The Histone Demethylase FBXL10 Regulates the Proliferation of Spermatogonia and Ensures Long-Term Sustainable Spermatogenesis in Mice

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ABSTRACT

The F-box and leucine-rich repeat protein 10 (Fbxl10) gene encodes a protein that catalyzes demethylation of H3K4 and H3K36. In this study, we show the important roles of FBXL10 as a histone demethylase in sustainable sperm production using mice in which the JmjC domain of Fbxl10 was deleted (Fbxl10ΔJmjC/ΔJmjC). In histological analysis, testis sections from 10-wk-old Fbxl10ΔJmjC/ΔJmjC mice appeared normal. On the other hand, testes from 7-mo-old Fbxl10ΔJmjC/ΔJmjC mice contained a greater ratio of seminiferous tubules exhibiting degeneration of spermatogenesis. Further analysis using an in vitro spermatogonia culture system, that is, germ line stem cells (GSCs), revealed that Fbxl10ΔJmjC/ΔJmjC GSCs expressed a significantly higher level of P21 and P19 mRNAs, cyclin-dependent kinase inhibitors and also known as cellular senescence markers, than wild-type (WT) GSCs. Furthermore, the ratio of Fbxl10ΔJmjC/ΔJmjC GSCs in G0/G1 phase was higher and the ratios in S and G2/M phases were lower than the corresponding ratios of WT GSCs, and the doubling speed of Fbxl10ΔJmjC/ΔJmjC GSCs was significantly lower than that of WT GSCs. In addition to these in vitro results, an in vivo study indicated that recovery of spermatogenesis after a transient reduction in the number of testicular germ cells by busulfan treatment was significantly slower in Fbxl10ΔJmjC/ΔJmjC mice than in WT mice. These data suggest that Fbxl10 plays important roles in long-term sustainable spermatogenesis via regulating cell cycle.

cell cycle, epigenetics, spermatogenesis, spermatogonia, spermatogonial stem cells

INTRODUCTION

In male mice, spermatogenesis starts as early as Postnatal Day 3 (PD3). After several rounds of mitotic cell proliferation, spermatogonia enter meiosis. Meiotic cells termed spermatocytes undergo two successive rounds of cell division and become haploid spermatids. After an additional maturation period termed spermiogenesis, mobile and fertile spermatozoa first appear at around PD35. Spermatogenesis is then sustained throughout life under the control of molecular mechanisms, including epigenetics, which orchestrate proper gene expression [1–3].

Epigenetic modifications such as DNA methylation and histone modifications control gene expression either positively or negatively, play essential roles in the proper development of specific tissues, and confer cellular identity. The methylation status of histone lysine residues, which is spatiotemporally balanced by the activities of histone methyltransferases and histone demethylases, is an important epigenetic modification. A number of studies using gene knockout mouse models revealed that proper histone modifications are crucial for germ cell development and sustainable spermatogenesis [1, 2, 4]. For example, a lack of Mjmlc, which encodes a H3K9 demethylase, causes age-dependent infertility accompanied by a progressive reduction in the number of germ cells [5]. Proper maintenance of H3K4 methylation is crucial for fertility because knockout of the H3K4 methyltransferase-encoding gene Kmt2b/Mll2 or Prdm9/Meisetz results in an infertile phenotype in both males and females [6, 7]. Similarly, a lack of Kdm1b/Lsd2, which encodes a histone lysine demethylase that catalyzes demethylation of H3K4, causes defects in proper genomic imprinting, resulting in infertility in female mice, whereas male gene knockout mice show no distinct abnormal phenotype and their reproductive performance is unaffected [8].

F-box and leucine-rich repeat protein 10 (FBXL10, also called KDM2B and JHDM1B) is a histone lysine demethylase possessing the histone lysine demethylase catalytic domain JmjC and catalyzes demethylation of H3K4 [9, 10] and/or H3K36 [11, 12]. In mice, Fbxl10 is expressed in many tissues such as the thymus, brain, heart, and lung, but its expression intensity is more pronounced in the testis [13]. Fbxl10 has two different isoforms, a longer full-length (FL) isoform contains the catalytic domain for histone demethylation JmjC and a short-form (SF) isoform that lacks the JmjC domain although other catalytic domains such as the F-box domain (protein-binding), CXXC-zinc finger domain (DNA-binding), PHD domain, and leucine-rich repeats are the same as in the FL isoform. Recently, we established a gene knockout mouse model in which JmjC domain-containing Fbxl10-FL was deleted (Fbxl10 ΔJmjC/ΔJmjC, Fbxl10ΔΔ) and demonstrated that a lack of Fbxl10-FL causes a significant increase in the occurrence of a neural tube defect during prenatal development resulting in prenatal death, although some Fbxl10ΔΔ adult mice do not exhibit this lethal phenotype and reach adulthood [13]. Interestingly, Fbxl10ΔΔ adult male mice have significantly fewer sperm in the epididymis [13]. Our results, together with those of reports showing the importance of proper histone
modifications for germ cell development and/or spermatogenesis, led us to hypothesize that Fbxl10-FL might be important for sustainable spermatogenesis. Therefore, in the present study, we aimed to investigate the roles of Fbxl10-FL in spermatogenesis. A gene knockout mouse model that we previously established revealed that although Fbxl100/−/− males were fertile at least when they were 1 yr old, a lack of Fbxl10 caused a progressive increase in the number of seminiferous tubules showing degeneration of spermatogenesis, accompanied by a drastic alteration in the distribution of H3K4me3 in testicular germ cells. Furthermore, cultured spermatogonia, that is, germine stem cells (GSCs), lacking Fbxl10-FL expressed a significantly higher level of the cellular senescence markers P21 and P19 and proliferated significantly slower than wild-type (WT) cells. Our present data indicate that FBXL10 ensures long-term sustainable spermatogenesis via regulating the cell cycle.

