Epigenetic factors in the regulation of prospermatogonia and spermatogonial stem cells

Yen-Tzu Tseng¹, Hung-Fu Liao¹, Chih-Yun Yu¹, Chu-Fan Mo¹ and Shau-Ping Lin¹,²,³,⁴

¹Institute of Biotechnology, National Taiwan University, Taipei 106, Taiwan, ²Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan, ³Research Centre for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei 106, Taiwan and ⁴Centre for Systems Biology, National Taiwan University, Taipei 106, Taiwan

Correspondence should be addressed to S-P Lin; Email: shaupinglin@ntu.edu.tw

Abstract

Appropriate regulation of epigenome within cells is crucial for the determination of cell fate and contributes to the lifelong maintenance of tissue homeostasis. Epigenomic re-establishment during embryonic prospermatogonia development and fine-tune of the epigenetic landscape in postnatal spermatogonial stem cells (SSCs) are two key processes required for functional male germ cell formation. Repression of re-activated transposons and male germline-specific epigenome establishment occur in prospermatogonia, whereas modulations of the epigenetic landscape is important for SSC self-renewal and differentiation to maintain the stem cell pool and support long-term sperm production. Here, we describe the impact of epigenome-related regulators and small non-coding RNAs as well as the influence of epigenome modifications that result from extrinsic signaling for controlling the decision between self-renewal, differentiation and survival in mouse prospermatogonia and SSCs. This article provides a review of epigenome-related molecules involved in cell fate determination in male germ cells and discusses the intriguing questions that arise from these studies.


Introduction

Epigenetics has been defined as the heritable changes in gene expression that alters phenotypes without changing genotypes. Sophisticated epigenomic regulation is critical for proper cellular functions and determine the differentiation potential of stem cell and progenitor cells (Cedar & Bergman 2009). The regulatory molecules that contribute to establishment and rearrangement of the epigenome include DNA methylation mediators, chromatin modulators and small non-coding RNAs. DNA methylation, performed by DNA methyltransferases (DNMTs), is an important epigenetic event for transcriptional regulation. DNMTs can exert biochemical effects by adding a methyl group to the fifth carbon of cytosine to generate 5'-methylcytosine. The maintenance of DNA methylation is performed by DNMT1 during semiconservative DNA replication, while de novo DNA methylation is catalyzed by DNMT3s (Bestor 2000, Chedin et al. 2002, Gowher & Jeltsch 2002). In addition to DNA methylation, post-translational modifications of specific amino acids of histone tails, including acetylation, phosphorylation, methylation and ubiquitination, are involved in the regulation of gene expression. For example, H3K4 di/trimethylation (H3K4me2/3) and H3K27 acetylation (H3K27ac) modifications at the transcription start sites are usually associated with active transcription by facilitating the binding of positive transcription factors, whereas H3K9me3 and H3K27me3 modifications result in condensed chromatin that frequently links to gene repression (Chen & Dent 2014). Moreover, accumulating evidence reveals that small RNAs such as the Piwi-interacting RNAs (piRNAs), function cooperatively with histone modifiers to facilitate heterochromatization, implicating their roles in epigenetic regulations (Pezic et al. 2014).

Among the various cell types in an organism, germ cells are uniquely capable of transmitting biological information across generations after natural fertilization (Oatley & Brinster 2008). The proper spatial-temporal modulation of chromatin is tightly associated with germ cell fate determination. Throughout germ cell development, there are at least two critical stages that require extensive epigenetic regulation to ensure proper germ cell development and function. One of these is the genome-wide epigenome reprogramming event that occurs during prospermatogonia development before birth. The second is the epigenomic fine-tuning that occurs in spermatogonial stem cells (SSCs) at the postnatal stage (Sasaki & Matsui 2008, Song & Wilkinson 2014).

Prospermatogonia are derived from primordial germ cells (PGCs) at the embryonic stage (Fig. 1). After birth,
prospermatogonia develop into undifferentiated spermatogonia (Asingle, Apair and Aalign), which are a heterogeneous population consisting of SSCs and spermatogonial progenitor cells (SPCs) (Fig. 2). SSCs are suggested to be the foundation for spermatogenesis, and they display self-renewal and differentiation abilities to maintain the stem cell pool, whereas SPCs are not only competent for differentiation, but also capable of regaining self-renewal potential for regeneration (Oatley & Brinster 2008, Nakagawa et al. 2010). Gaining insight into the mechanisms for the development of functional germ cells from prospermatogonia and SSC/SPCs is important partly because a significant portion of male-related infertility cases is thought to result from the impaired maintenance and differentiation of male germ cells (Boivin et al. 2007, Matzuk & Lamb 2008).

Accumulating evidence indicates that epigenetic factors play important roles in cell fate decisions and the commitment to germ cell development. In this review, we briefly summarize the importance of some epigenome-associated factors for the regulation of mouse prospermatogonia development and SSC/SPC maintenance and differentiation.

