An epigenetic switch is crucial for spermatogonia to exit the undifferentiated state toward a Kit-positive identity

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SUMMARY
Epigenetic modifications influence gene expression and chromatin remodeling. In embryonic pluripotent stem cells, these epigenetic modifications have been extensively characterized; by contrast, the epigenetic events of tissue-specific stem cells are poorly understood. Here, we define a new epigenetic shift that is crucial for differentiation of murine spermatogonia toward meiosis. We function of these epigenetic changes in spermatogonia in vivo, the DNA methylation machinery was destabilized by ectopic Dnmt3b expression or Np95 ablation. Forced Dnmt3b expression induced expression of Kit; whereas ablation of Np95, which is essential for maintaining DNA methylation, interfered with differentiation and viability only after spermatogonia become Kit positive. These data suggest that the epigenetic status of spermatogonia shifts dramatically during the Kit-negative to Kit-positive transition. This shift might serve as a switch that determines whether spermatogonia self-renew or differentiate.

KEY WORDS: Stem cell differentiation, Epigenetics, Germ cells, Kit-negative identity, Kit-positive identity

INTRODUCTION
Stem cells in adults are distinct from progenitor cells in that they can self-renew (Brinster, 2002; Spangrude et al., 1988). Shortly after birth, murine male germ cells acquire stem cell identity; and the mitotic cells that maintain homeostasis through self-renewal during spermatogenesis are known as spermatogonia (Phillips et al., 2010). Spermatogonia are classified as either type A, intermediate or type B. Type A spermatogonia can be subclassified as single (A₁), paired (A₁₃), aligned (A₁₄, A₁₅, A₁₆, or A₁₇) or differentiating (A₁, A₂, A₃ or A₄; supplementary material Fig. S1) (Russell et al., 1999). The A₁ theory holds that only A₁ cells retain stem cell capacities (de Rooij and Russell, 2000), whereas A₁₃ and A₁₄ cells (known as proliferating spermatogonia) are thought to be progenitor cells that can no longer self-renew (de Rooij and Russell, 2000; Huckins, 1971; Oakberg, 1971). Nevertheless, the capacity to self-renew has never been tested in A₁₃-A₁₄ spermatogonia because, until now, the definitive assay was thought to require transplantation of purified A₁₃-A₁₄ cells, and no appropriate purification method is known. An alternative spermatogonia classification method relies on the differential expression of the marker gene Kit: only Kit-negative (Kit⁺⁻) spermatogonia show stem cell activity in both neonates and adults (Ohbo et al., 2003; Shinohara et al., 2000). Kit expression commences at the A₁ stage, whereas most A₁₃, A₁₄ and A₁₅-A₁₆ spermatogonia are Kit⁺⁺ (supplementary material Fig. S1) (de Rooij and Russell, 2000; Yoshinaga et al., 1991). In this article, we distinguish between A₁₃, A₁₄ and A₁₅-A₁₆ spermatogonia by Kit expression.

Other markers are expressed in A₁ cells, including GFRα1, Oct4, Plzf, Ngn3 and Nanos2; and their expression profiles assist in understanding stem cell-specific gene networks (supplementary material Fig. S1) (Buageaw et al., 2005; Buas et al., 2004; Costoya et al., 2004; Ohbo et al., 2003; Sada et al., 2009; Yoshida et al., 2004). All of these markers were detected in subpopulations of A₁, A₁₃ and A₁₄ cells, raising questions about the heterogeneity of A₁ spermatogonia. In mice, for example, only a small fraction of A₁ cells co-expresses GFRα1 and EGFP in Ngn3-EGFP, whereas the remainder express only one of these genes (Nakagawa et al., 2010; Suzuki et al., 2009). One marker changes its expression pattern during development. Ngn3 is specifically expressed in Kit-negative fraction of neonate spermatogonia, and its expression broadens in adult, overlapping both Kit-negative and Kit-positive spermatogonia (Yoshida et al., 2004; Yoshida et al., 2006; Suzuki et al., 2009). These variable expression patterns in A₁ cells could reflect oscillatory expression of lineage-associated genes, differences in cell-cycle status or differential responses to micro-environmental signals. This heterogeneous expression pattern was suggested to reflect a metastable state, in which cells maintain a ‘memory of stemness’ until they reach a point of no return, and lose stem cell potential (May and Enver, 2001; Graf and Stadtfeld, 2008).

Nuclear architectures have been reported to fix the gene expression program associated with a particular cell fate (Bártová et
al., 2008; Cremer and Cremer, 2001), so we focused on the chromatin architecture in type A spermatogonia. Undifferentiated spermatogonia typically lack heterochromatin (de Rooij and Russell, 2000), but heterochromatin forms as spermatogonia differentiate (Chiarini-Garcia and Russell, 2002). Heterochromatin formation largely involves accumulation of epigenetic modifications, such as DNA methylation (Grewal and Jia, 2007; Jeltsch, 2006). DNA methylation is mediated primarily by DNA methyltransferase 1 (Dnmt1). In copying the methylation pattern of the original strand in newly replicated DNA, Dnmt1 associates with PCNA and Np95 (Bostick et al., 2007; Sharif et al., 2007). By contrast, if the DNA methylation target is a previously unmethylated site, then de novo 5-methylcytosine (5mC) methylation is carried out by two proteins: Dnmt3a and Dnmt3b. In embryonic male germ cells, Dnmt3a and Dnmt3b target specific sites for methylation, including imprinted genes and retrotransposons (Sasaki and Matsui, 2008). Later, Dnmt3a and Dnmt3b are again highly expressed in type A spermatogonia (La Salle and Trasler, 2006). Notably, in embryonic stem (ES) cells, Dnmt3a and Dnmt3b are known to interact with Np95, suggesting a link between the two machineries, one that maintains methylation patterns and the other that introduces de novo DNA methylation (Mellingler et al., 2009).

