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Epigenetic Regulation of Spermatogonial Stem Cell Homeostasis: From DNA Methylation to Histone Modification

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Abstract

Spermatogonial stem cells(SSCs)are the ultimate germline stem cells with the potential of self-renewal and differentiation, and a dynamic balance of SSCs play an essential role in spermatogenesis. During the gene expression process, genomic DNA and nuclear protein, working together, contribute to SSC homeostasis. Recently, emerging studies have shown that epigenome-related molecules such as chromatin modifiers play an important role in SSC homeostasis through regulating target gene expression. Here, we focus on two types of epigenetic events, including DNA methylation and histone modification, and summarize their function in SSC homeostasis. Understanding the molecular mechanism during SSC homeostasis will promote the recognition of epigenetic biomarkers in male infertility, and bring light into therapies of infertile patients.

Keywords Epigenetics · DNA methylation · Histone modification · Spermatogonial stem cells · Spermatogenesis

Introduction

Life-long male fertility relies on the continuous production of sperm that derive from spermatogonial stem cells (SSCs). On the one hand, as reproductive stem cells, SSCs need to proliferate and differentiate like embryonic stem cells and maintain a relatively stable number through self-renewal. On the other hand, SSCs undergo mitosis, meiosis, spermiogenesis, and finally produce sperm [1]. SSCs are located at the basement membrane of seminiferous tubules. In the first week after birth in mice, some primordial germ cells (PGCs) produced SSC, and the other part directly developed progenitor and differentiating spermatogonia, which participated in the first wave of spermatogenesis [2–4].

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Epigenetics refers to heritable changes in gene expression without changing the DNA sequence. DNA methylation and histone modification are two critical epigenetic events that regulate SSC homeostasis by transcriptional regulation. DNA methyltransferases (DNMTs) catalyze DNA methylation by adding a methyl group to the fifth carbon of cytosine to produce 5-methylcytosine (5mC). DNA methyltransferase 1(DNMT1) maintains DNA methylation that has been methylated, while DNA methyltransferase 3 s (DNMT3s) catalyze *de novo* DNA methylation [5]. Histone tail modification, including methylation, acetylation, ubiquitination, phosphorylation, take part in both transcriptional activation and gene silencing. For example, H3K4 methylation at the transcription start sites promotes active transcription, while H3K9 and H3K27 methylation contributes to gene repression [6]. More importantly, emerging studies reveal that the crosstalk between DNA methylation and histone modification is essential for SSC homeostasis [7, 8].

Recent studies have discovered numerous DNA methyltransferases, histone modifiers that are crucial for SSC homeostasis [9–12]. In this review, we focus on the studies of epigenetic regulation in SSC homeostasis in mammals. We mainly summarize and discuss the chromatin modifications, including DNA methylation and histone modification, that occur during SSC homeostasis. Our discussion keeps pace with the latest studies in this field and we illustrate chromatin modification changes and their function in SSC homeostasis. This review would advance our understanding of chromatin modification changes during SSC homeostasis, and inspire male infertility treatment in the clinic.

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SSC Developmental Models

Based on the morphological features, spermatogonia can be classified to type A, intermediate, and type B spermatogonia. A-single (A_s), A-paired (Ap), and A-aligned (A_{al} 4–16) spermatogonia are seen as undifferentiated spermatogonia, while A1-A4 plus intermediate and type B spermatogonia are differentiating spermatogonia. Undifferentiated spermatogonia can be subdivided into SSCs and progenitors. Maintenance of male fertility relies on homeostatic SSCs [13, 14]. Until now, three models have been proposed to describe the characteristics of SSCs (Fig. 1a-c). In the male germline, SSCs are the only stem cells that maintain self-renewal ability, and therefore the SSC model proposed is crucial for us to study male fertility.

"Classic A_s model" classifies spermatogonia according to morphological characteristics. In this model, A_s are actual stem cell that can either self-renew by complete cytokinesis or produce A_{pr} by incomplete cytokinesis, while A_{pr} and A_{al} are progenitors that lose stem cell capabilities and differentiate into A1 spermatogonia. Therefore, chain length is the only criterion for distinguishing spermatogonia from SSC and differentiation-committed spermatogonia [1, 15, 16]. This model has been challenged by numerous studies in recent years due to the emergence of advanced technologies particularly the application of transplantation and lineage tracing assay [17, 18]. For example, multiple studies demonstrated that not all A_s are equivalent, and some A_s can express NGN3 and KIT, which represent differentiation priming and differentiation, respectively [18]. Therefore, the 'revised A_s model' and 'dynamic A_s model' are became the two most popular models at present and summarized as below sections.

According to the "revised A_s model," also called 'A_s hierarchical model,' only a specific subset of A_s within ID4-GFP (or LHX1, BMI1, ETV5, BCL6B, PAX7, etc.) signal contributes to SSC pool [13, 19–22]. A_s within bright ID4-GFP signals are named as $SSC_{ultimate}$, it can either self-renew or differentiate. Undifferentiated spermatogonia within dim ID4-GFP signal are named as $SSC_{transitory}$, it hardly renews itself but mainly produces progenitor cells [23]. ID4 (inhibitors of DNA binding 4) belongs to the inhibitor of DNA binding (ID) family and can be unregulated by GDNF signaling [24]. Lineage tracing studies revealed restricted expression patterns of ID4 in A_s and A_{pr} spermatogonia, and quantitative assessment showed that all $ID4^+$ cells are SSCs [25]. Analogously, the transplantation assay showed that stem cell potential is limited to $ID4^+$ undifferentiated spermatogonia, and $ID4^-$ cells are differentiation-committed [20, 26]. Nonetheless, this model suffers from limitations because the niche of SSCs in neonatal mice differs from that in adulthood [27]. The ID4 signal can be detected in 93% of $PLZF^+$ spermatogonia in neonatal mice but rapidly decrease, and only 2% of $PLZF^+$ cells express ID4 in adult testis [20].

The "dynamic A_s model" proposes that $GFR\alpha1^+$ spermatogonia and $NGN3^+$ spermatogonia represent SSCs and progenitors, respectively. GFRα1 is the co-receptor for GDNF and present in the majority of A_s and A_{pr} [17, 28]. The lineage-tracing study revealed that under homeostatic conditions all stem cell activities are limited in $GFR\alpha1^+$ spermatogonia, but transplantation assay showed that under regenerative conditions $GFR\alpha1^+$ cells are not the only for long-term spermatogenesis and a proportion of $GFR\alpha1^-$ cells qualify stem cell potential [17, 18, 29]. Additionally, intravital live imaging revealed that the fragmentation of A_{pr} and A_{al} produces A_s, and $GFR\alpha1^+$ A_s incompletely divide to generate A_{pr} rather than completely divide to generate two A_s, which largely differ from "revised A_s model" [17]. NGN3, a progenitor maker, is present in A_{al} and a small proportion of A_{pr} but rarely detected in A_s [18, 30]. In homeostatic testes, $NGN3^+$ spermatogonia usually differentiate to A1 with a tiny minority function as SSCs [31]. Once tissue damage occurs, they can reverse back to stem cells and reconstruct spermatogenesis [30].

The question of what are SSCs has brought a lot of arguments in the reproductive medicine field, but until now neither 'revised A_s model' nor "dynamic A_s model" totally defined SSCs. Different materials and technologies drive two widely divergent viewpoints between these two models. One is how to maintain SSCs: complete cytokinesis or syncytial fragmentation. Another is whether progenitors contribute to the stem cell pool. Despite the contrasting stem cell models, these two models confirm that chain fragmentation and self-renewal activities exist in $GFR\alpha1^+$ spermatogonia, and undifferentiated spermatogonia are the functional hierarchy and environmental dependent.

