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類 3 號 DNA 甲基轉移酶 (Dnmt3L) 對小鼠精原幹細胞功能的影響

The role of Dnmt3L in maintaining the property of spermatogonial stem cells during mouse germ cell development

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中文摘要

基因表現的後生遺傳調控是維持細胞正常功能之重要因素。部分體內之幹細胞被維持在一種「不活躍」的「休眠」狀態，並等待其適合自我複製或向下分化的時機。類 3 號 DNA 甲基轉移酶 (Dnmt3L) 是一個表現在哺乳動物生殖細胞發育過程中不可或缺的後生遺傳調控者，缺少此基因會對內源反轉錄病毒反轉錄子序列造成過低的 DNA 甲基化的情形，造成進入減數分裂之生殖細胞的凋亡，並隨著小鼠年齡增長,逐漸造成完全喪失生殖細胞之性狀(Sertoli cell-only syndrome)，無法繁殖後代。然而 Dnmt3L 對於精原幹細胞功能之影響目前尚不清楚，因此本研究針對精原幹細胞發育之時期探討 Dnmt3L 對於維持精原幹細胞之功能之影響。

第一部分之研究顯示 Dnmt3L 對精原幹細胞之休眠狀態之建立或維持有重要的影響。此結果主要從 Dnmt3L$^{-/-}$之初生幼鼠表達 Plzf 的細胞數減少與兩個異染色質標記 H3K9me3 和 H4K20me3 之環狀染色特徵減少結果得知。另經過生殖細胞移植之功能性測試實驗發現 Dnmt3L$^{-/-}$之生殖細胞無法至宿主之睪丸中增生與分化。因此，此部分試驗顯示出 Dnmt3L 除了對 DNA 甲基化很重要外，也參與在異染色質之建立與/或維持之過程中。第二部分試驗顯示缺乏 Dnmt3L 至少會間接造成支持精原幹細胞正常功能之微環境受到影響。從 VASA 與 STRA8 之染色中，看見異常的生殖細胞與微環境細胞分離結果顯示，在 Dnmt3L$^{-/-}$五週大之小鼠睪丸中，精原幹細胞與其體細胞微環境 (niche) 間之交互作用出現問題。另外此部分之功能性測試的預先試驗顯示 Dnmt3L$^{-/-}$之睪丸無法支持野生型之精原細胞重新在生精細管中增生與分化。此試驗結果意味著 Dnmt3L 不僅在生殖細胞中佔有重要的角色，並可能對於支持精原幹細胞正常功能之微環境亦有其影響。

綜合以上所述,此研究利用了精原幹細胞的模式將Dnmt3L之角色從調控DNA甲基化聯結至影響組蛋白修飾之功能上，並且在功能性測試試驗中顯示 Dnmt3L 不僅是維持精原幹細胞功能之重要因子，而且也可能直接或間接性影響精原幹細胞之微環境。

ABSTRACT

Epigenetic regulation of gene activities is crucial for proper cellular function. Some of the stem cells *in vivo* are kept in a quiescent status, waiting for their turn to proliferate or differentiate. DNA methyl- transferase 3-like (Dnmt3L) is a very important epigenetic regulator expressed during germ line development. Deficiency of Dnmt3L in male germ cells causes severe hypomethylation in retrotransposons. This leads to germ cell depletion after the pachytene stage and eventually sertoli-cell only syndrome in mice. However, how Dnmt3L affects the function of spermatogonial progenitor/stem cells (SSCs) is not well understood. This study focuses on the developmental stages of SSCs in mice, and investigates the role of Dnmt3L in maintaining SSCs property.

The first part of the study showed that Dnmt3L was essential for the establishment and/or maintenance of the quiescent status of SSCs. It was concluded from the reduced Plzf positive cells and perinuclear staining pattern of two heterochromatin markers H3K9me3 and H4K20me3 in Dnmt3L$^{-/-}$ neonatal mice testes. Our preliminary functional transplantation study also demonstrated that Dnmt3L$^{-/-}$ spermatogonia could not colonize the wild-type host testes. Taken together, the first part of the study has demonstrated a new role of Dnmt3L on keeping the quiescent status of SSCs. Also, Dnmt3L seems to be an epigenetic regulator not only important for DNA methylation, but also involved in proper heterochromatin establishment and/or maintenance in SSCs. The second part of this study showed that deficiency of Dnmt3L may affect the niche environment essential for sustain in the normal function of SSCs. Based on Vasa and Stra8 staining patterns, we've observed an abnormal germ cell-niche/somatic cell segregation, the niche structure that was supposed to hold the spermatogonia seemed to

be disrupted in Dnmt3L$^{-/-}$ 5 wks-old testes. Preliminary functional transplantation studies also demonstrated that Dnmt3L$^{-/-}$ testes failed to support the colonization repopulation of wild-type spermatogonia. The second part of our study gives a hint that Dnmt3L is not only essential for germ cell function, but may also affect normal niche environment that supports SSCs.

In conclusion, this study has used SSCs as a model to connect the epigenetic modifier Dnmt3L from DNA methylation to histone modification. Also, functional analysis and molecular staining demonstrate that Dnmt3L is not only necessary for normal SSC function, but may also directly or indirectly maintain the niche environment of SSCs.

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Chapter 1 Literature review

1.1 The importance of epigenetics in stem cell study

Stem cells have the ability to keep a tissue homeostasis well by its potential to self-renew and differentiate at the same time. Epigenetics, including DNA methylation, histone modification, and non-coding RNA-mediated events (Goldberg *et al*., 2007) can help us understand the "status" of stem cells, and thus disclose the mystery of how stem cells sustain the tissue function. Many epigenetic regulatory factors, which are the "players" for epigenetic mechanisms have been discovered to play important roles in stem cell self-renew or maintenance, and have also drawn attention to be important in cancer research.

1.2 Reprogramming and *de novo* DNA methylation in the mammalian germ line

In mammals, a genome-wide epigenetic event of reprogramming occurs during the pre-implantation stage (Hajkova *et al*., 2002; Kato *et al*., 1999; La Salle *et al*., 2004; Lee *et al*., 2002; Szabo *et al*., 2002; Szabo and Mann, 1995). Most of the DNA methylation marks are erased and re-established (Kafri *et al*., 1992; Monk *et al*., 1987). By this time, DNA methyltransferase 3a (Dnmt3A) and DNA methyltransferase 3b (Dnmt3B) take place to establish DNA methylation marks (Bourc'his *et al*., 2001; Okano *et al*., 1999). Interestingly, however, another wave of reprogramming on DNA methylation marks occurs in the mouse germ line around 11.5 to 12.5 days post coitum (dpc) when PGCs start to localize into the gonad. These events include the erasure and re-establishment of parental imprinting marks, de-repression and silencing of endogenous retrotransposon sequences, and X-chromosome re-activation in females

(Bourc'his *et al*., 2001; Hajkova *et al*., 2002; Hata *et al*., 2002; Kaneda *et al*., 2004b; Slotkin and Martienssen, 2007) (Fig. 1-1). *De novo* DNA methylation in the male gonad starts prenatally from the beginning of spermatogenesis around 12.5 dpc, whereas *de novo* DNA methylation happens after birth in female when oocytes finish the first meiotic division and start to enter the GV stage (Cedar and Bergman, 2009; La Salle *et al*., 2004; Reik, 2007; Sasaki and Matsui, 2008). During the window of *de novo* DNA methylation of both male and female, Dnmt3-like (Dnmt3L), a non-catalytic paralogue and regulator for DNA methylation, has a very specific expression window matching the *de novo* DNA methylation event (La Salle *et al*., 2004) (Fig. 1-2).

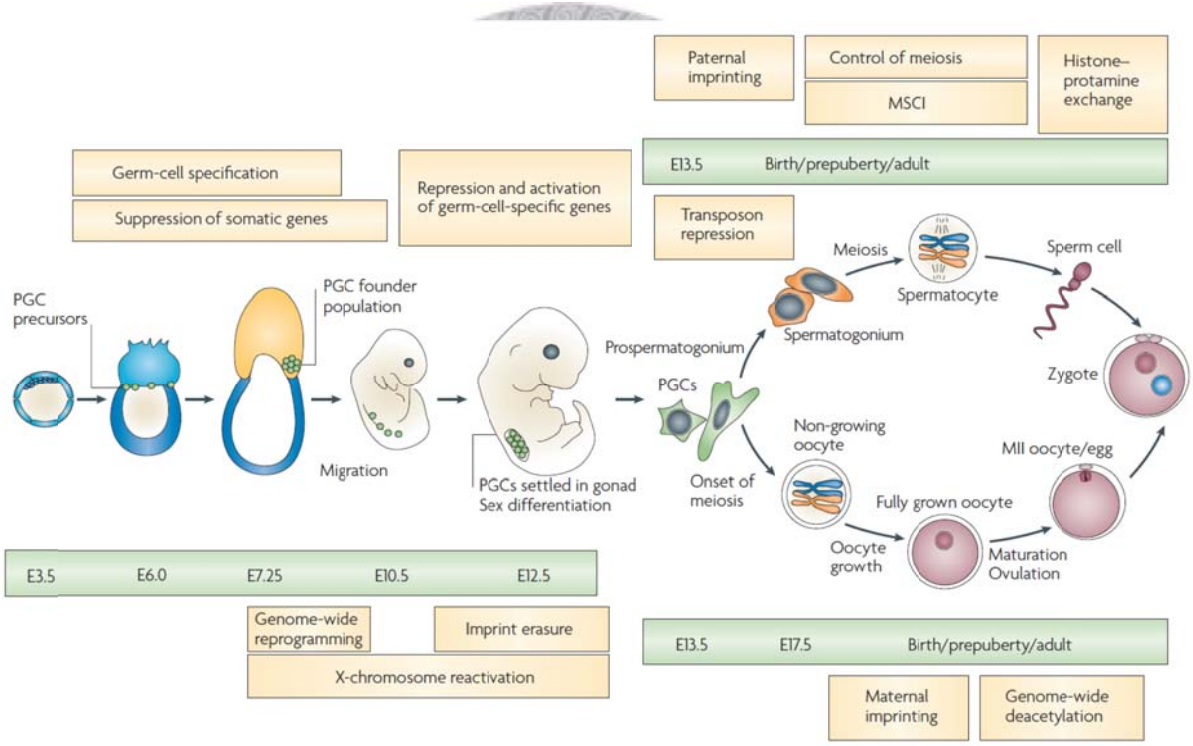

Fig. 1-1 Summary of the epigenetic events in the mouse germ line development

The mouse germ line development starts from the blastocyst by E3.5, the germ-cell precursors start to migrate and proliferate at the same time towards the genital ridge of the embryo, and become PGCs by E13.5 when they reach the genital ridge. A cell cycle arrest of the germ cells happens at this stage when imprinting erasure and re-establishment take place. The onset of meiosis differs between spermatogenesis in male and oogenesis in female (Sasaki and Matsui, 2008).

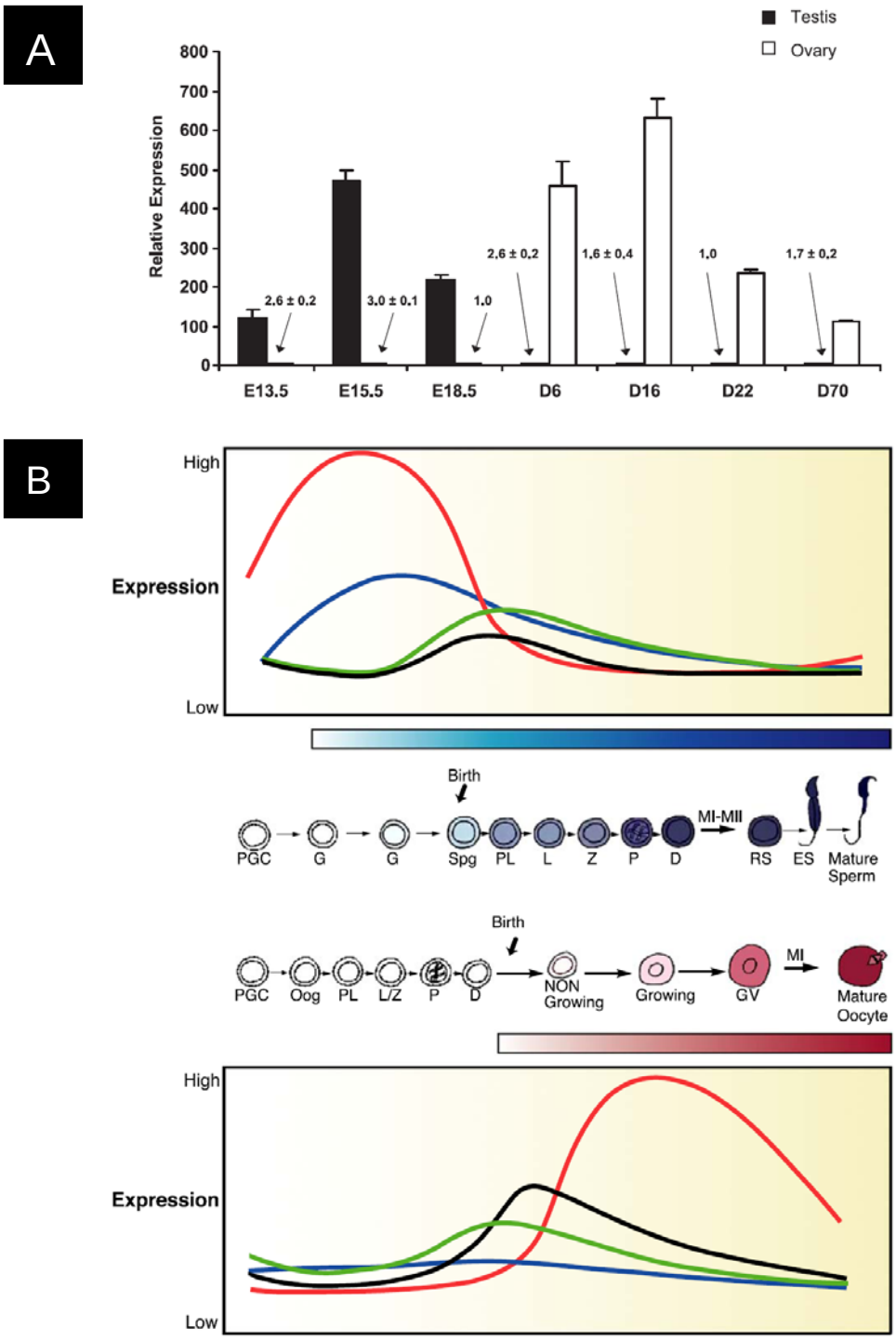

Fig. 1-2 Expression windows for Dnmts during male and female germ line development

A. The mRNA expression pattern of Dnmt3L in male and female germ line.
B. The combined expression patterns of Dnmt1(black line), Dnmt3a (blue line), Dnmt3b (green line), and Dnmt3L (red line). The intensity of the shading in the boxes represent the level of DNA methylation of the paternal (blue) and maternal (red) genomes. (La Salle *et al*., 2004)

1.3 Dnmt3L's discovery and its function in the germ line

Dnmt3L is an epigenetic regulatory protein of similar sequence with Dnmt3A and Dnmt3B, but lacking the methyltransferase domain (Aapola *et al*., 2000; Okano *et al*., 1998) (Fig. 1-3), and shown to be essential in both male and female gametogenesis (Bourc'his and Bestor, 2004; Bourc'his *et al*., 2001; Hata *et al*., 2006; Hata *et al*., 2002; Webster *et al*., 2005). Dnmt3L$^{-/-}$ mice generated separately by Bestor's and Hata's group have shown a failure in establishing maternal methylation imprinting marks during oogenesis. Progenies derived from Dnmt3L$^{-/-}$ oocytes fail to develop pass embryonic day 9.5, due to biallelic expression of genes that originally should be maternally methylated (Bourc'his *et al*., 2001; Hata *et al*., 2002). Dnmt3L$^{-/-}$ male are sterile, with an absence of germ cells developing later than pachytene spermatocytes, due to defected homologous chromosome pairing during meiotic synapsis, which then results in apoptotic cell death (Fig. 1-4). The inappropriate pairing of chromosomes is found due to the reactivation of endogenous retrotransposons, namely LINE1 and IAP transcripts, which should be methylated during male gametogenesis (Fig. 1-5) (Bourc'his and Bestor, 2004; Hata *et al*., 2006). Imprinted genes associated with paternally methylated DMRs (differentially methylated region) have also been affected in Dnmt3L$^{-/-}$ male in Kaneda's study (Kaneda *et al*., 2004a), but Bourc'his's group had shown only partial demethylation (about 50%) in *H19* DMR, and no significant demethylation in *Dlk1-Gtl2* DMR (Bourc'his and Bestor, 2004). In spite of the controversy, the role of Dnmt3L in DNA methylation in the mammalian germ line cannot be ruled out.

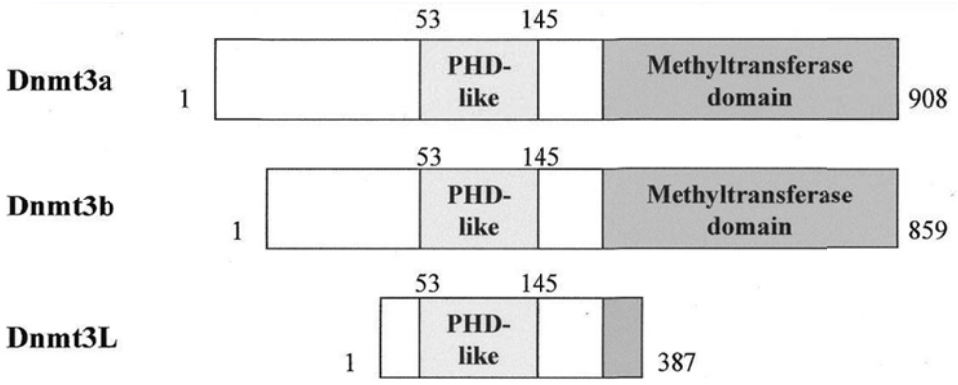

Fig. 1-3 Representation of the Dnmt family protein

The N-terminal PHD-like domain is conserved in all three Dnmt homologues, while the C-terminal methyltransferase domain is absent in Dnmt3L. (Deplus *et al*., 2002)

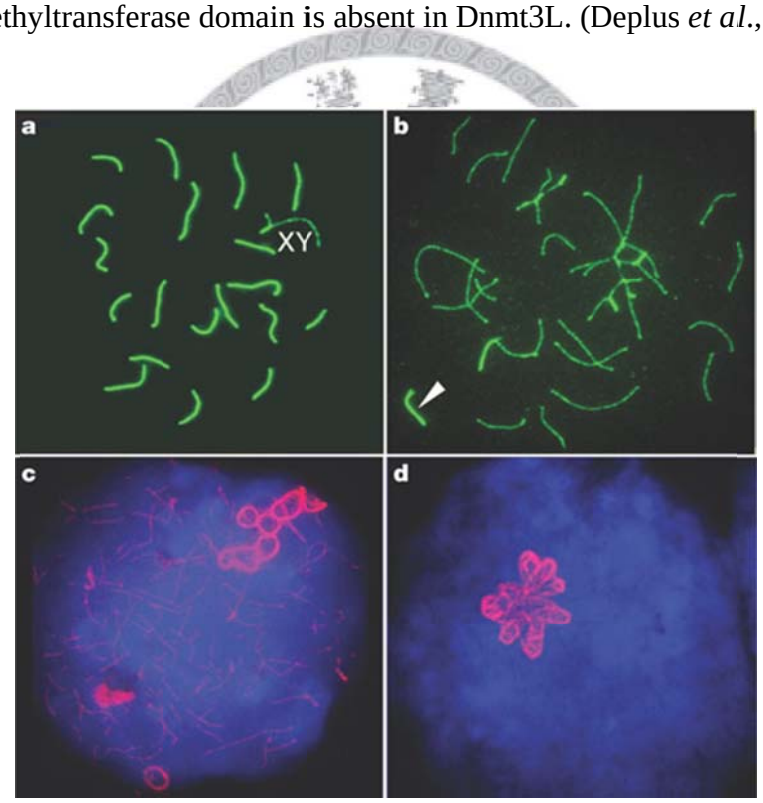

Fig. 1-4 The aberrant chromosome pairing during meiotic synapsis in most Dnmt3L$^{-/-}$ leptotene and zygotene spermatocytes

a. Normal sister chromatin paring during meiotic synapsis.
b. Aberrant chromosome paring in Dnmt3L$^{-/-}$ spermatocytes
c. and d. types of abnormal synaptonemal complex in Dnmt3L$^{-/-}$ spermatocytes. synaptonemal complex protein 1 and 3 antibodies were used for labeling the synaptic chromosomes.

(Bourc'his and Bestor, 2004)

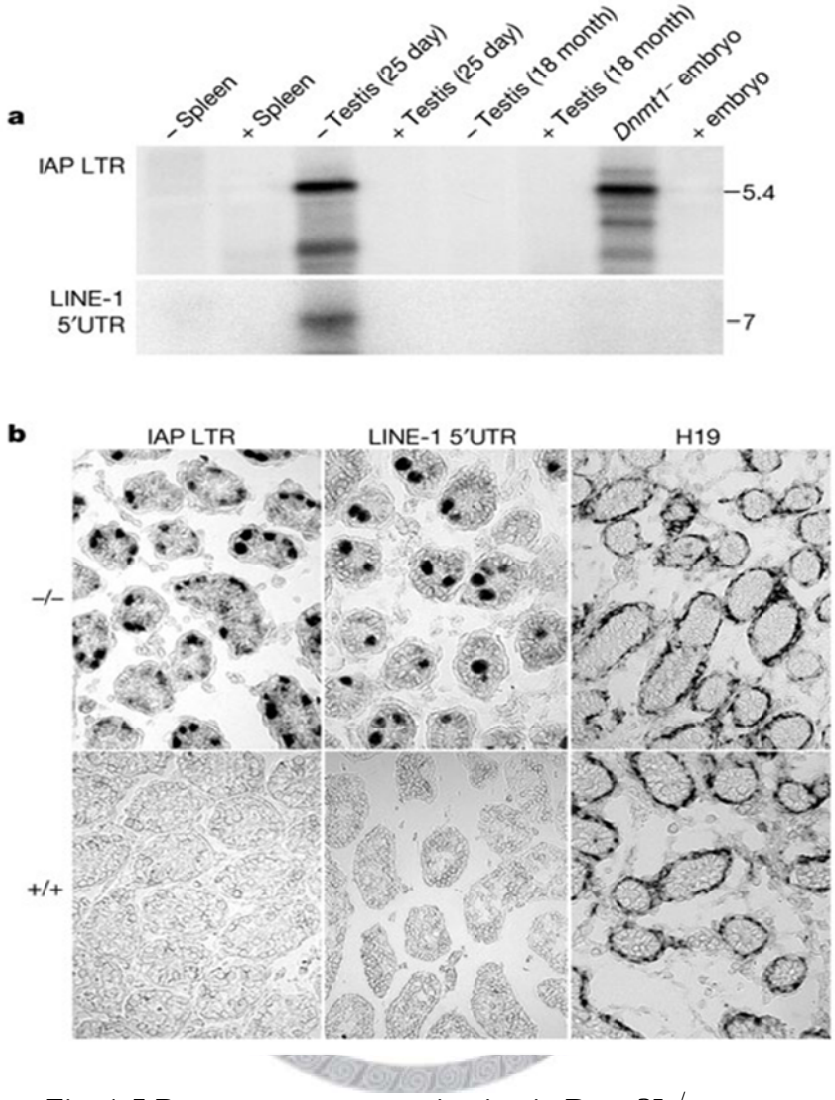

Fig. 1-5 Retrotransposon reactivation in Dnmt3L$^{-/-}$ testes

a. Northern blot of IAP and LINE1 LTR showed a reactivation in Dnmt3L$^{-/-}$ testis of 9.5 days and Dnmt1$^{-/-}$ embryos.
b. *In situ* hybridization shows an activation of the IAP and LINE1 sequences in the neonatal testis. There are no significant difference between Dnmt3L$^{-/-}$ and wild type germ cells in the paternally expressed gene *H19*, whose expression is associated with the methylated *H19* DMR.

