA methylation may be required to the transition from the Kit-negative SSCs to Kit-positive differentiated spermatogonia. It has been demonstrated that the transcripts of DNMT3a2 and DNMT3b remains at the highest level in type A spermatogonia compared to other types of male germ cells. Shirakawa *et al.* (2013) have recently reported that DNMT3a2 and DNMT3b are not present in PLZF-positive and KIT-negative SSCs. Conversely, DNMT3a2 and DNMT3b expression is parallel with the Kit expression in spermatogonia. These observations suggest that DNA methylation is less prevalent in SSCs vs differentiated spermatogonia. Furthermore, overexpression of DNMT3b in the Kit-negative SSCs can induce Kit expression (Shirakawa *et al.* 2013). Altogether, the DNMT3a2 and DNMT3b may methylate genes essential for maintaining SSCs in an undifferentiated state and facilitating the transition from the undifferentiated spermatogonia to the differentiated state. It is of great interest to further explore the interaction between the DNA methylation dynamics and SSC differentiation in a global scale, which can shed light on the epigenetic causes of the non-obstructive azoospermia with spermatogenesis arrest in the SSC phase.

The role of DNA methylation on meiosis and spermiogenesis

The *de novo* DNA methylation and maintenance methylation are associated with meiosis in male germ cells, as illustrated by the observation that the expression of DNMT1, DNMT3a2 and DNMT3b is up-regulated in leptotene and zygotene spermatocytes (Oakes *et al.* 2007). The methylation of testis-specific genes PRM1 and PRM2 has been shown in meiotic prophase I (Trasler *et al.* 1990), and the imprinted genes and non-CpG island sequences are methylated in meiosis (Oakes *et al.* 2007). Demethylation of testis-specific genes may be required for meiotic initiation, since DNA methyltransferases are attenuated significantly in type B spermatogonia and preleptotene spermatocytes. Recently it has been reported that down-regulation of DNMT3L may increase the expression of the premeiotic-specific gene-STRA8, leading to the onset of meiosis prematurely (Vanhoutteghem *et al.* 2014). In addition, it has been revealed that 5mC-specific dioxygenase (TET1) can mediate the demethylation of the locus-specific genes, including the meiotic genes, and promotes the meiotic initiation through the activation of these genes in oocytes (Yamaguchi *et al.* 2012). It remains to be determined whether the function of TET1 in spermatogenesis resembles oocytes. Nevertheless, it is tempting to speculate that the meiotic initiator may be demethylated and activated in spermatogonia and preleptotene spermatocytes. Overexpression of DNMT3a2 and DNMT3b during this phase or knockout of TET1, Aid and Apobec1 can be helpful to identify the essential factors in meiotic initiation.

In mice, the expression of DNMT1, DNMT3a2 and DNMT3b is enhanced in leptotene and zygotene spermatocytes and reduced in pachytene spermatocytes (La Salle & Trasler 2006). In contrast to rodents, the expression of DNMT1, DNMT3a2 and DNMT3b peaks in pachytene spermatocytes in humans (Marques *et al.* 2011). These differences in the expression of DNMTs in rodent vs human male germ cells may result from the different isolation approaches used for separating these cells. The former utilized the STA-PUT method by cell gravity, while the latter employed the micromanipulation based upon the cellular diameters from testicular biopsy. However, the methylation activity in human pachytene spermatocytes is limited to the expression level and needs to be verified by functional assays. Additionally, the mechanisms that maintain epigenetic marks through meiosis especially in pachytene spermatocytes require additional studies and verification. Notably, during spermiogenesis in both mice and humans, DNMT1, DNMT3a2 and DNMT3b are highly expressed in round spermatids (La Salle & Trasler 2006, Marques *et al.* 2011). Interestingly, DNMT1 is present in non-proliferative round spermatids whilst DNMT3a2 and DNMT3b are expressed after the establishment of paternal methylation pattern. Thus, DNMT3a2 and DNMT3b may function in maintaining the methylation patterns through the *de novo* methylation pathway, although the roles of DNMT1 in round spermatids remain to be elucidated in future studies.

Summary

In summary, we have discussed recent advances in the field regarding the roles of novel epigenetic regulators, including miRNAs and DNA methylation, in mitosis, meiosis and spermiogenesis. Although much progress has been made, there are many issues remaining to be investigated. First, more studies should be conducted in uncovering the spatiotemporal and sequential expression of these epigenetic regulators in male germ cells during spermatogenesis. Also, the roles of these epigenetic mediators in controlling spermatogenesis remain unknown. Secondly, it remains unclear which epigenetic regulators are the actual initiators for the onset of mitosis, meiosis and/or spermiogenesis. Thirdly, most of the information on the epigenetic regulators in spermatogenesis are derived from rodents, very little is known about epigenetic regulation on human spermatogenesis due to the difficulties in obtaining human testis tissues. Since cell types and stages of spermatogenesis are distinct between humans and rodents, it is essential to identify whether epigenetic regulators in rodent spermatogenesis are similar to humans. Recently it has

been shown that human SSCs can be induced to differentiate into haploid spermatids which were used successfully for fertilization with full developmental capacity (Yang *et al*. 2014). It is interesting to explore what epigenetic regulators (miRNAs and DNA methylation) are involved in human SSC mitosis and differentiation. Finally, it is postulated that there are certain check points in the spermatogenesis, e.g. the progress from SSCs to differentiating spermatogonia, the entering of mitotic germ cells to meiotic process, and the transition from the canonical histone to the PRM in spermatids. During these critical processes, the interactions or crosstalk among epigenetic regulators remain unknown. Significantly, the stringent epigenetic regulation in spermatogenesis would yield novel insights into the etiology of sterility/infertility and offer new targets for gene therapy of male infertility. To uncover new epigenetic regulators on spermatogenesis will also facilitate the development of new approaches for male contraception since it is feasible to control the epigenetic regulation artificially without changing DNA sequences and the process is reversible and safe.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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