MATERIALS AND METHODS

Animals and Ethics Statement

Mice were housed under pathogen-free conditions in the experimental animal facility at the University of Tokyo. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Tokyo (approval number PA10-59) and performed in compliance with their guidelines. DBA/Jcl mice were purchased from Clea-Japan, and C57Bl6J mice were purchased from Japan SLIC. Fbxl100/−/− mice used in this study were produced in our laboratory as previously reported [13] and have a mixed genetic background (129, C57BL/6j, and DBA/Jcl).

Isolation of Testicular Germ Cells for Development of GSCs

For development of GSCs, testes from mice at Postpartum Day 5–7 were harvested and digested into single cells as reported previously [14–16]. Single cells were washed twice with PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) bovine serum albumin (BSA), and THY1.2+ cells, the fraction in which spermatogenial stem cells are enriched [14, 15, 17], were sorted by magnetic-activated cell sorting. Collected cells were cultured to develop into stably self-renewing GSCs (Fig. 3A) on mitotically inactivated feeder cells, that is, x-ray-irradiated mouse embryonic fibroblasts (MEFs), in medium as previously reported [18] with slight modifications. Specifically, 2 mM GlutaMax or 5 mg/ml AlbuMax II (both from Life Technologies) were included in the RNA extraction procedure in this kit. Total RNA was extracted from testes using Sepasol-RNA I super G (Nacalai Tesque) according to the manufacturer’s instructions and treated with DNasel (Takara) to digest potentially contaminating genomic DNA. Total RNA was extracted from cultured GSCs using Nucleospin RNA II (Macherey-Nagel) according to the manufacturer’s instructions. DNase treatment was included in the RNA extraction procedure in this kit. Total RNA (500–800 ng) was used to synthesize cDNA using SuperScript VILO (Life Technologies) according to the manufacturer’s instructions in a reaction volume of 10 μl, which was diluted 2-fold with water after the reverse transcription reaction. The synthesized cDNA was used for quantitative real-time PCR analysis (Applied Biosystems StepOne; Life Technologies) in a PCR reaction mixture of 10 μl containing 1X TaqSybr Green mix (Life Technologies) and 0.3 μM each of the forward and reverse primers. The fold difference was calculated using the ΔΔCt method [19] with Gapdh as the reference. The primer sequences used in this study are shown in Table 1.

Histological Analysis

Tests were fixed in PBS containing 4% (w/v) paraformaldehyde overnight at 4°C and then dehydrated by serial treatment with gradient ethanol solutions (from 20% to 100% [v/v] ethanol). Thereafter, dehydrated testes were embedded in paraffin for sectioning. Sections were cut at a thickness of 5 μm. For histological staining, sections were deparaffinized in Lemosol A (Wako), rehydrated by serial treatment with gradient ethanol solutions (from 100% to 70% [v/v] ethanol), and stained with hematoxylin-eosin (HE). Paraffin-embedded sections were also used for immunohistochemistry as described below.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and rehydrated as described above. Rehydrated sections were boiled in sodium citrate buffer (10 mM sodium citrate and 0.05% [v/v] Tween-20 prepared in water, pH 6.0) or Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, and 0.05% [v/v] Tween-20 prepared in water, pH 9.0) using autocloaking (105°C, 15 min) to reactivate antigens, blocked in PBS containing 0.1% (v/v) Triton-X and 5% (w/v) BSA for 1 h at room temperature, and exposed to primary antibodies overnight at 4°C. Immunoreactivity was visualized using Alexa Fluor 488-, 555-, or 647-conjugated host animal-specific secondary antibodies (Life Technologies) and observed using a microscope capable of detecting fluorescein (BZ-9000; Keyence). The primary antibodies used were as follows: rabbit anti-FBX110 (1:200; 09-864; Merck Millipore), rabbit anti-H3K36me1 (1:100; ab9048; Abcam), rabbit anti-H3K36me2 (1:25; 2901; Cell Signaling Technology), rabbit anti-H3K36me3 (1:100; 4909; Cell Signaling Technology), rabbit anti-Fbxl10 (1:200; 9727; Cell Signaling Technology), rabbit anti-PLZF (1:50; sc-22839; Santa Cruz), goat anti-GATA4 (1:400; sc-1237; Santa Cruz), rabbit anti-WT1 (1:50; sc-192; Santa Cruz), rat anti-germ-cell-specific nuclear antigen (GENA) (1:2000; clone TRA98; BioAacademia), goat anti-SCP3 (1:100; sc-20845; Santa Cruz), and rat anti-Ki67 (1:100; clone 16A8; BioLegend).