**Epigenome reprogramming in prospermatogonia in mice**

Prospermatogonia, also termed gonocytes, are developed from PGCs during embryonic development (Fig. 1). Mouse PGCs, originating from the epiblasts, are specified in response to BMP4 and BMP8B from the extraembryonic ectoderm and BMP2 from the visceral endoderm (Lawson et al. 1999, Ying et al. 2000, Ying & Zhao 2001), and they are induced through the tripartite network of BLIMP1/AP2a/PRDM14 to establish the
germline epigenome during early postimplantation development at approximately embryonic day (E) 6.5–7.25 (Saitou et al. 2002, Yamaji et al. 2008, Ohinata et al. 2009, Grabole et al. 2013, Magnusdottir et al. 2013). After their formation at E7.25, PGCs move to the endoderm, migrate during hindgut endoderm expansion and eventually locate to the genital ridges. PGCs exhibit a burst of mitotic activity after colonization of the genital ridges in E12.5 mice. Female PGCs then mature into oogonia and arrest in meiotic prophase, whereas male PGCs give rise to prospermatogonia during the G0/G1 mitotically arrested stage until birth (Nagano et al. 2000, Suzuki & Saga 2008, Western et al. 2008).

As they proliferate and migrate through the hindgut endoderm, PGCs undergo genome-wide DNA demethylation and alteration of histone modifications. The genome-wide DNA methylation level is decreased from 70% at E6.5 to 30% at E9.5, and it is further reduced to ~10% in male PGCs after entering developing gonads at E13.5 when male PGCs are exceedingly DNA hypomethylated (Seisenberger et al. 2012, Kobayashi et al. 2013). Transposable elements (TEs) are reactivated in PGCs at this stage consequent to the progressive loss of DNA methylation and reduced H3K9me2 levels (Seki et al. 2007, Hajkova et al. 2008). Other repressive histone modifications, including H2/H4R3me2S, H3K27me3 and H3K9me3 on TEs are suggested to protect genome integrity from bursts of up-regulated TE activity during this developmental window (Ng et al. 2013, Kim et al. 2014, Liu et al. 2014).

Re-establishment of the epigenome from E13.5 to birth is essential for preparing the male germ cell-specific epigenetic modifications, ensuring functional prospermatogonia development (Kato et al. 2007,
Kobayashi et al. 2013, Singh et al. 2013). Global DNA methylation levels rise from ~10% in E13.5 PGCs to ~50% in E16.5 prospermatogonia, coinciding with redistributing the active marks of H3K4me2/3 and H3K36me3 in prospermatogonia (Singh et al. 2013, Morselli et al. 2015). The elevated expression of DNA methyltransferase 3A/3B/3L (DNMT3s) during these stages suggests the potential contribution of de novo DNA methylation at this stage (La Salle et al. 2004, Sakai et al. 2013). The sequence specificity for de novo DNA methylation may be partly guided by the pre-existing chromatin context. Methylated H3K4 protects the underlying DNA sequence from de novo DNA methylation at gene promoter regions, as methylated H3K4 inhibit the interaction between the H3 histone tail and the ADD domain of DNMT3s (Jia et al. 2007, Ooi et al. 2007, Zhang et al. 2010, Singh et al. 2013). On the other hand, H3K36me3 is positively correlated with de novo methylation within gene bodies to promote transcriptional elongation in fetal and neonatal prospermatogonia (Morselli et al. 2015). Interestingly, DNA methylation is found more frequently in regions of low CpG density than in regions with high CpG density (Kobayashi et al. 2013). In addition, methylation within non-CpG sequences is only observed in prospermatogonia, not in somatic cells and PGCs (Kobayashi et al. 2013, Vlachogiannis et al. 2015). The global loss of DNA methylation at transcriptional start sites does not lead to a dramatic increase in gene expression levels between E13.5 PGCs and E16.5 prospermatogonia, suggesting that prospermatogonia execute a DNA methylation-independent mechanism for transcriptional control. Indeed, in male germ cells at these stages, developmentally-regulated genes are marked with both active H3K4me3 and repressive H3K27me3 modifications at the promoter regions, priming these genes for subsequent activation upon the occurrence of suitable developmental cues (Ng et al. 2013, Sachs et al. 2013).

Chromatin states also influence the establishment of parental-origin-specific expression of imprinted genes. Imprinted genes are controlled by parental-origin-specific DNA methylation marks. After erasure of these imprinting marks during PGC development, sperm- and oocyte-specific methylation marks need to be re-established to avoid the occurrence of imprinting disorders associated with altered dosage of imprinted gene expression in the offspring (Davis et al. 2000, Lucifero et al. 2002, Arnaud 2010). In prospermatogonia, maternally methylated imprinting control regions (ICRs) are methylated at H3K4 to protect against DNA methylation. In contrast, paternally methylated ICRs lack methylated H3K4, which correlates with the acquisition of DNA methylation (Henckel et al. 2012). An exception is observed at the Rasgrf1 locus, which contains a transposon-derived sequence and depends on a small RNA-mediated mechanism (Watanabe et al. 2011, Henckel et al. 2012), suggesting that some repetitive elements are regulated through distinct pathways during germline reprogramming.

TEs comprise ~40% of mammalian genomes and exhibit potentially deleterious effects on cell survival and differentiation when activated (Slotkin & Martienssen 2007). Appropriate epigenetic mechanisms for long-term TE suppression are crucial for the maintenance of genominc stability in male germ cells (Bourc’his & Bestor 2004, Aravin et al. 2008, Kuramochi-Miyagawa et al. 2008). Accumulating evidence indicates that the vast majority of TE reacquire DNA methylation through a default mechanism from E13.5 to E16.5. For instance, 95% of long interspersed nuclear element-1 (LINE-1) and long terminal repeat (LTR) retrotransposons acquire de novo DNA methylation through a non-selective mechanism (Molaro et al. 2014). Some TEs evade de novo DNA methylation and display constitutive hypomethylation associated with H3K4me2 modulation, whereas a small proportion of TE subfamilies undergo small RNA-guided transposon silencing (Kobayashi et al. 2013, Singh et al. 2013, Molaro et al. 2014, Pezic et al. 2014). In addition to recruiting histone methyltransferase for H3K9 trimethylation establishment, a portion of the small RNA-dependent TE silencing is correlated with DNA methylation. The enrichment of H3K9me3 modifications at these evolutionarily young full-length TE families implicates the crucial role of this repressive mark in TE silencing in prospermatogonia (Pezic et al. 2014).