Heterochromatin harbors transcriptionally repressive histone tail modifications, such as dimethylated Lys 9 of histone H3 (H3K9me2) (Bårtová et al., 2008; Grewal and Jia, 2007). H3K9me2 is mainly associated with facultative heterochromatin, whereas H3K9me3 is observed at constitutive heterochromatin, particularly at pericentromeric heterochromatin (PH). In meiotic spermatogonia, both H3K9me2 and H3K9me3 reportedly are crucial for pairing of homologous chromosomes (Peters et al., 2001; Takada et al., 2011); and although H3K9, H3K27 and H4K20 modifications during the mitotic phase have been characterized histologically (Payne and Braun, 2006), their biological significance with respect to spermatogonial differentiation remains unclear.

To begin to analyze the epigenetic changes occurring in spermatogonia, we took advantage of the fact that germ cells remain connected after division and that their chain length reflects their cell-division history. We exploited this property, and stained the chains for various marker genes, to characterize the epigenetic modifications of type A spermatogonia. Our aim was to extend a marker-based characterization through epigenetic approaches and determine the molecular ‘point of no return’ for stem cells as they differentiate.

MATERIALS AND METHODS

Mice

The following mice were used: C57BL/6N (Japan SLC), Neurogenin3 (Ngn3)-EGFP (Yoshida et al., 2004), Rosa26m1/mGflox (Rosa26-tdTomato-EGFP) (Muzumdar et al., 2007), Ngn3-Cre (Yoshida et al., 2004), CAG-loxp-DsRed2-loxp-Flag-Dnmt3b-ires-EGFP (CAG-DsRed-Flag-Dnmt3b), Oct4-EGFP (Ohbo et al., 2003), Rosa26R-CreER12-Np95fox/fox (Np95 cKO) and GFRα1-EGFP (Usaka et al., 2007). The animals were housed in a barrier facility at Yokohama City University. Rosa26m1 into mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Genotyping PCs were carried out using tail DNA with the primers listed in supplementary material Table S1.

Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was performed as described previously (Ohmura et al., 2004). Briefly, encapsulated testes were incubated in PBS supplemented with 1 mg/ml collagenase (Sigma-Aldrich) and 100 units of DNase I (Invitrogen) for 15 minutes at 37°C with agitation. For Kit staining, suspended cells were incubated at 4°C with Fc-block for 15 minutes and then with APC-anti-Kit antibody (eBiosciences) for 30 minutes. Propidium iodide (2 μg/ml; Sigma-Aldrich) was added, and cells were sorted and analyzed using MoFlo (Beckman-Coulter).

Antibodies

Primary antibodies used for immunohistochemistry were sheep anti-5mC (Capralogics), mouse anti-DNA (AbD Serotec), rabbit anti-Cyp450 (Chemicon), rabbit anti-DDDDK (Abcam), mouse anti-Dnmt3a (IMGENEX), mouse anti-Dnmt3b (IMGENEX), goat anti-Edcad (R&D systems), goat anti-Gata4 (Santa Cruz Biotechnology), rabbit anti-GFP (Millipore), goat anti-GFRA1 (R&D systems), mouse anti-G9a (Perseus Proteomics), mouse anti-GLP (Perseus Proteomics), mouse anti-H3K9me2 (Abcam), rabbit anti-H3K9me2 (Millipore), rat anti-Ki67 (Dako), goat anti-Kit (R&D Systems), rat APC-anti-Kit (eBioscience), mouse anti-Np95 (Abcam), mouse anti-Pflz (Santa Cruz Biotechnology), rabbit anti-Pflz (Santa Cruz Biotechnology), rabbit anti-Sox9 (a gift from Dr Kanai, The University of Tokyo, Tokyo, Japan) and rat anti-TRα98 (a gift from Dr Nishimune, Osaka University, Osaka, Japan) (Tanaka et al., 1997). Secondary antibodies used were goat anti-mouse conjugated to AlexaFluor 488, goat anti-mouse conjugated to AlexaFluor 546, goat anti-rabbit conjugated to AlexaFluor 488, goat anti-rabbit conjugated to AlexaFluor 546, goat anti-rabbit conjugated to AlexaFluor 647, donkey anti-goat conjugated to AlexaFluor 488 (all from Invitrogen) and donkey anti-goat conjugated to biotin (Jackson ImmunoResearch).