Western Blot Analysis

Protein was extracted from GSCs by sonication in RIPA buffer and analyzed by standard Western blot analysis protocols with the same set of methylation-specific anti-H3 primary antibodies as used in the immunohistochemistry. Rabbit anti-H3 antibody (1:5000; ab1791; Abcam) was also used to detect total H3. Secondary horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (GE healthcare Japan) was used to visualize each protein with Luminata Forte (Merck Millipore).

Flow Cytometry

For cytometric analysis to detect cell surface proteins, cells were incubated with PerCP/Cy5.5-conjugated anti-EpCAM (1:2500; clone G8.8; BioLegend), APC-conjugated anti-c-KIT (1:25000; clone 2B8; BioLegend), and Alexa Fluor 488-conjugated anti-ITGA6 (1:500; clone GoH3; BioLegend) antibodies at 4°C for 45 min in PBS containing 2 mM EDTA and 1% (w/v) BSA. To detect intracellular cleaved CASPASE-3, cells were fixed with 1% (v/v) paraformaldehyde-dehyde prepared in PBS for 10 min at room temperature and then permeabilized using 90% (v/v) ice-cold methanol for 30 min. Permeabilized cells were incubated with a rabbit anti-cleaved CASPASE-3 antibody (1:1000; 6664; Cell Signaling Technology) at 4°C for 45 min and then exposed to Alexa Fluor 647-conjugated anti-rabbit immunoglobulin G (Life Technologies) at 4°C for 45 min. For cell cycle analysis, cells were fixed and permeabilized in 90% (v/v) ethanol prepared in PBS overnight at −20°C. Thereafter, DNA was stained with 200 μg/ml propidium iodide (Sigma-Aldrich) and endogenous RNA was simultaneously digested with 50 μg/ml RNase A (Merck Millipore) for 20 min at 37°C. Stained cells were analyzed using the FACS Calibur system (BD Biosciences).

Busulfan Administration to Adult Male Mice

Male mice aged 8 wk old were intraperitoneally administered busulfan (B2635; Sigma-Aldrich) at a dose of 30 μg/g body weight. Testes were collected 2, 4, or 8 wk after administration and subjected to histological or flow cytometric analysis.

Statistical Analysis

All numerical data are shown as the mean ± SEM of three independent replications. Differences between genotypes were tested using the Student t-test. P values less than 0.05 were considered significant.

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Fbxl10 Expression in Testes During Postnatal Development

To investigate the fluctuation in Fbxl10 expression in testes during postnatal development, both isoforms of Fbxl10 mRNA (i.e., Fbxl10-FL and Fbxl10-SF) extracted from WT C57B6/J strain were measured using quantitative PCR. In addition, mRNA expression of a homologous gene of Fbxl10, termed Fbxl11, which shares the same set of functional domains including the histone demethylase JmJc domain [20], was also measured. Although Fbxl10-FL expression did not change much between PD6 and PD10, it was 9.07 ± 0.73 fold higher at PD21 than at PD6 and was further increased at 6 wk of age (23.17 ± 2.25 fold higher than at PD6) (Fig. 1A). On the other hand, although expression of Fbxl10-SF was also higher at PD30 than at PD6, this increase was less evident than that in Fbxl10-FL. The fluctuations in Fbxl11 and Fbxl10-SF exhibited a similar pattern, that is, expression was slightly increased until PD21–PD30 and remained roughly constant thereafter until 6 wk of age (Fig. 1A). These different expression patterns suggest that Fbxl10-FL and Fbxl10-SF are differentially regulated in different types of testicular cells. Next, to determine the expression of FBXL10 protein in testes, immunohistochemistry was performed using testes from 9-wk-old WT male. FBXL10 labeling was detected in spermatogonia and spermatocytes as well as GATA4+ somatic Sertoli cells, whereas the signal was low or absent in haploid spermatids (Fig. 1B and Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org).