DNA Methylation in SSCs

Among epigenetic events, DNA methylation is one of the most investigated and usually involves heterochromatin formation and gene repression [32]. The methylation reaction includes two types: the maintenance of methylation and *de novo* methylation [33]. The former occurs during DNA replication process. When the methylated double-stranded DNA undergoes replication to generate two new double-stranded DNA, only the parental strand is methylated and the newly-synthesized daughter strand is unmethylated. DNA methyltransferase 1 (DNMT1) uses asymmetric methylated DNA as a substrate to identify the methylated CpG sites on the single parent strand of the newly formed DNA double-strand and then catalyzes the methylation of cytosine (C) at the newly-synthesized DNA strand [5]. *De novo* methylation is a reconstruction of the methyl-state of DNA [34]. It catalyzes methylation at unmethylated DNA double-strand and does not rely on DNA replication [35]. The maintenance of DNA

methylation depends on the activities of DNMT1, while *de novo* methylation needs DNMT3A and DNMT3B [34].

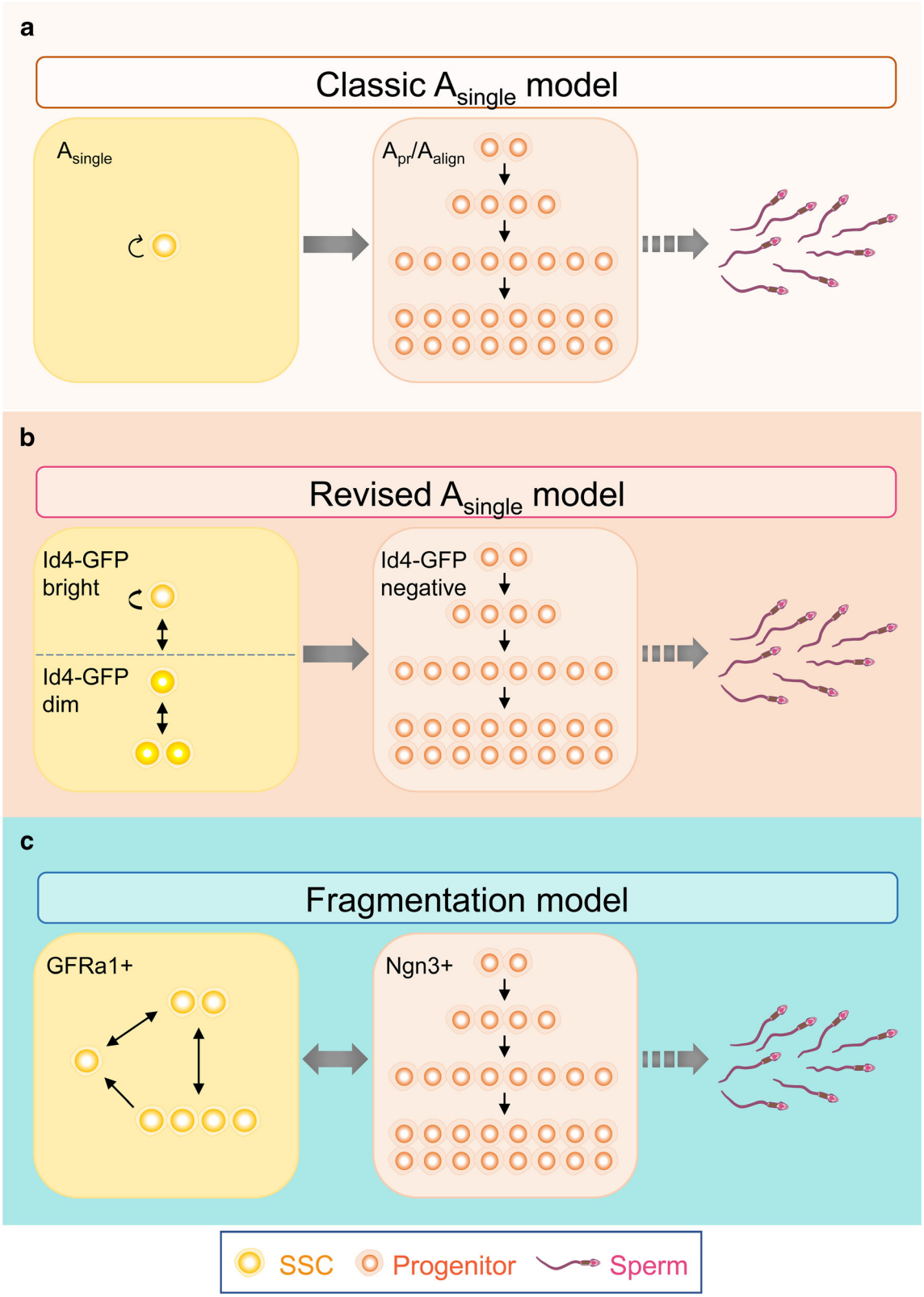
DNMT1 is abundant in spermatogonia and the depletion of DNMT1 induces apoptosis of SSCs because global DNA hypomethylation trigger defective reprogramming [36, 37]. UHRF1, a multifunctional protein, can recruit DNMT1 on DNA replication forks to maintain DNA methylation [38, 39]. Our recent study showed that UHRF1 is highly expressed in the cytoplasm of prospermatogonia at embryo day 15.5 and translocated to the nuclei of spermatogonia after birth [40]. This cytoplasmic-nuclear translocation implies that UHRF1 may play a role in spermatogonial stem cells. Inducible knockout of UHRF1 at two weeks destructs KIT^{-} to KIT^{+} spermatogonia transition, but one-month-old mutant mice still contain the same amount of undifferentiated spermatogonia as compared to the control group [41]. It seems like UHRF1 only functions in spermatogonial differentiation, but we cannot rule out that UHRF1 may play a potential role in SSC homeostasis. The first reason is that UHRF1 suppresses retrotransposon in the germline [40], and maintaining transposon silence is essential for spermatogonial stem cell homeostasis [9]. The second reason is that some genes such as KDM2B affect SSCs in adult mice instead of underage mice [9]. Therefore, further studies, including a conditional knockout of UHRF1 at an earlier age or analyzing the phenotype at an older age, need to be performed.

DNMT3A is abundant in the gonocytes at postnatal day 0 (P0), significantly downregulated by P7 and upregulated again along with spermatogonia differentiation [42]. Contrary to DNMT3A, DNMT3B is rarely detected in P0 gonocytes but appears in differentiating spermatogonia at P7 [41]. Consistent with DNMTs expression, the 5mC signal is 2–3 times stronger in differentiating spermatogonia compared with undifferentiated spermatogonia [41]. Loss of DNMT3A in mice results in progressive loss of spermatogonial stem cells, meiotic defects and male infertility, while DNMT3B is dispensable for spermatogenesis [43].

DNMT3L, another DNMT3 family member, also participates in *de novo* DNA methylation [44]. DNMT3L itself does not qualify catalytic activity, but it works with DNMT3A and DNMT3B to facilitate the *de novo* methylation. Besides, DNMT3L has a PHD domain at N-terminal, which is associated with histone modification [45–47]. DNMT3L is abundant in prospermatogonia and the precursors of spermatogonial stem/progenitor cells (SPCs), but cannot be detected from postnatal day 4–6. *Dnmt3l* knockout mouse testes show unchanged spermatogonia number at P7, germ cell loss at P14 and Sertoli-cell-only phenotype at 8–10 weeks [48–50]. Further, DNMT3L regulates the expression of CDK2 that plays a key role in PLZF degradation. Moreover, the loss of DNMT3L causes upregulated expression of cell proliferation-promoting factor Sallike protein 4B(SALL4B) and PLZF antagonist Sallike protein 4A (SALL4A) levels [51]. Both PLZF and SALL4 are spermatogonial stem/progenitor cells (SPCs)-specific protein and the SALL4-to-PLZF ratio determine SSC fate [52].