1.4 The Relationship between DNA methylation and histone modification in gene silencing

Dnmt3L's role has mainly been focused on its regulation in DNA methylation and its interaction between DNA methyltransferases. However, Dnmt3L is not a methyltransferase itself, but an epigenetic regulator related to transcriptional silencing (Aapola *et al*., 2002). Transcriptional silencing is a serial event on the chromatin that involves histone modification and sometimes DNA methylation. Important epigenetic modifications such as histone deacetylation, trimehtylation of histone 3 at lysine 9 (H3K9me3), trimehtylation of histone 4 at lysine 20 (H4K20me3), and DNA methylation are all considered important repressive chromatin marks (Cedar and Bergman, 2009; Fuks, 2005; Schotta *et al*., 2004). Studies on the epigenetic modifiers responsible for gene silencing demonstrated that DNA methyltransferases, histone methyltransferases, histone deacetylases, and other proteins related to transcriptional silencing have close interactions between each other, and might even form a positive reinforcing loop to keep the chromatin in a repressive state (Fig. 1-6) (Becker, 2006; Jurkowska *et al*., 2008; Law and Jacobsen, 2010; Otani *et al*., 2009; Taverna *et al*., 2007; Vaissiere *et al*., 2008).

For Dnmt3L, although it does not contain a methyltransferase domain, there is a plant homeodomain (PHD)-like domain at its amino terminal (Aapola *et al*., 2000; Okano *et al*., 1998). This PHD-like domain can interact with other Dnmts during *de novo* DNA methylation. In particular, Dnmt3L recruits the catalytic domain of Dnmt3A through its own carboxyl-terminal domain to form a double Dnmt3L, and double Dnmt3A tetramer, which recognizes the unmethylated histone 3 lysine 4 (H3K4)

through the amino-terminal PHD-like domain of Dnmt3L, or the cysteine rich ATRX-DNMT3-DNMT3L (ADD) domain. With this tetrameric structure, the CpG islands during *de novo* DNA methylation have a methylation spacing pattern of 8 to10 base pair (Fig. 1-7) (Aapola *et al*., 2002; Bachman *et al*., 2001; Deplus *et al*., 2002; Fuks *et al*., 2003; Glass *et al*., 2009; Jia *et al*., 2007; Ooi *et al*., 2007) Besides the recognition of unmethylated H3K4, the PHD-like domain of Dnmt3L also recruits another transcriptional repressor histone deacetylase1 (HDAC1), which gives further evidence that Dnmt3L facilitates transcription repression in cooperation with other histone modifiers, and is independent of DNA methylation (Aapola *et al*., 2002; Burgers *et al*., 2002; Deplus *et al*., 2002).

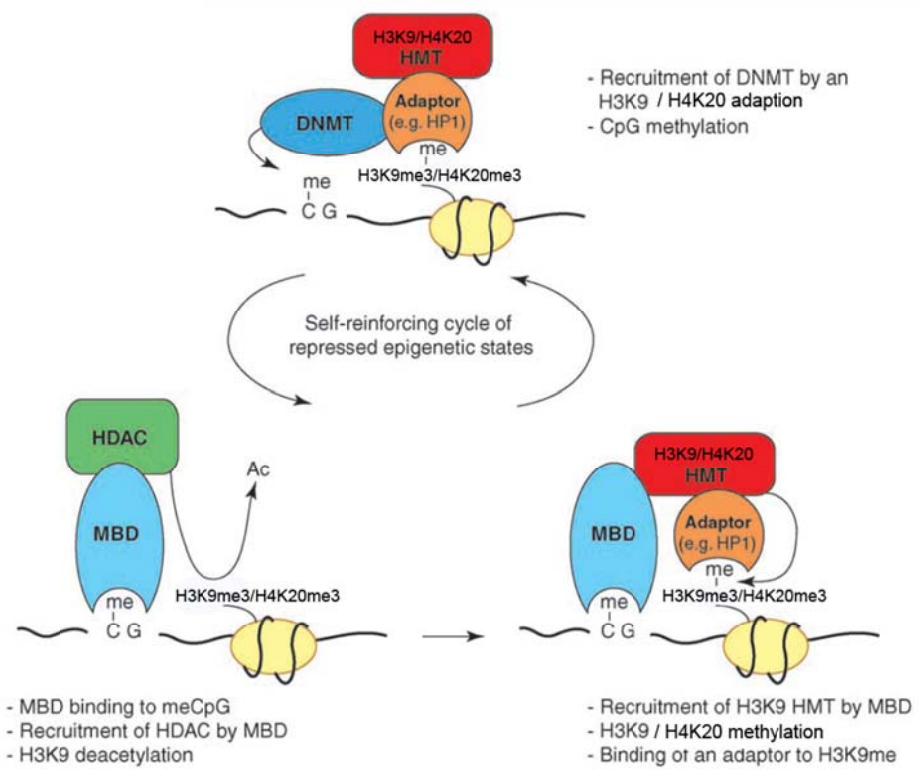

Fig. 1-6 Model for a self-reinforcing cycle of histone modification and DNA methylation

Starting from the upper complex, Dnmts adapt with an adaptor protein that recognizes the repressive histone mark such as H3K9me3 and H4K20me, and methylates the CpG region near by. The methyl CpG with attract a certain MBD (methyl-binding protein) protein, which recruits HDAC. The reinforcing cycle forms by the fact that the de-acetylation of histone caused by HDAC will reinforce histone methylation. (Fuks, 2005)

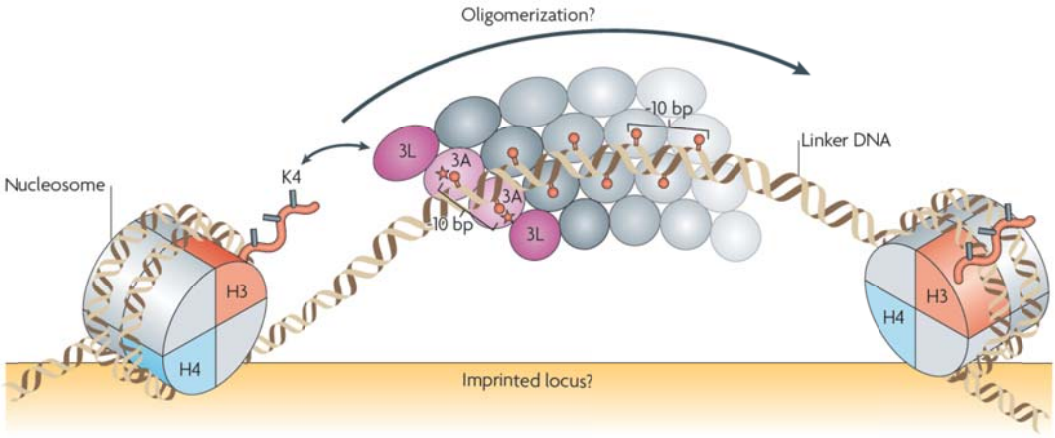

Fig. 1-7 A schematic model of the Dnmt3a-3L complex recognizing unmethylated H3K4 in *de novo* DNA methylation

(Law and Jacobsen, 2010)

1.5 Study of spermatogonial stem cells

Knowing the importance of Dnmt3L in the germ line, and its role in transcriptional silencing, this study aims to focus on the role of Dnmt3L on spermatogonial stem cells (SSCs), which are stem cells in the male germ line that are mostly in a transcriptional/ cell cycle repressive status. Although the germ line stem cells in females has just been identified recently (Zou *et al*., 2009), SSCs have been studied in many aspects through the past several decades. SSCs are a group of cells that are maintained within the male testes that populate the seminiferous tubule by self-renewing itself and differentiating into spermatocytes that undergo meiosis. These cells are a perfect model nowadays for studying about stem cell property and stem cell-niche cell interaction, which has implications to organ development, stem cell property and cancer research (Fig. 1-8).

SSCs are derived from gonocytes, which are derived from PGCs once they colonize to the male gonad around 13.5 dpc. Once colonized the testis, the gonocytes become spermatogonia after birth (Bendel-Stenzel *et al*., 1998; De Rooij and Russell, 2000; McLaren, 2003). Spermatogenesis is a differentiation process from spermatogonium to mature spermatozoa. The first wave of spermatogenesis begins right after birth from day 0, where spermatogonium directly differentiate into spermatozoa. However, SSCs appear from the second wave of spermatogenesis around 3~4 day after birth, where spermatogonia locate back to the basal membrane of the seminiferous tubules and differentiate from then (McLean *et al*., 2003; Yoshida *et al*., 2006).

Studies about SSCs have been started with germ cell transplantation experiments, in which only the stem cells transplanted have the capability to repopulate the germ cell-depleted testis environment (Fig. 1-9) (Brinster and Zimmermann, 1994; Ogawa *et al*., 1997). The re-populating ability has further been improved significantly from

spermatogonia that were purified using some molecular markers (Fig. 1-10), such as α6-integrin, β1-integrin, Thy1, CD9, Ep-CAM, and Gfrα1, which have been reported to be essential for SSC property (Buageaw *et al*., 2005; Kanatsu-Shinohara *et al*., 2004b; Kubota *et al*., 2004a; Ryu *et al*., 2004; Shinohara *et al*., 1999). In addition to the surface markers important for SSCs, some signaling pathways essential for the self-renewal and maintenance for SSCs have been identified (Fig. 1-11). Briefly, the signaling pathways can be divided into extrinsic pathways and intrinsic pathways. Identified mostly by in vitro culture systems, the most important extrinsic pathway for SSC self-renewal is the glial cell line-derived neurotrophic factor (GDNF) pathway (Airaksinen and Saarma, 2002; Kanatsu-Shinohara *et al*., 2004a; Kubota *et al*., 2004b; Meng *et al.*, 2000b), which begins with the excretion of GDNF by the sertoli cells, niche cells that host spermatogonia at the basement membrane of the seminiferous tubule, then passed down into the SSCs through either PI3K or SFK pathways; FGF and EGF, have also been shown to support the role of GDNF for SSC self-renewal, but the mechanisms are still unknown (Kanatsu-Shinohara *et al.*, 2006; Kanatsu-Shinohara *et al.*, 2005a; Kanatsu-Shinohara *et al.*, 2003; Kanatsu-Shinohara *et al.*, 2005b; Kubota *et al.*, 2004c). Extrinsic mechanisms are better known compared with the intrinsic regulatory systems. Influenced by the SFK pathway, Bcl6b, Etv5, and Lhx1 are internally regulating SSC self-renewal. The machinery of the above factors are mostly studied by *in vitro* culture systems; on the other hand, targeted disruption studies of Plzf (promyelocytic leukemia zinc finger protein) and Taf4b (TATA box-binding protein-associated factor 4b) severely impaired spermatogenesis and resulted in azoospermia in the male testis (Buaas *et al*., 2004; Costoya *et al*., 2004; Falender *et al*., 2005).

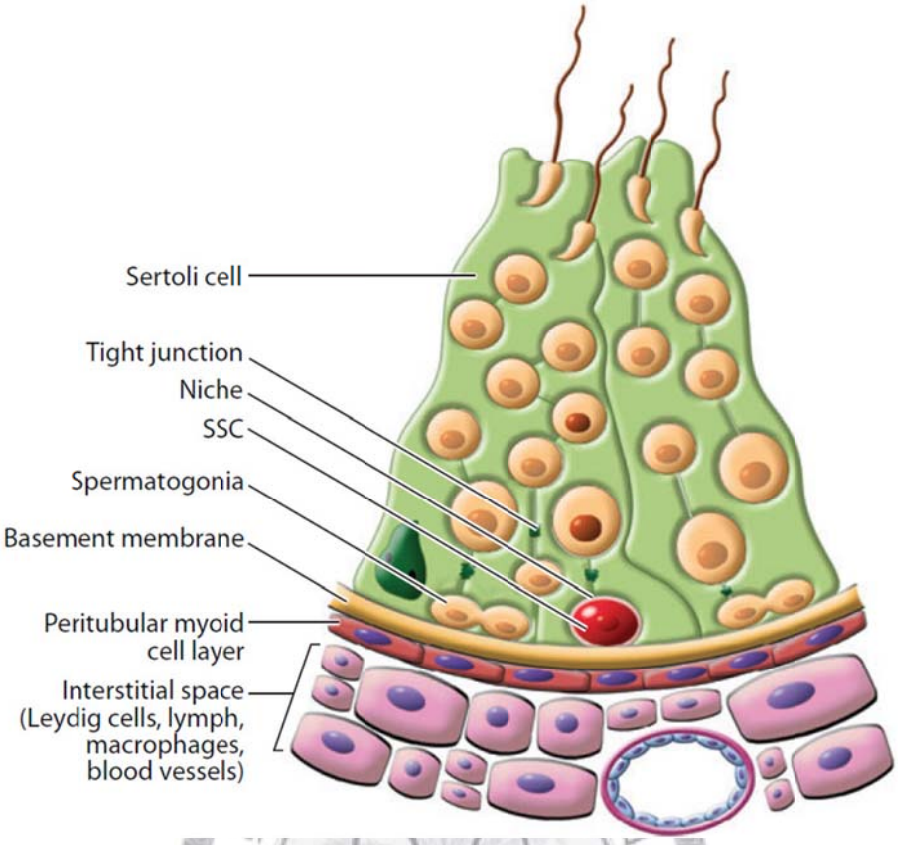

Fig. 1-8 The SSC and its niche

SSCs sit at the basal membrane of the seminiferous tubule. Surrounded by its niche cells--- sertoli cells, and kept behind the tight junction. SSCs either proliferate into Apr spermatogonia or differentiate into later germ cell stages. Blood supply flows through the interspaces between seminiferous tubules. The peritubular myoid cells are also proposed to be influential to the maintenance of SSCs. (Oatley and Brinster, 2008)

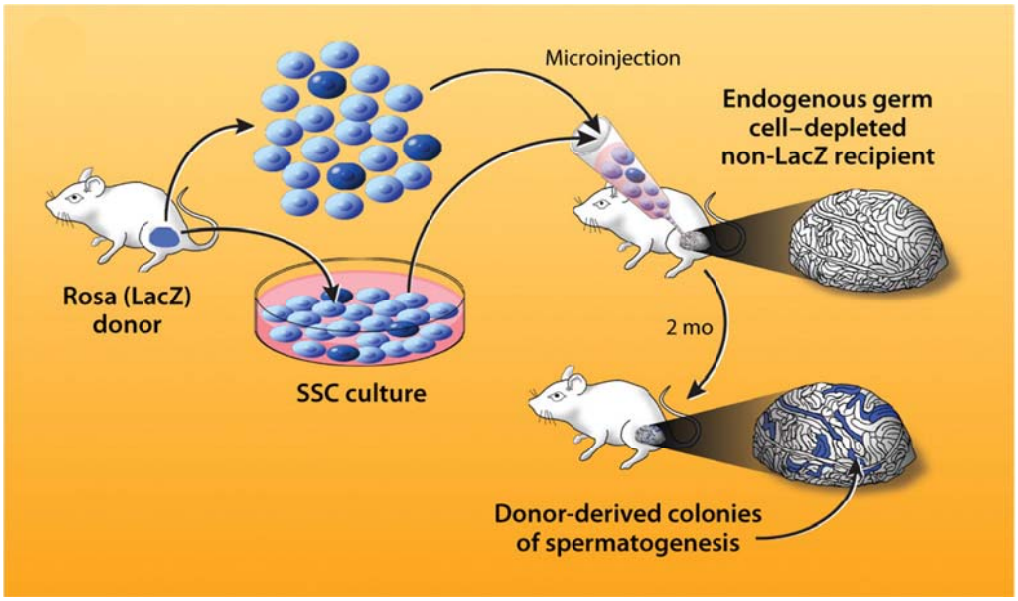

Fig. 1-9 Transplantation of SSCs

Functionally evaluation of the property of SSCs can be done by transplanting the SSCs into a germ-cell depleted mice testes. The SSCs for transplantation can be obtained from LacZ or GFP labeled transgenic male testis. After transplantation of the germ cells, either cultured SSCs or freshly isolated germ cells, colonization can be found after at least one spermatogenic cycle (approximately 35 days). Colonization can be detected by X-gal staining with a Lac-Z donor, or GFP detection from a GFP-transgenic donor. (Oatley and Brinster, 2008)

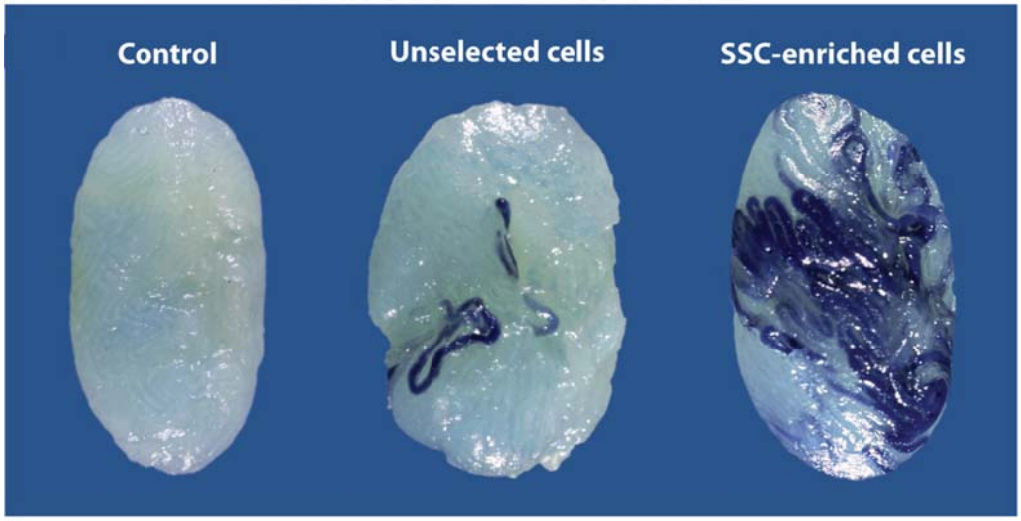

Fig. 1-10 Colonization ability of unselected germ cells and SSC-enriched cells

(Oatley and Brinster, 2008)

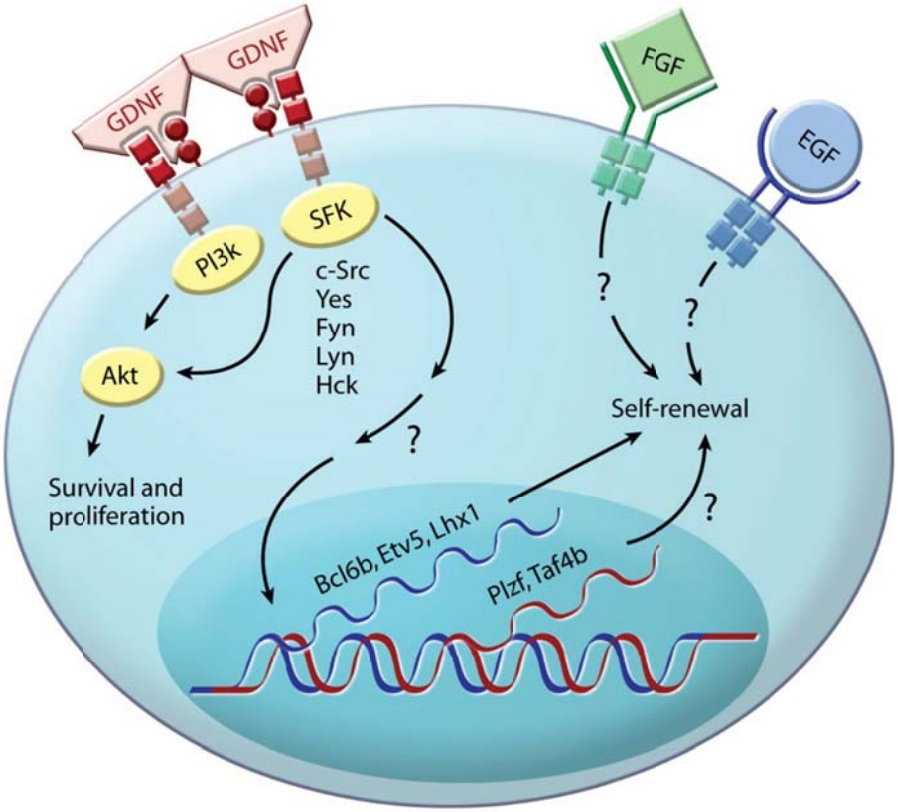

Fig. 1-11 Pathways regulating SSC self-renewal

(Oatley and Brinster, 2008)

Plzf is a transcriptional repressor gene that is reported to be essential for the maintenance and differentiation of stem cells in many tissues. In 2004, two groups have back-to-back reported that Plzf is required for SSC self-renewal and maintenance (Buaas *et al.*, 2004; Costoya *et al.*, 2004; Kotaja and Sassone-Corsi, 2004). In cellular experiments, Plzf attracts transcriptional repressor proteins such as Bmi1 and HDAC (Barna *et al.*, 2002). In addition, Plzf expression is restricted in CyclinD1 and CyclinD2 negative cells on mouse testes sections. This further shows that Plzf expressing cells are also mitotically inactive and restricted to undifferentiated spermatogonia (Buaas *et al.*, 2004). In 2006, Payne and Braun had correlated PLZF-positive spermatogonia with different types of histone modification. Specifically, $PLZF^{+}$ spermatogonia in the testis

sections appear to have a “perinuclear” staining of H3K9me3 and H4K20me3, two histone modifications of pericentric heterochromatin (Fig. 1-12). However, in PLZF$^{lu/lu}$ testis, the perinuclear staining does not exist in the spermatogonia, and turns to a “punctate” staining pattern that is indicative of lost of the quiescent ability, which leads to differentiation. Thus, the perinuclear staining pattern of H3K9me3 and H4K20me3 was associated with the quiescent status of spermatogonial stem cells (Fig. 1-13) (Payne and Braun, 2006).