HISTONE DEMETHYLASE FBXL10 AND SPERMATOGENESIS

Several studies reported that FBXL10 specifically catalyzes demethylation of H3K36me1/me2/me3 or H3K4me3 [9, 11, 21]. Thus, to determine the global methylation patterns of H3K36 and H3K4 in Fbxl10-FL/SF testes, immunohistochemistry using specific antibodies was performed. No significant differences in the distribution of H3K36me1/me2 or me3 between WT and Fbxl10-FL/SF testes was detected (Fig. 2A). On the other hand, the distribution pattern of H3K4me3 differed between Fbxl10-FL/SF testes and age-matched WT testes. In WT testes, the H3K4me3 signal was most evident in SCP3+ primary spermatocyte (semiferous stage I–III) or spermatogonia (semiferous stage IX–XI), and the signal was weaker in cells that went beyond the first meiosis. By contrast, although the H3K4me3 signal was detected in SCP3+ primary spermatocyte or spermatogonia in Fbxl10-FL/SF testes, it was stronger in postmeiotic spermatids (Fig. 2B). We previously reported that the number of sperm is significantly lower (~2-fold) in Fbxl10-FL/SF mice than in age-matched controls [13]. Thus, in the present study, we determined the importance of Fbxl10-FL in spermatogenesis. Testes from Fbxl10-FL/SF mice were histologically analyzed. No significant difference in HE-stained testis sections was observed between 10-wk-old WT and Fbxl10-FL/SF mice, whereas the number of seminiferous tubules exhibiting degeneration of spermatogenesis was significantly higher in testes of 7-mo-old Fbxl10-FL/SF mice than in those of age-matched WT mice (n = 3 different mice for each age and genotype, and 108–247 tubules were counted; Fig. 2, C and D). We also stained for the Sertoli cell marker WT1. WT1+Sertoli cells were present even in abnormal seminiferous tubules that lacked germ cells (Supplemental Fig. S2). These results suggest that FBXL10 is not essential for spermatogenesis, at least during younger age, but regulates the H3K4me3 status in testicular germ cells and plays a role(s) in sustaining spermatogenesis for a long period.

Fbxl10-FL/SF GSCs Exhibit Slower Growth In Vitro and Increased Expression of the Cyclin-Dependent Kinase Inhibitor (CDKI) P21 and P19

FBXL10 reportedly regulates cell proliferation through CDKIs. Enhanced expression of Fbxl10 activates the proliferation of human cancer cells [22, 23], whereas downregulation of Fbxl10 induces an increase of P16 expression in embryonic fibroblasts of mice [24]. These studies and our present