In addition, methylation of transposons cannot be ignored and transposon silencing plays a vital role in protecting germline genome integrity (Fig. 2a-b). Genome demethylation in mice occurs at primordial germ cells (PGCs) from embryo day 9.0 to 13.5 [53]. Subsequently, the first *de novo* DNA methylation leads to abundant methylation of transposons. However, some transposons such as IAP and LINE1 avoid *de novo* DNA methylation and are activated, thereby threatening genomic integrity [54]. To against this effect, the piRNAs and their associated PIWI proteins destruct cytoplasmic transposon RNAs and generate secondary antisense transposon-derived piRNAs to cooperate with MIWI2 (PIWIL4) to establish DNA methylation [55–57]. Loss of *de novo* DNA methyltransferase DNMT3L results in a bulk reduction of CpG DNA methylation across all genomic classes, whereas MIWI2 deficiency reduces DNA methylation at transposons [9]. Global knockout either DNMT3L or MIWI2 leads to similar phenotype: progressive loss of spermatogonia in adult testes [51, 58]. The fact that DNMT3L is absent in adult spermatogonia implies a non-direct molecular role for DNMT3L in spermatogonia. MIWI2 is present in adult spermatogonia but the conditional knockout of MIWI2 in adult mouse testes has no effect on spermatogenesis compared to control for up to a year [9]. Therefore, these results suggested that the loss of spermatogonia in adult mice may derive from defective reprogramming at an earlier age. Interestingly, TE analyses of isolate undifferentiated spermatogonia revealed that *Dnmt3l* and *Miwi2* mutants share the similar deregulated transposons [9]. However, the deregulation of *de novo* transcriptome genes is more apparent in DNMT3L mutant mice, and the up-regulated genes in DNMT3L mutant mice almost overlap with the up-regulated genes in MIWI2 mutant mice [9]. More importantly, most upregulated genes exhibited IAPs dominantly association, indicating that the upregulated genes primarily derive from IAP elements [9]. Besides, LINE1 keeps silent in both *Dnmt3l* and *Miwi2* mutant mice, which further confirms the studies by Di Giacomo et al. that only DNA hypomethylation is insufficient to activate LINE1 [8, 9].

In contrast to repressive DNA methylation, DNA hydromethylation is a sign of activated gene expression, which is catalyzed by ten-eleven translocation (TET) enzymes, including TET1, TET2, and TET3. The loss of TET1 in mice speeds SSC aging and results in premature reproductive aging, manifesting reduced SSC numbers, meiotic recombination defects, increased apoptosis, decreased proliferation and subfertility [61]. TET1 introduces DNA demethylation by oxidization of 5-



methylcytosine (5mC) to 5-hydroxymethylcytosine(5hmC). The deletion of TET1 results in lower 5hmC and higher 5mC expression levels in spermatogonia and down-regulated cell-cycle related genes. Given that both TET1 and 5hmC regulate Wnt and PI3K-Akt signaling pathway, autophagy, and stress response gene, the down-regulated genes may be related to a

◀ **Fig. 1** Spermatogonial stem cell models. (**a**) Classic A_s model proposes that the SSC pool is constituted by all A_s, which can either renew itself by completely cytoplasmic division or convert to A_{pr} by incompletely cytoplasmic division. A_{pr} further incompletely divide to form A_{al} and then irreversibly enter a differentiating pathway. (**b**) Compared with classic A_s model, revised A_s model deems that only a particular subset of A_s represents SSC pool. $SSC_{ultimate}$ marked by ID4-GFP ^{bright} qualify self-renewal ability, while $SSC_{transitory}$ marked by ID4-GFP ^{dim} primarily generate progenitors. ID4-GFP ^{negative} progenitors differentiate in an irreversibly linear direction. (**c**) In the dynamic SSC model, GFR$\alpha1^+$ undifferentiated spermatogonia represent the SSC pool. A_s hardly undergo complete division to support itself and is replenished by chain fragmentation. Most $NGN3^+$ spermatogonia are differentiation-committed and possess limited self-renewal potential under homeostatic conditions, whereas $NGN3^+$ cells can reverse back to stem cell state by fragmentation under regenerative conditions

hypermethylation state [62, 63]. Moreover, in middle-aged TET1 deficient mice, telomeres are shortened as old wild-type mice. Further, natural aging mice and TET1 deficient mice show similar downregulated pathways containing Wnt, Ras/MAPK, PI3K-Akt, AMPK, and retinol metabolism (which are associated with SSC homeostasis), indicating that loss function of TET1 could mimic natural aging process to some extent [61, 64, 65].

Histone Modification in SSCs

Histones are the basic protein in eukaryotic chromatin and are rich in basic amino acids such as arginine and lysine. Arginine and lysine together make up a quarter of all amino acid residues. Histones contain a large number of positively charged basic amino acids, thus it can interact with negatively charged phosphate groups in DNA to form DNA-histone complexes. Each basic histone has two domains: the folding spherical region and the amino-terminal (N-terminal) domain. The folding region of histones mediates the interaction of histones and entangled DNA; The amino-terminal domain is like a "tail", located outside the spherical core structure of the nucleosome (the extension of the nucleosome) and interact with other regulatory proteins and DNA [66]. Histone tail modifications regulate chromatin structure and transcription. Common histone tail modifications mainly include methylation, acetylation, phosphorylation, ubiquitylation, and carbonylation, etc. [67]. We will mainly focus on histone methylation and acetylation to discuss in this review as below.

Histone Methylation

Histone methylation attracts a lot of research interest, as it marks active and inactive chromatin. Histone methylation occurs at lysine and arginine residues of the amino-terminal tails. In the term of arginine, the guanidine group in the side chain can be symmetrically or asymmetrically mono-methylated and di-methylated, while the e-amino group in lysine can be mono- di- or tri-methylated [68–70]. Multiple histone methylations are identified in SSCs or undifferentiated spermatogonia, including H3K4me1, H3K4me2, H3K4me3, H3K9me1, H3K9me3, H3K27me2, H3K27me3, H4K20me2, H4K20me3 (Table 1) [71–73]. Among them, the methylation of H3K4 promotes transcriptional activation, whereas H3K9, H3K27, and H4K20 methylation contribute to transcriptional repression (Table 2) [74–76]. The dynamic balance of histone methylation relies on the coordination of histone methyltransferase and demethylase [69]. Although the mouse models are few for us to study methylated histones directly, some studies have made remarkable advances in histone methyltransferases/ methyltransferases. We summarized the mouse models associated with histone methylations in spermatogonial stem cells in Table 3.

H3K4

Methylation of histone H3K4 contributes to transcriptional activation and a more-open chromatin configuration. KMT2B (also called MLL2), one of H3K4 methyltransferase, is highly expressed in SSCs and essential for male germ cell development [77]. The deletion of KMT2B leads to SSC-to-progenitor transition failure and a progressive loss of spermatogonia at a later age [12, 77]. In addition, KMT2B is identified to be a critical SSC priming factor and deposits H3K4me3 to both monovalent and bivalent promoters, which prepare for gene activation at late spermatogenesis and embryonic development. However, whether H3K4me3 priming is essential for SSC differentiation or whether other mechanisms exist for SSC differentiation remain open questions [12].