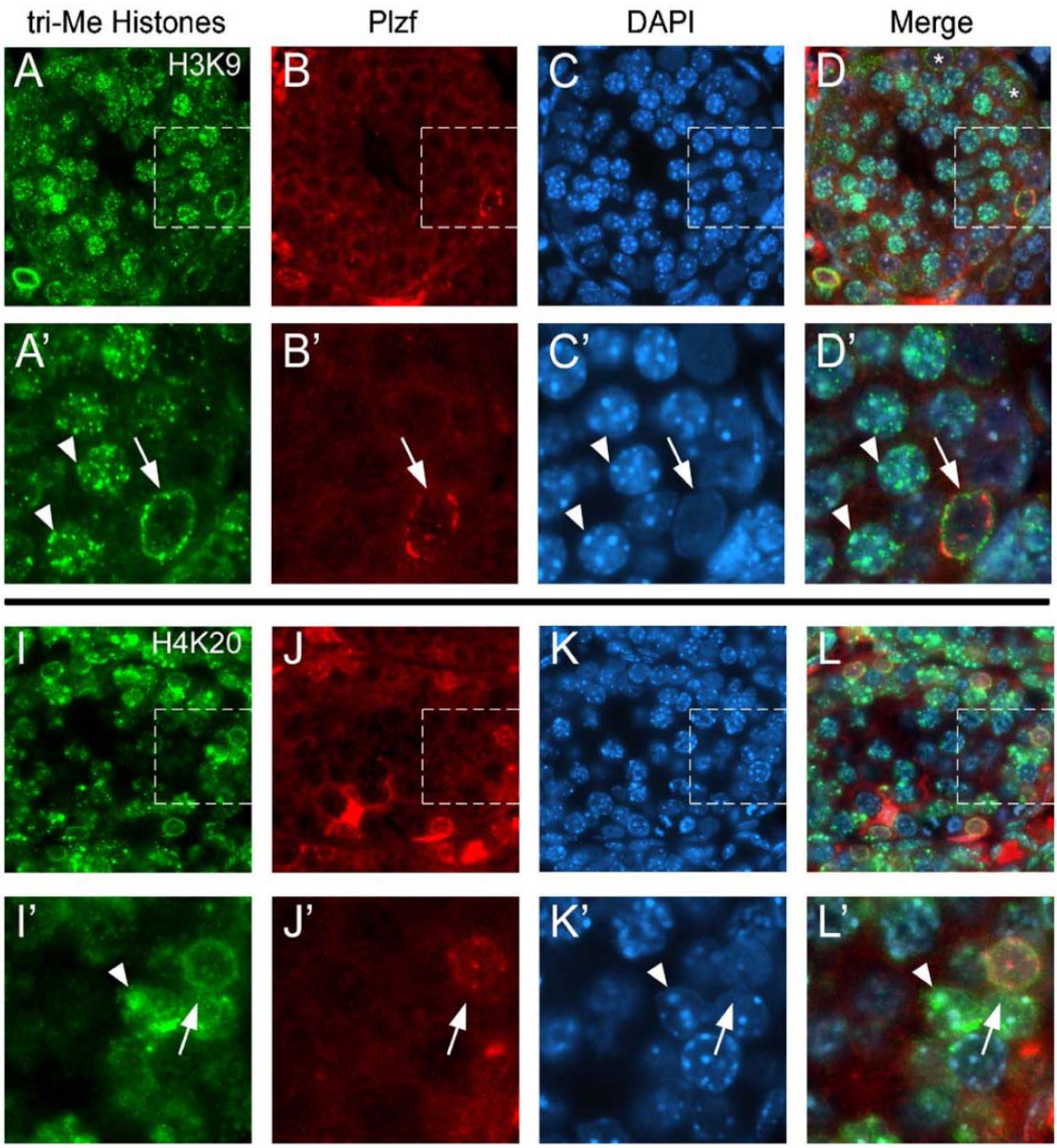

Fig. 1-12 Perinuclear staining of H3K9me3 and H4K20me3 in PLZF^{+} spermatogonia

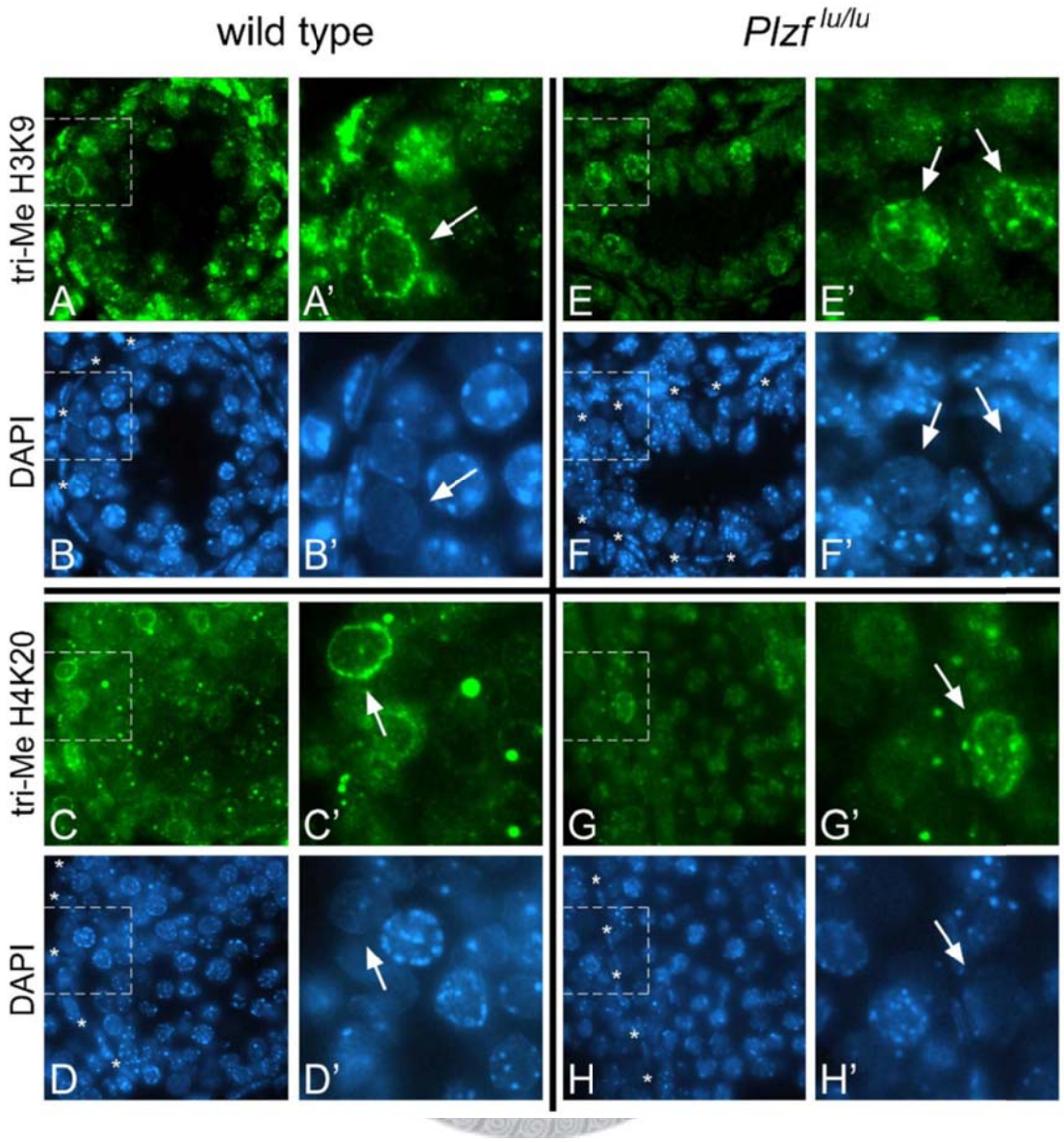

Fig. 1-13 Loss of perinuclear staining of H3K9me3 and H4K20me3 in PLZF$^{lu/lu}$ testes

Chapter 2 Background and hypothesis

Stem cells are a group of cells with self-renewal and differentiation ability. They can proliferate themselves and differentiate into the desired cell type in the tissue. These characteristics make stem cells the key types of cells sustaining the tissue longevity. Studies of stem cell characteristics have found that many stem cells *in vivo* are in fact in a transcriptional/ cell cycle quiescent status waiting for its turn to self-renew or differentiate (Arai *et al*., 2004; Li and Clevers, 2010). For instance, a majority of bone marrow stem cells paused at a G_0 state unless being activated by external stimulation (Cheshier *et al*., 1999; Sorrentino, 2010). The regulation of this quiescent status may involve a combination of transcription factors, cell signaling molecules, epigenetic modifiers of gene expression, and molecular regulators of cell cycle progression (Sorrentino, 2010).

Dnmt3L is an epigenetic modifier that is important for transcriptional repression in the germ line. The major discovered function of Dnmt3L is in facilitating *de novo* DNA methylation through interacting with Dnmt3A (Glass *et al*., 2009; Jia *et al*., 2007; Ooi *et al*., 2007). Dnmt3L also has the PHD-like domain that can recruit other histone modifiers such as HDAC to regulate transcriptional repression (Aapola *et al*., 2002). Dnmt3L's expression is also restricted to pluripotent cells and in germ cells. Dnmt3L's expression window is concentrated at the establishment of *de novo* methylation, when primordial germ cells undergo cell cycle arrest (Bourc'his and Bestor, 2004; Bourc'his *et al*., 2001; Hata *et al*., 2006; Hata *et al*., 2002; La Salle *et al*., 2004; Webster *et al*., 2005). Studies of Dnmt3L revealed its importance in maintaining germ cell function. In addition, Dnmt3L is not only related with DNA methylation, but also associated with histone modification, all regarding transcriptional repression (Aapola *et al*., 2002).

Spermatogonial stem cells (SSCs) are also found to be mostly at a transcriptional repressed status (Takubo *et al*., 2008; Yoshioka *et al*., 2009). Plzf, a marker for SSCs, has a restricted expression in CyclinD1 and CyclinD2 negative cells, and a co-expression with H3K9me3 and H4K20me3 (Costoya *et al*., 2004; Payne and Braun, 2006). Knowing the fact that SSCs have a high ratio of transcriptional quiescent cells, and Dnmt3L is a germ line-specific epigenetic regulator that is expressed during the cell cycle arrest of germ cell development, and is responsible for transcriptional repression, we consider SSCs as a perfect model for us to understand the role of Dnmt3L in the quiescent status of SSCs. We propose that Dnmt3L is responsible for the quiescent status of SSCs through regulating other histone modifiers that are also responsible for transcriptional repression.

The intimacy between niche/somatic cells and stem cells has been found to be essential in many tissues (Li and Xie, 2005). Abnormality of the niche cells can cause severe disruption to the maintenance of stem cells, and vice versa. The niche of SSCs is composed of sertoli cells, basal laminin and myoid cells. Many factors secreted by Sertoli cells have been identified to be essential for SSC self-renewal and maintenance (Chen *et al.*, 2005; Hess *et al.*, 2006; Kubota *et al.*, 2004c; Meng *et al.*, 2000a; Ohta *et al.*, 2000). As for the germ cell depletion phenomenon in Dnmt3L$^{-/-}$ testes, we also think Dnmt3L may somehow influence the niche function of SSCs through epigenetic pathways related to stem cell- niche cell interaction.

In this study, we used Dnmt3L$^{-/-}$ male mice as an experiment material. We first focused on the influence of Dnmt3L on SSCs themselves, using histological observations, immune-staining and testis transplantation experiments. Second we focused on the influence of Dnmt3L on the niche environment to sustaining SSCs, also using immune-staining and testis transplantation experiments.

Chapter 3 Materials and Methods

3.1 Sample collection

3.1.1 Dnmt3L$^{-/-}$ animal breeding

Dnmt3L$^{+/-}$ transgenic mice with a C57/BL6 plus a little 129SV genetic background were kindly provided by Dr. Hata. We've bred them first to wild type C57/BL6 mice, selected the ones carrying the Dnmt3L^{-} allele, and crossed Dnmt3L$^{+/-}$ offspring to obtain Dnmt3L$^{-/-}$ male with a 12.5% chance according to the Mendel's law (25% to get Dnmt3L$^{-/-}$, and 50% to get male). Following 21 days of pregnancy, we recorded the date of birth as 0 days post partum (p0 or 0 dpp) at the first date of birth, and so on. A time course of p4, p6, p8, p10, p13, 3 wks, 4 wks, and 5 wks of testes samples were collected (Fig. 3-1).

3.1.2 Testes sample collection and genomic DNA extraction

Animals born from the same litter were collected and labeled as one unit. Mice litters of different ages were sacrificed and the abdomen was opened with sharp forceps. The gonad/testes were clipped off from the connecting tissues and sunk into ice-cold Phosphate Buffered Saline (PBS). For immunohistochemistry, one testis was then sunk into 4% Paraformaldehyde (PFA) in 4℃ overnight with gentle invertion. The other testis was sunk into Trizol reagent (Invitrogen), and kept in -80℃ for RNA isolation.

The tail tips from every mice were collected and cut into very small segments into a third tube containing 100μl of 1x PCR buffer. After adding 80 pg of proteaseK (4μl from stock), the tissues were incubated and gently shaken in 55℃ overnight. ProteaseK activity was terminated by heating the tubes in 95℃ for 15 mins. After centrifuging for 5 mins, 13000 rpm, genomic DNA was separated from the supernatant and kept in 4℃

before use.

3.1.3 **Genotyping**

The Dnmt3L$^{-/-}$ animals from the litter were genotyped with PCR reaction. Primers were designed by Dr. Hata's lab, with two primers from the wild-type exons 2 and 3 of the *Dnmt3L* gene, and another designed that crosses over exon2 and to the constructed IRES-β-geo gene to detect the null allele (Fig. 3-2). PCR results of both bands of 414 bps and 530 bps are the heterozygous pups, bands with the 530 bps are the wild-type pups, and bands with only 414 bps are the homozygous Dnmt3L$^{-/-}$ samples (Fig. 3-3). primer sequences are as follows:

Primer 1: 5'-GACAGCTCTAGCCCTGATGC-3';

Primer 2: 5'-CTGTCGGAGGCGAGGAGGGG-3';

Primer 3: 5'-CACACTCCAACCTCCGCAAACTCCT-3'

3.1.4 **Testes sections preparation**

After inverting in 4% PFA at 4℃ overnight, testes were washed in ice-cold PBS for three times for 5mins, then transferred into 10% sucrose in PBS buffer until the testes sinks to the bottom of the eppendorf tube. Testes were then transferred to 20% sucrose in PBS buffer until it sank to the bottom of the eppendorf tube. The testes were then transferred to 30% sucrose until it sank to the bottom of the eppendorf tube. At last, the testes were embedded in the OCT compound (Sakura), and frozen in -80℃ before sectioning. Sections were made into 8 to 5 um thick, and the sections were kept in -20℃ before immunostaining.

For whole mount staining, tissues were digested in 1mg/ml collegenase IV for 5 to 10 mins in room temperature, washed with PBS, and fixed at 4% PFA in 4℃ overnight. After rinsing off the PFA for 3 times with PBS, the tissues were dehydrated with a

methanol gradient, and stored in 100% methanol before use.

3.2 Immunohistochemistry

For fluorescent staining, frozen sections were washed in 3xPBS for dissolving OCT, and rehydrated in 1xPBS and PBST. The sections were blocked in 10% goat serum for 1hr mins in room temperature. Primary antibodies were diluted in 10% goat serum and added to the samples and kept at 4℃ overnight. Primary antibodies (PLZF, H3K9me3, H4K20me3 and Tra98) were washed in PBST for 3 times. Secondary antibodies diluted in 10% goat serum and added to the samples in the dark for 30 mins at room temperature. After washing secondary antibodies with PBST, the slides were stained with Hoechst33342 (Sigma) in the dark, and mounted with florescent mounting medium (Sigma).

Rabbit polyclonal IgG antibody against PLZF was 1:100 diluted (Santa Cruz sc22839). Rabbit polyclonal IgG antibodies against H3K9me3 (Abcam ab8898) and H4K20me3 (Abcam ab9053) were 1:500 diluted (Abcam). Rat monoclonal IgG antibody against Tra98 was 1:300 diluted (Abcam ab82527). The secondary antibodies include goat anti rabbit polyclonal IgG conjugated with Alexa488 (Invitrogen), and goat anti rat polyclonal IgG conjugated with TexasRed (Abcam).

Paraffin embedded samples were deparaffinized with xylene for 30 mins twice, rehydrated with an ethanol gradient. Antigen retrieval was performed by heating samples in 95℃ in citric acid based retrieval solution (Vector) for 10 mins, washed in PBS buffer, and treated with 3% H_2O_2 for 15 mins. Samples were blocked in 10% goat serum for 1 hr in 37℃, then treated with primary antibodies (Vasa and Stra8) at 4℃ overnight. Primary antibodies were washed away in PBST three times, and the samples were treated with secondary antibodies for 30 mins at room temperature. The secondary

antibodies were removed with three time washes, and the samples were treated with ABC solution (Vector ABC kit). DAB solution was applied after washing ABC for three times. The slides were counterstained with the Hematoxylin solution (Aldrich) for 30 seconds, washed with PBS, dehydrated, mounted with mounting medium (Sigma) and dried overnight.

Primary antibodies for paraffin embedded sections were rabbit polyclonal anti-Vasa (Abcam ab13840), and rabbit polyclonal anti-Stra8 (Abcam ab49602). Secondary antibodies are universal secondary antibody conjugated with biotin (Vector ABC kit).

For whole mount staining, tissues in 100% methanol were rehydrated in an ethanol gradient, washed with PBS, treated with 3% H_2O_2 for 10 mins. After washing off with PBS for 3 times, tubules were treated with 0.5% TritonX-100 in PBS, washed for three times, and blocked in 10% goat serum for 1~2 hrs at room temperature. They were then incubated with primary antibody (rabbit polyclonal IgG anti-Plzf, Santa Cruz sc22839) at 4℃ overnight. Primary antibodies were washed in PBS containing 0.2% Tween 20 and 1% goat serum for 5 times. Secondary antibody (goat anti-rabbit polyclonal IgG conjugated with Alexa488, Invitrogen) was diluted in PBS containing 2% BSA and 0.1% Tween20 for 1 hr in room temperature in the dark. Samples were washed three times in PBST, and counterstained with Hoechst33342, washed in PBST, and mounted with fluorescent mounting medium (Sigma).

3.3 Germ cell transplantation

3.3.1 Animal preparation

Recipient animals for transplantation were ICR wild-type male mice or C57/BL6

Dnmt3L$^{-/-}$ mice and their control littermates. At 6 wks of age and above, all recipient mice were treated with 40 mg/kg busulfan, a chemical that can destroy internal germ cells (Bucci and Meistrich, 1987). Dnmt3L$^{-/-}$ recipients were also treated since there is still remaining germ cells in the tubules that might influence the transplantation results. Busulfan was dissolved in DMSO to a concentration of 8mg/ml, and diluted with PBS to 4mg/ml at 37℃ before being used. Busulfan treated mice were kept for 30 days for complete depletion of germ cells, before germ cell transplantation (Fig. 3-4).

Animals for donor cells were prepared from C57/B6 Dnmt3L$^{+/-}$ mice were bred with ICR β-actin-GFP (Dnmt3L wt) mice. The offspring detected with Dnmt3L$^{+/-}$ genotype were selected, intercrossed and their pups with GFP+ phenotype were genotyped for Dnmt3L$^{-/-}$ and littermate control of either Dnmt3L$^{+/-}$ or wild-type (Fig. 3-5, Fig. 3-6).

3.3.2 **Germ cell isolation**

Germ cells of Dnmt3L$^{-/-}$ and littermate control were isolated following modified procedures of previous studies (Buaas *et al*., 2004; Ogawa *et al*., 1997). The tunica albuginea were removed from the testis exposing the seminiferous tubules. The tubules were then incubated in 10 volumes of 1X Hank's buffer containing 1mg/ml collegenase IV, and 200~500mg/ml DNase at 37℃ with gentle agitation for 15 mins or until the tubules separated into small fragments. The separated tubules were put on ice for 5 mins to sediment. The tubules were washed with Hank's buffer and centrifuged for 1min at 500 rpm, and the washing was repeated 5 times. The washed tubules were gently pipeted into Hank's buffer containing 1m MEDTA and 0.25% trypsin and incubated at 37℃ for 5 mins with gentle agitation. Trypsin digestion was terminated by adding 10~20% FBS. 200~700 μg/ml DNase was added to prevent the cells from adhering

together. The cells were then filtered through 40μm mesh, centrifuged at 600 g for 5 mins at 16℃. The supernatant was removed and the cells were resuspended in Hank's buffer containing 1% PVA to a concentration of around 1.5×10^6 cells/ml. The cells were kept on ice with no longer than 4 hrs before the transplantation procedure (Fig. 3-7).

3.3.3 **Germ cell transplantation**

The transplantation procedure was in accordance to previous studies (Ogawa *et al*., 1997). The capillary glass tubes for cell injection had an outer diameter of 1.0 mm and 0.75mm inner diameters. They were pulled and cut to a 40μm diameter wide opening, and the blunt ends were grinded into a 30°angle. Air flow was pumped into the filaments during the grinding process to maintain the sanitation of inner-side of the capillary glass. The tip of the capillary glass finished grinding was washed with hydrofluoric acid, and rinsed with dd water and 75% ethanol. After all of the capillaries are done grinding, they were sterilized with an autoclave and kept sealed before the procedure.

The recipient animal was treated with antibiotics 30 mins before the procedure. Animals were anesthetized with Avertin (Sigma), the abdomen were shaved off the fur with an electric razor, and sterilized with 75% ethanol. The abdomen of the animal was cut to make an opening of 5mm from the outer skin, and 3mm of the inner muscular layer. The testis was withdrawn from the abdomen by pinching the fatty tissue. After finding the focus of the testis under the dissecting microscope, the efferent duct was located at the same position with the major vessels that runs through the testes. The fatty tissue that embraces the efferent duct was eliminated to make the efferent duct expose better.