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**FIG. 1.** Spatiotemporal expression of Fbxl10 in murine testes. A) Quantitative real-time PCR analysis of Fbxl10-FL, Fbxl10-SF, and Fbxl11 in testes during postnatal development. Gene expression was standardized using Gapdh, and the expression level of each gene at PD6 was designated as 1-fold (n = 3 per point). B) Immunohistochemical analysis of FBXL10 and GATA4 (Sertoli cells) in 9-wk-old testes. Nuclei were stained with 4′,6-diamidino-2-phenylindole. SC, Sertoli cell; Spg, spermatogonia; and PS, pachytene spermatocyte (bar = 50 μm).
FIG. 2. Histological analysis of testes from Fbxl10<sup>−/−</sup> mice. A and B: Distribution of H3K36 and H3K4 methylation patterns in testes. Testes from Fbxl10<sup>−/−</sup> mice and age-matched WT mice were stained with an anti-H3K36me1/me2/me3 antibody (A) or an anti-H3K4me3 antibody (B) as well as for a first meiosis marker (SCP3) or a germ cell marker (GENA). 4',6-Diamidino-2-phenylindole staining for nuclei is shown in the right panel (bar = 50 μm). C) HE staining of testes from 10-wk-old and 7-mo-old Fbxl10<sup>−/−</sup> mice and age-matched WT mice. Asterisks indicate seminiferous tubules exhibiting degeneration of spermatogenesis (bar = 100 μm). D) The graph shows the ratios of degenerating seminiferous tubules in Fbxl10<sup>−/−</sup> and WT mice. The asterisk depicts a significant difference (n = 3, P < 0.05).
histological analysis that showed a progressive increase in the degeneration of spermatogenesis led us to hypothesize that cell cycle and/or proliferation of spermatogonia of Fbxl10<sup>DJ</sup>/DJ mice might be compromised. Undifferentiated spermatogonia can self-renew in vitro under stimulation with FGF2 and GDNF on feeder MEFs and possess stem/progenitor characteristics (GSCs) [14, 15, 25]. We took advantage of this culture system to evaluate the role of Fbxl10-FL in spermatogonia in vitro. GSCs have morphological features that are distinct from those of well-known pluripotent stem cells, such as embryonic stem cells (tightly aggregated and three-dimensional) and epiblast stem cells (flat and two-dimensional), and have a grapelike morphology [25]. GSCs lacking Fbxl10-FL also formed typical grapelike colonies, their shape was indistinguishable from that of WT GSCs (Fig. 3A), and they could proliferate for more than 25 passages. Using this GSC, we analyzed intensities of H3K4me3 as well as H3K36me1, 2, and 3 by Western blot analysis. There were no distinct differences in H3 methylations between Fbxl10<sup>DJ</sup>/DJ GSCs and WT GSCs (Fig. 3B). Next, we investigated the mRNA expression of six known spermatogonial marker genes by quantitative PCR. Fbxl10<sup>−/−</sup> mRNA was also measured to confirm knockout of Fbxl10-FL in our model. Little amplification of this gene was observed in Fbxl10<sup>DJ</sup>/DJ GSCs, confirming that these cells did not express Fbxl10-FL (Fig. 3C). Expression of c-Ret, a marker of undifferentiated spermatogonia, tended to be lower (P =
The average number of PLZF marker, and Ki67, a mitotically active cell marker (Fig. 5A). Therefore, we next determined mitotic activities of undifferentiated spermatogonia in vivo by observing cellular recovery. The results from the present in vitro study using a gene knockout mouse model. Our results demonstrated that a lack of Fbxl10-FL drastically altered the H3K4me3 distribution in testicular germ cells and there was an age-dependent increase in the number of seminiferous tubules exhibiting degeneration of spermatogenesis. An in vitro GSC culture model showed that Fbxl10-FL/WT GSCs expressed a significantly higher level of P21, P19, and P16, respectively; Fig. 4B). The average expression levels of Fbxl10-FL were higher in Fbxl10-FL and WT GSCs, respectively; Fig. 4A). CDKIs negatively regulate cell proliferation, and their enhanced expression causes the slower growth of many cell types [28]. Thus, we hypothesized that GSCs lacking Fbxl10-FL might exhibit altered proliferation. As anticipated, the doubling speed of Fbxl10-FL GSCs was significantly slower than that of WT GSCs (Fig. 4B). We also checked the frequency of apoptotic cells by immunostaining with an antibody against cleaved CASPASE-3, an indicator of apoptosis, and then performing flow cytometry. The frequency of apoptotic cells did not significantly differ between Fbxl10-FL and WT GSCs (Fig. 4C). Furthermore, cell cycle analysis using flow cytometry showed that the ratios of cells in G1/G0 phase and G2/M phase were higher and lower, respectively, among Fbxl10-FL GSCs than among WT GSCs, and these differences were significant (Fig. 4D). These results suggest that Fbxl10-FL regulates GSC proliferation by controlling progression of the cell cycle through CDKIs.

Loss of Fbxl10-FL Reduces the Proliferation of Undifferentiated Spermatogonia In Vivo after Busulfan Treatment

The results from the present in vitro study using Fbxl10-FL/WT GSCs led us to hypothesize that the proliferative activity of spermatogonia might be suppressed in Fbxl10-FL/WT mice. Therefore, we next determined mitotic activities of undifferentiated spermatogonia by immunohistochemistry with antibodies against PLZF, an undifferentiated spermatogonia marker, and Ki67, a mitotically active cell marker (Fig. 5A). The average number of PLZF+ undifferentiated spermatogonia per tubule did not differ between Fbxl10-FL/WT and age-matched WT (n = 3 different mice for each genotype, and 29–70 tubules were counted, Fig. 5B). In contrast, almost a half of PLZF+ undifferentiated spermatogonia in WT testes were also Ki67+, whereas mitotically inactive spermatogonia (PLZF+/Ki67−) were more evident in Fbxl10-FL/WT testes (Fig. 5A, arrows), and the ratio was significantly higher in Fbxl10-FL/WT than WT (n = 3 different mice for each genotype, and 303–685 PLZF-positive spermatogonia were counted; Fig. 5C). A transient reduction in the number of undifferentiated spermatogonia by administration of the cytotoxic alkylating agent busulfan reportedly causes an increase in mitotic division of undifferentiated spermatogonia [29, 30]. We also used this experimental model to determine the proliferation speed of undifferentiated spermatogonia in vivo by observing cellular recovery. The number of undifferentiated spermatogonia (EpCAM+/ITGA6+/c-KIT−) [29, 31] at 2 wk after busulfan treatment did not differ between Fbxl10-FL/WT and WT mice, whereas the number of these cells at 4 wk after administration was lower (P = 0.08) in Fbxl10-FL/WT mice than in WT mice (Fig. 6A). Consistently, histological analysis showed that at 8 wk after injection, significantly fewer seminiferous tubules showed reconstitution of spermatogenesis in Fbxl10-FL/WT mice than in WT mice (Fig. 6B). To assess the mitotic activity of undifferentiated spermatogonia, we stained busulfan-treated testes collected at 4 wk after injection with antibodies against PLZF and Ki67. The majority of PLZF-undifferentiated spermatogonia in WT testes were also Ki67+. By contrast, spermatogonia that were PLZF+ but Ki67− were more evident in Fbxl10-FL/WT testes (Fig. 6C), and there were significantly more of these cells in Fbxl10-FL/WT than in WT testes (n = 3 different mice for each genotype, and 193–474 PLZF-positive spermatogonia were counted, Fig. 6D). Together, the results from our Fbxl10-FL/WT mouse model indicate that FBLX10 ensures long-term sustainable spermatogenesis by regulating cell proliferation.