In contrast, KDM1A (also known as LSD1, AOF2) can remove gene-activating H3K4me1/2 and is associated with H3 deacetylase HDAC1/2 [90]. KDM1A is abundant in spermatogonia, absent in leptotene spermatogonia, and subsequently, appears in pachytene spermatocytes but disappears again in round spermatids [71]. Conditional knockout *Kdm1a* in mice just before birth results in compromised SSC maintenance, failed differentiation [78]. Immunofluorescence showed that the staining signal of H3K4me2 is increased whereas the signal of H3K4me1 is comparable between control and mutant spermatogonia. Further analyses revealed that the loss of KDM1A down-regulates PLZF, NANOS2, and BCL6B, which are critical regulators for SSC self-renewal [78]. However, specific reasons for the down-regulation of these genes and whether KDM1A is required for adult mouse SSC homeostasis are still unclear. Moreover, one group found that inducible deletion of KDM1A in adult mice leads to progressive loss of germ cells, abnormal accumulation of spermatocytes, and increased apoptosis [11]. Stem cell marker OCT4 is ectopically distributed on differentiating spermatogonia, and increased H3K4me2 is detected at the OCT4 locus.

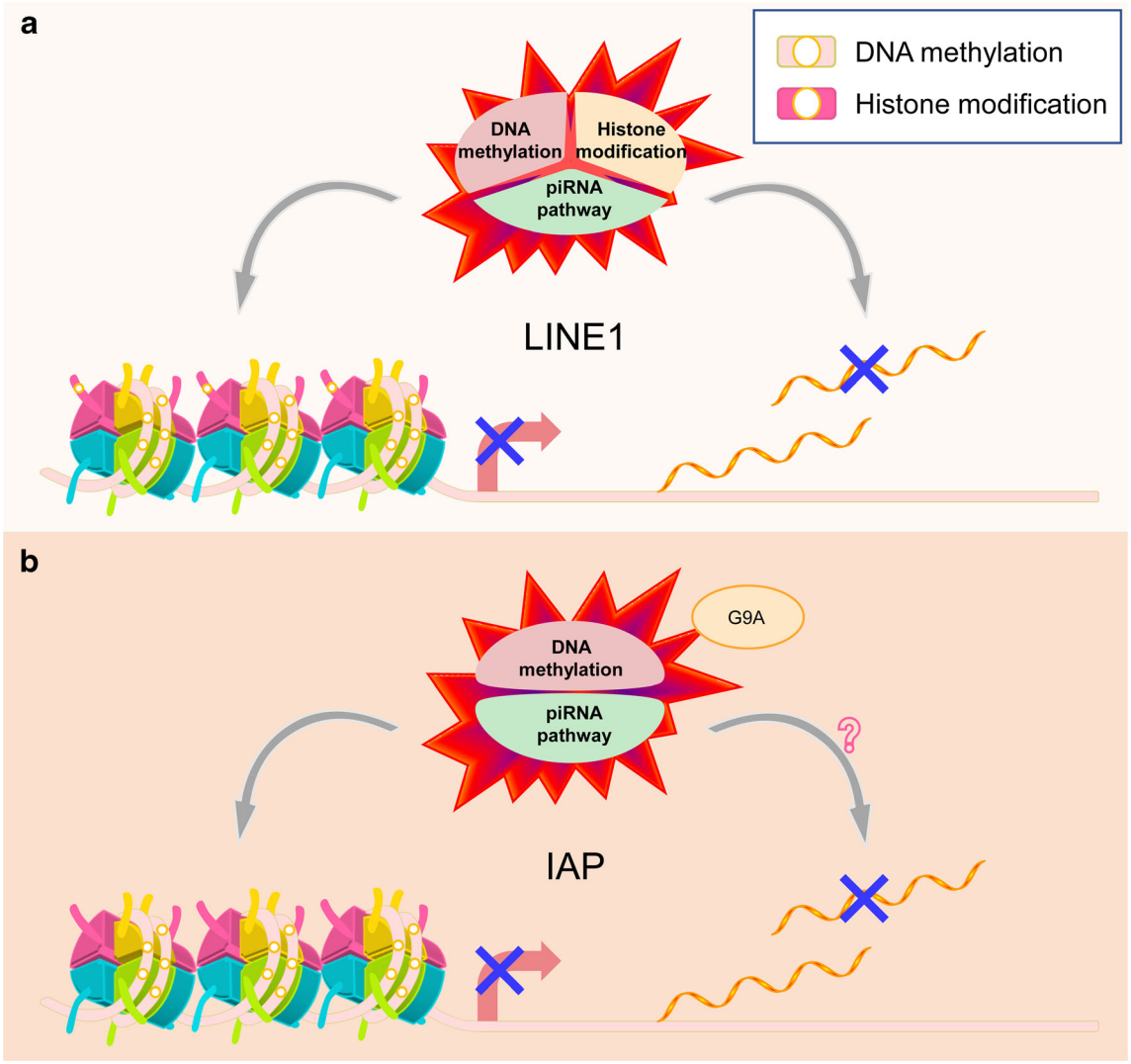


Fig. 2 Propose model of transposon repression in spermatogonial stem cells. (**a**) The silencing of LINE1 in spermatogonial stem cells (SSCs) is unique compared to other germ cells [8, 59]. Three mechanisms DNA methylation, histone modification and the piRNA pathway are involved in the process of LINE1 silencing in spermatogonial stem cells. The H3K9me2-mediated LINE1 silencing is sufficient and indispensable to maintain LINE1 silencing when DNA methylation and piRNA pathway are lost [8, 59]. (**b**) Slightly different LINE1 silencing, DNA methylation is essential for silencing IAP [9]. However, whether the piRNA-mediated post-transcriptional silencing pathway is required for transposon silencing remains an open question. Interestingly, G9A catalyzes the dimethylation of H3K9 at both IAP and LINE1 elements in spermatogonia, but this repression is redundant for silencing IAP [8, 9, 59, 60]

Besides, over 6-fold enrichment of KDM1A is observed at the promoter of OCT4 in the adult testes. Thus, this study suggested that KDM1A may repress OCT4 by removing H3K4me2 [11]. The accumulation of spermatocytes in adult

Table 1 Distribution patterns of methylated histones in spermatogonia of mouse testes

	Undifferentiated spermatogonia				Differentiating spermatogonia				Reference
	H3K4	H3K9	H3K27	H4K20	H3K4	H3K9	H3K27	H4K20	
Mono-Me Histones	++	+	–	–	+++	+	+++	+++	[41, 71–73]
Di-Me Histones	++	+	+++	+++	++	+	+++	+++	
Tri-Me Histones	++	+++	+	+++	++	+++	+	+++	

+++, ++, +, and – signify strong, intermediate, weak, and negative signal, respectively

Table 2 Histone methyltransferases involved in spermatogonial stem cells

Type	Gene	Alias	Biological role	Function	Reference
H3K4	MLL2	KMT2B	Priming	(1) Convey H3K4me3 at both monovalent and bivalent promoters involved in late spermatogenesis and embryonic development; (2) Down-regulate DDX4, Oct4 etc.	[12, 77]
	LSD1	KDM1A, AOF2	Homeostasis	(1) Demethylate H3K4me2; (2) Deacetylate H3; (3) Repress maintenance genes; (4) Downregulate Oct4 by removing H3K4me2.	[11, 71, 78]
	FBXL10	KDM2B, JHDM1B	Homeostasis	(1) Not contribute to the demethylation of H3K4me3 and H3K36me1/2; (2) Affect cell recovery under damage conditions through upregulating cell cycle related cyclin-dependent kinase inhibitor (CDKI) P19 and P21.	[79]
H3K9	G9A	KMT1C	Homeostasis	(1) Di-methylate H3K9; (2) Suppress LINE 1 elements by cooperation with DNA methylation and the piRNA pathway.	[8]
	SETDB1	KMT1E, ESET	Homeostasis	(1) Methylate H3K9; (2) Regulate PTEN/AKT/FOXO1 pathway; (3) Increase DNA methylation and H3K9me2 at the Cox4i2 locus.	[7, 10, 80]
	FANCB	FA2, FAB, FACB	Homeostasis	(1) Possibly maintain SSCs by regulating H3K9 methylation.	[81]
	JMJD1C	KDM3C, TRIP8	Homeostasis	(1) Not contribute to the demethylation of H3K9; (2) Upregulate Oct4 expression.	[82, 83]
H3K27	EZH2	KMT6, KMT6A	Homeostasis	(1) Not contribute to the methylation of H3K27; (2) Repress the expression of Ngn3 and Kit.	[84]
	EED	HEED, WAIT1	Homeostasis	(1) Not contribute to the methylation of H3K27; (2) Regulate SSC maintenance though down-regulating undifferentiated spermatogonia marker genes, such as Ngn3, Nanos3, and Line28a.	[85]
	SCML2		Priming	(1) Promote the formation of bivalent chromatin domains by binding to H3K4me2/3 enriched hypomethylated promoters to facilitate H3K27me3 deposition accumulation; (2) Build a complex with PRC2 for the deposition of H3K27me3.	[86, 87]
	JMJD3	KDM6B	Homeostasis	(1) Not contribute to the methylation of H3K27; (2) Increase the abscission of intercellular bridges (3) Not caused by an intercellular bridge formation problem.	[88]