Molecules contributing to epigenomic reprogramming and TE silencing in prospermatogonia

Roles of DNMTs in prospermatogonia development

A combination of active and passive DNA demethylation pathways contribute to a global DNA methylation loss in PGCs (Popp et al. 2010, Hackett et al. 2013, Ohno et al. 2013, Vincent et al. 2013). Some CpG dinucleotides in specific contexts, particularly those found in introns and intergenic regions, retain partial DNA methylation in E13.5 PGCs (Kobayashi et al. 2013). When the wave of DNA methylation occurs after E13.5, the vast proportion of the genome progressively regains the methylation marks before birth (Molaro et al. 2014). The two de novo methyltransferases, DNMT3A and DNMT3B, as well as their facilitator DNMT3L participate in this process (La Salle et al. 2004, Sakai et al. 2004). DNMT3L is unable to add a methyl group to cytosine by itself due to the lack of essential components on its C-terminus that enable enzymatic activity, but it can act as a facilitator and cooperate with DNMT3A/3B to perform de novo DNA methylation (Chedin et al. 2002, Suetake et al. 2004). Analyses of conditional Dnmt3a and Dnmt3b deficiency in male germ cells show that both of them are crucial for the establishment of DNA methylation at paternally
methylated imprinted loci and for retrotransposon silencing. Notably, in addition to having the same effects on the Rasgrf1 locus and the LINE-1 and intracisternal A particle (IAP) retrotransposons, DNMT3A preferentially methylates the differentially methylated H19 and Dlk1-Gtl2 imprinted loci, whereas DNMT3B is important for methylation of satellite repeats, suggesting that these two DNMTs display common and differential target specificities in prospermatogonia (Kato et al. 2007). Male mice with a conditional Dmnt3a deficiency exhibit defective spermatogenesis and adult testis that lack germ cells (Kaneda et al. 2004), indicating that DNMT3A is critical for male germ cell development and maintenance. In contrast, Dmnt3b-mutant male germ cells develop normally and are able to fertilize eggs and generate offspring, suggesting a mechanism of compensation for defective Dmnt3B during postnatal germ cell development (Kaneda et al. 2004).

The phenotype of Dnmt3l knockout (KO) prospermatogonia is similar to that of Dmnt3a conditional mutants that display defective methylation on paternally methylated imprints and TEs, which are suggested to cause germ cell loss and infertility in adult Dnmt3l mice (Bourc’his & Bestor 2004, Kaneda et al. 2004). A recent study showed that the ADD domain of Dnmt3l is important for CpG methylation at repeat sequences and non-promoter CpGs as well as non-CpG methylation in prospermatogonia (Vlachogiannis et al. 2015). Non-CpG methylation is uniquely observed in prospermatogonia in the perinatal testes, but it is progressively lost during spermatogonial proliferation after birth (Ichiyanagi et al. 2013, Kobayashi et al. 2013). The ADD domain of Dnmt3l is demonstrated to be crucial for the interaction with HDAC1 and unmethylated histone H3K4 (Aapola et al. 2002, Deplus et al. 2002, Ooi et al. 2007). Therefore, in addition to mediating DNA methylation, Dnmt3l may modulate and rearrange the epigenome through histone modifications, as Dnmt3l is essential for facilitating the interactions among HDAC1, SETDB1, TRIM28 and Dnmt3A in mouse embryonic testes (Kao et al. 2014).

### Epigenetic mechanisms of piRNA-dependent TE silencing in prospermatogonia

Most of TEs can be repressed during the first wave of the de novo DNA methylation period. However, some retrotransposons, including several subfamilies of the LINE and LTR classes, can evade default DNA methylation and remain transcriptionally active at E16.5 (Seisenberger et al. 2012, Kobayashi et al. 2013, Molaro et al. 2014). A small RNA-guided TE silencing mechanism is suggested to play a role in suppressing those active TEs from E16.5 to birth (Aravin et al. 2007, Carmell et al. 2007). In E13.5 PGCs with a nadir of global DNA methylation, transiently reactivated retrotransposons are converted into a pool of primary piRNAs. These TE transcripts provide templates for secondary piRNA amplification (Molaro et al. 2014). In turn, these piRNAs have been suggested to work with the piRNA-interacting protein Miwi2, which further recruits histone modifiers for the deposition of repressive histone marks, and facilitate a secondary wave of de novo DNA methylation in these evolutionarily young and active TEs in the nucleus (Aravin et al. 2008, Kuramochi-Miyagawa et al. 2008). A recent study also demonstrated that in Miwi2-null prospermatogonia, subsets of LINE-1 families are aberrantly associated, associated with an increase in the active histone mark H3K4me2/3 (Pezic et al. 2014).

piRNAs are 25–32 nucleotide single-strand RNAs that interact with PIWI family proteins and are mainly expressed in the developing germline (Aravin et al. 2006, Girard et al. 2006). The biogenesis of piRNAs can be divided into primary piRNA biogenesis and secondary piRNA amplification; the latter is also known as the ping-pong cycle. Primary piRNAs are generated from single-stranded precursors that are cleaved by an endonuclease into piRNA intermediates. The intermediates are incorporated into a piRNA-interacting MILI protein complex and are then trimmed by an unknown endonuclease and methylated at the 2' O position of their 3' end by HEN1 to generate mature piRNAs (Chuma & Nakano 2013, Weick & Miska 2014). In a ping-pong cycle of amplification to enhance transposon silencing, primary piRNAs, which usually have uridine as the 1st nucleotide, interact with the MILI complex to guide the cleavage of complementary transcripts at the 10th overlapping nucleotide to produce secondary piRNAs with a hallmark of adenine at their tenth position (Aravin et al. 2008). Secondary piRNAs interact with Miwi2 and re-enter the nucleus to target TE transcripts and recruit histone methyltransferases (Aravin et al. 2008, Kuramochi-Miyagawa et al. 2008).

