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Notch Signaling in Sertoli Cells Regulates Cyclical Gene Expression of Hes1 but Is Dispensable for Mouse Spermatogenesis

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Mammalian spermatogenesis is a highly regulated system dedicated to the continuous production of spermatozoa from spermatogonial stem cells, and the process largely depends on microenvironments created by Sertoli cells, unique somatic cells that reside within a seminiferous tubule. Spermatogenesis progresses with a cyclical program known as the "seminiferous epithelial cycle," which is accompanied with cyclical gene expression changes in Sertoli cells. However, it is unclear how the cyclicity in Sertoli cells is regulated. Here, we report that Notch signaling, which is known to play an important role for germ cell development in *Drosophila* and *Caenorhabditis elegans*, is cyclically activated in Sertoli cells and regulates stage-dependent gene expression of Hes1. To elucidate the regulatory mechanism of stage-dependent Hes1 expression and the role of Notch signaling in mouse spermatogenesis, we inactivated Notch signaling in Sertoli cells by deleting protein O-fucosyltransferase 1 (Pofut1), using the *cre-loxP* system, and found that stage-dependent Hes1 expression was dependent on the activation of Notch signaling. Unexpectedly, however, spermatogenesis proceeded normally. Our results thus indicate that Notch signaling regulates cyclical gene expression in Sertoli cells but is dispensable for mouse spermatogenesis. This highlights the evolutionary divergences in regulation of germ cell development.

Mammalian spermatogenesis is based on a highly specialized stem cell system that largely depends on microenvironments within a seminiferous tubule. Spermatogonia, immature male germ cells containing the stem cell population in mammalian testes, proliferate several times and then differentiate into meiotic spermatocytes. After meiosis, these cells sequentially transform to round spermatids and elongated spermatids and are finally released into the lumen as spermatozoa (25). Spermatogenesis progresses with a cyclical program known as the "seminiferous epithelial cycle" (33). During spermatogenesis, germ cells at different developmental stages form groups and synchronously differentiate. In the mouse, 12 germ cell groups, known as seminiferous epithelial stages I to XII, are identifiable and are arranged in order along the length of tubules. Sertoli cells, which are unique somatic cells in the seminiferous tubules, directly interact with a germ cell group and support the constant differentiation of these cells by creating microenvironments essential for self-renewal and differentiation (3, 22, 32). It has also been shown that Sertoli cells change their gene expression patterns in response to alterations in the interacting germ cell groups (40, 50), and such gene expression changes suggest that Sertoli cells create stage-specific microenvironments. It has been known that retinoic acid (RA) signaling is involved in the seminiferous epithelial cycle (43, 44). However, it remains unclear whether other signaling pathways are implicated in the cyclical gene expression change and how the cyclicity in Sertoli cells is regulated.

Notch signaling is an evolutionarily conserved system that is implicated in cell fate decisions and various developmental processes in invertebrates and vertebrates (2, 15, 20, 21). The activation of Notch signaling is induced by the interaction between the extracellular domain of Notch receptor and the ligands Delta and Jagged expressed on neighboring cells. This leads to the cleavage of the Notch intracellular domain (NICD) by gamma-secretase and its translocation to the nucleus, where it forms a complex with a transcriptional regulator RBP-jk and other coregulators. This, in turn, results in the transcriptional activation of specific target genes, such as Hes family genes (11). All four identified Notch receptors (Notch1 to -4) are modified posttranslationally by protein O-fucosyltransferase 1 (Pofut1), an enzyme that transfers fucose to epidermal growth factor (EGF) repeats of the extracellular domain and may act as a chaperone protein for Notch receptors (28, 29, 36). These regulatory mechanisms have been shown to be important for the cellular localization of Notch receptors and their binding to ligands, and the inactivation of Pofut1 thus causes severe Notch signaling defects (30, 31).

It has been reported previously that Notch signaling is important for germ cell development in *Caenorhabditis elegans* (13) and *Drosophila* (1, 14, 37, 46). In mammalian testes, other group of investigators have also reported the immunohistological detection of Notch receptors and ligands (5, 8, 23, 45), but these findings are not consistent. Furthermore, there have been no reports that have examined the significance of Notch signaling in mouse spermatogenesis using genetic approaches. Thus, it remains unclear whether Notch signaling is implicated in mouse spermatogenesis and whether there is any functional difference of Notch signaling in mammalian germ cell development compared to that in invertebrates. In our present study, we have found that Notch signaling is cyclically activated in Sertoli cells during stages VII to VIII and regulates stage-dependent expression of Hes1. However, inactivation of Notch signaling in Sertoli cells and germ cells by deletion of Pofut1 does not cause abnormal spermatogenesis during the periods we examined. These findings suggest that cyclical

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activation of Notch signaling in Sertoli cells regulates stage-dependent gene expression of Hes1 but that both are dispensable for mouse spermatogenesis.