testes may be due to a meiotic checkpoint happened in adult spermatogenesis versus the first wave of spermatogenesis. Together, it is worth noting that the phenotype of KDM1A deficiency in neonatal and adult mice is not the same.

In addition, F-box and leucine-rich repeat protein 10 (FBXL10) is a JmjC-domain-containing protein and catalyzes the demethylation of H3K4me3 and H3K36me1/2/3 [91–93]. The deficiency of FBXL10 does not compromise fertility at a young age in mice, but *Fbxl10* mutants suffer from degenerated spermatogenesis in 7-month-old mouse testes and defective cell recovery under regenerative conditions. Functional analyses using an *in vitro* spermatogonia culture system showed that the loss of FBXL10 in germline stem cells (GSCs) upregulates the level of *P21* and *P19* mRNA, cell cycle-related cyclin-dependent kinase inhibitors (CDKI), rather than affecting histone methylation [79].

H3K9

Histone H3K9 methylation plays an important role in heterochromatin formation and gene transcriptional regulation. H3K9me3 is one of the most important histone methylation modifications, which shows a unique nucleus distribution in undifferentiated spermatogonia and DAPI-dense area distribution in differentiating spermatogonia [41]. Exposure to hexavalent chromium (Cr(VI)) increases the expression of H3K9me3, causing apoptosis of SSCs. Interestingly, melatonin(N-acetyl-5-methoxytryptamine) with anti-apoptotic function can rescue the apoptosis of SSCs and reduce the level of H3K9me3 level [80, 94]. Further, Cr(VI) can induce an increase in SETDB1 expression, while melatonin pretreatment causes a decrease in SETDB1 expression [80]. SETDB1 has been suggested to establish H3K9me3 to repress gene expression through histone modification [95], thus Cr(VI)-induced H3K9me3 overexpression may be due to the upregulation SETDB1. Similarly, another group found that the knockdown of SETDB1 *in vitro* leads to a decreased number of SSCs because of the upregulation of genes involved in cell apoptosis and differentiation [96]. Moreover, SETDB1 knockdown SSCs can not reconstruct spermatogenesis in recipient mice treated with busulfan [96], suggesting that SETDB1 is also essential for regeneration under regenerative conditions. Functional analysis showed that the apoptosis of SETDB1-KD cells can be partially rescued by the knockdown of cytochrome *c* oxidase subunit IV isoform(COX4I2), which is related to the onset of apoptosis [96]. Furthermore, ChIP and bisulfite sequencing PCR revealed that SETDB1 inhibits the expression of COX4I2 by promoting DNA methylation and H3K9me3 accumulation [7]. Additionally, a subsequent

Table 3 Mouse models associated with chromatin modification in spermatogonial stem cells

Gene	Model	Method	Cre type	Phenotype	References
Dnmt1	SSCs	KD		(1) Apoptosis of SSCs	[89]
Dnmt3a	Mouse, SSCs	CKO	*Tnap*-Cre	(1) Infertile; (2) Progressive loss of germ cells and meiotic defects in knockout mice; (3) Normal self-renewal and spermatogenic defects after double knockout *Dnmt3a* and *Dnm3b* in SSCs	[43, 89]
Dnmt3b	Mouse, SSCs	CKO	*Tnap*-Cre	(1) Fertile; (2) Normal self-renewal and spermatogenic defects after double knockout *Dnmt3a* and *Dnm3b* in SSCs	[43, 89]
Dnmt3l	mouse	KO, OE		(1) Infertile; (2) Progressive loss of spermatogonia in knockout mice; (3) Normal self-renewal after overexpression of *Dnmt3l* in SSCs; (4) Meiotic defects	[50, 89]
Kmt2b	Mouse	CKO	*Rosa26*-CreERT2	(1) Infertile; (2) SSC-to-progenitor transition defects; (3) Progressive loss of spermatogonia at late age	[12, 77]
Lsd1	Mouse	CKO	*Ddx4*-Cre	(1) Infertile; (2) Progressive loss of SSCs; (3) Spermatogonial differentiation defects; (4) Blocking in zygotene-like state	[11, 71, 78]
Fbxl10	Mouse	KO		(1) Age-dependent infertile, fertile before 1 month of age; (2) Normal spermatogenesis at a young age; (3) Degeneration of spermatogenesis at 7-month-old mouse testes (4) Cell recovery failure under damage conditions	[79]
G9a	Mouse	CKO	*Rosa26*-CreERT2	(1) Infertile; (2) Progressive loss of germ cells inclusive of SSCs in the *Mili* null background; (3) Loss of spermatocytes.	[8]
Setdb1	SSCs	KD		(1) Progressive loss of SSCs; (2) SSC apoptosis	[7, 10, 80]
Fancb	Mouse	KO		(1) Infertile; (2) Progressive loss of $PLZF^{+}$ spermatogonia from 1 month;	[81]
Jmjd1c	Mouse	KO		(1) Age-dependent infertile, fertile before 3 months of age; (2) Progressive loss of germ cells including SSCs from 3 months of age	[82, 83]
Ezh2	Mouse	CKO	*Ngn3*-Cre	(1) Subfertile; (2) Progressive loss of SSCs; (3) Precocious spermatogonial differentiation	[84]
Eed	Mouse	CKO	*Ddx4*-Cre	(1) Infertile; (2) Progressive loss of SSCs	[85]
Scml2	Mouse	KO		(1) Infertile; (2) Normal self-renewal of undifferentiated spermatogonia; (3) Spermatogonial differentiation defects	[86, 87]
Jmjd3	Mouse, SSCs	CKO, KD	*Stra8*-Cre	(1) Prolonged fertility; (2) Larger testes and an increase of undifferentiated spermatogonia; (3) Frequent fragmentation of spermatogonial cysts	[88]

KD: knockdown, OE: overexpression, KO: knockout, CKO: conditional knockout

study found the PTEN/AKT/FOXO1 pathway participates in the regulation of SETDB1 on SSC apoptosis [10]. Although *in vitro* studies mentioned above provide credible evidence about the regulatory mechanism of SETDB1 on SSC maintenance, but until now, no *in vivo* studies such as research on loss-of-function mouse models are reported.