The expression levels of the majority of the LINE and LTR family members are not affected in the Miwi2-deficient prospermatogonia, suggesting the Miwi2-dependent TE silencing participates in a small subset of TEs, particularly in the transcriptionally and positionally active subfamilies (Carmell et al. 2007, Kuramochi-Miyagawa et al. 2008). In mouse prospermatogonia, high levels of H3K9me3 are detected on the evolutionarily young and transposable LINE and LTR families, such as L1-Gf, L1-T, L1-A and IAPEz, and IAPEy. The H3K9me3 modification is enriched in upstream (5' end) of these LINE families, while LTRs show higher H3K9me3 levels in both upstream and downstream regions, suggesting that there may be a distinct regulatory mechanism between them in embryonic male germ cells (Singh et al. 2013, Pezic et al. 2014). Dysfunction of the piRNA-dependent pathways in Miwi2-mutant prospermatogonia leads to de-repression of young LINE families (L1-Gf, L1-T, L1-A), without changing the expression of the LTR families (IAPEz, and

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Epigenome modulations for SSC/SPC self-renewal and differentiation

During the first week after birth, spermatogonia migrate from the luminal position to the basal membrane for proper self-renewal and differentiation in the seminiferous tubules (Dettin et al. 2003, McLean et al. 2003). A subset of spermatogonia become biologically active SSCs when they re-enter the cell cycle between 3 and 6 days postpartum (dpp), whereas others undergo apoptosis or directly transition into c-KIT+ differentiating spermatogonia for the first spermatogenic wave in pre-pubertal mice (Yoshida et al. 2006). In adult testes, SSCs are fundamental for steady state spermatogenesis. SSCs mature into SPCs, which then differentiate into A1 spermatagonia. A1 spermatagonia become A2, A3, A4, intermediate and type B spermatogonia through mitotic divisions, followed by meiotic maturation, resulting in primary and secondary spermatocytes. Eventually, spermatocytes are transformed into haploid spermatid and spermatooza (Oatley & Brinster 2008) (Fig. 2).

Numerous studies reporting the results of gene expression profile analysis, whole-mount immunostaining and cell transplantation indicate that SSC/SPCs are heterogeneous in their expression of markers, including GFRA1, ID4, PLZF and THY1 (Kubota et al. 2003, Sada et al. 2009, Suzuki et al. 2009, Gassei & Orwig 2013, Chan et al. 2014, Hermann et al. 2015). SSC/SPCs display similar morphological characteristics, but differential gene expression patterns among SSC/SPC subpopulations suggest that a shift in the epigenetic state contributes to determination of their cell fates, which may include quiescence, self-renewal or differentiation (Shirakawa et al. 2013, Liao et al. 2014, Hermann et al. 2015).

Rare differences in DNA methylation at promoter regions between undifferentiated THY1+ SSC/SPCs and the differentiation-committed c-KIT+ spermatogonia suggest that histone modifications are predominantly responsible for the transition between self-renewal and differentiation (Hammoud et al. 2014). Compared to PLZF+c-KIT− or GFRA1+c-KIT− SSC/SPCs, c-KIT+ cells exhibit different subnuclear localization of H3K9me3, indicating heterochromatin reorganization, and exhibit extended histone H3K9 dimethylation (H3K9me2) on a global level. These observations and the up-regulated DNMT3A2 and DNMT3B expression levels in differentiation committed spermatogonia suggest that epigenome rearrangements participate in the differentiation of SSC/SPCs (Shirakawa et al. 2013). In addition, adult THY1+ SSC/SPCs and c-KIT+ spermatogonia both exhibit chromatin bivalency with H3K4me3 and H3K27me3 at their promoters of development-related genes, accompanying DNA hypomethylation (Hammoud et al. 2014). Notably, a differentiation important factor, STRA8, converts its promoter from the bivalent chromatin state in THY1+ SSC/SPCs to the active chromatin state that retain only H3K4me3 modification in c-KIT+ spermatogonia (Hammoud et al. 2014). These data suggest that epigenetic modulations are important for the cell fate determination of spermatogonia.