MATERIALS AND METHODS

Animals. The *N1IP*::*Cre*^{HI} *Rosa-YFP*, Pofut1 conditional knockout (cKO), *Cag-Cat-GFP Nanos3-cre* mice have been described previously (19, 30, 34, 38). For the generation of an *Amh-cre* transgenic mouse, the 720-bp promoter region and entire first intron of the *Amh* gene were amplified from the tail DNA of a C57BL6/j mouse by PCR, and this product was used to drive Cre recombinase as previously described (9). Transgenic mice were generated by pronuclear injection of the purified transgene into F2 hybrid oocytes from B6/C3H parents (CLEA Japan), and six founders were obtained. The wild-type mice used in this study were the MCH strain purchased from CLEA Japan. All mice were maintained in accordance with National Institute of Genetics (NIG) guidelines, and all procedures were carried out with approval from the Committee for Animal Care and Use in NIG.

Histology. For histological analysis, mouse testes without tunica albuginea and epididymides were fixed in Bouin's solution at room temperature for more than 24 h and embedded in paraffin. Sections (4 μm thick) were stained using hematoxylin and the periodic acid-Schiff (PAS) method. Seminiferous epithelial stages were determined as described previously (33).

Immunostaining. After removal of the tunica albuginea, the mouse testes were fixed with 4% paraformaldehyde (PFA) at 4°C overnight and embedded in paraffin or 22-oxacalcitriol (OCT) compound (Sakura Finetek). After a blocking step, sections were incubated with goat anti-Gata4 antibody (Santa Cruz), rabbit anti-Plzf antibody (Santa Cruz), or chick anti-green fluorescent protein (anti-GFP) antibody (Aves) overnight at 4°C. The resulting signals were detected by incubation with Alexa 488- or Alexa 594-conjugated IgG antibodies (Molecular Probes). For the detection of rabbit WT1 antibody (Abcam), rabbit anti-Notch1 ICD (N1ICD) antibody (Cell Signaling Technology), rabbit anti-β-galactosidase antibody (Serotec) or rabbit anti-Hes1 antibody (30), EnVision anti-rabbit antibody (Dako), and Tyramid Signal Detection Reagent (Perkin Elmer) were used. For the double immunostaining of N1ICD and WT1 and Hes1 staining, the appropriate antibodies were separately incubated after antigen retrieval (26). All sections were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI), and images were processed with Adobe Photoshop, version 10.

***In situ* hybridization.** *In situ* hybridizations were performed as previously described with some modifications (49). The probes for *Notch1*, *Notch4*, *Jagged1*, *Jagged2*, *Dll3*, and *Dll4* have been previously described (18, 30), and those for *Dll1* and for *Notch2* and *Notch3* were kindly provided by A. Gossler (Institute for Molecular Biology, Germany) and T. Gridly (The Jackson Laboratory), respectively. *Lgals1* (0.5 kbp), *Stra6* (2.3 kbp), and *Stra8* (1.1 kbp) were subcloned from testis cDNA by PCR. Digoxigenin (DIG)-labeled cRNA probes were synthesized with RNA labeling mix (Roche). Mice were perfusion fixed with 4% PFA under anesthesia with Avertin, and their testes were dissected. After removal of the tunica albuginea, the testes were further fixed in 4% PFA at 4°C overnight and embedded in paraffin. Sections were hybridized with DIG-labeled probe and incubated with alkaline phosphatase (AP)-conjugated anti-DIG Fab fragments (Roche). Signals were then detected using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) solution (Roche) mixed with 5% polyvinyl alcohol (Wako) to enhance the AP reaction (4).