In order to keep track on the cells once they were injected into the tubules, the cell

solution was mixed with Trypan Blue dye (Gibco) before injection (Fig. 3-8). The cell solution was loaded into a sterilized silicon pipe with a 0.5 mm inner diameter, connected to a blunt-end 18G syringe needle. A 0.5 ml glass syringe (Hamilton 1750) was connected to the syringe head and used to inhale the cells. 100μl of cell solution was prepare for each animal for transplantation, cell solution injected was confirmed to fill-up 70~80% of the testis surface. Animals were stitched with continuous stitches and discontinuous stitches of the inner muscular layer and the out skin layer, respectively. Antibiotics were given one time daily for three days after the surgery. GFP^+ colonies were detected with a dissecting microscope with blue light excitation and a green-light filter (Leica).

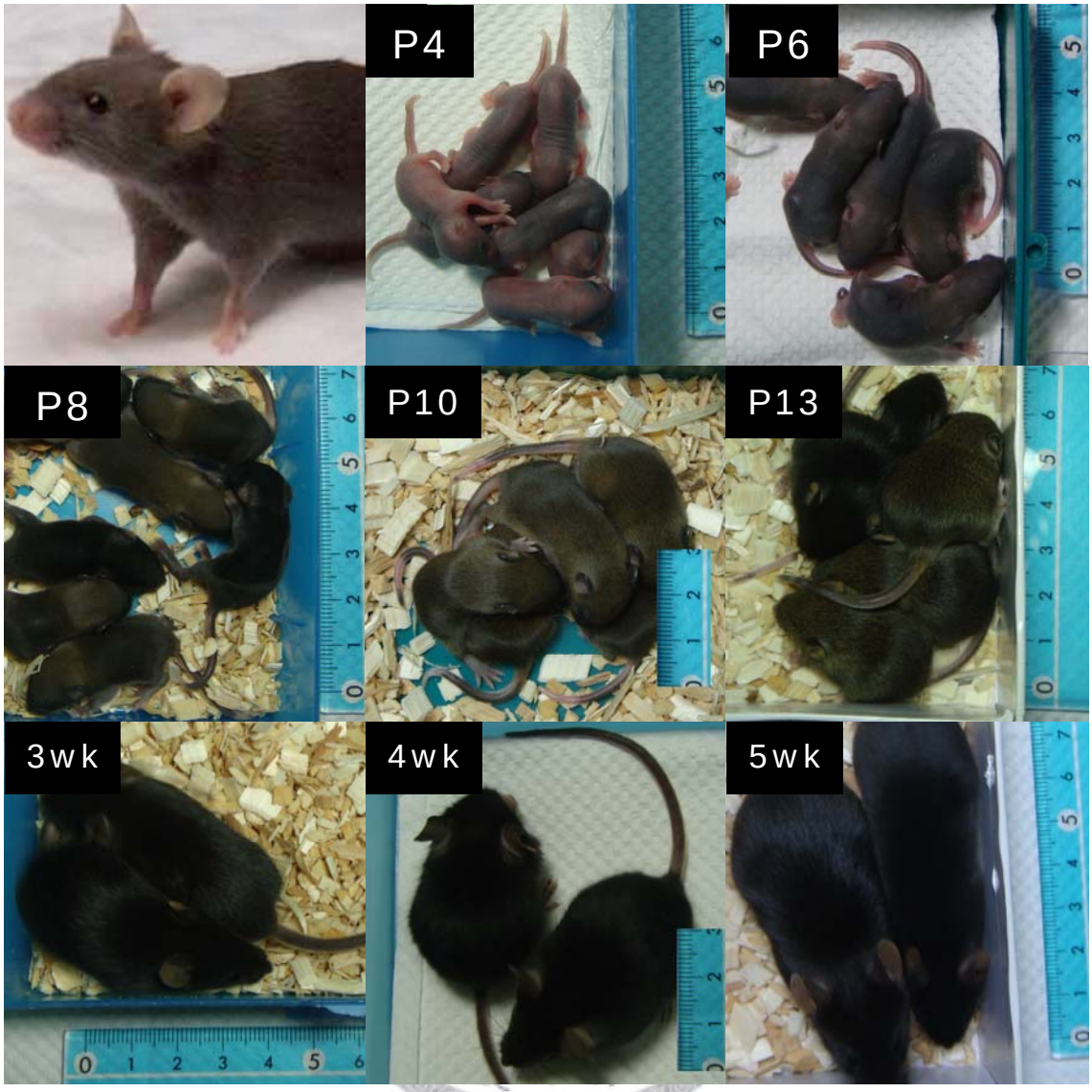

Fig. 3-1 Chronologically collected DNMT3L$^{+/-}$ × DNMT3L$^{+/-}$ offspring.

The genetic backgrounds of the parental origin of the samples are C57/BL6/129SV DNMT3L$^{+/-}$ heterozygous knock-out mice, which mostly has a black coat color, some of them a little brown. P4: 4 days postpartum, the day of second round of spermatogenesis and SSCs formation. P6: 6 days postpartum, the day when SSCs home to the basal membrane of the seminiferous tubule. P8: 8 days post partum, the day when type A spermatogonia first differentiate into spermatogonia type B. P10: 10 days post partum, the day when spermatogonia enter meiosis and form spermatocytes. P13: 13 days post partum, the day when meiotic spermatocytes enter pachytene stage. 3wk: three weeks post partum. 4wk: four weeks post partum. 5wk: 5 weeks postpartum, this is when the first group of mature sperm forms.

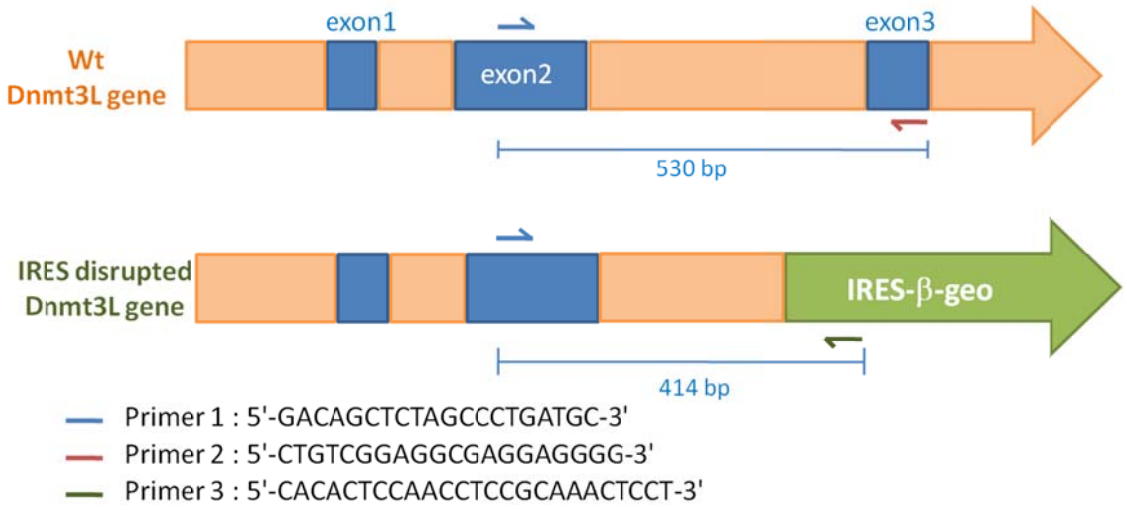

Fig. 3-2 Dnmt3L-detecting primer provided by Dr. Hata.

The upper allele shows exon1 to exon3 of the wild-type Dnmt3L gene. The first two primers are designed from exon2 and exon3. The bottom allele is the mutated Dnmt3L construct, which replaces the exon3 and parts of the intron sequences with IRES-β-geo, where the third primer is designed.

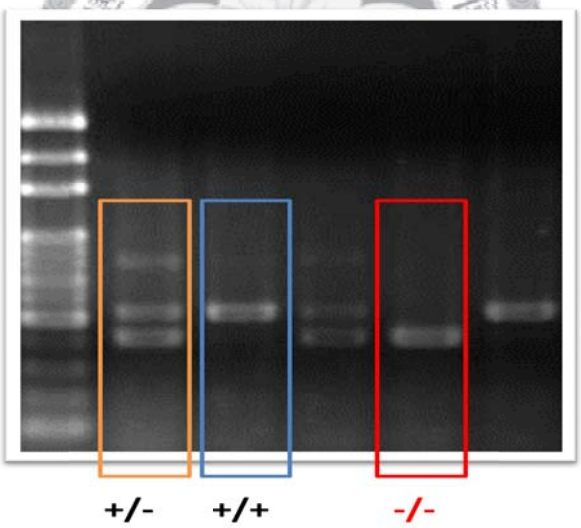

Fig. 3-3 PCR results from a Dnmt3L$^{+/-}$ ♂ × Dnmt3L$^{+/-}$ ♀ litter

A Dnmt3L$^{+/-}$ animal will show two bands after PCR, wild-type will show one 530 bp band and Dnmt3L$^{-/-}$ will show one 414bp band.

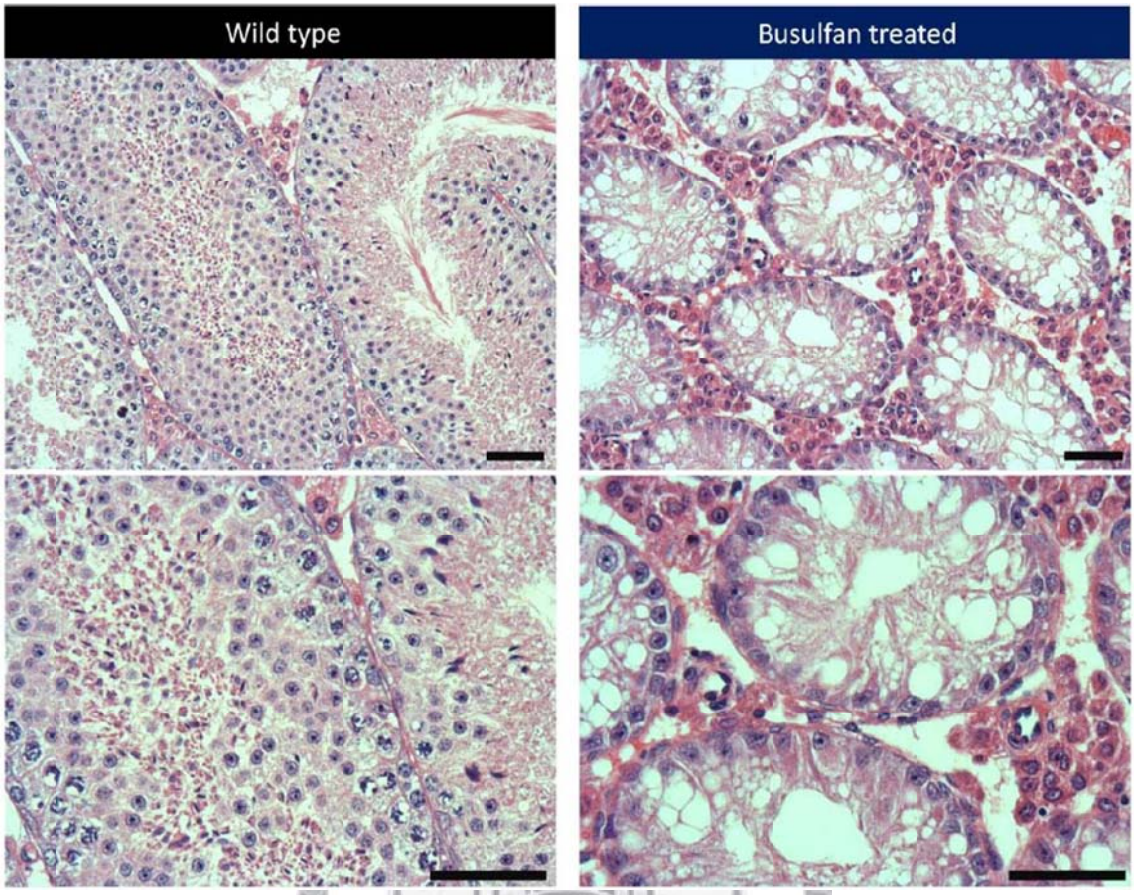

Fig. 3-4 Busulfan treated testis after one month.

The left pictures show a 10-week-old wild-type mice testis section; The right picture show a 10-week-old wild-type mice treated with busulfan when the mouse is 6 wks-old (now also 10 wks). The germ cells are almost thoroughly depleted after busulfan treatment. Scale bars: 50μm

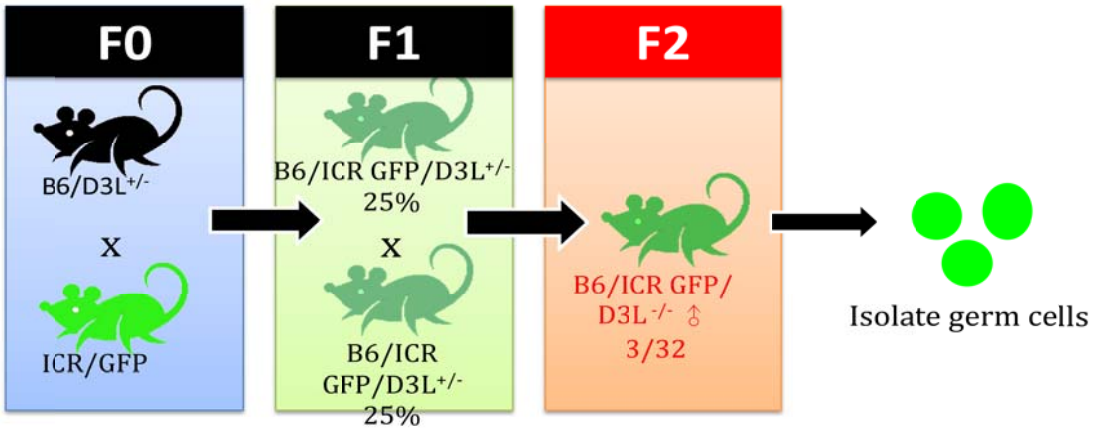

Fig. 3-5 Flow chart of the Dnmt3L$^{-/-}$ GFP^{+} mice breeding process.

At F0, ICR GFP^{+} mice were bred with B6 Dnmt3L$^{+/-}$ mice. GFP^{+}, Dnmt3L$^{+/-}$mice were

selected for F1 and breed together. F2 GFP^+ males were selected for genotyping, and after identifying $Dnmt3L^{-/-}$ from $Dnmt3L^{+/-}$ and wild-type littermates, the germ cells were isolated as described.

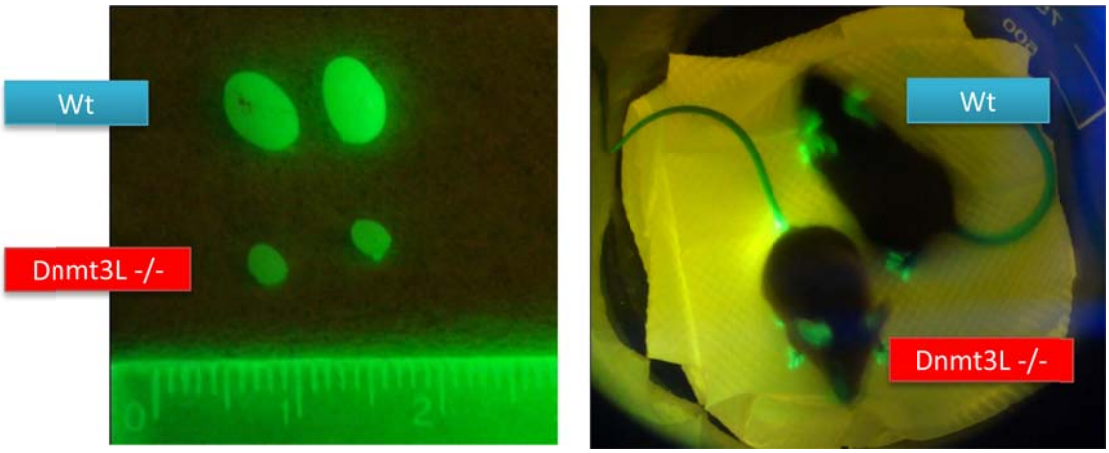

Fig. 3-6 Evidence of $Dnmt3L^{-/-}$ GFP^+ mice

The left picture is the testis of wild-type and $Dnmt3L^{-/-}$ GFP^+ mice testes. the right is the live animals with GFP expression.

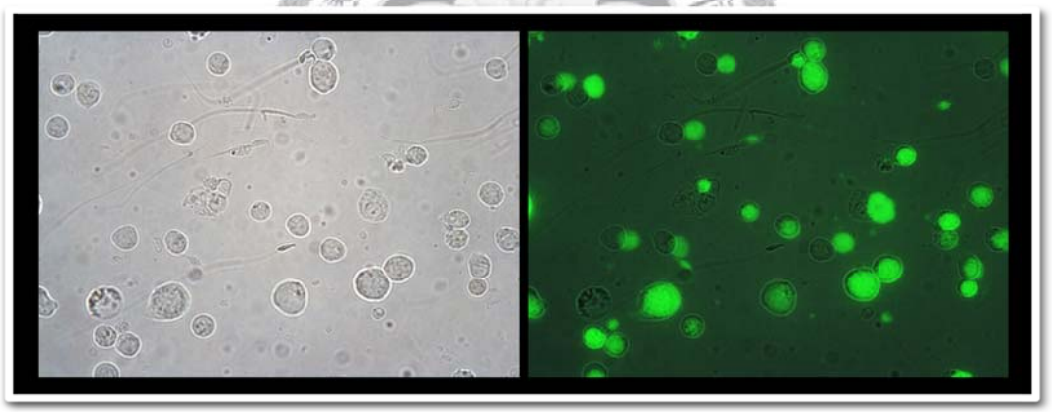

Fig. 3-7 GFP^+ germ cells isolated from the GFP^+ mouse testes.

The left panel is the bright field, and the right with GFP. The germ cells isolated were from 4-wk-old GFP^+ mouse testes. Thus almost-mature spermatozoa could be seen in the figure.

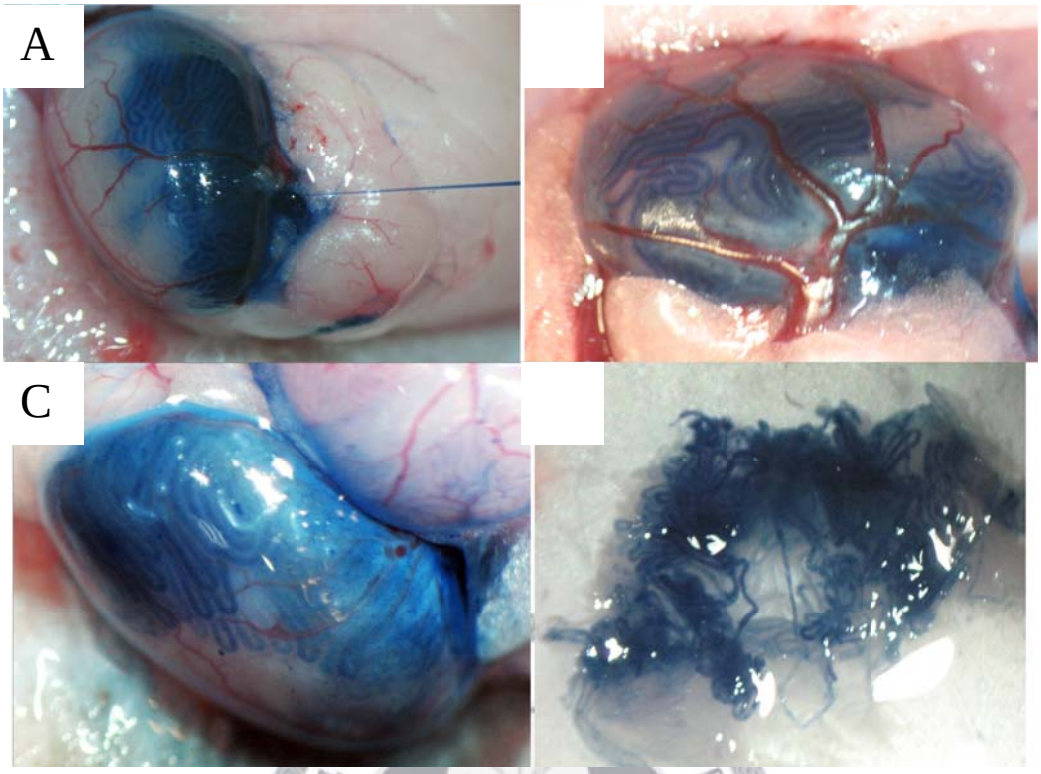

Fig. 3-8 Germ cell transplantation procedure.

A. A 40μm-wide sharp-ended glass capillary was used to inject the cells into the efferent duct. Trypan blue dye was used to visualize the cell solution flow into the seminiferous tubules.
B. 50~60% of the testis surface covered with Trypan blues dye after injection.
C. 80% of the testes covered with trypan blue dye
D. Testis' tunica albuginea removed and the seminiferous tubules dissolved after injected with Trypan blue dye.

Chapter 4 Results

4.1 Altered testicular size and a gradual germ cell loss in 5-weeks-old testes of Dnmt3L$^{-/-}$ mice.

At 5 wks of age, these was no difference in terms of size and health condition at this time point between Dnmt3L +/-, +/+ and -/- mice (Fig. 4-1A). However, Dnmt3L-/- testicular size is a lot smaller than that of the wild type; while the heterozygote littermate did not show any difference compared to the wild type in testicular size (Fig. 4-1B). According to the phenotypic data described above, in addition to previous studies showing no significant difference between Dnmt3L$^{+/+}$ (wild type) and Dnmt3L$^{+/-}$ in molecular level (La Salle *et al*., 2007), we've further used either Dnmt3L$^{+/+}$ and Dnmt3L$^{+/-}$ as littermate controls in all our following experiments for Dnmt3L$^{-/-}$ male mice testes.

The H and E staining sections of 5-week-old wild type and heterozygous testis also demonstrates similar morphology (data not shown), which reveals full spermatogenesis up until mature sperm has formed (Fig. 4-1C). However, Dnmt3L$^{-/-}$ testis has a severe depletion of germ cells. A portion of the seminiferous tubules still remain layers of germ cells that are processing to the meiotic stages (15%), another portion have only one to two layers of germ cells left at the basal membrane of the seminiferous tubule (33.3%) (Fig. 4-1D, star; Table. 4-1), and the last but almost half complete depletion of germ cells, leaving only the sertoli cells remaining in the seminiferous tubules (51%) (Fig. 4-1D, asterisk; Table. 4-1). This severe germ cell loss implies that Dnmt3L may have an important role in the maintenance/ self-renewal of SSCs, or may also be caused by a secondary effect from a disruption of the remaining niche cells, including sertoli cells and myoid cells.