Immunohistochemical analysis

Immunohistochemistry of tissue sections was performed as described previously with minor modifications (Ohmura et al., 2004). After sections were incubated with either PBS supplemented with 1% BSA (PBS/B) or with MOM blocking reagent (Vector Laboratories), the sections were incubated for either 2 hours at room temperature or overnight at 4°C, with an appropriate primary antibody and then a relevant secondary antibody for 1 hour at room temperature. Nuclear staining was conducted with either TOPRO3 (Invitrogen) or DAPI (Sigma-Aldrich). For 5mC staining and signal quantification, we followed previously reported protocols (Hajkova et al., 2010; Loukínov et al., 2002; Okada et al., 2007; Seki et al., 2005). Briefly, after staining with anti-Pflz or anti-Kit antibody, the specimens were re-fixed with 4% PFA for 30 minutes at room temperature, permeabilized, denatured for 30 minutes in 2 N HCl, and incubated with anti-5mC antibody for 2 hours at room temperature. DAPI served as a nuclear stain. Images were analyzed as reported with previously with modifications (Hajkova et al., 2010). The same protocol was applied to the anti-DNA staining. The total intensity of 5mC staining of each nucleus was calculated by NIH Image using the 2-stack images and normalized to the nuclear area after subtracting the average background pixel intensity. Whole-mount immunohistochemistry was performed as described previously (Ohmura et al., 2004). Briefly, seminiferous tubules were incubated with a relevant primary antibody after blocking, and then reacted with an appropriate secondary antibody. Both section and whole-mouse specimens were covered with Vectashield, and observed by confocal laser microscopy (Carl Zeiss LSM510 or Olympus FV-1000). The TSA Biotin System was used to detect Kit, according to the manufacturer’s recommendations (Perkin Elmer).

Bisulfite sequencing

Bisulfite treatment of genomic DNA was performed using EZ DNA Methylation Kit (Zymo Research) as described previously (Hirasawa et al., 2008) with minor modifications. Briefly, DNA extracted from 5 x 10^6 cells was denatured at 0.3 M NaOH for 20 minutes at 37°C and then treated with 9 M sodium bisulfite. Purified DNA was subjected to desulphonation followed by elution. Bisulfite-treated DNA was then used for PCR, and cloning was performed using pGEM-T Easy Vector Systems (Promega) prior to sequencing. The methylation levels and efficiency of bisulfite conversion were calculated using Quantification Tool for Methylation Analysis (Quma) software (http://quma.cdb.riken.jp). Primer sequences used are listed in supplementary material Table S1.

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from mouse testes using RNeasy Micro Kit (Qiagen) and treated with DNase I (Promega) at 37°C for 20 minutes.
strand cDNA was synthesized using Superscript III (Invitrogen). Primers are listed in supplementary material Table S1. The thermal cycling conditions were 94°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds; and 72°C for 7 minutes.

Quantitative real-time RT-PCR (qPCR)

Dnmt3a1, Dnmt3a2 and Hprt cDNAs were amplified using specific primers (supplementary material Table S1) and cloned with pGEM-T Easy Vector Systems (Promega) to use as templates for determining absolute mRNA levels (supplementary material Fig. S2A,B). Total RNA from sorted cells was isolated and DNase treated as described above. First-strand cDNA synthesis was performed using 100 ng of total RNA, random hexamers and SuperScript III reverse transcriptase. Real-time PCR was carried out using SYBR Premix Taq II (Takara) and Dnmt3a1 and Dnmt3a2 copy numbers were determined by absolute quantitation. A standard curve was drawn using 10-fold serial dilutions (10 to 10⁷ copies) of pGEMT-Dnmt3a1 and pGEMT-Dnmt3a2 plasmids. The thermal cycling conditions used were 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. Experiments were conducted on each subpopulation of cells purified from three independent sorting experiments, with PCRs performed in triplicate.

Western blotting

Western blot analysis of Np95 was performed according to a published method (Sharif et al., 2007). Whole-cell extracts from testis were subjected to 10% sodium dodecyl sulfate PAGE (SDS-PAGE) and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with the Np95 antibody overnight at 4°C, followed by incubation with an appropriate horsradish peroxidase (HRP)-conjugated secondary antibody (Invitrogen) for 1 hour at room temperature. Positive signals were detected and visualized with Chemi-Lumi One reagents (Nacalai tesque, Kyoto, Japan).

RESULTS

A₅ to A₅₋₈ spermatogonia are largely Dnmt3a2 negative

To characterize the epigenetic machineries in spermatogonia, Dnmt3a2 protein expression was analyzed by immunohistochemistry; in parallel, we analyzed expression of the two markers Plzf and Kit. Triple staining of adult testes revealed that Plzf-positive (Plzf⁺) but Kit-negative (Kit⁻) spermatogonia were negative for Dnmt3a2 (supplementary material Fig. S2E). Conversely, Plzf-negative (Plzf⁻) but Kit-positive (Kit⁺) spermatogonia were positive for Dnmt3a2 (supplementary material Fig. S2E). We noted that Plzf weak-positive and Kit⁺ spermatogonia also expressed Dnmt3a2, (supplementary material Fig. S2F). Reportedly, differentiating spermatogonia express Kit up to the early spermatocyte stage (Schrans-Stassen et al., 1999; Yoshinaga et al., 1991), so our results indicate that a subpopulation of type A spermatogonia (by definition Kit⁻) do not express the DNA methylation protein Dnmt3a2.