Quantitative RT-PCR. Seminiferous tubules during stages I to III, IV to VI, VII to VIII, and IX to XII were isolated under dissection microscopy as previously described (16). Total RNA was purified with an RNeasy kit (Qiagen), and cDNA was synthesized using oligo(dT) primer and SuperScript III (Invitrogen) according to manufacturer's instructions. Real-time reverse transcription-PCR (RT-PCR) was performed using a KAPA SYBR Fast qPCR kit (KAPA Biosystems), Thermal Cycler Dice (Takara), and the following PCR primers: *Hes1*, 5′-CCAGCCAGTGTCAACACGA-3′ and 5′-ATGCCGGGAGCTATCTTTCT-3′; *Gapdh*, 5′-CATGTTCCAGTATGACTCCACTC-3′ and 5′-GGCCTCACCCCATTTGATGT-3′. Signals were normalized by the *Gapdh* expression.

Preparation and injection of lentivirus. The packaging plasmids pCAG-HIVgp and pCMV-VSV-G-RSV-Rev (where CMV is cytomegalovirus, VSV-G is vesicular stomatitis virus G protein, and RSV is Rous sarcoma virus) and the expression plasmids CSII-EF-MCS-IRES2-Venus (where EF is elongations factor, MCS is multiple cloning site, and IRES2 is internal ribosome entry site 2) and CSII-EF-Venus were kindly provided by H. Miyoshi (Riken BRC, Japan). Lentiviral vectors were prepared as previously described with some modifications (17, 41). A total of 5×10^6 HEK293T cells were seeded on a 10-cm dish 24 h prior to transfection. A total of 10 μg of plasmid DNA (2.7 μg of pCAG-HIVgp, 2.7 μg of pCMV-VSV-G-RSV-Rev, and 4.6 μg of transfer vector plasmid) mixed with 15 mM (based on monomer units) polyethylenimine (PEI) solution (Polysicence) was added to the culture medium. After 36 h of cultivation, the medium containing lentivirus vector was collected, and lentiviruses were purified with sequential centrifugation at $20{,}000 \times g$ for 5 h and resuspended in Hanks' balanced salt solution (Invitrogen). Lentiviruses were injected into testes from postnatal day 7 (P7) to P10 via efferent ductules (10).

Isolation and culture of primary Sertoli cells. Primary Sertoli cells were isolated as previously described with some modifications (12). Two- to three-week testes without tunica albuginea were sequentially treated with 0.5 mg/ml collagenase (WAKO), 1 mg/ml hyaluronidase (Sigma) plus 1 mg/ml collagenase, and 1 mg/ml hyaluronidase in Dulbecco's modified Eagle's medium (DMEM) containing DNase I. Small pieces of seminiferous tubules were removed via filtering through a 100-μm-pore-size filter, and isolated Sertoli cells were cultured with F12-DMEM mixed with 10 μg/ml insulin (Invitrogen), 5 μg/ml transferrin (Sigma), and 5 ng/ml epidermal growth factor (Becton Dickinson) at 34°C. Medium was changed at days 2 and 4, and genomic DNA was isolated at day 5.

Sperm count. Cauda epididymides were dissected into small pieces and incubated in potassium simplex optimized medium (KSOM) at 37°C for 1 h under 5% CO_2 to allow the sperm to exude. The collected sperm were then fixed with 4% PFA and counted with a hemacytometer.

RESULTS

Notch signaling is cyclically activated in stage VII to VIII Sertoli cells. We first examined the expression pattern of each of the Notch receptors (*Notch1* to *-4*) and Notch ligands (*Jagged1* and *-2* and *Delta-like1*, *-3*, and *-4*) in adult mouse testes by *in situ* hybridization and observed stage-dependent expression for some of the genes (Fig. 1A to C and data not shown). These expression profiles included *Notch1* in stage VII to VIII Sertoli cells (Fig. 1A), *Jagged1* in stage I to VI and IX to XII elongated spermatids (Fig. 1B), and *Jagged2* in stage VII and VIII spermatogonia and preleptotene spermatocytes (Fig. 1C). We did not detect expression of genes encoding other Notch receptors and ligands. Since the genes encoding Notch1 receptor and ligands are expressed in adult mouse testes, we next examined the activation of Notch signaling using a specific antibody for the cleaved Notch1 intracellular domain (N1ICD) (26). We detected signals for cleaved N1ICD in the nuclei of stage VII to VIII tubules, and these nuclei also stained positively for WT1, a Sertoli cell-specific transcription factor in the adult testis, thus indicating Notch1 activation in Sertoli cells (Fig. 1D to F). Furthermore we found that Hes1, a well-known target of Notch signaling, was also expressed in stage VII to VIII Sertoli cells together with the N1ICD (Fig. 1G to I). This stage-dependent expression of Hes1 was also confirmed by real-time RT-PCR using stage-specific seminiferous tubules (Fig. 1J). These results suggest that simultaneous expression of *Notch1* and *Jagged2* in stage VII to