Extrinsic stimulation and SSC/SPC-associated microenvironments

Growing evidence has shown that extrinsic stimulation from neighboring cells also contributes to the modulation of the SSC/SPC transcriptome. In the testis, SSC/SPCs reside in a special microenvironment, also known as a niche, which is composed of blood vessels, Leydig cells, macrophages and myoid cells, as well as Sertoli cells, which surround the SSC/SPCs to provide paracrine signals and intercellular communication to regulate SSC/SPC cell fate decision (Hai et al. 2014, Chen & Liu 2015). Sertoli cells secrete glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2) and retinoic acid (RA) to influence the

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capacity for self-renewal or differentiation in SSC/SPCs (Ref). GDNF and FGF2 regulate many downstream genes, including a subset of transcription factors such as Etv5, Bcl6b, Lhx1, Pou3f1, Nanos2, and Id4, which are all important for SSC/SPC maintenance (Oatley et al. 2007, Schlesser et al. 2008, Sada et al. 2009, Wu et al. 2011, Chan et al. 2014). The involvement of GDNF in SSC/SPC proliferation was first discovered by Meng et al. (2000) in genetically engineered mice. GDNF heterozygous KO mice display a gradual loss of SSC/SPCs, whereas GDNF-overexpressing mice exhibit SSC/SPC accumulation, indicating that the dosage of GDNF is important for SSC/SPC proliferation (Meng et al. 2000). GDNF can activate the PI3K/AKT pathway by binding to the GDNF receptor components GFRA1 and RET to stimulate SSC/SPC proliferation (Lee et al. 2007, Oatley et al. 2007). FGF2 can also significantly increase SSC/SPC proliferation (Ishii et al. 2012), whereas the balance between the FGF2 and GDNF levels mediates SSC self-renewal, as an increased level of GDNF associated with an elevated stem cell number is observed in FGF2-depleted testes (Takashima et al. 2015). In addition to stimulating self-renewal, Sertoli cells are the main source of RA in mouse testes. RA is essential for spermatogonia differentiation and can lead to down-regulation of Plzf and Oct4, which are essential factors for self-renewal (Gaemers et al. 1996, Vernet et al. 2006, Dann et al. 2008). RA predominately affects NGN3⁺ SSC/SPC, which have a greater competence for differentiation compared with GFRA1⁺ SSC/SPCs (Ikami et al. 2015). The differential responses of different SSC/SPC subpopulations to paracrine stimulation suggest that alternation of the epigenome occur during the transition from an undifferentiated to a differentiated state.

### Factors involved in epigenome modulations for SSC/SPC maintenance and differentiation

#### Histone modifiers and transcription factor-associated epigenetic modulation in SSC/SPC maintenance and differentiation

PLZF is a transcriptional regulator that binds to DNA through Kruppel-like zinc fingers at its C-terminus and is shown to interact with several epigenetic regulators to affect the epigenome. This protein can recruit HDACs and DNMTs to promote gene silencing and inhibit cell proliferation and differentiation in some adult stem cells (Doulatov et al. 2009, Puszyk et al. 2013). Plzf-nonsense-mutant and Plzf-null mice show a progressive loss of germ cells and depletion of SSC/SPCs in adult testes, indicating that PLZF is essential for SSC/SPC maintenance (Buas et al. 2004, Costoya et al. 2004). PLZF⁺ SSC/SPCs are a relatively quiescent subpopulation in postnatal testes (Costoya et al. 2004, Sada et al. 2009, Liao et al. 2014). PLZF co-localizes with H3K9me3 and may participate in modulating the distribution of H3K9me3; mitotically arrested PLZF⁺ SSC/SPCs exhibit a perinuclear distribution of H3K9me3 and diffuse chromatin, whereas proliferative PLZF⁺ SSC/SPCs and differentiated spermatogonia present punctate foci of H3K9me3 and chromatin (Fig. 3A and B).

![Epigenetic regulations in the male germline](https://www.reproduction-online.org)

**Figure 3** The characteristics of quiescent and active THY1⁺ SSC/SPCs. (A) Schematic illustrating the status of THY1⁺ SSC/SPCs. Perinuclear staining of PLZF/H3K9me3 (in orange frame) indicates relative quiescent SPCs. Punctate staining of PLZF/H3K9me3 (in purple frame) represents proliferating SPCs. (B) The majority of the mitotically arrested THY1⁺ SSC/SPCs is associated with perinuclear PLZF/H3K9me3 signals and diffuse chromatin; whereas the cell cycling THY1⁺ SSC/SPCs are correlated with punctate PLZF/H3K9me3 foci and punctate chromatin (lower). Scale bar: 5 μm. The luxoid/lu⁺ mutation was used to validate the specificity of the PLZF antibody. (C) (Upper) DNMT3L is predominantly expressed in a subpopulation of THY1⁺ PLZF⁺ SSC/SPCs associated with quiescent characteristics. Dnmt3l-knockout (Dnmt3l⁻/-) samples were used to validate the specificity of the DNMT3L antibody. The strongest signal intensity from Dnmt3l-knockout THY1⁺ cells was used as the background. Arrows indicate the DNMT3L⁺ PLZF⁺ THY1⁺ cells. Scale bar: 10 μm. (Lower) The percentages of DNMT3L⁺ PLZF⁺ in 8 dpp THY1⁺ SSC/SPCs. Approximately 25% of the THY1⁺ PLZF⁺ SSC/SPCs are DNMT3L⁺ cells.
(Payne & Braun 2006, Yoshioka et al. 2009, Liao et al. 2014). PLZF is suggested to be an important factor for self-renewal based on its ability to regulate the c-Kit gene, which participates in spermatogonia differentiation (Filippioni et al. 2007), and its ability to positively regulate Redd1, which is a negative regulator of the mammalian target of rapamycin complex 1 (mTORC1) pathway (Gan et al. 2008, Hobbs et al. 2010). mTORC1 is critical for the balance of self-renewal and differentiation due to its role in regulating downstream gene translation and repressing the GDNF receptors GFRA1 and RET in PLZF−/− SSC/SPCs (Hobbs et al. 2010). Furthermore, PLZF can antagonize the transcription factor SALL4A, probably by restricting SALL4 to a perinuclear localization to prohibit SSC/SPC differentiation (Hobbs et al. 2012), suggesting that coordinated modulation in the nucleus may help fine-tune SSC/SPC cell fate determination.