As others have shown semi-purified spermatogonia express predominantly Dnmt3a2 (La Salle and Trasler, 2006), our immunohistochemistry probably detected expression of Dnmt3a2, although Dnmt3a1, a longer isoform, can be recognized by the same antibody. To verify that Dnmt3a2 is the isoform predominantly expressed, highly purified spermatogonia from either postnatal day 7.5 (P7.5) Oct4-EGFP (Ohbo et al., 2003) or adult Ngn3-EGFP mice (Yoshida et al., 2004) (supplementary material Fig. S2A-D) were analyzed by qPCR for Dnmt3a1 and Dnmt3a2 expression. Confirming expectations, significantly more Dnmt3a2 was expressed (supplementary material Fig. S2B,D).

Strong Dnmt3a2 expression has been noted in P0.5 gonocytes (Sakai et al., 2004), so expression was tracked in developing newborn mice. Dnmt3a2 was highly expressed in the gonocytes of P0.5 testes (Fig. 1A) and then was rapidly downregulated by P7.5 in Plzf⁺/Kit⁻ spermatogonia, when it became undetectable (Fig. 1B). We and others have shown that, at stage Dnmt3a2 expression is downregulated in Plzf⁺/Kit⁻ spermatogonia, tissue-specific stem cells emerge in testes (McLean et al., 2003; Ohbo et al., 2003). Conversely, Dnmt3a2 is expressed in Plzf⁻/Kit⁺ spermatogonia that have lost stem cell capacity (Fig. 1B). Ohbo et al., 2003).

To track the cell division history of the Kit⁻/Dnmt3a2⁺ spermatogonia, we examined expression profiles of various
spermatogonial markers. Immunohistochemistry of whole-mount testes tracked co-expression of Dnmt3a2 with three markers expected on A1 spermatogonia, Kit (Fig. 1C, D; reporter driven by the Ngn3 regulatory sequence Ngn3-EGFP), and one marker expected on A1 spermatogonia, Kit (Fig. 1C, D; supplementary material Fig. S2G). Double-staining of adult testes with Plzf and Dnmt3a2 antibodies revealed that not only A1 but also A1-4, A1-4-8 and A1-8 spermatogonia lacked Dnmt3a2 expression (Fig. 1C, Table 1). This finding was confirmed in GFRα1pos spermatogonia, Kit (Fig. 1C, D; supplementary material Fig. S2G). The Dnmt3a2 expression patterns in adult spermatogonia are summarized in Table 2: collectively, spermatogonia from A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). The Dnmt3b expression patterns in adult spermatogonia are summarized in Table 2: collectively, spermatogonia from A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In contrast to Dnmt3a2, Dnmt3b was barely detectable in P0.5 gonocytes (Fig. 2A), but by P7.5, Dnmt3b appeared in Plzfpos/Kitpos spermatogonia but not in Plzfpos/Kitpos spermatogonia (Fig. 2B). Double staining for Plzf and Dnmt3b of adult testes revealed that from stages A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C). Similar findings were observed in GFRα1pos and Ngn3-EGFPpos spermatogonia (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K). The Dnmt3b expression patterns in adult spermatogonia are summarized in Table 2: collectively, spermatogonia from A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D).

### Table 1. Dnmt3a2pos chain distribution in A1-A1-8 spermatogonia

<table>
<thead>
<tr>
<th>A1</th>
<th>A1-4</th>
<th>A1-4-8</th>
<th>A1-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt3a2pos/GFRα1pos</td>
<td>0/267</td>
<td>0/151</td>
<td>0/55</td>
</tr>
<tr>
<td>Dnmt3a2pos/Plzfpos</td>
<td>0/166</td>
<td>0/131</td>
<td>0/78</td>
</tr>
<tr>
<td>Dnmt3a2pos/Ngn3-EGFPpos</td>
<td>0/36</td>
<td>0/31</td>
<td>0/56</td>
</tr>
<tr>
<td>Total</td>
<td>0/469</td>
<td>0/313</td>
<td>0/189</td>
</tr>
</tbody>
</table>

The number of Dnmt3a2pos chains that are GFRα1pos, Plzfpos or Ngn3-EGFPpos among A1, A1-4, A1-4-8 and A1-8 spermatogonia counted in the whole-mount immunohistochemistry images in Fig. 1.

### Table 2. Dnmt3bpos chain distribution in A1-A1-8 spermatogonia

<table>
<thead>
<tr>
<th>A1</th>
<th>A1-4</th>
<th>A1-4-8</th>
<th>A1-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt3bpos/GFRα1pos</td>
<td>0/280</td>
<td>0/133</td>
<td>0/89</td>
</tr>
<tr>
<td>Dnmt3bpos/Plzfpos</td>
<td>0/219</td>
<td>0/165</td>
<td>0/92</td>
</tr>
<tr>
<td>Dnmt3bpos/Ngn3-EGFPpos</td>
<td>0/23</td>
<td>0/46</td>
<td>0/72</td>
</tr>
<tr>
<td>Total</td>
<td>0/522</td>
<td>0/344</td>
<td>0/253</td>
</tr>
</tbody>
</table>

The number of Dnmt3bpos chains that are GFRα1pos, Plzfpos or Ngn3-EGFPpos among A1, A1-4, A1-4-8 and A1-8 spermatogonia counted in the whole-mount immunohistochemistry images in Fig. 2.