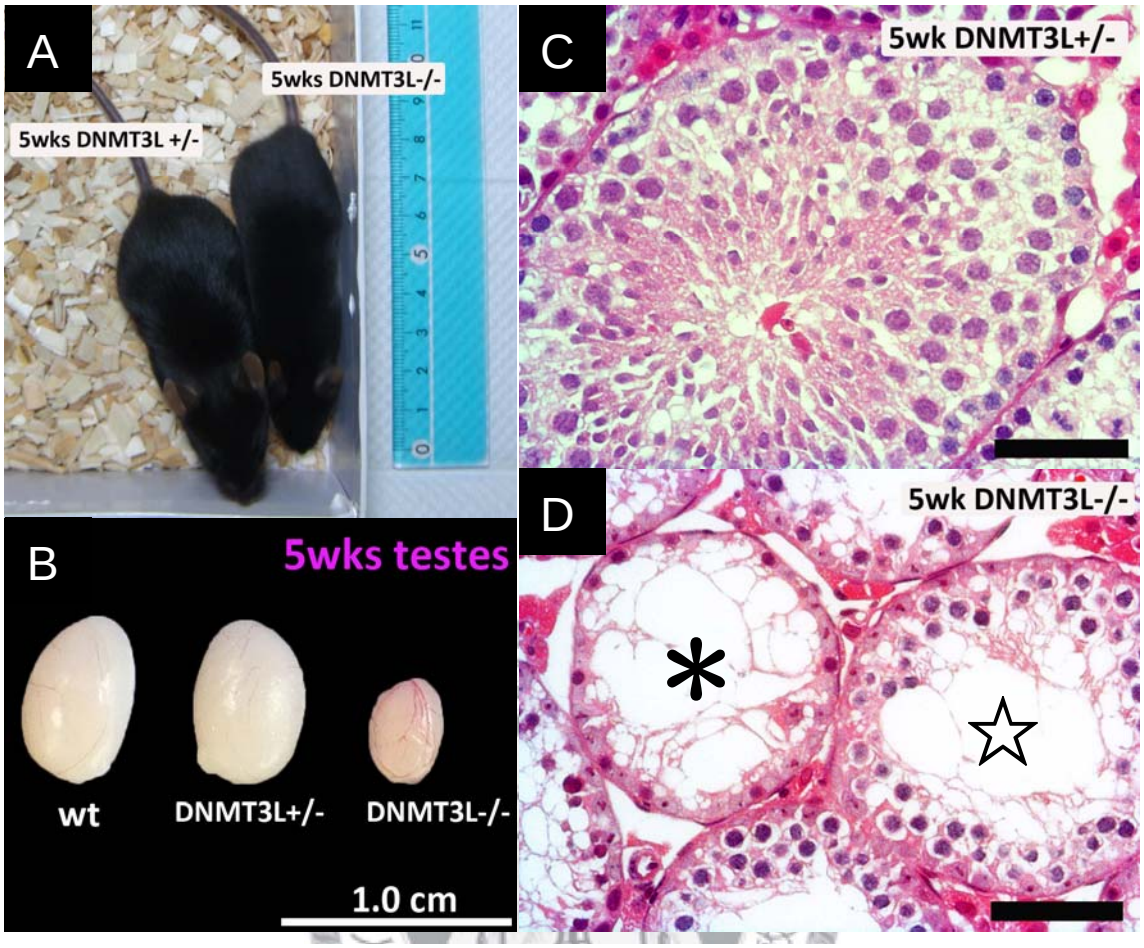

Fig. 4-1 Morphological and histological difference 5-wk-old Dnmt3L$^{-/-}$ and Dnmt3L$^{+/-}$ littermate

A. 5-wks-old living animals showing no difference in size and health condition between Dnmt3L$^{-/-}$ and its Dnmt3L$^{+/-}$ littermate.
B. Morphological comparison in wt (Dnmt3L$^{+/+}$), Dnmt3L$^{+/-}$, and Dnmt3L$^{-/-}$ testes. Wild type testes and the Dnmt3L$^{+/-}$ show no significant difference in testicular size; but Dnmt3L$^{-/-}$ is significantly smaller in size. wt: wild type.
C. Hematoxylin and Eosin (H and E) staining of the Dnmt3L$^{+/-}$ testis section. A complete spermatogenesis can be observed from the basal membrane to the middle of the seminiferous tubule. Scale bar: 100μm.
D. H and E staining of the Dnmt3L$^{-/-}$ testis. Severe germ cell depletion can be seen in all of the seminiferous tubules. The tubule with the star shows 2~3 layers of germ cells remaining in the seminiferous tubule; the tubule with the asterisk specifically has no germ cells, even absence of spermatogonia, with only sertoli cells remaining. Scale bar: 100μm.

Table. 4-1 Statistical count on the percentage of different types of seminiferous tubules in Dnmt3L$^{-/-}$ 5-wk-old testes

Types of seminiferous tubules	>3 layers of germ cell	1~2 layers of germ cell	~0 layers of germ cell
Littermate control	98%±1%	1%±1%	1%±1%
Dnmt3L$^{-/-}$	11%±4%	36%±4%	52%±5%

Total percentage per seminiferous tubule type were calculated from 6 different sections from 2 biological repeats. 23 to 49 and 15 to 18 seminiferous tubules were counted from each Dnmt3L$^{-/-}$ and its heterozygous littermate's sections respectively. Results are presented as mean ± SEM.

4.2 No significant morphological difference was shown in Dnmt3L$^{-/-}$ and its littermate control mice from 4 to 8 dpp

To examine whether SSCs have been affected in Dnmt3L$^{-/-}$ mice testes, 4 dpp to 8 dpp samples were used as a representative of earlier stages of spermatogenesis. In terms of histological appearance, no significant difference was observed in the 4dpp to 8dpp windows between the Dnmt3L$^{-/-}$ and the control testes (Fig. 4-2).

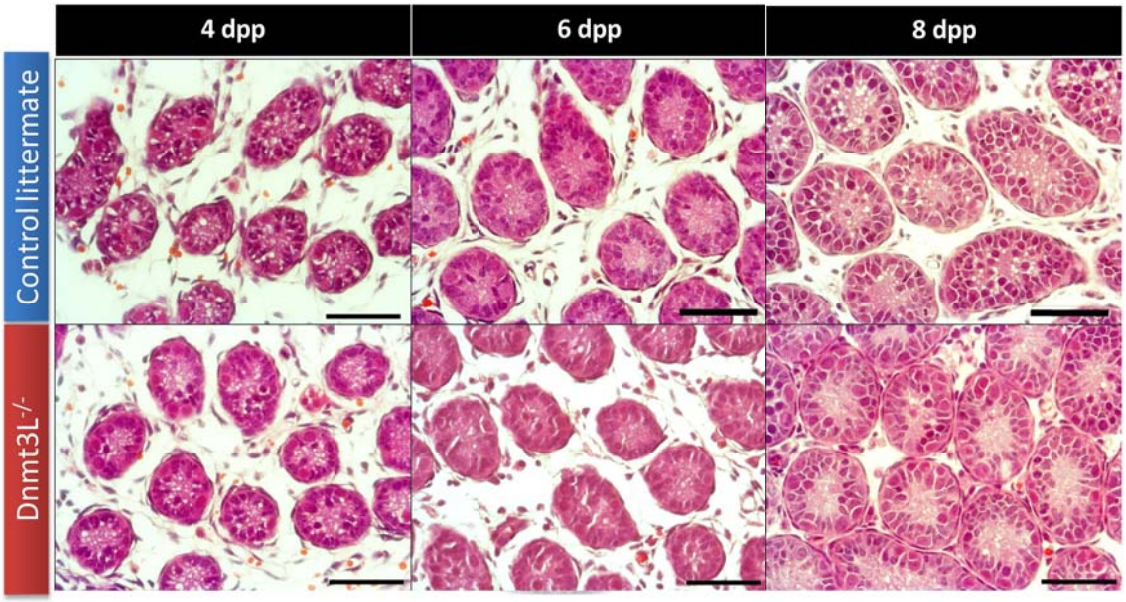

Fig. 4-2 Histological observations in DNMT3L$^{-/-}$ and their littermate control testes at neonatal stages.

DNMT3L$^{-/-}$ mice testes did not show significant difference in terms of histological morphology during early spermatogenic stages before entering meiosis. Scale bar: 50μm.

4.3 **Reduced PLZF^{+} cells found in Dnmt3L$^{-/-}$ testes**

Due to the above results, we further examined whether the SSC have actually been affected in the seminiferous tubules from 4 dpp to 8 dpp. PLZF is an essential factor for normal SSC self-renewal and maintenance (Buaas *et al*., 2004; Costoya *et al*., 2004; Kotaja and Sassone-Corsi, 2004; Taverna *et al*., 2007). Also being a transcriptional repressor (Barna *et al*., 2002), it has been shown to maintain SSCs in a quiescent status associated with a perinuclear staining pattern of H3K9me3 and H4K20me3 (Payne and Braun, 2006). We thus first chose PLZF as a marker to identify the quiescent SSCs within the mouse testes.

Immunostaining was used to detect PLZF^{+} germ cells in 4dpp, 6dpp, and 8dpp testes of Dnmt3L$^{-/-}$ and the control littermates. Our results showed that Dnmt3L$^{-/-}$ testes had a reduction in the number of PLZF^{+} germ cells in all stages of 4dpp to 8dpp (Fig. 4-3). Whole mount tissue staining revealed a very homogenous pattern of PLZF^{+} cells throughout the seminiferous tubule; while in Dnmt3L$^{-/-}$ seminiferous tubules, PLZF^{+} cells not only were globally decreased in number, but also appeared with “empty spaces” in between the tubules (Fig. 4-4). Quantification results showed a significant reduction in PLZF^{+} cell number in 4 and 6 dpp stages (Fig. 4-5). The PLZF staining results indicates that Dnmt3L may affect the transcriptional repressive state (or the so-called “quiescent” status) of SSCs.

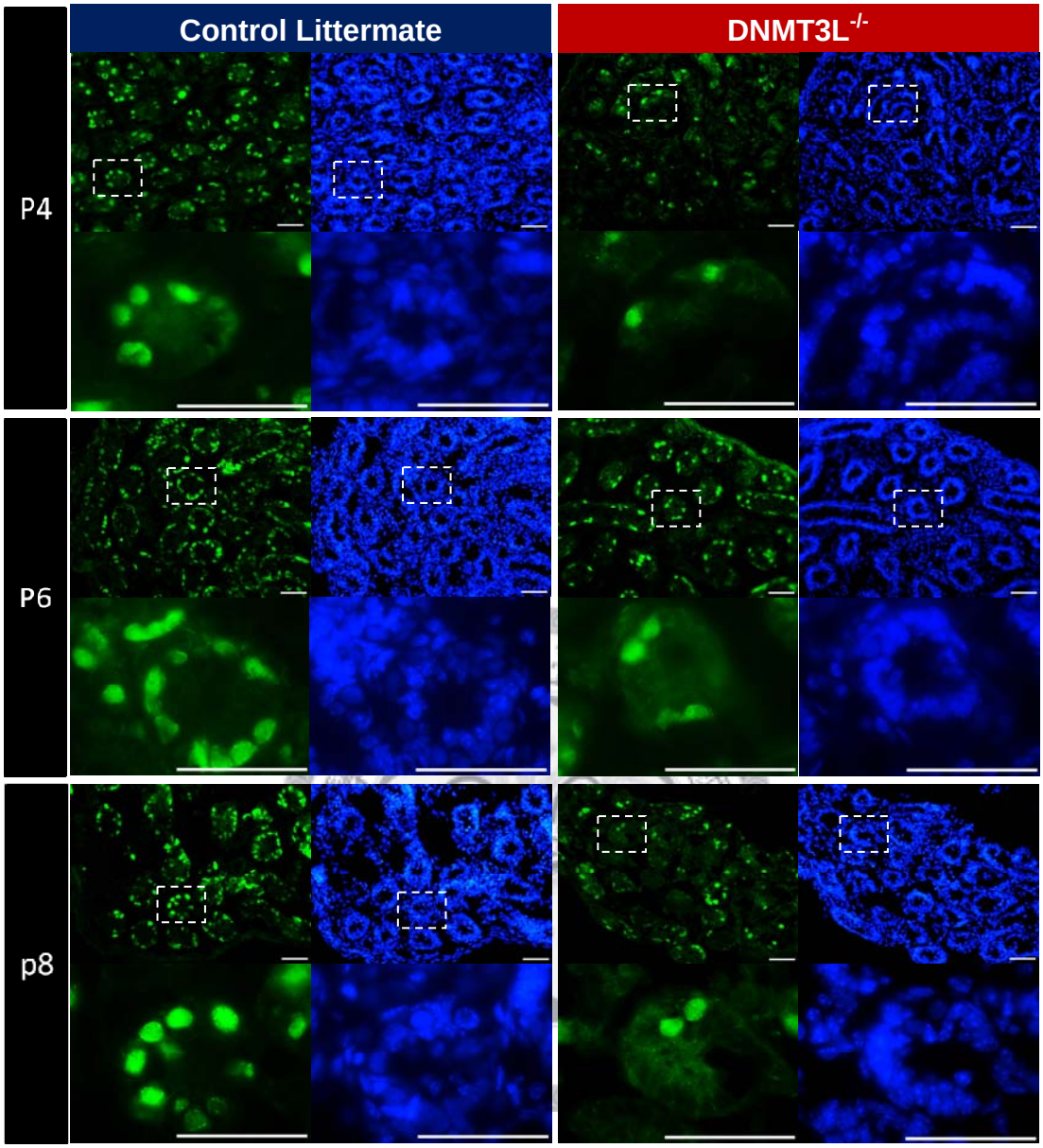

Fig. 4-3 PLZF expression patterns in p4 to p8 DNMT3L$^{-/-}$ and its littermate control testes

Representative photographs for PLZF staining of p4 to p8 seminiferous tubule of DNMT3L$^{-/-}$ testes and its littermate control. Green, PLZF; blue, Hoechst 33342. The white dotted boxes are enlarged in the bottom picture. Scale bars: 50μm

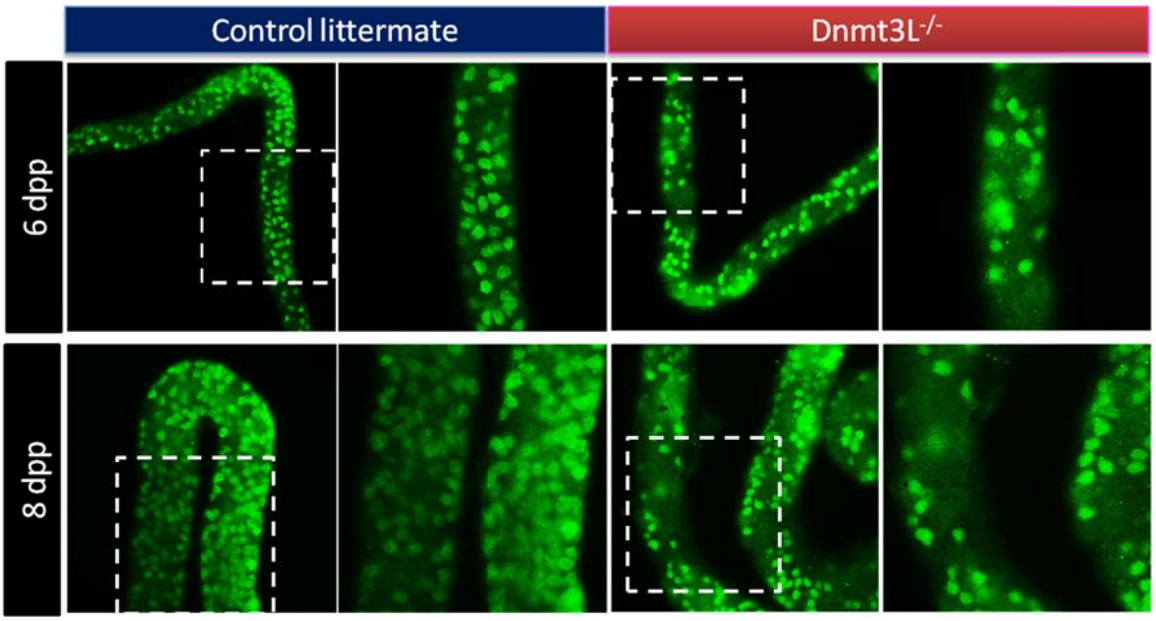

Fig. 4-4 whole mount morphology PLZF staining on DNMT3L$^{-/-}$ and littermate control seminiferous tubules.

Homogenous staining pattern can be seen throughout the seminiferous tubules of the control littermate's group; while chunks of negative staining were shown in the Dnmt3L$^{-/-}$ testes. The areas boxed are enlarged in the pictures on the right.

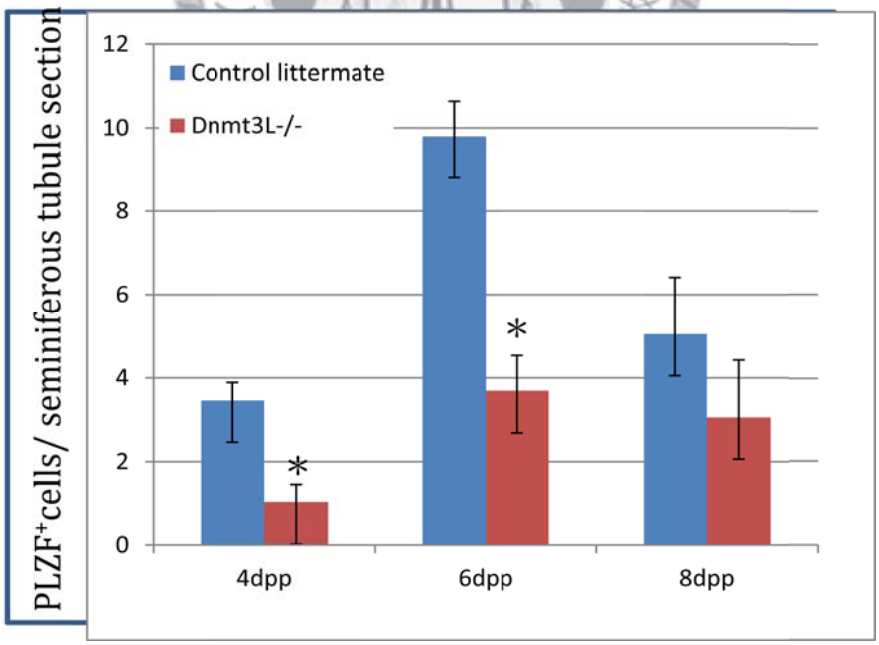

Fig. 4-5 Quantification of PLZF staining results from all testes sections.

25 round-shaped seminiferous tubules sections were counted with each animal, and 3, 4, and 3 animals were counted for 4, 6, and 8 dpp samples respectively. Result are presented as mean ± SEM ＊0.1>p-value>0.05.

4.4 Loss of H3K9me3 and H4K20me3 perinuclear staining in Dnmt3L$^{-/-}$ spermatogonia in ages from 4 dpp to 4 wks mouse.

After observing the decreased PLZF staining number, we further examined the epigenetic status that represents transcriptional quiescent status more specifically. The two histone modifications H3K9me3 and H4K20me3 are heterochromatin marks that indicate highly repressive transcriptional levels (Schotta *et al*., 2004). Previous studies of SSCs function have connected PLZF expression and these two repressive marks that are considered highly associated with the quiescent status of SSCs. The staining patterns of H3K9me3 and H4K20me3 in PLZF^{+} spermatogonia has distinguished from other differentiated cell types with a perinuclear staining pattern, which means the two marks appeared to stain around the periphery of the nuclear envelope, indicating a more "organized" or "tighter" structure of the chromatin (Payne and Braun, 2006).

In the staining results from 4 dpp to 8dpp, H3K9me3 staining had shown a severe reduction of perinuclear staining patterns in the nucleus of the spermatogonia in all sample stages (Fig. 4-6), this phenomenon continues to 4 wks of age (Sup. 1) H4K20me3 staining also reveals a similar pattern with H3K9me3 staining (Fig. 4-7 and Sup. 2).

The loss of H3K9me3 and H4K20me3 perinuclear staining result suggests that the quiescent status of SSCs has been affected in Dnmt3L$^{-/-}$ spermatogonia.

The above study of Dnmt3L provides an insight of the connection between DNA methylation and histone modification. It also suggests that epigenetic modifiers of different levels can work together to perform transcriptional silencing *in vivo*.