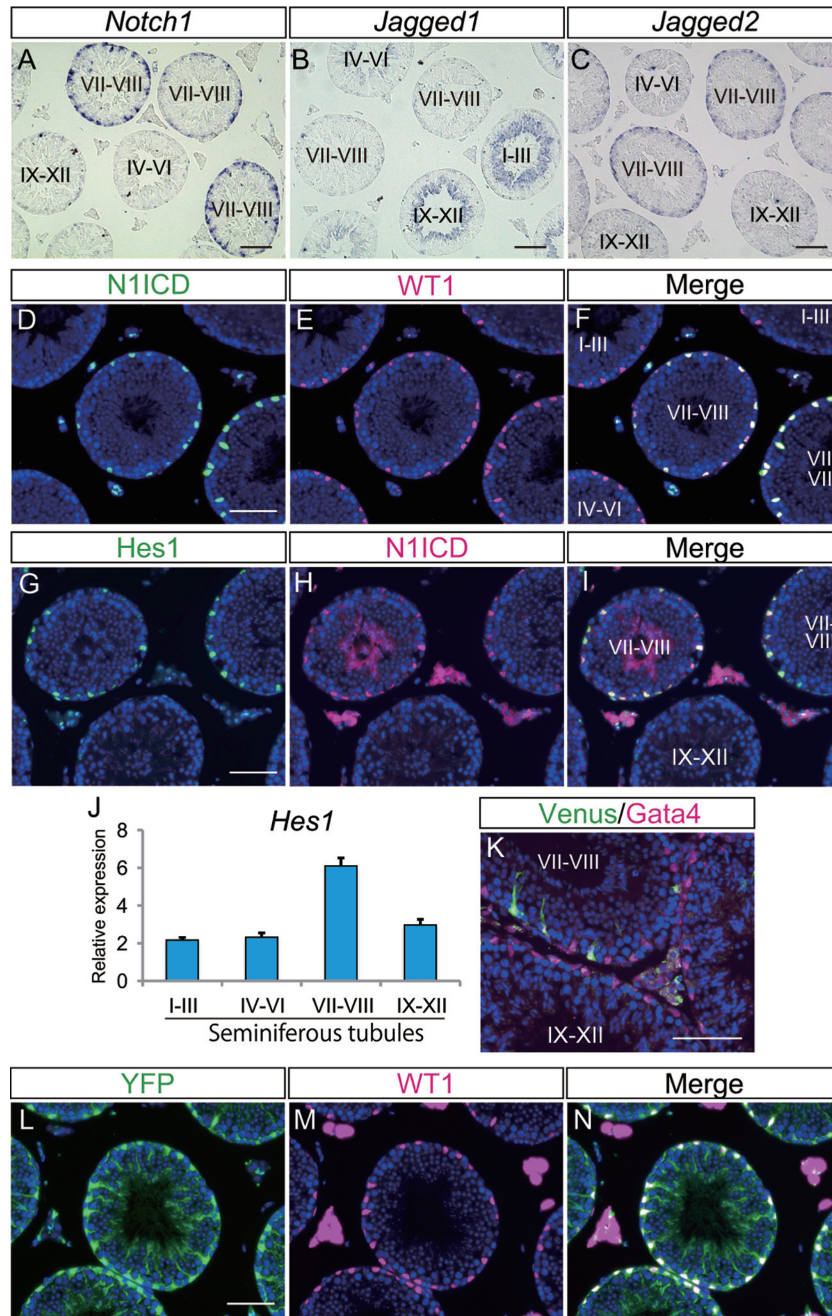

FIG 1 Expression pattern of Notch signaling-related genes and activation of Notch signaling in the adult mouse testis. (A to C) Expression of *Notch1*, *Jagged1*, and *Jagged2* in adult mouse testes, examined by *in situ* hybridization. (D to F) Immunostaining of cleaved-N1ICD and WT1 and their merged signal in the adult mouse testis. (G to I) Immunostaining of Hes1 and cleaved-N1ICD and their merged signal. (J) Expression of *Hes1* mRNA in stage-specific tubules. *Gapdh* was used as the internal control. Error bars, standard deviations. (K) Immunostaining of Venus (green) and Gata4 (magenta) in adult testis from a TP1-Venus transgenic mouse. (L to N) Immunostaining of YFP and WT1 and their merged signals in an *N1IP*::*Cre*; *Rosa-YFP* testis. Nuclei were stained with DAPI (blue). The seminiferous epithelial stages were determined by staining of serial sections with PAS and hematoxylin. Scale bar, 80 μm.