**Spermatogonia in neonatal and adult mice from A1 to A1-8 are also Dnmt3b-negative**

In contrast to Dnmt3a2, Dnmt3b was barely detectable in P0.5 gonocytes (Fig. 2A), but by P7.5, Dnmt3b appeared in Plzfpos/Kitpos spermatogonia but not in Plzfpos/Kitpos spermatogonia (Fig. 2B). Double staining for Plzf and Dnmt3b of adult testes revealed that from stages A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C). Similar findings were observed in GFRα1pos and Ngn3-EGFPpos spermatogonia (supplementary material Fig. S2K), with Dnmt3b expression positively correlated with Kit expression (Fig. 2D). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K). The Dnmt3b expression patterns in adult spermatogonia are summarized in Table 2: collectively, spermatogonia from A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K). The Dnmt3b expression patterns in adult spermatogonia are summarized in Table 2: collectively, spermatogonia from A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C). In a similar manner to Dnmt3a2, we noted a small number of chains of four spermatogonia existing at stages VIII-X is Kit positive (A1 spermatogonia), and they expressed Dnmt3b (supplementary material Fig. S2K). The Dnmt3b expression patterns in adult spermatogonia are summarized in Table 2: collectively, spermatogonia from A1 to A1-8, spermatogonia do not express Dnmt3b (Fig. 2C).

### DNA methylation status of Kitneg and Kiptpos spermatogonia

How does the expression of these DNA methylating proteins affect DNA methylation in spermatogonia? To evaluate global methylation of Plzfpos/Kitneg and Plzfpos/Kitpos spermatogonia,
cytosine 5 methylation was tracked with a previously characterized sheep-anti-5mC antibody, in 3D confocal immunohistochemical analysis (Loukinov et al., 2002) (supplementary material Fig. S3A). The results of this assay implied that the DNA was less methylated in Plzfpos/Kitneg spermatogonia (supplementary material Fig. S3A). To quantitate the difference in signal strength, we summed the anti-5mC signal from the z-section images spanning the nucleus (NIH Image). Twelve cells were analyzed from each cell type (i.e. Plzfpos/Kitneg and Plzfneg/Kitpos spermatogonia, and control Sertoli cells). Moreover, to confirm that the antibody indeed had access to the methylated DNA, the chromosomes were also stained with an anti-DNA antibody under denaturing conditions (supplementary material Fig. S3C,D). This careful analysis revealed that although Plzfpos/Kitpos spermatogonia and Sertoli cells stained equivalently, Plzfpos/Kitneg spermatogonia showed comparatively ~2- to 3-fold weaker staining for 5mC (supplementary material Fig. S3B). These data suggest that when spermatogonia undergo the Kitneg to Kitpos transition, the signal strength of 5mC increases with level of Dnmt expression. A future analysis could characterize this genome-wide methylation change at the sequence level.

To examine cytosine methylation of genes known to be crucial for spermatogonial differentiation, FACS-purified GFRα1-EGFPpos/Kitneg and Ngn3-EGFPpos/Kitpos spermatogonia from adults were analyzed by bisulfite sequencing. Methylation was assessed in the promoters and enhancer regions of two spermatogonial marker genes: plzf and kit; these might be methylated distinctly as Plzf, a repressor protein, and Kit have inverse expression patterns (Filipponi et al., 2007). The kit enhancer region includes two putative Plzf-binding sites (Filipponi et al., 2007; Spinello et al., 2009), but these sites were similarly methylated in GFRα1-EGFPpos/Kitneg and Ngn3-EGFPpos/Kitpos spermatogonia (supplementary material Fig. S3E). However, methylation of the plzf regulatory region was higher in Ngn3-EGFPpos/Kitpos spermatogonia than in GFRα1-EGFPpos/Kitneg spermatogonia (91.3% and 61.8%, respectively; supplementary material Fig. S3F), suggesting the plzf promoter is less likely to be permissive for transcription factors to bind in the Kitpos cells. Similarly, methylation of the plzf regulatory region was higher in Ngn3-EGFPpos/Kitpos spermatogonia than in GFRα1-EGFPpos/Kitneg spermatogonia (91.3% and 61.8%, respectively; supplementary material Fig. S3F), suggesting the plzf promoter is less likely to be permissive for transcription factors to bind in the Kitpos cells. Similarly, methylation of the enhancer of oct4 (a marker gene for undifferentiated cells) (Pesce et al., 1998) was higher in Ngn3-EGFPpos/Kitpos spermatogonia than in GFRα1-EGFPpos/Kitneg spermatogonia (52.3% and 16.0%, respectively; supplementary material Fig. S3G). These results suggest that DNA methylation might directly or indirectly regulate some of the genes guiding spermatogonial differentiation. In the future, whole-genome
bisulfite sequencing should be able to clarify the picture of methylation changes in differentiating spermatogonia.

H3K9me2 and H3K9me3 modification patterns are altered differently at the Kitneg to Kitpos transition in spermatogonia

H3K9 modifications are directly linked to large-scale organizations of chromosomal territories and chromocenter formation, which may fix a gene expression program associated with a particular cell fate (Bártová et al., 2008; Cremer and Cremer, 2001). To determine when H3K9me2 modification increases, whole-mount adult testes were probed immunohistochemically with the markers, Plzf, GFRα1, Ngn3-EGFP and Kit (Fig. 3A,B; supplementary material Fig. S4A). This analysis showed that, regardless of the spermatogonial marker used, As, Apr and Aal spermatogonia showed only weak levels of H3K9me2 modification compared with neighboring cells (Fig. 3A; supplementary material Fig. S4A). Double staining with anti-Kit and anti-H3K9me2 antibodies showed that all Kitpos spermatogonia exhibited strong H3K9me2 signals (Fig. 3B).