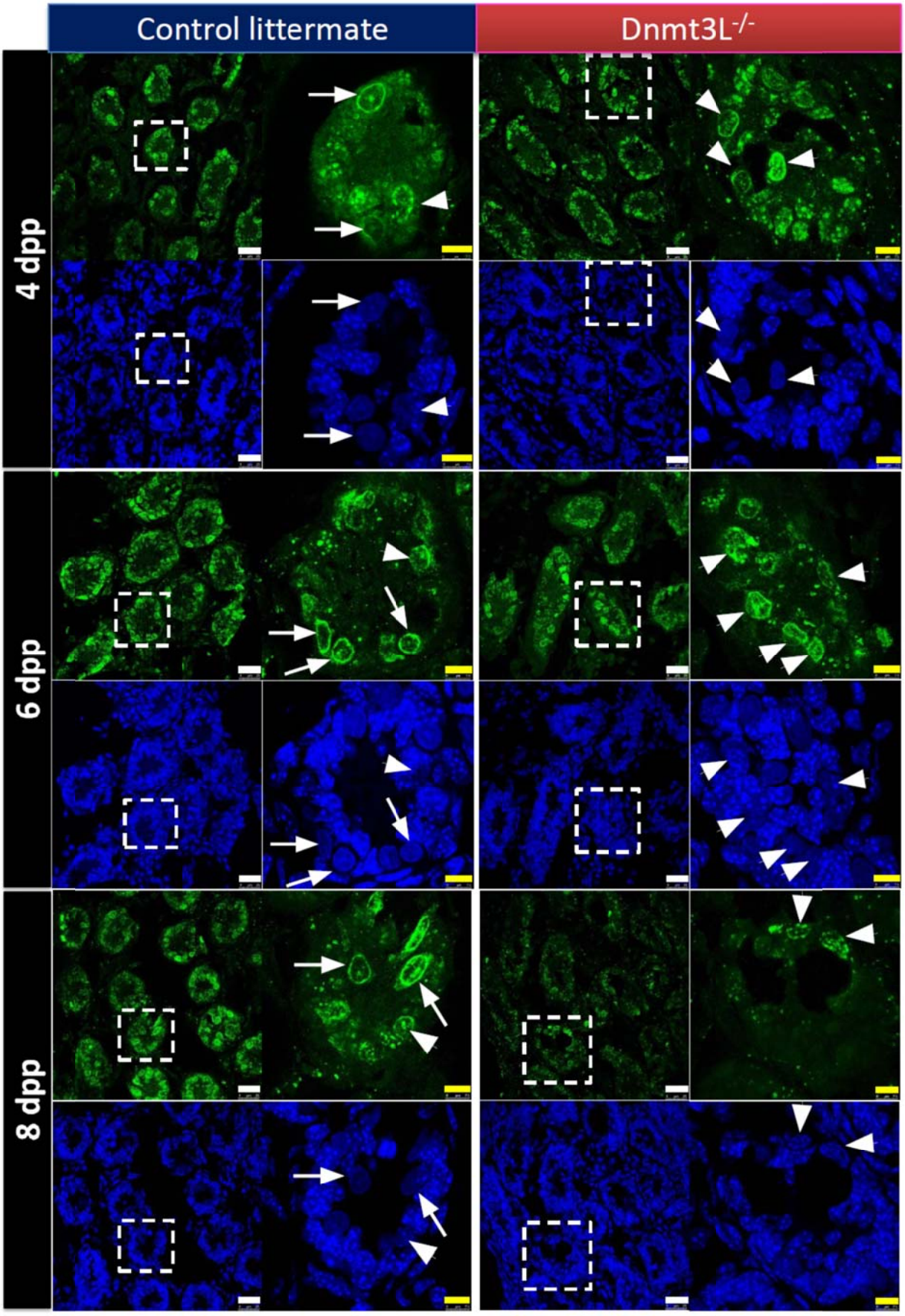
Control littermate
Dnmt3L-/-
4 dpp
6 dpp
8 dpp

Fig. 4-6 H3K9me3 staining patterns of Dnmt3L$^{-/-}$ and its control littermate's testes from 4 dpp to 8dpp

The arrows point to the perinuclear stained nuclei; the arrowheads point to the punctuate nuclei. Plenty of perinuclear staining were observed in the littermate control group, while most of the Dnmt3L$^{-/-}$ spermatogonia were found to have punctuate staining.

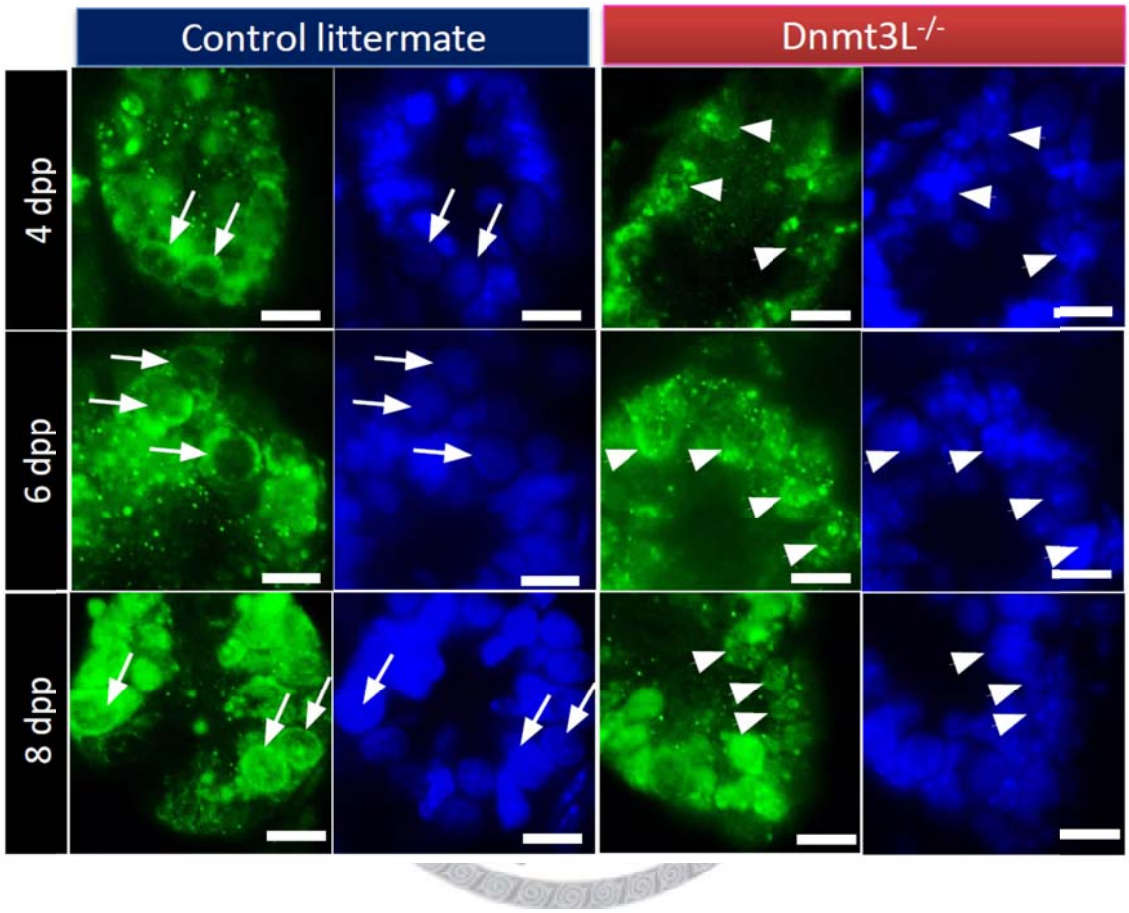

Fig. 4-7 H4K20me3 staining patterns of DNMT3L$^{-/-}$ and its control littermate's testes from 4 dpp to 8dpp.

The arrows point to the perinuclear stained nuclei; the arrowheads point to the punctuate nuclei. Alike the H3K9me3 staining pattern, plenty of perinuclear staining were observed in the littermate control group, while most of the Dnmt3L$^{-/-}$ spermatogonia were found to have punctuate staining.

4.5 Failure of colonization from Dnmt3L$^{-/-}$ germ cells to busulfan-treated recipient mice via germ cell transplantation

Dnmt3L's role on SSC maintenance and quiescent status helped us to understand Dnmt3L's function in the molecular and histological level. Functional studies can further back-up our molecular data on how Dnmt3L may affect the property of SSCs *in vivo*.

From the colonization result of GFP^{+} wild-type germ cells transplantation, we found obvious colonized seminiferous tubules with GFP expression; however, no GFP^{+} cells were detected in the recipient testis of Dnmt3L$^{-/-}$ donor germ cells (Fig. 4-8). This result showed a disruption of Dnmt3L$^{-/-}$ spermatogonia to colonize to the host testes, which indicates that Dnmt3L may not only influence the quiescent status of SSCs, but eventually could influence SSCs' ability to colonize to the basal membrane of the host testes as well.

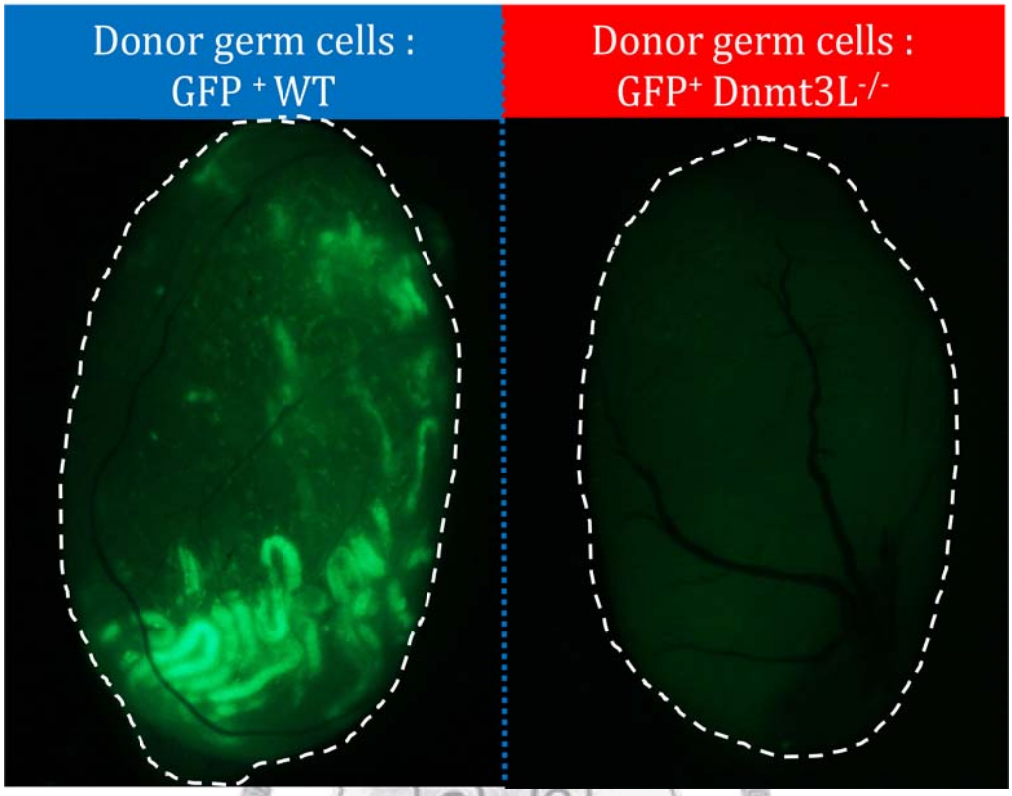

Fig. 4-8 Wild type-GFP germ cells verses Dnmt3L$^{-/-}$ GFP germ cell transplantation analysis

GFP+ wild-type and Dnmt3L$^{-/-}$ Germ cells were transplanted into wild-type recipients via germ cell transplantation. Around 25% of the surface seminiferous tubules were colonized with wild-type GFP^{+} germ cells 6 wks after transplantation. Dnmt3L$^{-/-}$ germ cells, however, did not show signs of colonization at the surface seminiferous tubules.

4.6 Dnmt3L and its effect on the niche holding normal SSC function

One other possibility for the spontaneous germ cell loss in Dnmt3L$^{-/-}$ testes may be the secondary effect caused by defected niche environment when Dnmt3L is absent. The interesting staining patterns of VASA (or MVH or DDX4) and STRA8 in the Dnmt3L$^{-/-}$ testis supports this hypothesis.

4.6.1 VASA staining pattern implies a disrupted SSC niche

In the 5 wks-old testes of the control littermate, VASA was expressed strongly on all stages of germ cells except the elongated spermatids (Fig. 4-9). Among all types of germ cells, only the early types of spermatogonia that are located around the basal membrane had a low level of VASA expression. This explains the theory for the transcriptional quiescence for SSCs (Fig. 4-9 B).

On the other hand, Dnmt3L$^{-/-}$ 5 wk old testes already had global germ cell loss throughout all seminiferous tubules. VASA staining pattern on Dnmt3L$^{-/-}$ 5 wks-old samples revealed several different types of patterns (Fig. 4-10), such as nearly sertoli-cell-only patterns with one to two remaining VASA^{+} cells adjacent to the basal membrane (Fig. 4-10A); or tubules with several layers of germ cells left all with strong VASA staining but with no weakly-stained, undifferentiated spermatogonia at the basal membrane (Fig. 4-10 B and C); Some tubules have their Sertoli cells outnumbered by VASA^{+} germ cells in the only remaining layer of cells left. From the above types described, one characteristic in common is that there are no normal undifferentiated spermatogonia that should be weakly stained with VASA, but only spermatogonia-like germ cells and early-stage spermatocytes that are strongly stained with VASA.

An implication from this observation is that all of the remaining germ cells are no longer early-stage spermatogonia, but already differentiated germ cells. The cause of this observation may be a combination of many secondary effects that eventually results in a collapse of the niche environment, without keeping the ability to maintain undifferentiated SSCs.

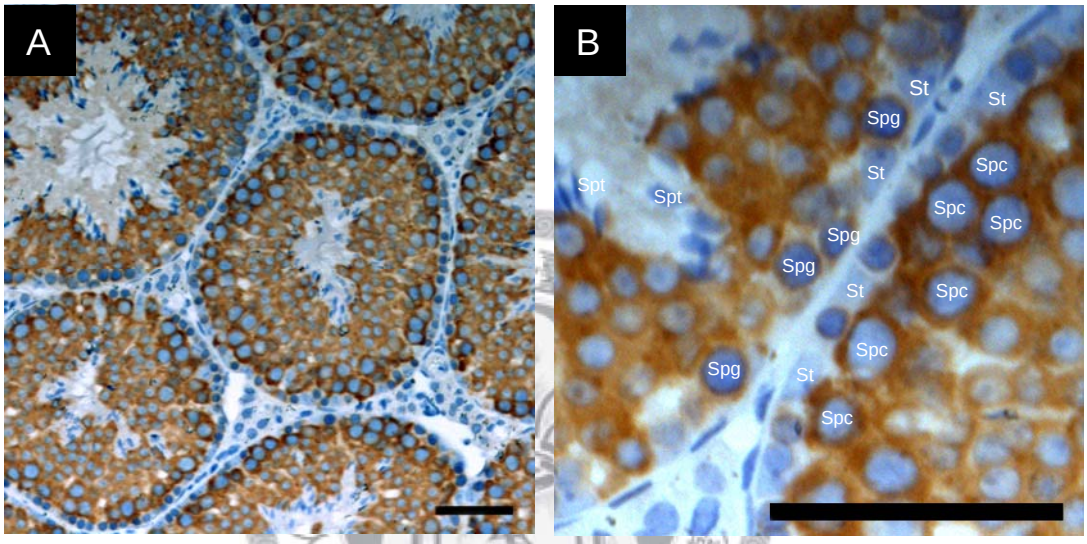

Fig. 4-9 Histological observation on VASA stained 5-wks-old mouse testis.

A. Broad view of the VASA staining pattern. All germ cells are stained except the spermatocytes in the middle of the seminiferous tubule. Scale bar: 50μm.

B. Enlarged view from A. showing different cell types differentially stained with VASA. Spermatocytes are the most heavily stained, Spermatogonia are weakly stained, and Sertoli cells and spermatocytes do not have VASA expression. Abbreviations: Spg: Spermatogonia, Spc: Spermatocyte, Spt: Spermatid, St: Sertoli cell. Scale bar: 50μm.

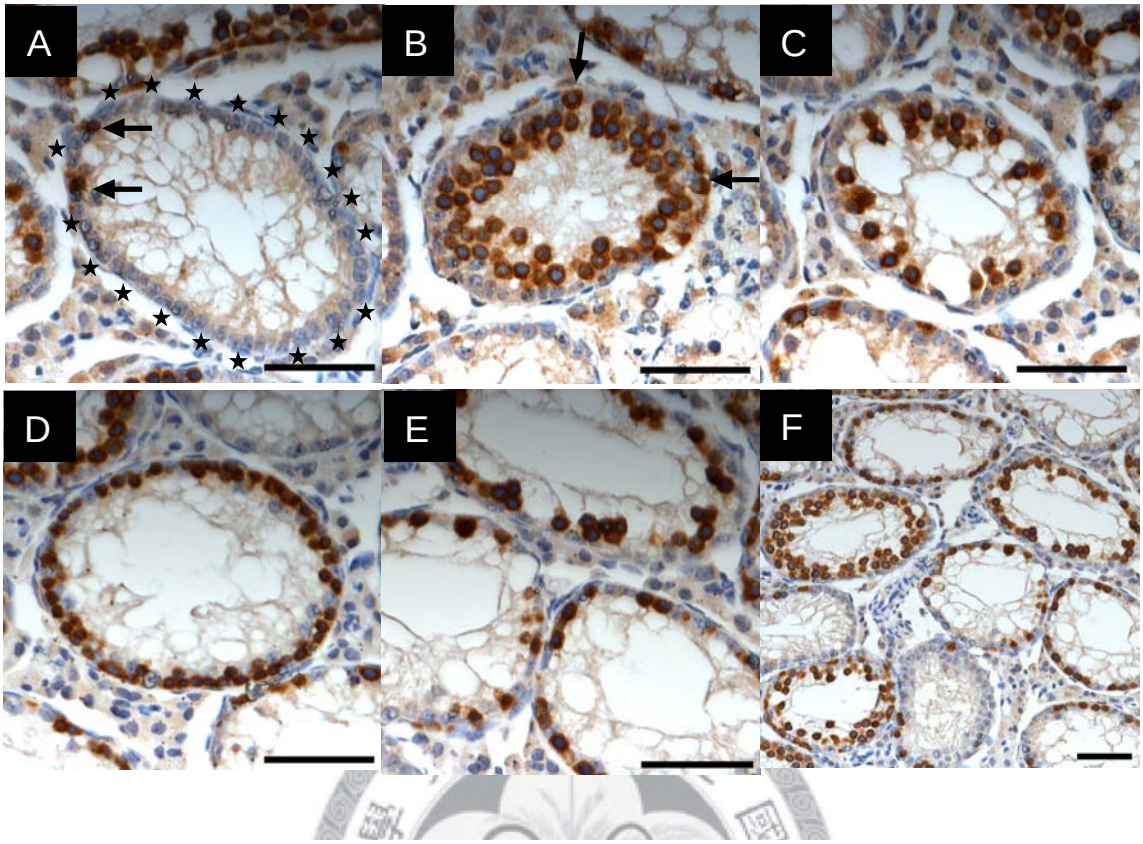

Fig. 4-10 Histological observations on VASA-stained Dnmt3L$^{-/-}$ 5-wks-old testis

A. Sertoli cell-only type of seminiferous tubule. Only two VASA^{+} germ cells remained (arrows). Stars surround the Sertoli cells of the seminiferous tubule.
B. C. Several layers of VASA^{+} germ cells still remain in the tubule, but little or no VASA^{+} germ cells sit in the basal membrane. Arrows: the only germ cell with strong VASA staining at the basal membrane.
D. E. One layer of germ cells at the basal membrane strongly stained with VASA.
F. Broad view of the VASA stained 5-wks-old Dnmt3L$^{-/-}$ testes section.

4.6.2 STRA8 staining implies a disrupted SSC niche but not spermatogenic cycle

Stra8 is a gene expressed in pre-meiotic spermatogonia, and indicates a communication between niche cells and germ cells. STRA8 staining in the 5 wks-old testes revealed a clear stage-specific and cell type-specific pattern, with 17% of seminiferous tubule stained with STRA8 (Fig. 4-11 A). On the other hand, In the Dnmt3L$^{-/-}$ testes, even though not able to tell the seminiferous stages due to the severe cell loss, the staining pattern of STRA8 only appeared in 24% of seminiferous tubules, indicating another “staging” machinery took over in the absence of spermatocytes and spermatids (Fig. 4-11 B).

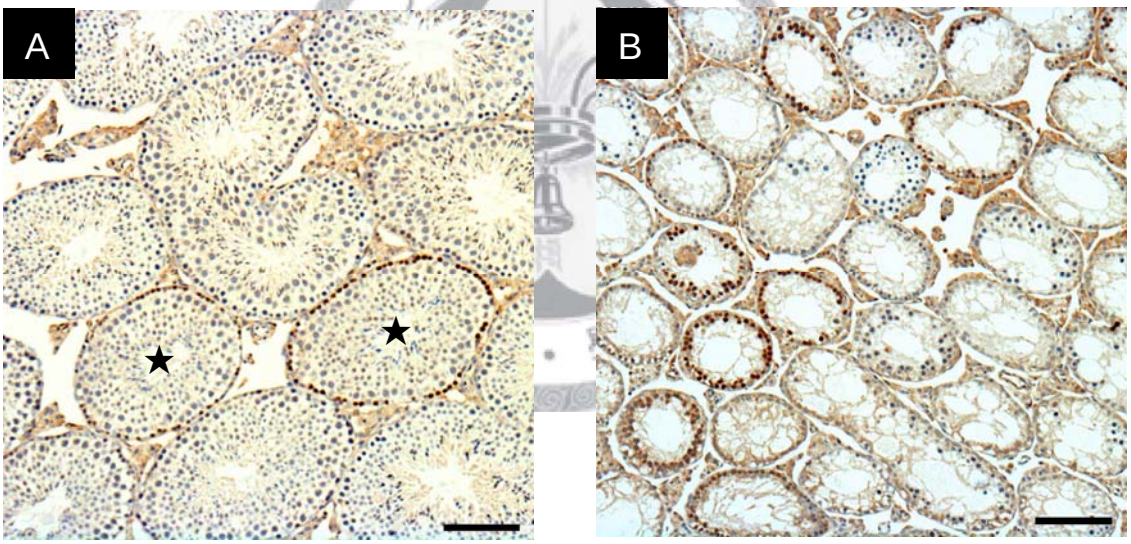

Fig. 4-11 STRA8 expression pattern in 5-wks-old Dnmt3L$^{-/-}$ and its littermate control.

A. 5-wks-old control testis stained with STRA8. Star refers to the two seminiferous tubules strongly stained with STRA8 in preleptotene spermatocytes. The percentage of STRA8+ tubules from total tubules is: 17%. Scale bar: 50μm
B. 5-wks-old Dnmt3L$^{-/-}$ testes stained with STRA8. The percentage of STRA8+ tubules from total tubules is: 24%.Scale bar: 50μm

Looking closer to the STRA8 staining patterns in DNMT3L$^{-/-}$ 5 wks-old testis, we observed a similar pattern with VASA staining, where many of the stained spermatogonia or spermatocytes are “detached” from the basal membrane of the seminiferous tubule, with the remaining cells at the basal membrane being mostly Sertoli cells (Fig. 4-12 A and B). Few of the STRA8^{+} seminiferous tubules have STA8^{+} germ cells at the basal membrane, but very few Sertoli cells were left at the same layer.

Taken together, from the results of VASA and STRA8 staining in 5 wks-old DNMT3L$^{-/-}$ testes, there is an implication that DNMT3L may have an effect on not only germ cells itself, but also the niche environment. Further examinations should be done to confirm this hypothesis.