VIII tubules leads to activation of the Notch signaling in stage-specific Sertoli cells.

We also confirmed Notch activation using a Notch reporter system, the *TP1-Venus* transgenic mouse, in which Venus expression is induced upon activation of Notch signaling via RBP-jk-binding sequence (35). Consistent with the expression pattern of N1ICD, reporter expression in this animal model was detected exclusively in stage VII to VIII Gata4-positive Sertoli cells (Fig. 1K). We next examined *N1IP*::*Cre* knock-in mice, in which Cre-dependent recombination is induced in cells that experienced activation of Notch1 signaling (19). To visualize the cells in adult testes, *N1IP*::*Cre* mice were crossed with *Rosa-YFP* reporter mice

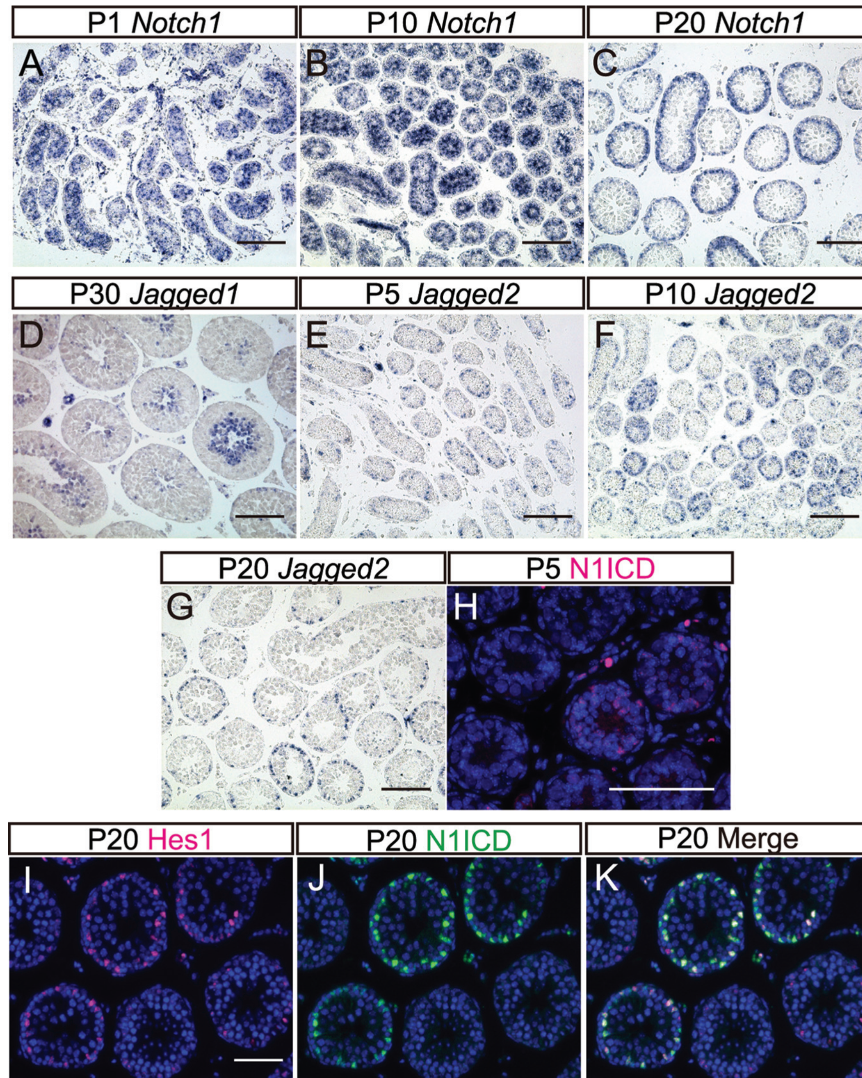

FIG 2 Expression patterns of Notch signaling-related genes and activation of Notch signaling in the developing mouse testis. (A to C) Expression patterns of *Notch1* in the testes at P1, P10, and P20. (D) Expression pattern of *Jagged1* in the testis at P30. (E to G) Expression patterns of *Jagged2* in the testes at P5, P10, and P20. (H) Distribution of cleaved-N1ICD in P5 testis. (I to K) Immunostaining of Hes1 and N1ICD and their merged signal in P20 testis. Nuclei were stained with DAPI (blue). Scale bars, 80 μm (A to G) and 40 μm (H and I).