### DISCUSSION

In this study, we investigated the roles of Fbxl10-FL in spermatogenesis using a gene knockout mouse model. Our results demonstrated that a lack of Fbxl10-FL drastically altered the H3K4me3 distribution in testicular germ cells and there was an age-dependent increase in the number of seminiferous tubules exhibiting degeneration of spermatogenesis. An in vitro GSC culture model showed that Fbxl10-FL/WT GSCs expressed a significantly higher level of P21 and P19, cell cycle inhibitors and known as cellular senescence markers, and proliferated slower than WT GSCs. Furthermore, mitotically inactive undifferentiated spermatogonia was more evident in Fbxl10-FL/WT mice than in WT mice and recovery of spermatogenesis in vivo after busulfan treatment was also significantly slower in Fbxl10-FL/WT mice than in WT mice.

### Table 1. Primer sequences used for quantitative real-time PCR.

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<th>Gene</th>
<th>Forward</th>
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* FL, full length; SF, short form.

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0.067), whereas expression of c-Kit, a differentiated spermatogonia marker, was significantly higher in Fbxl10-FL/WT GSCs than in WT GSCs, suggesting that Fbxl10-FL/WT GSCs are predisposed to undergo differentiation to some extent (Fig. 3C).

Previous studies indicated that FBXL10 negatively regulates cell proliferation of undifferentiated undifferentiated spermatogonia in vivo by observing cellular recovery. The number of undifferentiated spermatogonia (EpCAM+/ITGA6+/c-KIT−) [29, 31] at 2 wk after busulfan treatment did not differ between Fbxl10-FL/WT and WT mice, whereas the number of these cells at 4 wk after administration was lower (P = 0.08) in Fbxl10-FL/WT than in WT mice. Consistently, histological analysis showed that at 8 wk after injection, significantly fewer seminiferous tubules showed reconstitution of spermatogenesis in Fbxl10-FL/WT mice than in WT mice. To assess the mitotic activity of undifferentiated spermatogonia, we stained busulfan-treated testes collected at 4 wk after injection with antibodies against PLZF and Ki67. The majority of PLZF-undifferentiated spermatogonia in WT testes were also Ki67+. By contrast, spermatogonia that were PLZF+ but Ki67− were more evident in Fbxl10-FL/WT testes (Fig. 6C), and there were significantly more of these cells in Fbxl10-FL/WT than in WT testes (n = 3 different mice for each genotype, and 193–474 PLZF-positive spermatogonia were counted, Fig. 6D). Together, the results from our Fbxl10-FL/WT mouse model indicate that FBLX10 ensures long-term sustainable spermatogenesis by regulating cell proliferation.

### DISCUSSION

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Taken together, our present data suggest that FBXL10 in spermatogonia plays important roles in long-term sustainable spermatogenesis.

Some recent papers reported that FBXL10 loss-of-function causes much severe phenotype than our Fbxl10-DJ/DJ, for example, Fbxl10 mutant mice [32, 33] are totally embryonic lethal before 9–10 day postcoitum. In either previous case, loss of CxxC domain, through which FBXL10 binds to specific loci of the genome and regulates epigenetics of either DNA or histone as a component of Polycomb repressive complexes, is thought to be responsible for the lethality. In our mutant mouse, however, only the histone demethylase JmjC domain was deleted, and another Fbxl10 isoform, that is, b Fbxl10-SF, which harbors CxxC, still remains functional. This may be a reason for explaining the difference of phenotypic intensity between our present results and the previous studies.