Several epigenetic regulators are known to be important factors for SSC/SPC maintenance and differentiation. EED, JMJD1C, JMJD3 and SETDB1 are expressed in PLZF+ SSC/SPCs, suggesting epigenetic modulations may participate in determining cell fate of SSC/SPCs (Iwamori et al. 2013, Kuroki et al. 2013, An et al. 2014, Mu et al. 2014). JMJD1C is a histone H3K9me1/2 demethylase (Kim et al. 2010). A deficiency of JMJD1C results in the progressive reduction of SSC/SPCs in the testes, indicating a role for JMJD1C in SSC/SPC maintenance. Loss of JMJD1C does not cause global H3K9 demethylation, suggesting that the influence of JMJD1C may be restricted to specific regions of the genome (Kuroki et al. 2013). In contrast, the H3K27me2/3 demethylase JMJD3 is not essential for fertility but plays a role in the fragmentation of spermatogonial cysts without affecting SSC/SPC differentiation. The loss of JMJD3 leads to increased fragmentation of spermatogonial cysts and an increased proportion of Asingle spermatagonia (Iwamori et al. 2013). EED belongs to the polycomb-repressive complex 2 (PRC2), which can catalyze di- and tri-methylation of histone H3K27 (Mu et al. 2014). Germ cell specific deletion of EED using the Mvh-Cre recombinase from E15.5 causes germ cell exhaustion in adults, suggesting a role for PRC2 in SSC/SPC maintenance.

SETDB1 is a histone H3K9me3 methyltransferase and is essential for SSC/SPC survival. In vitro studies have shown that SETDB1 may modulate the expression of Cox4i2, an apoptosis-promoting molecule. Moreover, SETDB1 targets the promoter regions of Dazl and Sohlh2, which are two contributors to spermatogonia differentiation, implying a function for SETDB1 in the cell fate determination of SSC/SPCs (Schrans-Stassen et al. 2001, Hao et al. 2008, Suzuki et al. 2012, An et al. 2014). Of note, one in vitro study showed that, similar to the loss of H3K9me3, the down-regulation of SETDB1 causes a reduction in DNA methylation in the promoter region of Cox4i2, suggesting that SETDB1 may contribute to the regulation of gene expression not only by modifying histone proteins but also by influencing DNA methylation (An et al. 2014).

**Roles of DNMTs in SSC/SPC survival and germline homeostasis**

Down-regulation of Dnmt1 in cultured germline stem cells (GSCs) causes apoptosis, whereas double Dnmt3a/3b mutant GSCs display aberrant spermatogenesis when transplanted back into recipient testes (Takashima et al. 2009). Dnmt3l-deficient germ cells fail to establish in vitro GSC cell lines (Niles et al. 2013). Ectopic expression of Dnmt3l results in spermatogenic arrest at the spermatocyte stage associated with decreased expressions of Clgn, Piwil, Ccna1 and Crem, which are factors involved in germ cell differentiation and survival (Takashima et al. 2009).

In prospermatogonia, DNMT3L is predominantly localized in the nucleus (Sakai et al. 2004). In contrast, postnatal THY1+ SSC/SPCs display both nuclear and cytoplasmic expressions of Dnmt3l (Fig. 3C). DNMT3L is expressed in a proportion (~25%) of postnatal THY1+ SSC/SPCs that exhibit characteristics of a quiescent state with perinuclear PLZF and diffuse chromatin (Fig. 3C), demonstrating the heterogeneity of the SSC/SPC subpopulation. Deficiency of Dnmt3l results in enhanced proliferation of THY1+ cells in association with over-expressed CDK2 and reduced PLZF stability (Liao et al. 2014). Dnmt3l-deficient SSC/SPCs display increased expression of TE s and hypomethylation at paternally methylated ICRs (Liao et al. 2014, Vlachogiannis et al. 2015). Some of the genes that are deregulated in Dnmt3l-deficient OCT4+ SSC/SPCs are located close to transcriptionally active TEs, suggesting that their aberrant expression pattern may be due to epigenetic defects associated to TE de-repression. These abnormal gene expression activities and the germ cell exhaustion phenotype observed in adult Dnmt3l-deficient testes may be solely due to the accumulation of epigenomic defects in embryonic prospermatogonia. Whether postnatal DNMT3L, which is expressed in a subset of relatively quiescent SSC/SPCs (Fig. 3), also plays a significant role in SSC/SPC function will require clarification with conditional Dnmt3l-KO experiments.

**Small non-coding RNAs in SSC/SPC maintenance and differentiation**

Increasing evidence indicates that small ncRNAs, including miRNAs and piRNAs, are expressed in male germ cells and are important for spermatogenesis (Aravin et al. 2007, Carmell et al. 2007, Wu et al. 2012). miRNAs are generated from transcripts that are processed predominantly by RNA polymerase II. The pre-miRNAs are single-stranded RNAs that form hairpin structures and are processed into mature miRNAs by
DROSHA and DICER (Lee et al. 2003, Denli et al. 2004, Han et al. 2006). Mature miRNAs then associate with ARGONAUTE (AGO) proteins to form the RNA-induced silencing complex (RISC), which binds to mRNA and can lead to mRNA degradation or translational repression (Gregory et al. 2005, Diederichs & Haber 2007). Differentially expressed miRNAs among male germ cells have been reported, indicating that miRNAs are expressed in a cell type-specific manner. Recent reviews consolidating miRNAs for male germ cell development pointed to the miRNAs involved in SSC/SPC maintenance can be found in (Kotaja 2014, Yao et al. 2015). Here, we focus on the miRNAs involved in SSC/SPC maintenance and differentiation.