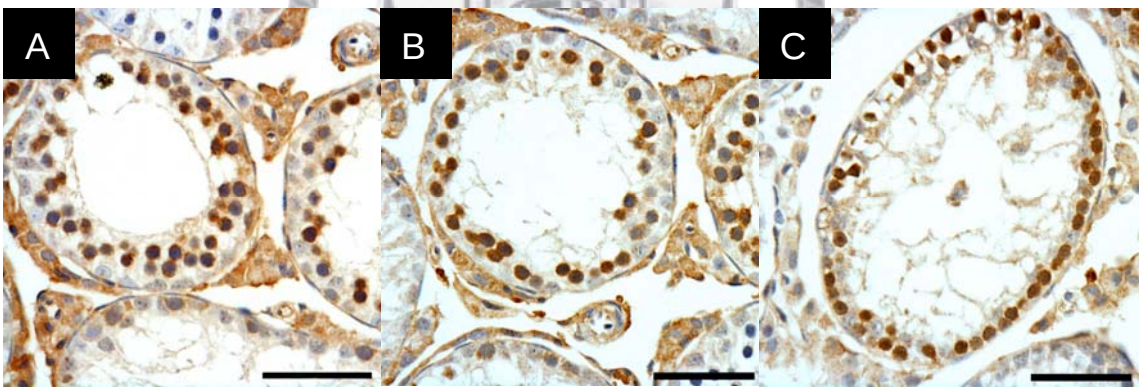

Fig. 4-12 STRA8 staining patterns on Dnmt3L$^{-/-}$ 5-wks-old testis

A and B. Germ cells stained with STRA8 are mostly distributed at the second layer or further from the basal membrane. The first layer is occupied with Sertoli cells.
C. The only layer of cells left in the tubule is mostly germ cells stained with STRA8, accompanied with very few Sertoli cells that are STRA8 negative.

4.6.3 Functional analysis of the Dnmt3L$^{-/-}$ niche by germ cell transplantation experiment

To test the hypothesis considering Dnmt3L's effect on the niche environment to support the function of SSCs, germ cell transplantation experiment revealed that when the littermate control testes has a few GFP^{+} colonies, Dnmt3L$^{-/-}$ testes lack any GFP^{+} colonies (Fig. 4-13 and Sup. 4).

The colonization ability is one of the key factors for SSCs function. This ability not only depends on the ability of germ cells, but also is highly dependent on the niche environment. The transplantation experiment shows that the niche of Dnmt3L$^{-/-}$ testes, at least from 6-10 weeks old does not support colonization of wild-type GFP^{+} germ cells.

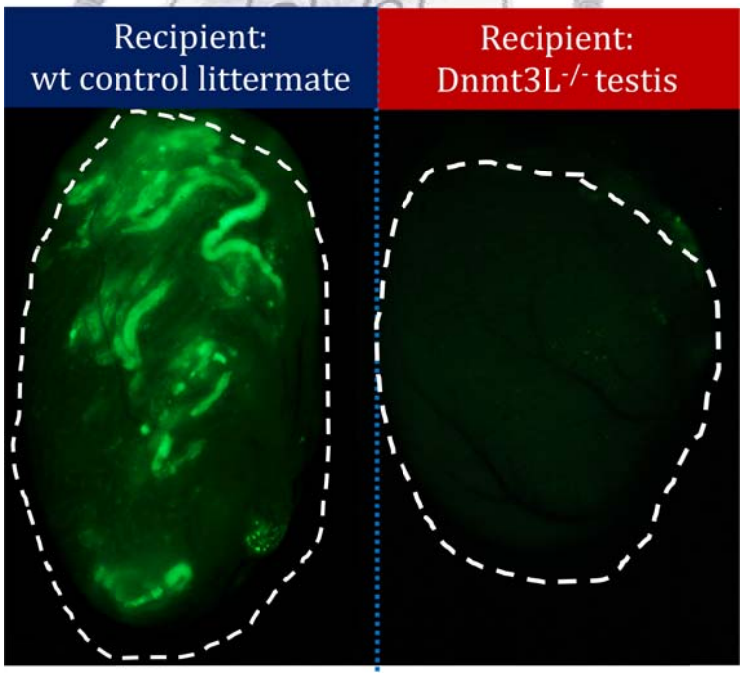

Fig. 4-13 Germ cell transplantation results of GFP^{+} germ cells transplanted into Dnmt3L$^{-/-}$ and its littermate control mice

Germ cells of GFP^{+} wild-type were transplanted into recipients of Dnmt3L$^{-/-}$ mice and its control littermate. The surface seminiferous tubules were colonized in the control littermate testis 6 wks after transplantation. Dnmt3L$^{-/-}$ testis, however, did not show signs of colonization at the surface of seminiferous tubules.

Chapter 5 Discussion

In this study, we have shown that Dnmt3L, an epigenetic regulator gene, has an impact on SSC property through influencing their quiescent status. This is also an evidence for Dnmt3L not only being an epigenetic regulator facilitating DNA methylation, but also affecting histone modifications (H3K9me3 and H4k20mes) *in vivo*. Germ cell transplantation experiments further reveal a failure of spermatogonia to colonize the host testes. In addition, the Dnmt3L$^{-/-}$ 6 to 10 wks-old testis environment failed to support external germ cells to repopulate its testes. Overall, Dnmt3L is shown to be responsible for SSC quiescence, SSC stemness, and the niche function.

Gradual germ cell loss in Dnmt3L$^{-/-}$ testis indicates abnormal SSC formation and/or maintenance

In previous studies of Dnmt3L, its function in the male germ line falls in the category of retrotransposon silencing. This is proposed to be the consequence of the "catastrophe" of meiotic synapsis in pachytene spermatocytes (Bourc'his and Bestor, 2004; Hata *et al*., 2006). Interestingly, in the first part of our study, when observing the 5 wks-old testes section of Dnmt3L$^{-/-}$ and its comparison to its control littermate, a gradual germ cell loss can be seen in the Dnmt3L$^{-/-}$ testes. In later stages (over 8 wks), almost all of the seminiferous tubules become sertoli-cell only (data not shown). This phenomenon supports the idea that in addition to the consequence of failure to progress after meiosis, the spontaneous cell loss may be caused by a loss of stem cell function in the niche, which eventually fails to sustain the cell population. Adding to the gradual germ cell loss phenomenon, previous studies have shown that Dnmt3L$^{-/-}$ neonatal testes have less GCNA-1^{+} germ cells (La Salle *et al*., 2007). This is consistent with our Tra98 staining results, which in neonatal testis from 4 dpp to 10 dpp show a reduction in

Tra98^{+} cells in Dnmt3L$^{-/-}$ pups (Sup. 3). This lower normal germ cell count could be a result from SSCs not properly formed in number and/or gene expression at the first place, or that SSCs not being able to sustain themselves once populating the seminiferous tubules.

Reduction of quiescent status of SSCs in Dnmt3L$^{-/-}$ mice testes shown from Plzf, H3K9me3 and H4K20me3 status

We've examined the quiescent status of SSC property with a major reason due to Dnmt3L's original identity --- transcriptional repression. Recent studies also have elucidated connection between stem cell property and quiescence (Arai *et al*., 2004; Moore *et al*., 2007; Moore and Lemischka, 2006; Sorrentino, 2010). In our study, we've used Plzf as a marker for quiescent SSCs, due to previous evidence of Plzf's relationship with transcriptional and cell cycle quiescence (Barna *et al*., 2002; Buaas *et al*., 2004; Payne and Braun, 2006). Chromatin structure is also important to indicate the transcriptional level and cell cycle of a cell. The two histone modifications, H3K9me3 and H4K20me3, have been reported to be essential for normal heterochromatin formation (Cedar and Bergman, 2009; Fuks, 2005; Schotta *et al*., 2004). The association between Plzf and the above two histone modifications has been reported to be essential for the maintenance of SSCs (Payne and Braun, 2006). Our results reveal that Dnmt3L$^{-/-}$ mice do have a reduced number in Plzf positive cell count and a reduced perinuclear staining patterns of H3K9me3 and H4K20me3. This is an indication that Dnmt3L does have an impact on the tighter chromatin structure of the cells that should originally be in a quiescent status. Further examination of the cell quiescence status can also be studied by analyzing the replication activity levels by staining with Brdu, in combination of CyclinD1 and CyclinD2; RNA polymerase markers representing the actual

transcriptional activity of a cell; on the other hand, markers of cell cycle arrest can reveal cells at G_0 stages. These above indicators could be studied in combination with PLZF staining and perinuclear staining of H3K9me3 and H4K20me3. We expect to see increased Brdu, CyclinD1, CyclinD2, and RNA polymerase expression in Dnmt3L$^{-/-}$ testes sections, also with a reduced ratio of G_0-stage spermatogonia. These will help us to further clarify Dnmt3L's effect on the quiescent status of SSCs.

Functional analysis shows lack of Dnmt3L affecting the property of SSCs

Functional analysis of the colonization ability of Dnmt3L$^{-/-}$ germ cells is the most direct evidence to analyze the effect of Dnmt3L on SSC function. Our results showed that the germ cells isolated from Dnmt3L$^{-/-}$ neonatal mice failed to colonize the host testes while the wild type germ cell could. The colonization and repopulation of externally injected SSCs to the host testes is a multiple process, including homing of SSCs to the basal membrane, replication of SSCs, parts of them differentiate to later stages of germ cells and reinitiate spermatogenesis. A successful repopulation of SSCs depends on the functionality of SSCs, the ratio of undifferentiated SSCs to differentiated germ cells, and the molecular interaction between SSCs and the niche environment. Our transplantation results back up the hypothesis that Dnmt3L may affect the property of SSCs.

Dnmt3L's possible influence on the SSC niche

Stem cells' functionality depends highly on their niches. Many studies have demonstrated the intimacy between SSCs and their niches, and how the interaction between them influences the self-renewal and differentiation ability of SSCs (Chen *et al.*, 2005; Hess *et al.*, 2006; Kubota *et al.*, 2004c; Meng *et al.*, 2000a; Ohta *et al.*, 2000). The most frequently studied niche cell for SSCs are Sertoli cells. Adhesion molecules for SSC and Sertoli cell communication such as β1-integrin, have been shown to be

essential for the homing ability of SSCs to the basal laminin after transplantation experiment. Since the niche function is essential for the normal function of SSCs, we've intended to test whether Dnmt3L may have an impact on the niche and/or the interaction between niche cells and SSCs. The most direct way was to transplant wild-type germ cells into Dnmt3L$^{-/-}$ testis. If only the germ cells are affected when lacking Dnmt3L, the wild-type germ cells should be able to reinitiate spermatogenesis in the Dnmt3L$^{-/-}$ testis. However, our results showed that the Dnmt3L$^{-/-}$ testis failed to support the repopulation of wild-type germ cells. Some possible explanations include the structure basis of the Dnmt3L$^{-/-}$ testis is already disrupted by the time of transplantation, or an imbalanced/ abnormal molecular interaction between the transplanted germ cells and the remaining somatic cells. Previous studies have showed that the extra-cellular matrix can also be an important factor for stem cells to "find their way home" (Lapidot *et al*., 2005).

Apart from our transplantation results, the VASA and STRA8 staining results gave us some indications of how the niche may be affected after depletion of Dnmt3L. VASA is expressed at all stages of germ cells, but has lower expression level in undifferentiated spermatogonia; and STRA8 is expressed in pre-meiotic spermatogonia and is an important factor for the onset of meiosis. From the VASA staining patterns of thr Dnmt3L$^{-/-}$ testes the characteristic in common is that there are no normal early spermatogonia that are stained weakly with VASA, but only spermatogonia-like germ cells and early-stage spermatocytes that are strongly stained with VASA. Two types of seminiferous tubules in which the outer layer of cells were out-numbered by sertoli cells (Fig. 4-10 A, B and C) revealed an phenomenon that all of the spermatogonia that should sit at the basal membrane in its niche environment were somehow "squeezed-out" from the niche and forced to continue with differentiation. This phenomenon may be a consequence of the spontaneous germ cell loss starting from 10 dpp, a date earlier than

the formation of pachytene spermatocytes, and the date when we've observed first loss of germ cells in TRA98 stained seminiferous tubules (Sup. 3). This eventually makes the seminiferous tubule a lot emptier compared with its original fully-packed state, and ending up having not enough cell volume to hold the normal tubule size and structure. The third type of seminiferous tubules tubules have only one layer of cells remained (Fig. 4-10 D and E). These tubules aren't Sertoli cell only, but have many of $VASA^{+}$ germ cells sitting at the basal membrane. The Sertoli cell: germ cell ratio at the basal membrane seems to be abnormal compared with normal seminiferous tubules. Interestingly, these germ cells have strong VASA staining, which are a lot different with normal seminiferous tubules where germ cells (or specifically spermatogonia) have weak VASA staining, but have stronger VASA at later stages of spermatogenesis. An implication of this observation is that all of the remaining germ cells are no longer early-stage spermatogonia, but already differentiated germ cells. A possible cause may be a collapse of the niche environment, without keeping the ability to maintain undifferentiated SSCs, which supports our transplantation results. Stra8 is a gene expressed in pre-meiotic spermatogonia, and indicates a communication between niche cells and germ cells. It is a gene highly responsive to retinoic acid, and its expression is highly specific with spermatogenic stages IV to VIII (Zhou *et al*., 2008a; Zhou *et al*., 2008b). Stra8 was also found to be induced by retinoic acid and regulate the onset of meiosis of spermatogonia and oogonia (Anderson *et al*., 2008). Thus Stra8 is an important gene for communication between the normal function of germ cells and its niche environment. The STRA8 staining results in our experiment, however, did not show an altered "staging machinery", but still had a similar pattern with the VASA stained sections, which both had an altered niche cell-germ cell segregation. This staining pattern gives an indication that the SSC niche may be affected by Dnmt3L

deficiency by a gradual disruption of the structure.

Dnmt3L is expressed specifically in germ cells. However, apart from influencing the structural basis of the SSC environment, how could depletion of Dnmt3L affect the niche cells if Dnmt3L is not expressed in them? Most studies regarding germ cells and niche cells demonstrate the essential influence of the niche cells to germ cells; on the other hand, however, recent studies on SSCs from Robert E Braun's group and Shosei Yoshida's group have showed implications on germ cells regulating their niche and influencing the self-renewal and differentiation ability of SSCs (data unpublished). Thus we think there is possibility for the affected germ cells in Dnmt3L$^{-/-}$ testes to influence their niche, with a feedback eventually influencing SSCs property.

To sum up, our study has revealed Dnmt3L's influence on SSCs, both on SSC's status themselves, and on the niche that supports them. Spermatogenesis is a very complex process that is involved with germ cells, somatic cells and many molecule exchanges. Our study provides an entry to understanding how the SSC property may be affected when the epigenetic status is altered after genetic depletion, and also provides insights to how germ cell depletion might influence the niche environment, and how the niche structure may affect SSCs homing and regeneration ability after transplantation. We expect future studies could reveal more precise insights on the mechanics of SSC function on germ line development.

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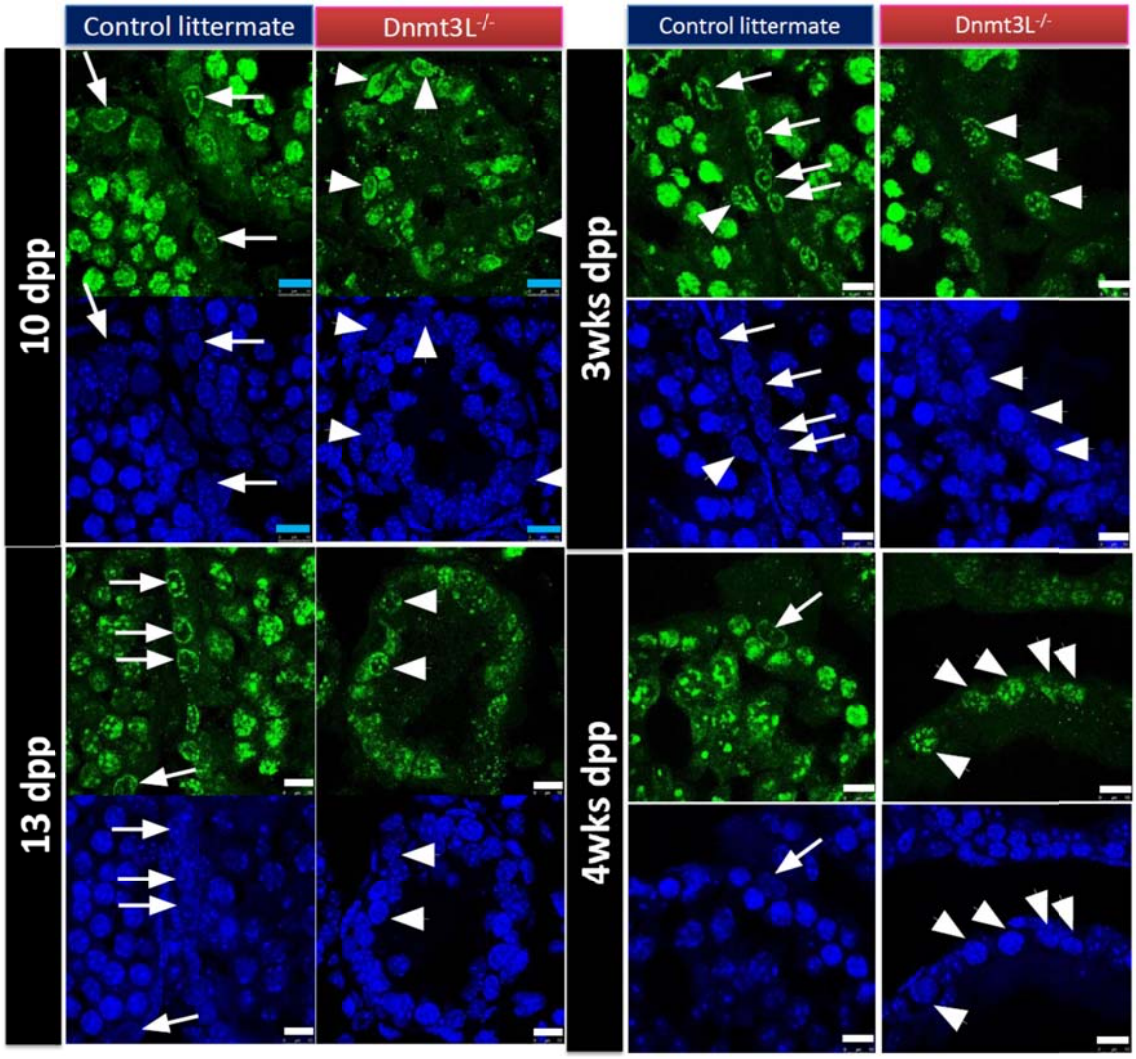
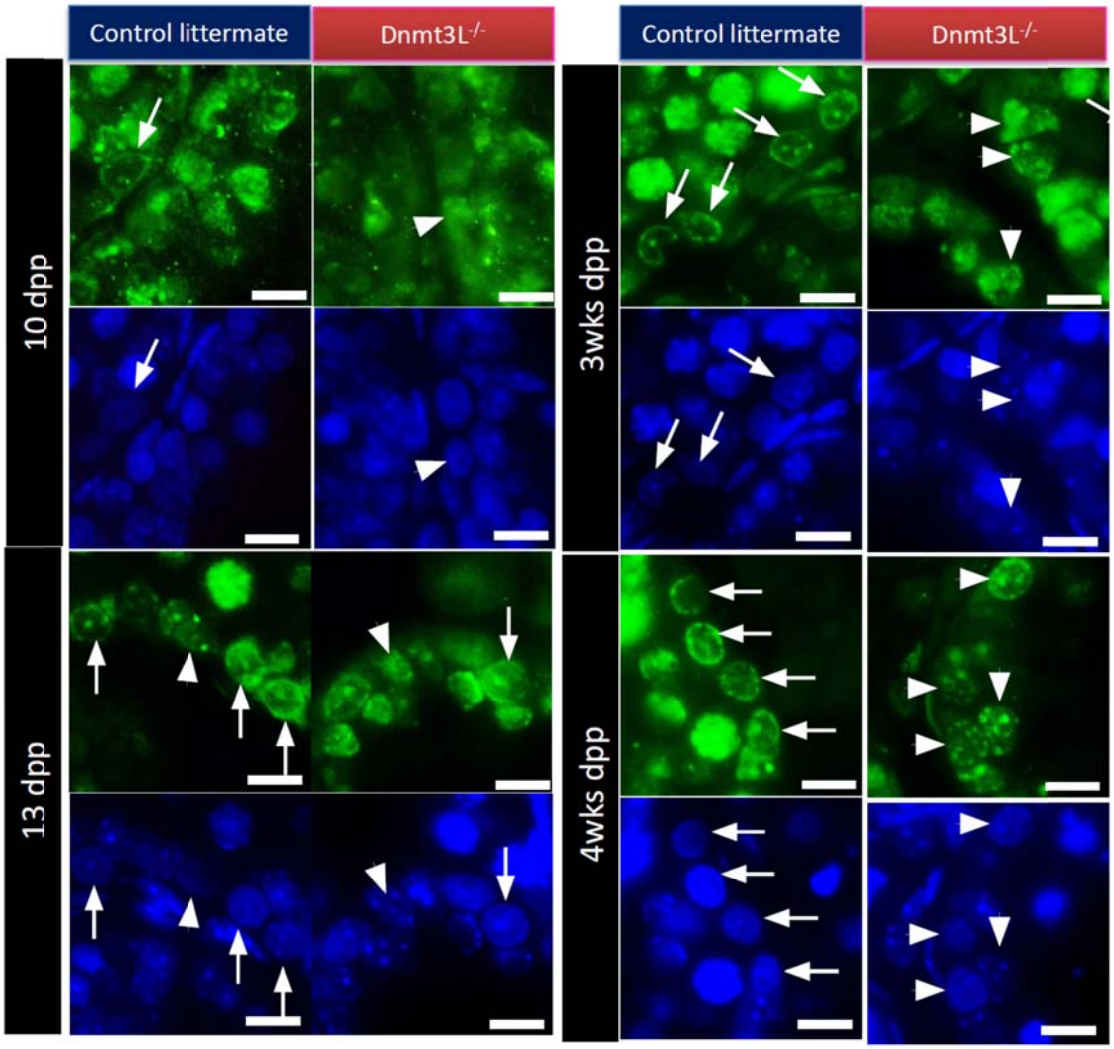
APPENDIX

SUPPLEMANTARY FIGURES



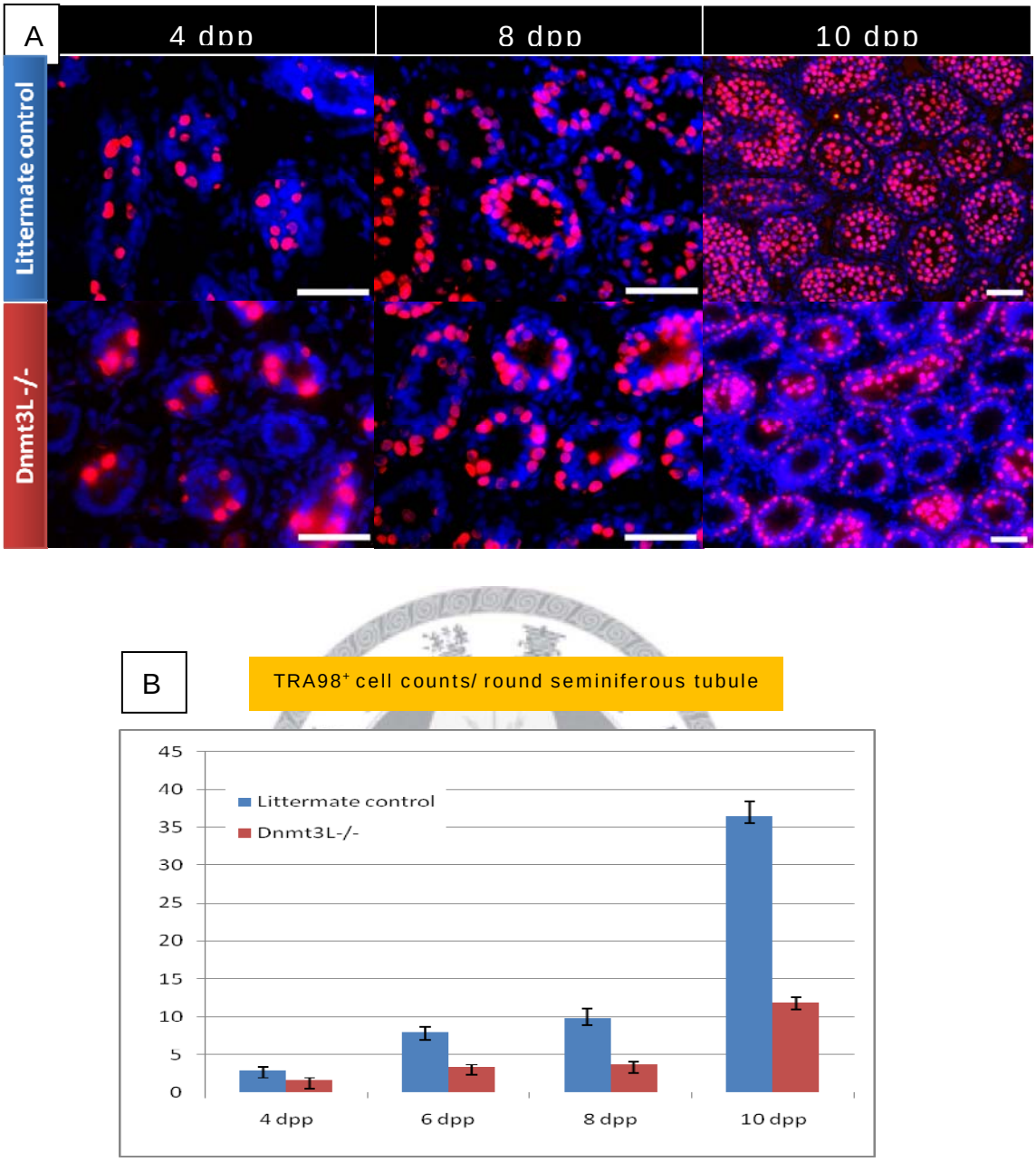
Sup. 1 10 dpp to 4 wks testes of Dnmt3L$^{-/-}$ and its littermate control sections stained by H3K9me3.