The H3K9me2 methylation is primarily catalyzed by two enzymes, G9a and GLP. So to confirm the results with the double staining of anti-Kit and anti-H3K9me2 antibodies, G9a and GLP expression was tracked in Kitneg and Kitpos spermatogonia. This experiment revealed that although G9a was ubiquitously expressed (Fig. 3C), GLP protein expression was stronger in Plzfneg/Kitpos spermatogonia compared with Kitneg spermatogonia (Fig. 3D).

Although G9a knockout mice manifest male germ cell abnormalities at the meiotic pachytene stage (Tachibana et al., 2007), our result suggests that H3K9me2 modification is already established in spermatogonia during the mitotic stage, in particular at the Kitneg to Kitpos transition. These data, then, suggest that H3K9me2 methylation rises simultaneously with Dnmt3a2 and Dnmt3b expression, and the enhancement of 5mC signals (supplementary material Fig. S4B).

By contrast, another repressive histone modification, H3K9me3, showed a distinct localization pattern: it is partitioned in a ring-like pattern, without a densely stained DAPI area (consistent with a previous report by Payne and Braun, 2006). This pattern was detected only in Plzfpos/Kitneg spermatogonia (Fig. 3Ea-f). Meanwhile, Plzfpos/Kitpos spermatogonia contained DAPI-dense areas that accumulated H3K9me3, a signal characteristic of PH regions (Fig. 3Eg-k). These data indicate that global levels of H3K9me2 increase...
during the Kitneg to Kitpos transition; and this change could mainly result from the activity of GLP rather than G9a. The appearance of the nuclear DAPI foci associated with changes in H3K9me3 localization suggests that, in interphase nuclei at the Kitneg to Kitpos transition, chromosomal organization might also significantly change.

Ectopic Dnmt3b expression in Kitneg spermatogonia induces Kit expression but no change in H3K9me2 modification

To assess the influence of Dnmt3 expression in vivo, Plzfpos/Kitneg spermatogonia were manipulated to conditionally express a Flag-tagged Dnmt3b. To achieve this, Ngn3-cre:CAG-DsRed2-Flag-Dnmt3b mice were induced to undergo a Cre-mediated recombination in vivo (Fig. 4A). Flag-Dnmt3b expression should be triggered only in cells expressing Ngn3 in neonates, which includes Oct4pos/Kitneg spermatogonia in newborn mouse testes (Yoshida et al., 2004). To confirm Cre-driven recombination, Ngn3-cre mice were crossed with R26R-tdTomato-EGFP mice and analyzed at P6.5 (Fig. 4B); EGFP/Plzf double staining confirmed that EGFPpos spermatogonia expressed Plzf (supplementary material Fig. S5Aa,b), and only a small number of them expressed Kit in P6.5 testes (supplementary material Fig. S5Ac,d), strongly suggesting recombination occurred predominantly in Plzfpos/Kitneg spermatogonia. We also performed cross-sectional analysis of Ngn3-EGFP mouse testis in which the same Ngn3 regulatory sequence drives EGFP. This analysis also revealed that most of the Ngn3-EGFPpos spermatogonia from P6.5 mice were Kitneg (supplementary material Fig. S5Ae,f).

To test whether Dnmt3b expression at P6.5 induced Kit expression, mice overexpressing Flag-tagged Dnmt3b were analyzed by immunohistochemistry, revealing that Flag-positive spermatogonia tended to co-express Kit in P6.5 testes (Fig. 4C). Cre and Flag-Dnmt3b expression was confirmed by RT-PCR (supplementary material Fig. S5B). Moreover, tissue sections showed that almost 50% of Flag-Dnmt3bpos spermatogonia were also Kitpos (Fig. 4C,F). By contrast, in EGFP reporter control mice, ~10% of EGFPpos spermatogonia were Kitpos (Fig. 4D,E). To exclude the possibility that these differences in frequency were due to differences in promoter strength or to distance between loxP sites, we assessed the recombination frequency of both transgenes and found the differences were negligible (see supplementary material Fig. S5C). Interestingly, Flag-Dnmt3bpos spermatogonia showed weak signals of H3K9me2, even in P8.5 testes (Fig. 4F); and subsequent analysis of G9a and GLP expression showed that GLP was not upregulated by the ectopic expression of Dnmt3b (Fig. 4G,H). It is also noteworthy that Flag-Dnmt3b-positive cells do not appear to undergo meiosis (data not shown), possibly owing to a disturbance in the physiological dynamic demethylation and methylation wave that occurs during meiosis (Loukinov et al., 2002).