In mice, the repressive H3K9me2 is present in spermatogonia and early stages of spermatocytes, and lost from pachytene spermatocytes [72, 97]. H3K9me2 may also be associated with transposon silencing, because it is resident across LINE1 elements [8, 59]. Di-methylation of H3K9 is catalyzed by G9A and GLP [97–99]. G9A is abundant in spermatogonia, weak in preleptotene spermatocytes and absent in the following spermatogenesis [8]. Appearing in spermatogonia and disappearing subsequently suggests that G9A may play a unique role in spermatogonia. The loss of G9A has no effect on spermatogonia and LINE1 [8, 100], thus, other mechanism maybe existed. Interestingly, MILI, a PIWI protein, participates in DNA methylation and the post-transcriptional silencing pathway [55, 56, 101]. The loss of MILI leads to the derepression of LINE1 in spermatocytes, but LINE1 in spermatogonia remains silent [59]. Considering that H3K9me2 resident across LINE1 elements, H3K9me2 and MILI-mediated DNA methylation and post-transcriptional silencing pathway may interact in maintaining LINE silencing. As expected, knocking out G9A in the MILI deletion background causes a complete loss of germ cells inclusive of spermatogonia, and immunofluorescence showed that LINE1 is activated in the G9A and MILI double knockout spermatogonia [8]. This finding suggested that H3K9me2-mediated LINE1 silencing is only essential in the absence of DNA methylation and piRNA pathway (Fig. 2b) [8]. Worth mention is that IAP is also di-methylated at H3K9 by G9A in spermatogonia [8, 59]. However, IAP may contain a powerful promoter that can eliminate the effect of H3K9me2, thus, H3K9me2 has little to do with the repression of IAP (Fig. 2b) [8, 9, 59].

In addition, KDM3A (also called JHDM2A or JMJD1A) is H3K9 demethylase that counters the enzymatic activity of G9A [97, 102, 103]. JMJD1C, a KDM3A homolog, also possesses H3K9 demethylase activities and its expression level is high in

undifferentiated spermatogonia [104]. JMJD1C deficient mice exhibit progressive loss of germ cells after three months of age due to failed long-term maintenance of SSCs [82]. Immunofluorescence showed that the OCT4 signal of JMJD1C mutant spermatogonia is weaker than that of the control group, suggesting that JMJD1C may affect the steady state of SSCs by increasing OCT4 level [83]. Interestingly, global H3K9 methylation is not changed in JMJD1C- null testes, which reminds us that JMJD1C works in extremely limited regions of the genome or not qualifies H3K9 demethylase activities actually [82, 104, 105]. FANCB is one of the eight Fanconi anemia proteins and plays various roles in mono-, di- and tri-methylated H3K9 during meiosis [81, 106]. For example, FANCB promotes the deposition of H3K9me2 but plays a negative role in the process of H3K9me3 accumulation. FANCB is essential for the long-term maintenance of undifferentiated spermatogonia and knocking out FANCB leads to progressive loss of $PLZF^{+}$ spermatogonia from one month [81]. Given that FANCB regulates H3K9 methylation in spermatocytes, the disturbed SSC homeostasis may be due to unstable H3K9 methylation. Furthermore, considering that FA/BRCA pathway functions in the maintenance of hematopoietic stem cells and pluripotent stem cells [107], other FA proteins probably also participate in SSC homeostasis.

H3K27

Given that H3K27me2/3 manifest perinuclear localization in A_s, A_{pr}, and A_{al} spermatogonia, the methylation of H3K27 probably functions in SSC homeostasis. Di- and tri-methylation of H3K27 can be catalyzed by polycomb repressive complex 2 (PRC2), which consists of four subunits: EED, SUZ12, EZH1/2 and RbBP 4/7. Disrupting PRC2 function via ablation EED in mice results in gradual loss of SSCs, leading to male infertility [85]. RT-qPCR shows that EED regulates SSC maintenance by downregulating NGN3, NANOS3, LINE28A, etc., rather than repressing the Ink4b–Arf–Ink4a locus, which results in cell cycle arrest on PRC2-null background in multiple tissues [85, 108, 109]. Since PRC2 plays a negative role in transcriptional activation, the downregulated genes cannot directly attribute the loss of PRC2. Considering that PRC2 deletion permit ectopic expression of neuron-type-specific transcription factors that can trigger a conversion of germ cells into neurons in *Caenorhabditis* elegans, the failure maintenance of SSCs may be due to the compromised spermatogonia-specific transcription induced by ectopic gene expression [85, 110].

EZH2 (also called KMT6), a catalytic component of PRC2, is abundant in undifferentiated spermatogonia, gradually decreases with differentiation, disappears in leptotene, and then reappears in pachytene spermatocytes [111]. Germline deletion of EZH2 in mice reduces the expression of ID4 and RET, and induces an ectopic expression of KIT in undifferentiated spermatogonia [84]. This finding suggested that EZH2 deletion disrupts SSC pool by impairing SSC self-renewal and promoting precocious spermatogonial differentiation. Consistent with the phenotype observed in mice, *in vitro* studies showed that ID4 is unregulated whereas NGN3, KIT, and STRA8 are downregulated when knocking down EZH2 in GSCs. Co-immunoprecipitation assay revealed that EZH2 interacts with NGN3 and KIT, implying that EZH2 binds these two proteins to repress SSC differentiation [84]. EZH2 deletion in mice also leads to the reduction of H3K27me3, but it is not completely absent, which reminds us that other histone methyltransferases are compensating for EZH2. Indeed, It has been demonstrated that the lack of EZH2-mediated-H3K27me3 deposition can be compensated by EZH1 in multiple tissues [112–114], thus EZH1/2 double-knockout model is essential to evaluate the function of H3K27me3 in spermatogonia.

Sex comb on midleg-like 2 (SCML2) is a germline-specific subunit of PRC1 that establishes H2AK119ub and H3K27me3 in the male germline. In undifferentiated spermatogonia, SCML2 builds a complex with EZH2(a subunit of PRC2) and binds to H3K4me2/3 enriched hypomethylated promoters for the deposition of H3K27me3 [86]. SCML2 is dispensable for SSC homeostasis, and it promotes the formation of bivalent chromatin domains (both H3K4me3 and H3K27me3 mark them) that prepare for embryo development. In contrary to Polycomb protein catalyzing the methylation of H3K27, UTX (also called KDM6A) and JMJD3 (also called KDM6B) erases H3K27 methylation. Loss of H3K27 demethylase JMJD3 in differentiating spermatogonia results in an increase in undifferentiated spermatogonia and frequent fragmentation of spermatogonial cysts, leading to prolonged male fertility [88]. Immunofluorescence revealed that the expression of H3K27me2 and H3K27me3 is no change in mutant spermatogonia, therefore, the phenotype of JMJD3 mutant mice is not due to H3K27me2/3 demethylation [88]. However, the genes that participate in the formation of the intercellular bridge show similar expression levels in mutant SSCs compared to control, suggesting that the increased fragmentation of spermatogonial cysts is not caused by intercellular bridge formation defects [88]. Therefore, there may be additional regulatory mechanisms accounting for frequent fragmentation [88].

Mouse *Cdyl* gene, universally considered as candidates for Y chromosome microdeletion (YCM), has been demonstrated function as a H3K27me3 reader associated with the formation of the transcriptional repressive complex [115, 116]. Conditional deletion of *Cdyl* using *Vasa*-Cre shortens male mouse fertility time due to progressive loss of germ cells [117]. Flow cytometry showed that the undifferentiated spermatogonia are significantly declined compared with the control littermates. Further, RNA-seq analysis revealed that a subset of spermatogonia maintenance genes is downregulated in *Cdyl* deletion samples. Moreover, differentiating spermatogonia manifest enhanced H3K27me3 signal compared with control littermates [117]. Although current studies show that

H3K27 methyltransferase affects SSC homeostasis, these enzymes usually work independently of H3K27 methylation in spermatogonia [84, 85, 88]. Therefore, the function of H3K27 methylation in SSC homeostasis needs to be further studied.