The proper regulation of H3K4 is important for fertility. For example, gene knockout of the H3K4 methyltransferase
Meisetz, which binds DNA at recombination hotspots and directs recombination during meiosis [34, 35], causes male infertility in mice, accompanied by a reduction in the H3K4me3 mark in testicular germ cells [6]. Our findings support the contribution of FBXL10-FL to the global H3K4me3 mark distribution in the testis (Fig. 2B). On the
other hand, spermatogenesis, at least at a young age, was not compromised by a lack of Fbxl10-FL. Therefore, Fbxl10-FL might be dispensable for spermatogenesis at this stage, despite the alteration in H3K4me3. Although FBXL10-FL and Meisetz share the same target histone residue, FBXL10-FL has demethylase activity, which is the opposite of Meisetz. Given that methylation of H3K4 is closely associated with transcriptional activation [36, 37], Meisetz is thought to function as a transcriptional activator whereas FBXL10 is thought to act as a transcriptional inhibitor. These differing roles might explain why our Fbxl10<sup>Δ/Δ</sup> male mice showed a different spermatogenesis phenotype than the previous generated knockout model. It is also possible that region-specific histone modifications of the genome are important for spermatogenesis. A mouse model lacking the histone H3K9-specific JmjC domain-containing protein JMJD1C exhibits an age-dependent abnormality in spermatogenesis, as in the present study, whereas no significant alteration in global histone modifications was detected by immunohistochemistry [5]. By contrast, Iwamori et al. [38] reported that knockout of a gene encoding another H3K9 demethylase, Kdm4a, clearly enhances the H3K9me3 mark in testicular germ cells, whereas no distinct

FIG. 6. Spermatogonial cell recovery after busulfan treatment is retarded in Fbxl10<sup>Δ/Δ</sup> mice. A: The numbers of undifferentiated spermatogonia (EpCAM<sup>+</sup>/ITGA6<sup>+</sup>/c-KIT<sup>−</sup>) in Fbxl10<sup>Δ/Δ</sup> and WT tests after busulfan treatment as determined by flow cytometry (n = 3). B: Histological sections of WT (top) and Fbxl10<sup>Δ/Δ</sup> (bottom) tests at 8 wk after busulfan treatment (bar = 100 μm). C: Immunohistochemistry of WT (top) and Fbxl10<sup>Δ/Δ</sup> (bottom) tests at 4 wk after busulfan treatment. Sections were stained with anti-PLZF (marker of undifferentiated spermatogonia) and anti-Ki67 (marker of mitosis) antibodies. Arrows indicate mitotically inactive undifferentiated spermatogonia (PLZF<sup>+</sup> and Ki67<sup>−</sup>) (bar = 50 μm). D: The graph shows the ratio of mitotically inactive undifferentiated spermatogonia. The asterisk depicts a significant difference (n = 3, P < 0.05).
abnormal phenotype was observed in spermatogenesis. A further study is required to investigate the loci-specific histone methylation status in $Fbxl10^{\Delta\Delta}$ testicular germ cells.

GSCs can self-renew in vitro and possess similar characteristics to in vivo undifferentiated spermatogonia, including stemness, under stimulation with GDNF and FGF2 [15, 25]. Interestingly, although $Fbxl10^{\Delta\Delta}$ GSCs grew slower than WT GSCs, they could proliferate for at least 25 passages, equivalent to about 6–8 mo in spite of their age-dependent spermatogenesis degeneration phenotype in vivo. Similar results reported that a lack of $Plzf$ increases the age-dependent degeneration of spermatogenesis, similar to the observation in our $Fbxl10^{\Delta\Delta}$ mice [39, 40], whereas $Plzf$-null GSCs can be developed and maintained for a long time, although they grow slower than WT GSCs [41].

Using this in vitro model, we revealed that expression of c-Ret tended to be weaker ($P = 0.067$) and expression of c-Kit was significantly stronger in $Fbxl10^{\Delta\Delta}$ GSCs than in WT GSCs (Fig. 3B). In testicular germ cells, c-Ret expression is restricted to early undifferentiated spermatogonia, whereas c-Ret dimerizes with GFRα1 and functions as a receptor for GDNF, an essential factor for spermatogonial stem cell self-renewal [42, 43]. By contrast, c-Kit is not expressed in undifferentiated spermatogonia, and its expression becomes evident in differentiating spermatogonia under stimulation with retinoic acid, a differentiation-initiating signal [44–46]. Therefore, $Fbxl10^{\Delta\Delta}$ GSCs might tend to undergo differentiation to some extent. Interestingly, artificially aged GSCs, in which aging is induced by serial transplantation into testes followed by magnetic-activated cell sorting isolation using an anti-THY1.2 antibody also express a higher level of c-Kit [47], similar to $Fbxl10^{\Delta\Delta}$ GSCs.