Interference with Np95-mediated DNA methylation in spermatogonia perturbs differentiation potential

Genomic methylation patterns are maintained in dividing cells, in part, because Np95 at DNA replication forks recruits Dnmt1 (Bostick et al., 2007; Sharif et al., 2007). In addition, Np95 binds H3K9me2/3 and associates with PH regions, thereby linking DNA methylation to heterochromatin formation (Nady et al., 2011; Papait et al., 2007; Papait et al., 2008; Rottach et al., 2010). To assess how DNA methylation-linked heterochromatin formation influences spermatogonial stem cell differentiation, we established a tamoxifen-inducible conditional knockout (cKO) of Np95 (supplementary material Fig. S6A). Tamoxifen-induced knockout of Np95 was confirmed by western blotting, immunohistochemistry and genomic PCR (Fig. 5C,D; supplementary material Fig. S6B).
Our results show that 2 weeks after the first tamoxifen injection, Np95-deficient mice had significantly smaller testes compared with littermate controls (Fig. 5A,B). Although Np95-deficient and littermate control testes contained a similar proportion of Plzfpos spermatogonia per seminiferous tubule, Np95-deficient testes contained far fewer Kitpos spermatogonia, suggesting Np95 is required for either the Kitneg to Kitpos transition or the maintenance of Kitpos spermatogonia (Fig. 6A-C). Additionally, Np95 cKO testes contained residual spermatogonia that showed weak signals of H3K9me2 modification and 5mC (supplementary material Fig. S7A,B). Np95 cKO testes analyzed by whole-mount immunohistochemical analysis revealed that the residual spermatogonia were primarily made up of single cells, paired cells, and chains of four and (less frequently) eight cells (Fig. 6D; supplementary material Fig. S8A,B). In contrast to controls, 16-cell chains were almost absent in Np95 cKO mouse testes (supplementary material Fig. S8B). To assess the cell-cycle status of these residual spermatogonia, they were stained with Ki67, a proliferation marker (Scholzen and Gerdes, 2000). In testes of 4-week-old Np95 cKO mice, 36% of Plzfpos spermatogonia were Ki67pos, which is similar to the proportion seen in control mice, suggesting that these Np95 cKO spermatogonia were proliferating normally (supplementary material Fig. S9). Thus, the residual spermatogonia are not quiescent, given that spermatogonia from As to Aal divide at least two or three times over 8.6 days (Tegelenbosch and de Rooij, 1993). To check the effect of Np95 cKO on testicular somatic cells, we analyzed expression of Gata4 and the cytochrome P450 side chain cleavage enzyme (Cyp450) in Leydig cells; Gata4 in Leydig and Sertoli cells; and Sox9 in Sertoli cells. Expression levels of these were similar in Np95 cKO mice and littermate controls (supplementary material Fig. S10A,B), probably because most somatic cells are post-mitotic (Clermont and Perey, 1957;
Kluin et al., 1984; Levine et al., 2000). Together, these data suggest that the influence of the Np95 cKO is restricted to germ cells in the testis. These results suggest that Np95 is necessary for either Kitneg to Kitpos transition or maintenance of Kitpos spermatogonia.

**DISCUSSION**

This study characterized a previously unidentified epigenetic shift crucial for spermatogonia to transition from Aa (Kitneg) to A1 (Kitpos). Multiple epigenetic changes arise, including an increase in Dnmt3a2/3b protein expression and changes in chromatin modifications (i.e. increase of 5mC signal, accumulation of the H3K9me2 mark and distribution changes of the H3K9me3 associated with DAPI-dense foci; Fig. 7). Our findings suggest significant epigenetic changes occur at the Aa1 to A1 transition, a period when spermatogonial nuclear architecture transforms. As this transition reportedly occurs without cell division (de Rooij, 2001; Russell et al., 1999), the changes in gene expression and resulting switches in cell identity might depend on such epigenetic dynamics.

In spermatogonia, disrupting Np95 probably blocks differentiation towards or after the Kitpos transition. Np95 mainly functions to maintain patterns of global DNA methylation; it does this by binding the histone modification H3K9me2/3, and by recruiting Dnmt1, a maintenance methyltransferase (Liu et al., 2013; this by binding the histone modification H3K9me2/3, and by recruiting Dnmt1, a maintenance methyltransferase (Liu et al., 2013; Nady et al., 2011; Rottach et al., 2010). Therefore, Np95 deficiency might trigger global hypomethylation and loss of association with H3K9me2/3 during the Kitneg to Kitpos transition, and impair spermatogonial differentiation. Nevertheless, Np95 KO testes contain some residual Aa to Aa1 spermatogonia that are Kitneg; these likely contain the stem cell population, and can probably tolerate low levels of cytosine methylation.

Similar effects were reported in pluripotent ES cells. Deficiency in Dnmts and Np95 does not alter the undifferentiated status of these cells, according to the studies using hypomethylated cell lines, namely, double-KO (Dnmt3a−/− and Dnmt3b−/−), triple-KO (Dnmt3a−/−, Dnmt3b−/− and Dnmt1−/−) and Np95−/− ES cells (Jackson et al., 2004; Sharif et al., 2007; Tsumura et al., 2006). Nevertheless, these hypomethylated ES cells cannot differentiate, even if leukemia inhibitory factor (LIF) is withdrawn, suggesting intrinsic cellular mechanisms protected these ES from extrinsic differentiation cues (Jackson et al., 2004). Spermatogonia are constantly moving in seminiferous tubules, in a manner different from other tissue-specific stem cells, which settle in niches (Morrison and Spradling, 2008; Nakagawa et al., 2010; Yoshida et al., 2007). Therefore, despite being constantly exposed to extrinsic signals, maintenance of a unique epigenetic state may prevent spermatogonia from differentiating.