Other Histone Modifications

Histone acetylation facilitates the dissociation of DNA from histones, then various transcription factors and co-transcription factors specifically bind to DNA binding sites and activate gene transcription. Histone acetylation and SSC homeostasis are closely linked. For example, the H4 acetylation is increased in human patients' testes within Sertoli-cell-only syndrome [118]. In the nucleus, the process of histone acetylation and histone deacetylation is in a dynamic balance, which is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) [119]. SIN3A is HDAC1 associated nuclear co-repressor protein and plays an essential role in SSC maintenance. The germ-cell specific deletion of SIN3A induces nearly all germ cells loss at two weeks, resulting in Sertoli-cell-only syndrome [120]. RNA-seq revealed that up-regulated transcripts are involved in developmental processes, and down-regulated transcripts are associated with nuclear receptor activity. The down-regulated transcripts include NR4A1 (NUR77), which is a nuclear receptor that works in cell proliferation and vanishes during the transition from undifferentiated spermatogonia to differentiating spermatogonia [121, 122]. Besides, Sertoli-cell specific deletion of SIN3A disrupts SSC niche and downregulates genes including PLZF, GFRα1, and OCT4 in fetal gonad, leading to fewer undifferentiated spermatogonia after birth [123]. In brief, SIN3A deletion damages SSC homeostasis by downregulating spermatogonial genes and disturbing the SSC niche.

Polycomb suppression complex1 (PRC1) generally participates in the establishment of H2AK119ub to suppress gene expression, but when working with ring finger protein 2 (RNF2), it plays a role in gene activation in SSCs [124, 125]. Conditional knockout RNF2 on a *Ring1*-null background (RNF2 can be partly compensated by RING1, and *Ring1*-null mice are fertile) erase the E3 ubiquitin ligases activity of PRC1, leading to the gradual loss of SSCs plus differentiation defects [124]. Interestingly, RNA-seq revealed that the most significantly downregulated gene is SALL4, a transcription factor critical for SSC maintenance and spermatogenic differentiation [52, 126]. More importantly, ChIP-seq analysis found that near the targeted start sites (TSSs) of these down-regulated genes, RNF2 and SALL4 are enriched instead of H2AK119ub, indicating that the gene activation activity of RNF2 is independent of H2AK119ub. In-depth functional characterization of RNF2 and SALL4 indicates that RNF2 activates SALL4, and then they work together for target genes activation [124].

Chromatin Remodeling

To ensure the dynamic combination of DNA and protein in chromatin, a series of specific chromatin remodeling complexes (also known as remodelers) are produced during process of intracellular evolution. The remodelers use the energy generated from hydrolyzing ATP to change the binding state of histones and DNA by sliding, reconstructing, removing nucleosomes, then protein factors can easily access the target DNA [127, 128]. According to the different domains of the catalytic subunit of ATPase, the remodelers can be divided into four families: SWI / SNF, ISWI, CHD, INO80 [129]. Although the current studies on SWI/SNF, INO80, and ISWI mainly focus on meiosis and spermiogenesis, CHD4-NURD complex has been confirmed to play a role in the homeostasis of SSCs [130–134]. CHD4-NURD complex contains MTA1, HDAC2A, RBBP4, and CHD4. All of them are abundant in the nuclei of $PLZF^{+}$ undifferentiated spermatogonia. Knocking out CHD4 in germ cells results in damaged maintenance and differentiation of spermatogonial stem cells, leading to infertility [130]. The functional analyses of CHD4 revealed that it binds to the promoters to repress the expression of genes related to SSC maintenance and differentiation [130]. The SPOC1 protein, also designated PHF13, contains a PHD domain associated with chromosome condensation [135, 136]. Immunofluorescence demonstrated that SPOC1 localizes in undifferentiated spermatogonia that are extremely similar to PLZF. Interestingly, the deletion of SPOC1 has no effect on SSCs at first, but it showed an age-dependent loss of germ cells in aged mice: a Sertoli-cell-only phenotype was observed at five months of age. Although SPOC1 was identified as a well-known histone remodeler and is required for SSC homeostasis, the mechanism of SPOC1 regulating SSC homeostasis still needs to be further investigated [137].

The Crosstalk Between DNA Methylation and Histone Modification in SSCs

Since DNA and histones form chromatin together, the synergistic effect of DNA methylation and histone modification is not surprising. Recent studies have demonstrated that crosstalk between DNA methylation and histone modification plays a vital role in SSC homeostasis [7, 8]. In consideration of both DNA methylation and H3K9 methylation are characteristics of heterochromatin, several studies aim to reveal their relationship. The first evidence linking DNA methylation with H3K9 methylation derives from H3K9me3 methyltransferase SETDB1,

in which knockdown of SETDB1 downregulates genes associated with SSC apoptosis via affecting DNA methylation and H3K9me3 [7]. Another study found that G9A-mediated H3K9me2 interacts with DNA methylation and the piRNA pathways to silence LINE1 in SSCs [8]. Therefore, histone modifiers are capable of cooperating directly or indirectly with DNA methyltransferase to correspond histone modification with DNA methylation in SSCs.

Additionally, UHRF1 maintains the stability of DNMT1 and binds to methylated H3K9 *in vitro* [138]. UHRF1 also interacts with other chromatin modifiers: DNMT3A/B, PRMT5, HDAC1, et al. [139–141]. Our recent study showed that UHRF1 serves as a jack of DNA methylation, histone modification, and the piRNA pathway in male germ cells [40]. Therefore, we speculate that UHRF1 plays a role in maintaining SSC homeostasis as well. Strikingly, polycomb repressive complex 2 (PRC2), a member of H3K27 methyltransferase, can be recruited into the CpG-rich sequence of embryonic stem cells [142]. Loss of PRC2 disrupts the homeostasis of SSCs and leads to male infertility [84, 85]. However, we still do not know whether PRC2 plays a role in DNA methylation and histone modification in SSCs.

Epigenetic Regulation in Human SSCs

Infertility has been identified as a global public health problem by the World Health Organization (WHO) [143]. At present, nearly 15% of couples at childbearing age suffer from infertility, of which male infertility accounts for about 50% [144]. Spermatogonial stem cells are unique, which have the dual capacity to self-renewal and differentiation during spermatogenesis [145]. Therefore, the research of human SSCs is of profound significance for the treatment of non-obstructive azoospermia (NOA) patients and the preservation and restoration of fertility in male tumor patients [146, 147]. In the past decades, the studies of human SSCs are few because human samples are difficult to collect and the method for long-term culture of human SSCs is still under research [148–153]. Several non-coding RNAs have been reported to play critical roles in human SSC homeostasis, but the studies about DNA methylation and histone modification are still in its infancy [154–157].