in which yellow fluorescent protein (YFP) is expressed after Cre-mediated recombination, and we found YFP expression in all Sertoli cells but not in any germ cells (Fig. 1L to N). These findings also support the idea that Notch signaling is cyclically activated in stage VII to VIII Sertoli cells.

Activation of Notch signaling is spatially and temporally regulated during the first round spermatogenesis. We next examined the time point at which Notch activation is established during mouse spermatogenesis. For this purpose, sections of mouse testes were prepared and subjected to *in situ* hybridization for *Notch1*, *Jagged1*, and *Jagged2* at 5-day intervals from postnatal day 1 (P1) to P30 (Fig. 2A to G). *Notch1* was detected in all Sertoli cells at P1 (Fig. 2A), and this expression was gradually restricted to some tubules through P10 to P20 and onward (Fig. 2B and C and data not shown). *Jagged1* transcription appeared to start at P30 (Fig. 2D and data not shown) when elongated spermatids differentiate. Although *Jagged2* transcripts were not detected in P1 testis (data not shown), they became weakly detectable in all seminiferous tubules at P5 (Fig. 2E), when the differentiation of gonocytes into spermatogonia takes place. At P10, just after initiation of meiosis, strong expression of *Jagged2* became apparent in some tubules (Fig. 2F), but this number decreased at P20 and onward (Fig. 2G and data not shown).

Notch activation was next examined using sections from P1, P5, and P20 testes. In the P1 testes, there was no evident N1ICD signal (data not shown), and N1ICD became faintly detectable in immature Sertoli cells at P5 when the nuclei of these cells were still located in the center of the tubules (Fig. 2H). This was further found to be coincident with the onset of *Jagged2* expression at around P5; however, Hes1 expression was not detectable, probably due to the low activation level of Notch signaling (data not shown). At P20, when Sertoli cells become mature and *Jagged2* expression increases in germ cells, N1ICD signals were detected in a proportion of Sertoli cell nuclei located on the periphery of the

tubules, and those cells also expressed Hes1 (Fig. 2I to K). These results thus suggest that Notch1 activation and Hes1 expression in Sertoli cells are temporally and spatially regulated by the expression of *Notch1* in Sertoli cells and *Jagged2* in germ cells.

Notch signaling regulates stage-dependent expression of Hes1. To clarify the role of Notch signaling in stage-dependent gene expression and mouse spermatogenesis, we used a conditional knockout (cKO) mouse carrying a *loxP*-flanked allele of *Pofut1*. *Pofut1* encodes O-fucosyltransferase which has an essential function in the proper folding and/or trafficking of all Notch receptors and therefore is required for activation of Notch signaling (30, 31). Hence, all Notch signaling will be abolished in this mutant mouse, independent of any other Notch receptor that may be expressed in Sertoli cells below the limit of detection by *in situ* hybridization.

It was reported that injection of lentivirus into seminiferous tubules resulted in Sertoli cell-specific transfection (10). Therefore, to remove the floxed allele specifically in Sertoli cells, we utilized lentivirus containing Cre recombinase followed by IRES Venus (LV-Cre) (Fig. 3A) to identify lentivirus-infected Sertoli cells. LV-Cre was injected into P7 to P10 testes of Rosa-LacZ mice, in which LacZ reporter expression would be initiated following Cre-dependent recombination. We found that Venus was specifically expressed in Sertoli cells that were also positive for LacZ, indicating that recombination is specifically induced in Sertoli cells (Fig. 3F to H). To inactivate Pofut1, we next injected LV-Cre into *Pofut1$^{flox/flox}$* testes at P7 to P10 and analyzed the testes at the age of 8 weeks. In those testes, expression of N1ICD in Venus-positive Sertoli cells became undetectable (Fig. 3I to N), suggesting that Notch signaling was inactivated in Sertoli cells. Furthermore, expression of Hes1 in stage VII to VIII Sertoli cells was also diminished (Fig. 3O to T). These results indicate that the stage-dependent expression of Hes1 is regulated by the cyclical activation of Notch signaling.