In postnatal male germ cells, expression of Dnmt3 may correlate inversely with the timescale of the stem cell activity level. Although in P0.5 testes, gonocytes express high levels of Dnmt3a2, where stem cell activity is very weak (McLean et al., 2003), Kitneg spermatogonia lose Dnmt3a2 expression by the end of the first postnatal week, strongly correlating with the timing of testicular stem cell emergence (McLean et al., 2003; Ohbo et al., 2003). Then, onset of Dnmt3a2 and also 3b expression in Kitneg spermatogonia correlates with the timing of the loss of stem-cell identity (Ohbo et al., 2003; Shinohara et al., 2000). Our methylation analysis with immunohistochemistry and locus-specific bisulfite sequencing suggests methylation changes are actually accompanied by Dnmt changes. Accordingly, in Aa to Aa1 spermatogonia, Ngn3-driven expression of Flag-Dnmt3b increased Kit expression, possibly via increased methylation at genes including p75 and oct4. In light of the abnormalities of Np95-depleted spermatogonia, these results suggest the DNA methylation machineries (including Np95-mediated molecular events) regulate the Kitneg to Kitpos transition. At this switching point, spermatogonia might lose their stem cell activity and/or the ability to differentiate. Nevertheless, as with a recent methylome analyses, which expanded immunohistochemistry-based views on global methylation patterns in early embryos (Smith et al., 2012; Smallwood et al., 2011), it is important to note that the accurate picture of methylation changes in spermatogonia remain undetermined; future studies should address this point.

H3K9me2 modification has been suggested to reshape the nuclear architecture in a previous study (Wen et al., 2009). In differentiated ES cells, H3K9me2 modifies large genomic regions, forming domains known as large organized chromatin K9-modifications (LOCKs), structures that are associated with nuclear
lamina. Conversely, LOCKs cover little of the genomes of undifferentiated ES cells. This suggests chromosomes assemble nuclear architectures that are differentiation-stage specific (Wen et al., 2009). Thus, as spermatogonia exit from the A1 to A2 stages and pass to the A1 stage, H3K9me2 modifications may reshape chromosome structure.

It is notable that H3K9me2 modification was not elicited by ectopic Dnmt3b expression in testes, although Kit expression was induced. This result suggests the Dnmt3b-mediated DNA methylation pathway does not trigger H3K9me2 accumulation in Kitpos spermatogonia. In addition, knocking out a major mammalian methyltransferase for H3K9me2, G9a, did not affect expression of Dnmt3a2 and Dnmt3b at the Kitneg to Kitpos transition in mouse testis (data not shown); rather, abnormal phenotypes do not arise until the meiotic phase (Tachibana et al., 2007). Nevertheless, as GLP greatly stimulates G9a activity (Tachibana et al., 2005), GLP might trigger genome-wide accumulation of H3K9me2 during Kitpos to Kitneg transition, as GLP becomes upregulated in Kitpos spermatogonia.

How do DNA methylation and histone modifications regulate spermatogonial differentiation? Hypothetically, such epigenetic modifications induce changes in nuclear architecture that globally alter gene expression networks, so particular sets of genes are expected to be targeted by DNA methylation and/or histone modifications. Indeed, we observed methylation changes at the regulatory regions of plzf and oct4, both of which are used as markers for undifferentiated spermatogonia (Pesce et al., 1998; Buaas et al., 2004; Costoya et al., 2004). Further studies might elucidate the mechanistic link between the epigenetic dynamics and differentiation of spermatogonia on a global scale. In particular, it is important to clarify the genomic targets of de novo Dnmts- and H3K9-modified regions, but it will be difficult to carry out these experiments owing to the current technical limitations.

In this study, we observed little epigenetic or phenotypic difference among A1, Aper, and Aal cells, regardless of where they localized on the seminiferous epithelium or the types of markers they expressed. This is in contrast to the dramatic differences seen at the Aal to A1 transition. Our finding of the uniform epigenetic similarity might indicate that A1 to A2 spermatogonia form a stem cell pool (Fig. 7). The hypothesis of a stem cell pool is supported by a differentiating from A1 cells undergoing synchronous divisions (data not shown); rather, abnormal phenotypes do not arise until the meiotic phase (Tachibana et al., 2007). Nevertheless, as GLP greatly stimulates G9a activity (Tachibana et al., 2005), GLP might trigger genome-wide accumulation of H3K9me2 during Kitpos to Kitneg transition, as GLP becomes upregulated in Kitpos spermatogonia.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
T.S. designed, performed and analyzed most experiments and wrote the manuscript. R.Y.-D., A.Y., Y.T.-H., K.U. and M.M. prepared and provided materials and performed experiments and wrote the manuscript. Y.K., K.N., H.S. and Y.S. performed immunohistochemical analysis. J.S. contributed to methylation analysis. Y.S. and H.K. provided materials and participated in discussion. T.S. supervised the study and participated in discussion. K.O. designed and supervised the study and wrote the manuscript. All authors were involved in the final stages of writing the article.

Supplementary material
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