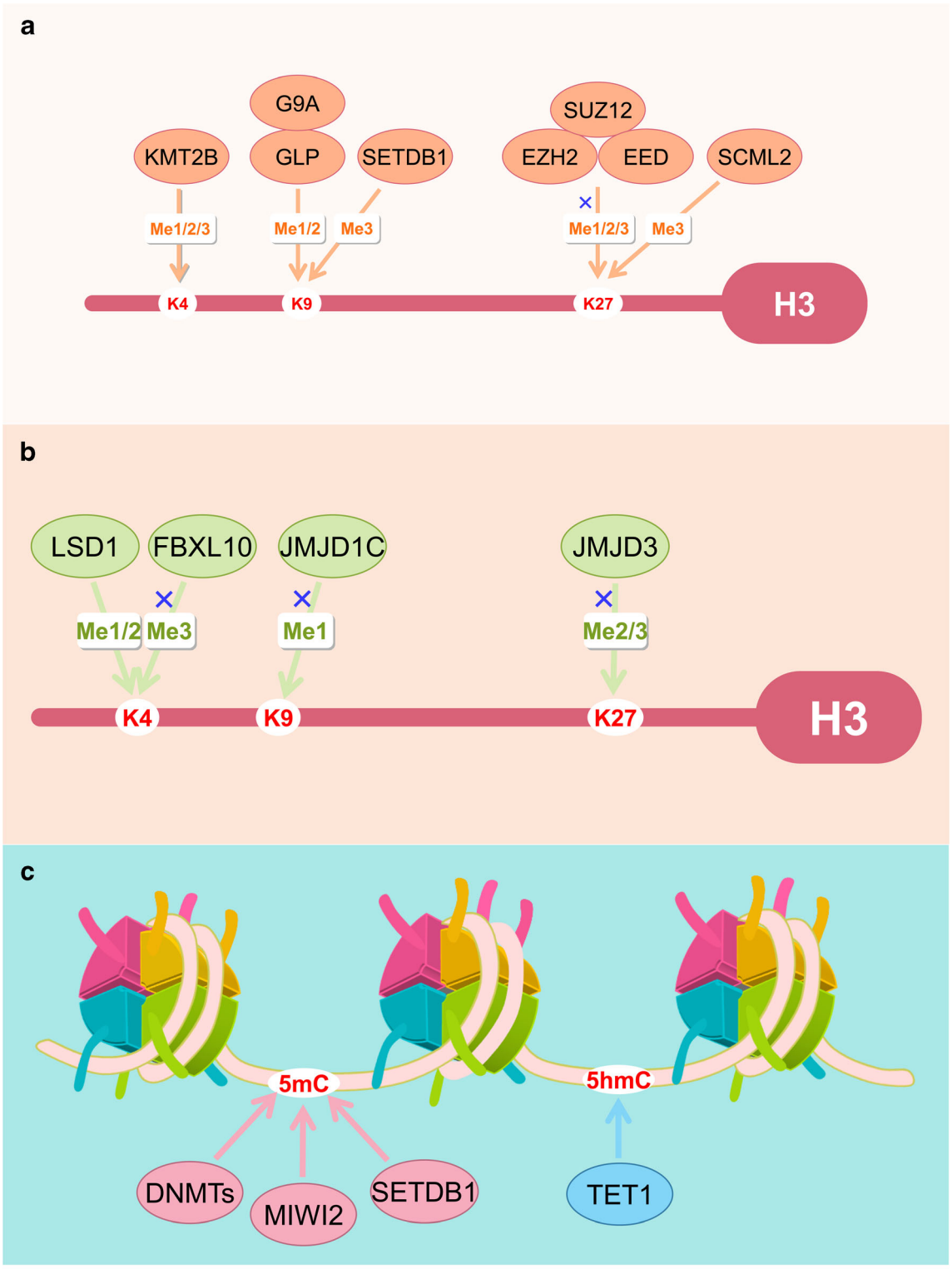
In humans, DNMT1 shares a dynamic nuclear-cytoplasmic localization during spermatogenesis, which exists in the nucleus and cytoplasm of spermatogonia, zygotene, and pachytene spermatocytes, and is imported into the nucleus of leptotene spermatocytes, secondary spermatocytes and exported to the cytoplasm of round spermatids [158]. DNMT3A is abundant in the nucleus of pachytene and secondary spermatocytes, whereas it localizes in the cytoplasm of other types of germ cells [158]. DNMT3B is primarily expressed in the nucleus of male germ cells [158]. Remarkably, DNMT1, DNMT3B, and DNMT3L are considered to be involved in human infertility [159–161]. However, further studies are still required to illustrate the relationship between DNA methyltransferases and human infertility.

There are few studies that focus on histone modification in human spermatogonial stem cell homeostasis. Loss of histone methyltransferases (such as *Mll2*, *Lsd1*, *Fancb*, *Eed*, and *Scml2*) in mice can impair the self-renewal or differentiation of SSCs and lead to male infertility. Interestingly, knockdown of JMJD3 *in vitro* can promote the proliferation of SSCs. *Jmjd3*-deficient mice manifest frequent fragmentation of spermatogonial cysts, larger testes, and prolonged fertility [88]. Although JMJD3 has not been identified in human testes, its function in humans may be similar to that in mice, and inhibiting the expression of JMJD3 through small molecule inhibitors may be a potential treatment method for male infertility [162]. In this review, we summarize the mouse model used to study epigenetic regulation (including DNA methylation and histone modification) of spermatogonial stem cells (Table 3), which may translate into human infertility research in the future.

Conclusion and Future Perspectives

In the past few decades, SSC models have been optimized with the occurrence of new technologies such as transplantation, lineage tracing, and intravital live imaging. Although the model provides valuable insights into SSC identity and function in spermatogenesis throughout development, neither 'revised A_s model' nor 'dynamic SSC model' can convince each other on the mechanism for SSC maintenance. One of the open questions in SSC biology is how epigenetic factors function in unipotency, self-renewal, differentiation, and preparation for future gene expression. Emerging studies have reported chromatin modification including DNA methylation and histone modification work in SSC homeostasis (Fig. 3a-c). Besides, the deposition of H3K4me3 and H3K27me3 in SSCs prepares the expression of later spermatogenesis genes by forming monovalent and bivalent chromatin domains. For example, KMT2B deposits H3K4me3 on both monovalent and bivalent promoters for late spermatogenesis and embryonic development [12]; SCML2 binds to H3K4me3 to deposit H3K27me3 at bivalent promoters for early embryo development [86].

Loss-of-function models are dominant methods to analyze the function of various genes in organisms. Multiple studies in mice have proved the value of chromatin modifiers in SSCs. However, the function of other chromatin modifiers in SSCs remains unclear.


a
G9A
KMT2B
GLP
SETDB1
SUZ12
EZH2
EED
SCML2
Me1/2/3
Me1/2
Me3
Me1/2/3
Me3
K4
K9
K27
H3
b
LSD1
FBXL10
JMJD1C
JMJD3
Me1/2
Me3
Me1
Me2/3
K4
K9
K27
H3
c
5mC
5hmC
DNMTs
MIWI2
SETDB1
TET1
Histone methyltransferase
DNA methyltransferase
Histone demethylase
DNA demethylase

◀ **Fig. 3** Histone modifiers function in spermatogonial stem cells. (**a-b**) Histone modifications on specific residues of H3 with various histone modifiers are shown. The basic amino acid–Lysine (K) is targeted for histone lysine methylation/demethylation by numerous histone-modifying enzymes –histone methyltransferase (KMT2B, G9A, GLP1, SETDB1, EZH2, EED, SCML2), histone demethylase (LSD1, FBXL10, JMJD1C, JMJD3). Although all of histone methyltransferases/demethylases listed function in spermatogonial stem cells (SSCs), some histone methyltransferases/demethylases (EZH2, SUZ12, EED, SCML2, FBXL10, JMJD1C, JMJD3) work through non-histone modification mechanisms in SSCs. (**c**) Colorful nucleosomes are wrapped by pink DNA. The red panel of nucleosome means H3. DNA methyltransferases (DNMTs) mediate DNA methylation by adding a methyl group to the fifth carbon of cytosine to produce 5-methylcytosine(5mC). MIWI2 can also establish methylation through an unknown mechanism. SETDB1 mediates the crosstalk between DNA methylation and histone modification in spermatogonial stem cells. TET1 introduces DNA demethylation by oxidization of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine(5hmC)

Remarkably, many histone modifiers such as SETDB1 and RNF2 also work in other ways in addition to histone modification [7, 124], thus the phenotype of these dysfunctional models may not be entirely attributed to the defects of histone modification. In addition, many genes in mammal have been proven to play essential roles in epigenetic events. Whether these genes are expressed and function in SSCs, or whether these genes have a unique role in SSCs, there is still a lot of room for research.

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Compliance with Ethical Standards

Ethics Approval and Consent to Participate Not applicable.

Conflict of Interest The authors declare no conflict of interest.

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