Several miRNAs are responsible for mouse SSC/SPC maintenance and differentiation, including miR-21, miR-17-92, miR-106b-25, miR221/222, miR-34c, miR-146, miR-let7s, miR-20 and miR-106a (Niu et al. 2011, Tong et al. 2011, 2012, He et al. 2013, Huszar & Payne 2013, Yang et al. 2013, Chakraborty et al. 2014, Wu et al. 2014, Yu et al. 2014). Compared with THY1+ spermatogonia, miR-21 is highly expressed in THY1+ SSC/SPCs and is regulated by ETV5 in THY1+ enriched cultured cells (Niu et al. 2011). ETV5, a downstream effector of GDNF signaling, is important for maintaining stemness (Schlesser et al. 2008). The inhibition of miR-21 leads to germ cell apoptosis in vitro and decreased colony numbers in transplantation assays, suggesting that miR-21 is important for SSC/SPC maintenance (Niu et al. 2011). miR-17-92 and miR-106b-25 are also expressed in THY1+ SSC/SPCs and are down-regulated after RA treatment both in vivo and in vitro, indicating a function for these miRNAs in SSC/SPC self-renewal (Tong et al. 2012). miR-221/222 is required for the maintenance of the undifferentiated state because it negatively regulates c-Kit expression and the inhibition of miR-221/222 results in the stimulation of differentiation associated with the loss of stem cell capacity in THY1+ cells in vitro (Yang et al. 2013). Similar to miR-221/222, miR-20 and miR-106a from the miR-17-92 cluster play roles in SSC/SPC self-renewal that may be partly mediated through targeting and down-regulating the spermatogonial differentiation factor STAT3 (He et al. 2013). miR-146 is highly expressed in THY1+ GFRA1+ cells compared with c-KIT+ differentiated spermatogonia. In vitro assays showed that miR-146 directly targets Med1, a transcription factor responsible for regulating and interacting with nuclear hormone receptors, including RA (Ren et al. 2000, Huszar & Payne 2013). Moreover, miR-146 can down-regulate c-Kit, Med1 and Sohlh2 in RA-treated spermatogonia in vitro, suggesting a function for miR-146 in modulating SSC/SPC differentiation via the regulation of RA (Huszar & Payne 2013). The simultaneous inactivation of the two functionally overlapping miRNAs, miR-34b/c and miR-449, causes sterility (Wu et al. 2014). The overexpression of miR-34c interferes with Nanos2 expression and increases the expression of Nanos3, Scp3 and Stra8 in THY1+ cultured cells, suggesting that miR-34c promotes differentiation in SSC/SPCs (Yu et al. 2014). In addition to miR-34c and miR146, which play roles in differentiation modulation, RA can up-regulate miR-let7s expression through LIN28 inhibition, implicating RA-induced miRNAs in spermatogonial differentiation (Tong et al. 2011). LIN28, an RNA-binding protein, suppresses miR-let7s biogenesis (Viswanathan & Daley 2010). The lack of LIN28A does not cause a significant reduction in the size of the functional stem cell compartment, indicating that LIN28A may not participate in maintaining the stem cell pool in mouse testes. However, LIN28A contributes to the regulation of Aal spermatogonia divisions without leading to premature differentiation. The influence of LIN28A in Aal spermatogonia expansion may be partly mediated by the let-7g (Chakraborty et al. 2014).

Endogenous siRNAs (endo-siRNAs) were recently found in mouse spermatogonia and are produced via DICER-dependent but DROSHA-independent pathways. Their capacity for targeted mRNA degradation in vitro suggests a potential function in post-transcriptional regulation (Song et al. 2011). Endo-siRNAs are also expressed in plants, where they function in the induction of DNA methylation and heterochromatin formation in the nucleus (Zhang & Zhu 2011, Matzke & Mosher 2014). However, the roles of endo-siRNAs in mouse male germ cells are still largely unknown. Future investigations of endo-siRNA-mediated gene silencing will yield many exciting discoveries.

It has been well-demonstrated that PIWI proteins and piRNAs are important for the repression of TE activity and re-establishment of epigenetic markers in fetal prospermatogonia (Aravin et al. 2008, Kuramochi-Miyagawa et al. 2008). In a comparison between two well-known types of piRNAs, pre-pachytene and pachytene piRNAs, piRNAs from 1-week old THY1+ SSC/SPCs and adult c-KIT+ spermatogonia exhibited high similarity with pre-pachytene piRNAs that contain high percentage (~50%) of transposon-derived piRNAs (Grivna et al. 2006, Aravin et al. 2007, Hammoud et al. 2014, HF Liao and SP Lin unpublished observation). Interestingly, we also observed an obvious increase of exon-derived piRNAs in 1-week-old THY1+ SSC/SPCs (HF Liao and SP Lin unpublished observation). Recent studies have shown that piRNAs can regulate the mRNA transcript level through a siRNA-mediated cleavage mechanism in spermatocytes and spermatids (Goh et al. 2015, Watanabe et al. 2015, Zhang et al. 2015). Further investigations of these exon-derived and intergenic region-derived piRNAs and their potential targets will be important to understand whether a piRNA-mediated mRNA degradation mechanism occurs in SSC/SPCs.