Dnmt3L$^{-/-}$ spermatogonia are mainly lost of the perinuclear staining pattern of H3K9me3. Arrows: perinuclear staining; arrowheads: punctate staining. Scale bars: 10mm



Sup. 2 10 dpp to 4 wks testes of Dnmt3L$^{-/-}$ and its littermate control sections stained by H3K9me3.

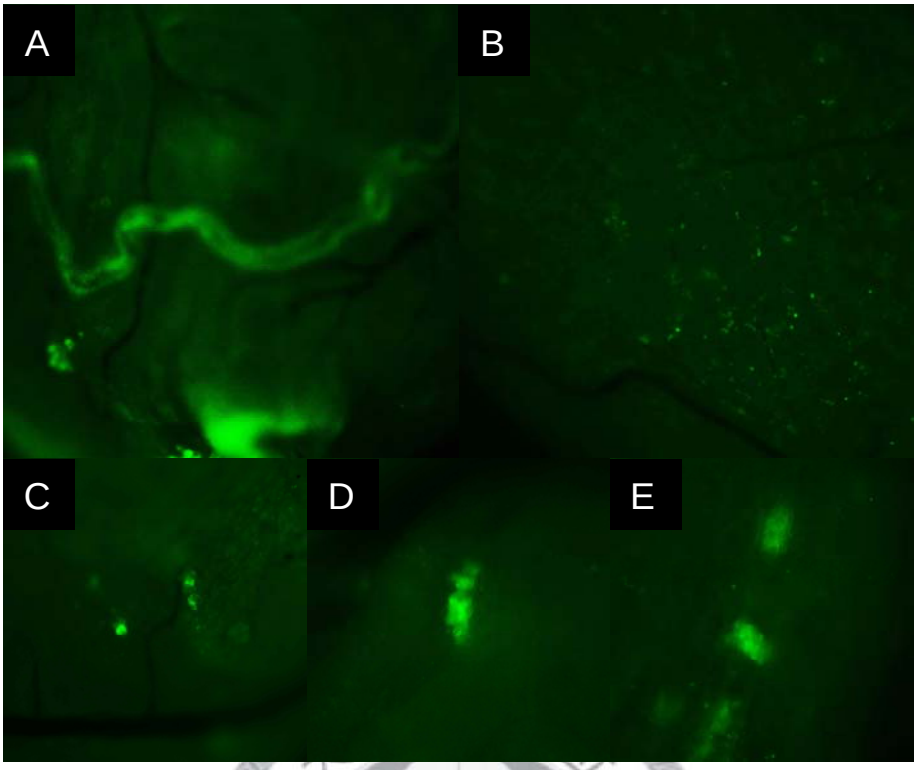
Dnmt3L$^{-/-}$ spermatogonia are mainly lost of the perinuclear staining pattern of H3K9me3. Arrows: perinuclear staining; arrowheads: punctate staining. Scale bars: 10mm



Sup. 3 TRA98 expression pattern in early stages of DNMT3L$^{-/-}$ and their littermate control testes.

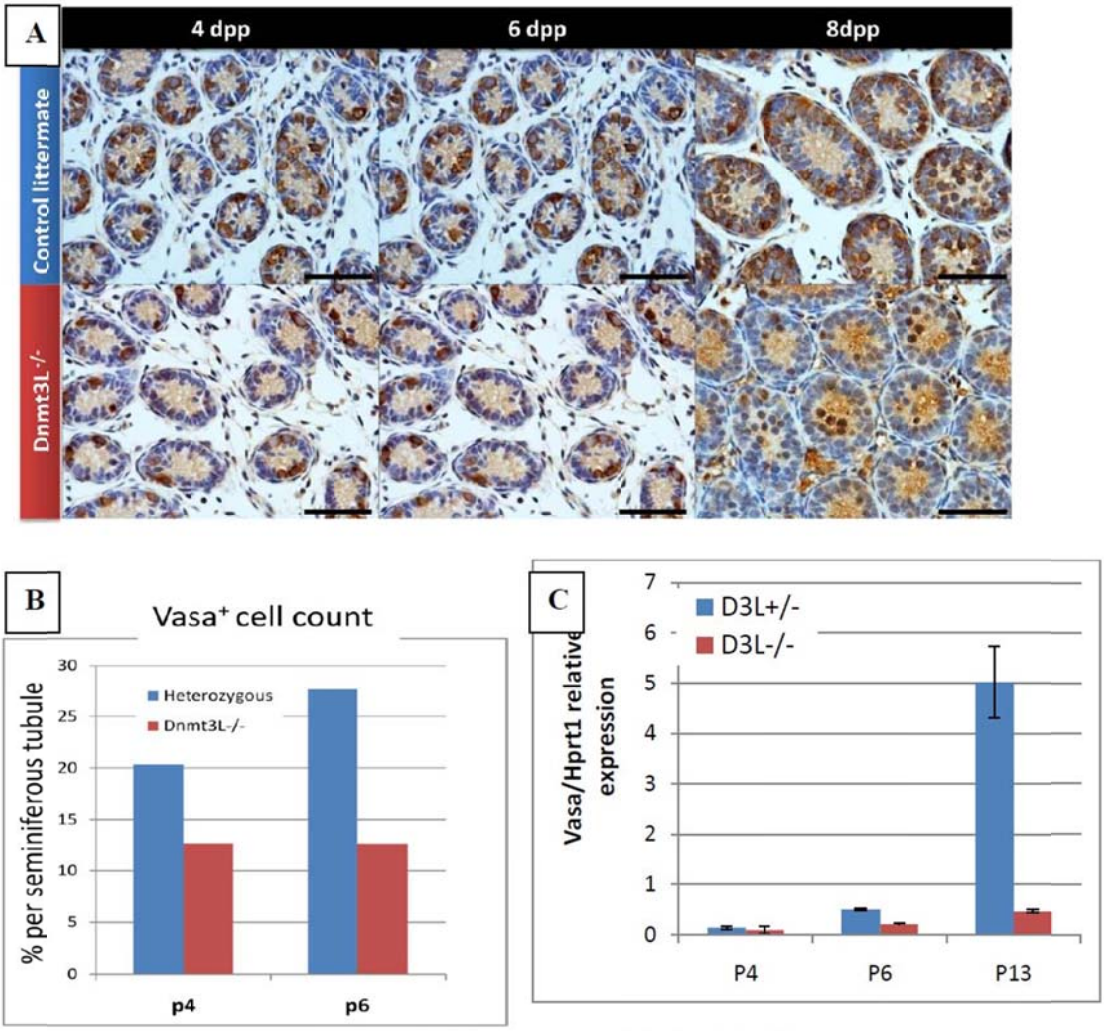
A. TRA98 staining patterns of 4 dpp, 8dpp and 10 dpp. TRA98+ cells stained in red, and Hoechst33342 blue. Scale bars: 50μm.

B. Quantification of TRA98 staining results from 4 dpp to 10 dpp.



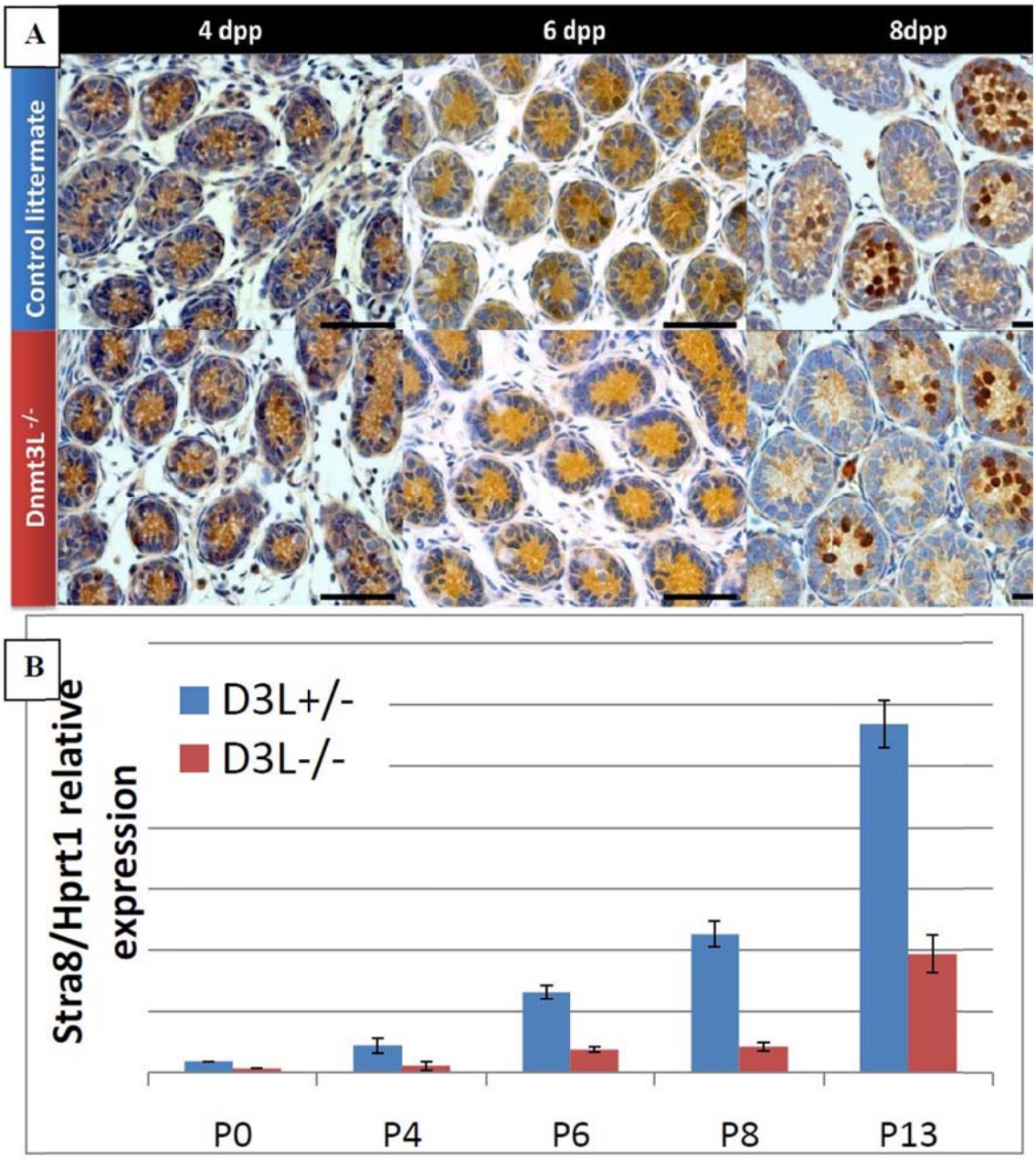
Sup. 4 Germ cell transplantation results of GFP^+ germ cells transplanted into $DNMT3L^{-/-}$ and its littermate control mice

A. Colony formation of GFP^+ germ cells in the littermate control testes treated with busulfan.
B. No sign of colony forms in the $DNMT3L^{-/-}$ testes treated with busulfan.
C. Other view of GFP^+ germ cell colony in the littermate control testis.
D. And E. Enlarged view of GFP^+ germ cell colony in the littermate control testis.



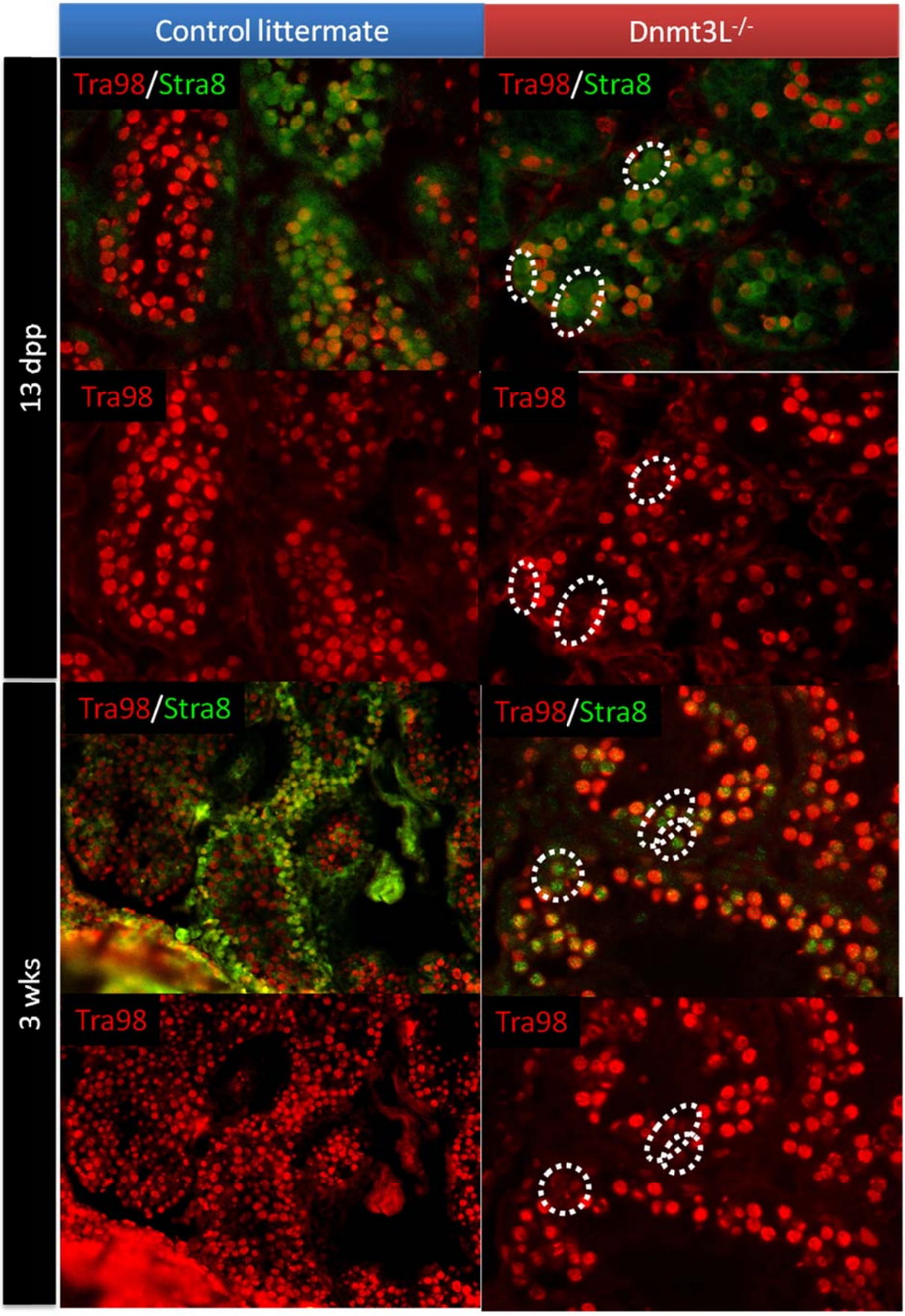
Sup. 5 VASA+ cell counts and relative expression level

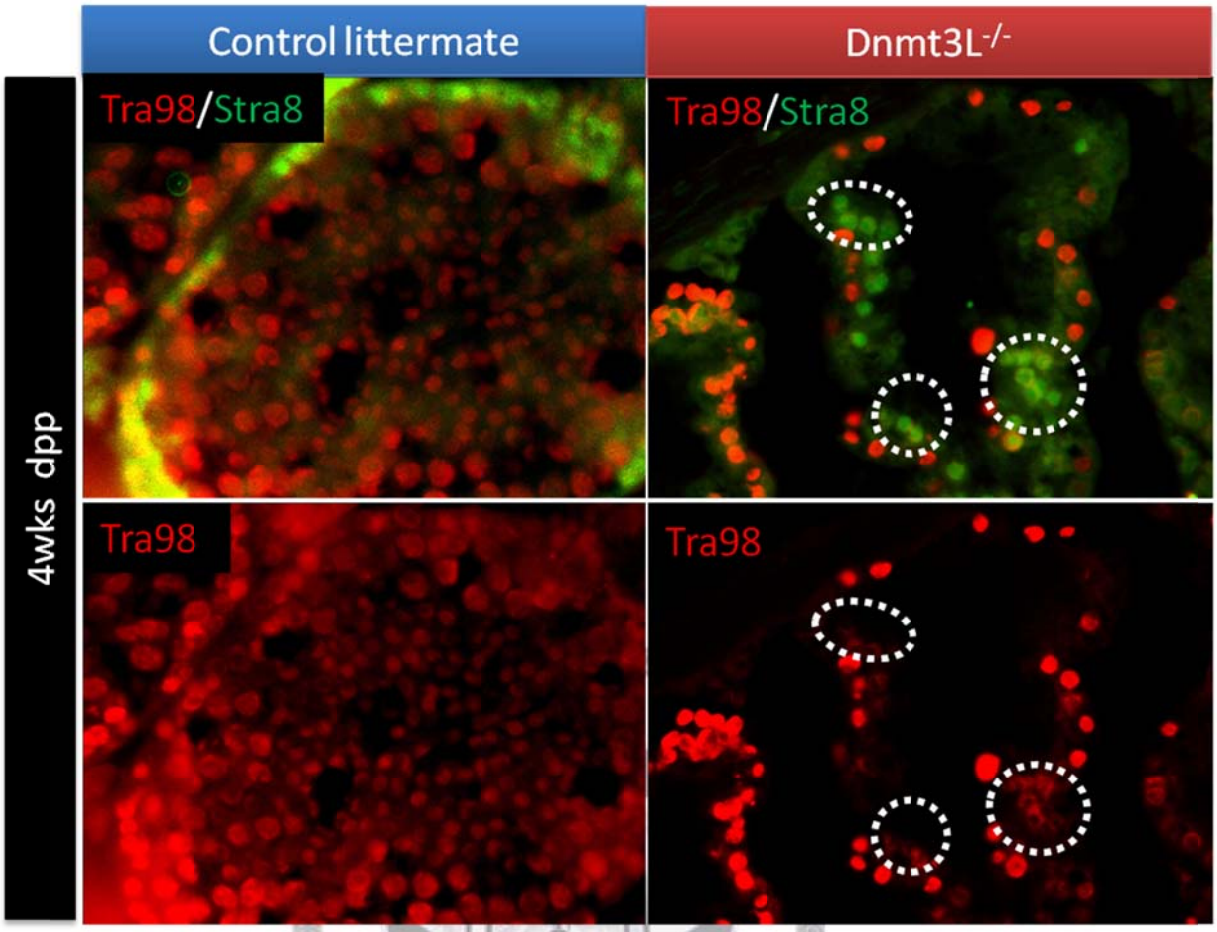
A. 4 dpp to 8 dpp VASA staining pattern of Dnmt3L$^{-/-}$ and its control littermate. VASA+ cells have reduced in number throughout 4 to 8 dpp in Dnmt3L$^{-/-}$ testes.
B. Quantification of VASA^{+} cells in VASA stained sections.
C. Vasa mRNA relative expression level in 4dpp, 6dpp and 13dpp whole testis.



Sup. 6 Stra8 expression in neonatal testes of Dnmt3L$^{-/-}$ and their control littermates.

A. STRA8 staining results in 4 dpp, 6dpp and 8dpp stages of Dnmt3L$^{-/-}$ and their control littermates' testes sections.
B. Stra8 mRNA relative expression level from 0 dpp to 13 dpp.


Control littermate
Dnmt3L-/-
13 dpp
3 wks
Tra98/Stra8
Tra98
Tra98/Stra8
Tra98
Tra98/Stra8
Tra98
Tra98/Stra8
Tra98



Sup. 7 STRA8/TRA98 double staining results in 13 dpp, 3 wks and 4 wks testis of Dnmt3L-/- and their control littermates testes.

Stra8 should co-localize with Tra98 expression, however, in Dnmt3L$^{-/-}$ testes, the white-dotted circles point-out Stra8^{+} cells with Tra98^{-} expression.