Aging or cellular senescence is a stress response that accompanies stable exit from the cell cycle [28]. A number of studies showed that $Fbxl10$ has anitvesenesce roles in many types of cells through suppression of CDKI expression. For example, overexpression of $Fbxl10$ can immortalize MEFs, and this effect is associated with suppression of the senescence-associated upregulation of either P16 or P15 [38, 47]. Similarly, forced expression of $Fbxl10$ prevents exhaustion of the long-term repopulation potential of hematopoietic cells [48]. By contrast, gene knockdown models revealed that $Fbxl10$ reduces cellular proliferation activity in MEFs and mesenchymal stem cells, accompanied by increased expression of P16 [24]. In the present study, the average expression levels of P16, P15, P19, and P21 were higher, the latter two significantly so in $Fbxl10^{\Delta\Delta}$ GSCs than in WT GSCs (Fig. 4A). This result is consistent with our previous report that a lack of $Fbxl10$-FL causes an increase in P19 and P21 expression in MEFs [13, 27] or Embryonic Day 8.5 embryos in vivo [13]. P21 and P19, which are encoded by the Cdkn1a and Cdkn2a genes, respectively, and are markers of cellular senescence [28, 49], are negative regulators to prevent cell cycle progression from G1 to S phase. This is in agreement with the findings of the present study that in comparison to WT GSCs, the proliferation of $Fbxl10^{\Delta\Delta}$ GSCs was significantly slower and the ratio of $Fbxl10^{\Delta\Delta}$ GSCs in G1/G0 phase was significantly higher. On the other hand, no significant difference was observed in the ratio of apoptotic cells between $Fbxl10^{\Delta\Delta}$ and WT GSCs, as determined by flow cytometry using an anti-cleaved CASPASE-3 antibody (Fig. 4C). This suggests that the slower proliferation of $Fbxl10^{\Delta\Delta}$ GSCs is related to slower cell cycle progression but not to more pronounced cell death. It is notable that the correlation between $Fbxl10$ and apoptosis seems to be cell type-dependent. For example, a lack of $Fbxl10$ significantly increases the ratio of MEFs that undergo apoptosis [13], whereas downregulation of $Fbxl10$ by gene knockdown does not significantly promote apoptosis in prostate adenocarcinoma LNCaP cells [22]. Similarly, drug-induced apoptosis is not mitigated by exogenous overexpression of $Fbxl10$ in MEFs [10].

Spermatogonial cell cycle in vivo was transiently hyperactivated by treating mice with the cytotoxic agent busulfan at a modulated dose [29, 30]. By using this in vivo model, we observed that the number of undifferentiated spermatogonia did not differ between $Fbxl10^{\Delta\Delta}$ and WT mice at 2 wk after busulfan administration, whereas there were fewer of these cells ($P = 0.08$) in $Fbxl10^{\Delta\Delta}$ mice than in WT mice at 4 wk after treatment (Fig. 6A). Furthermore, the number of mitotically inactive undifferentiated spermatogonia, which were detected as PLZF$^+$ and Ki67$^+$ cells, was 10-fold higher in $Fbxl10^{\Delta\Delta}$ mice than in WT mice, suggesting that loss of $Fbxl10$-FL dampens spermatogonial cell proliferation. Given that spermatogonial stem cells are seeds for all germ cell lineages in the testis and indispensable for sustainable spermatogenesis, the disordered proliferation of spermatogonia found in our $Fbxl10$ mutant testis might be one cause of the degeneration of spermatogenesis.

We previously reported that the mRNA expression of $Fbxl10$-FL is much higher in the testis than in other organs [13]. In addition, the present data revealed that $Fbxl10$-FL was expressed in mitotic spermatogonia and meiotic spermatocytes as well as in somatic Sertoli cells (Fig. 1B). Sertoli cells provide an essential micro-environment that supports testicular germ cell to complete the complicated process of spermatogenesis [3, 50]. Although we did not determine the function of Sertoli cells in $Fbxl10^{\Delta\Delta}$ mice in the present study, these cells were present in seminiferous tubules that exhibited degeneration of spermatogenesis in older mice (Supplemental Fig. S2).

Our in vitro study, in which the supportive effects of Sertoli cells were absent, revealed that $Fbxl10^{\Delta\Delta}$ GSCs exhibited significantly slower proliferation and higher $P21$ and $P19$ expression than WT GSCs (Fig. 4, A and B). In addition, mitotically active spermatogonia was significantly fewer as well as spermatogonial cell proliferation after busulfan treatment was clearly retarded in young mice (Figs. 5 and 6) even when $Fbxl10^{\Delta\Delta}$ mice displayed no distinct abnormality in spermatogenesis in the absence of treatment (Fig. 2C). Thus, we hypothesize that the age-dependent progression of abnormal spermatogenesis in $Fbxl10^{\Delta\Delta}$ mice is owing, at least in part, to characteristic changes in testicular germ cells, especially in spermatogonia. Next, the contribution of Sertoli cells to spermatogenesis in this $Fbxl10^{\Delta\Delta}$ model should be examined by transplanting $Fbxl10^{\Delta\Delta}$ spermatogonia into WT testes or vice versa.

ACKNOWLEDGMENT

The authors thank Dr. Hirotake Ichise, Dr. Taeko Ichise, Dr. Takahiko Chimura, and Dr. Takayuki Shibasaki for their valuable advices and discussions while carrying out the research.

REFERENCES

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