**Perspectives and conclusion**

A precise epigenomic profile is generally accepted to be required for transcriptome establishment and small RNA

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**Epigenetic regulations in the male germline**

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production during germ cell development. An important issue for germ cell development is to safeguard the integrity of the genome through repressing TE mobility and propagation (Siomi & Kuramochi-Miyagawa 2009, Ishizu et al. 2012). Miwi2 deficient male germline cells display de-repressed retrotransposons associated with an increase of in the level of the active histone mark H3K4me2/3 and a loss of DNA methylation on their sequences (Aravin et al. 2007, Carmell et al. 2007), suggesting that piRNAs may mediate TE silencing associated with DNA methylation and histone modification in fetal prospermatogonia (Fig. 4). At the postnatal stage, PLZF expression begins to be up-regulated and is crucial for LINE retrotransposon repression (Costoya et al. 2004, Puszyk et al. 2013). PLZF can interact with DNMT1 and HDAC1, and the co-localization of PLZF and H3K9me3 suggests that DNMTs and PLZF may cooperate with an H3K9 methyltransferase to regulate gene expression in SSC/SPCs (Payne & Braun 2006, Puszyk et al. 2013, Liao et al. 2014). In addition, PLZF co-localizes with SETDB1 in SSC/SPCs (An et al. 2014). These results imply that PLZF may cooperate with certain epigenetic factors and a piRNA-guided pathway to safeguard the germ line at the postnatal stage. A proposed model for LINE transposon silencing in prospermatogonia and SSC/SPCs is summarized in Fig. 4.

Furthermore, although DNMT3L, PLZF and SETDB1 may have overlapping functions in the acquisition of the correct biological characteristics of SSC/SPCs, the differences in the transcriptional profiles among Dnmt3l-KO, Plzf-KO and Setdb1-KO SSC/SPCs reveal that these three factors may regulate distinct gene targets (Hobbs et al. 2010, An et al. 2014, Liao et al. 2014).

**Figure 4** A proposed model for evolutionarily young LINE silencing within the nucleus of prospermatogonia and SSC/SPCs. Two waves of epigenetically modulated silencing of transposable elements (TEs) occur in developing prospermatogonia to safeguard the male germline. The first wave of default de novo methylation occurs between E13.5 and E16.5 to silence the majority of the TEs. piRNA-guided pathway is suggested to be responsible for repressing a portion of repetitive elements, such as evolutionarily young, transposition active, full-length long interspersed nuclear elements (LINEs), which can evade the first wave of de novo DNA methylation. Here, we propose a model of full-length LINE silencing in perinatal prospermatogonia and postnatal SSC/SPCs. In perinatal prospermatogonia, MIWI2 and the piRNA complex can recognize the transcriptionally and transposition active LINE TEs and recruit histone methyltransferases to deposit a repressive H3K9me3 mark in the upstream region of LINE TEs. Some of the TEs that are regulated by piRNA-mediated epigenetic repression will undergo de novo DNA methylation for long-term and stable silencing. In contrast, the constitutively active TEs may maintain an active H3K4me2/3 mark and DNA hypomethylation in their promoter regions during male germ cell development. At the postnatal stage in SSC/SPCs, the piRNA-mediated silencing mechanism may still occur for TE silencing because SSC/SPCs contain large amounts of transposon-derived piRNAs. In addition, PLZF and some epigenetic factors are suggested to silence full-length TEs, such as the evolutionarily young and potentially active elements of the LINE family. PLZF and its associated DNMT/HDAC proteins are required to establish or maintain repressive epigenetic modifications on full-length LINEs to suppress their expression. A deficiency of PLZF results in the transcriptional activation of these LINEs and correlates with active histone modifications and DNA hypomethylation.
Identifying their targets through chromatin immuno-precipitation sequencing (ChIP-Seq) will identify their overlapping and unique functions.

Deficiencies of the epigenetic factors JMD1C, PLZF and DNMT3L result in progressive germ cell exhaustion in mouse testes (Buaas et al. 2004, Kuroki et al. 2013, Liao et al. 2014). Some segments of seminiferous tubules contain many germ cells, whereas other segments display very few germ cells or Sertoli cell-only, raising the question of whether these epigenetic factors have the significant effect on seminiferous tubules at specific stages of spermatogenic cycle.

Comparing to prospermatogonia, postnatal SSC/SPCs are more heterogeneous, even within subpopulations with identical surface markers, for instance GFRA1+ , PLZF+ and THY1+ SSC/SPCs (Kubota et al. 2003, Sada et al. 2009, Gassei & Orwig 2013, Liao et al. 2014, Hermann et al. 2015, Takashima et al. 2015). The differences in isolation protocols and the affinity of antibodies may have effects on the enrichments of subpopulations with specific cellular states (Buageaw et al. 2005, Ebata et al. 2005, Grisanti et al. 2009, Liao et al. 2014, Hermann et al. 2015, Takashima et al. 2015, Vlachogiannis et al. 2015). In addition, a suitable 3D culture system of SSC/SPCs with living cell tracking and epigenome-wide single cell analysis without PCR amplification will lead to further understanding of SSC/SPC self-renewal and differentiation decisions.

In conclusion, this review summarized recent findings on epigenome-related factors essential for prospermatogonia development and SSC/SPC maintenance and differentiation. Future studies concentrating on the cross-talk and molecular functions of epigenome-related factors will further advance our understanding of the biology of prospermatogonia and SSC/SPCs to improve the diagnosis of infertility.

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

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