***Amh-cre* mouse successfully deletes Pofut1 and inactivates Notch signaling in Sertoli cells.** Despite the inactivation of Notch signaling and the loss of Hes1 expression in Sertoli cells, spermatogenesis in the Pofut1 cKO testes injected with LV-Cre was not affected (Fig. 3B to E). We hypothesized that the absence of any noticeable abnormality in the Pofut1 cKO testes injected with LV-Cre might be due to nonautonomous rescue of Notch-deficient cells by the Notch-expressing population. Therefore, to inactivate Pofut1 in most Sertoli cells, we generated an *Amh-cre* mouse, which induces Cre recombinase in embryonic Sertoli cells (9). The specificity of *Amh-cre* was confirmed by crossing with *Cag-Cat-GFP* reporter mice in which GFP is expressed after *cre*-mediated recombination. In 6-week-old *Amh-cre*; *Cag-Cat-GFP* mice, more than 95% of Sertoli cells became GFP positive (Fig. 4A). In addition, a small number of cells in other tissues, including liver, heart, and kidney, also expressed this reporter gene, suggesting that some leaky Cre expression takes place (data not shown). When *Pofut1$^{flox/flox}$* mice were crossed with *Amh-cre*; *Pofut1$^{+/-}$* mice, the resulting *Pofut1$^{flox/-}$*; *Amh-cre* mice were not born at the expected Mendelian frequency. This suggested that leaky deletion of *Pofut1* led to embryonic lethality. However, we were able to obtain enough viable, adult *Pofut1$^{flox/-}$*; *Amh-cre* mice to be analyzed.

To confirm that *Pofut1* was actually deleted in Sertoli cells by *Amh-cre*, we analyzed the *Pofut1* locus in cultured Sertoli cells isolated from *Pofut1$^{flox/-}$*; *Amh-cre* mice by PCR and found that deletion of the *Pofut1* locus was complete, as expected (Fig. 4B). Consistent with this, adult *Pofut1$^{flox/-}$*; *Amh-cre* testes lost N1ICD immunoreactivity in Sertoli cells (Fig. 4C). These results confirmed the successful inactivation of Notch signaling.

The majority of the stage-dependent gene expression remains unchanged in Sertoli cell-specific Pofut1 cKO mice. We assessed the possibility that Notch signaling is involved in the regulation of stage-dependent gene expression other than Hes1 in mouse Sertoli cells. We first examined the expression pattern of *Lgals1*, whose expression peaked in stage IX to XII Sertoli cells (40), and we found that *Lgals1* was normally expressed in *Pofut1$^{flox/-}$*; *Amh-cre* mice (Fig. 4D and E). We next analyzed the expression of *Stra6* and *Stra8*, which are originally identified as targets of RA signaling in embryonic carcinoma cells and highly expressed in stage VII to VIII Sertoli cells and stage VII to VIII germ cells, respectively (44). In *Pofut1$^{flox/-}$*; *Amh-cre* mice, the expression pattern of these genes was also comparable to that of the control mice (Fig. 4F to I). Finally, to investigate a feedback regulation of Notch signaling, we examined *Notch1* and *Jagged2* expression in the *Pofut1$^{flox/-}$*; *Amh-cre* mice, and we found that their stage-dependent expression levels were also normal (Fig. 4J to M). These results suggest that the stage-dependent gene expression in seminiferous tubules except for Hes1 would be independent of Notch signaling and that the stage-dependent expression of *Notch1* and *Jagged2* is not regulated by Notch signaling itself.

Activation of Notch signaling in Sertoli cells is dispensable for mouse spermatogenesis. We next examined fertility of *Pofut1$^{flox/-}$*; *Amh-cre* mice and found that 8-week-old mutant males were fertile and produced normal litter sizes, comparable to those of the control males mated with wild-type females (Table 1). We confirmed that testis size (Fig. 5A), the histology of the testis (Fig. 5B and C) and epididymis (Fig. 5D and E), and the number of spermatozoa in the epididymides (Table 1) in *Pofut1$^{flox/-}$*; *Amh-cre* testis were comparable to those of the control. We also checked a possibility of any changes in the seminiferous epithelial cycle in *Pofut1$^{flox/-}$*; *Amh-cre* mice due to the lack of Notch activation in stages VII to VIII. However, we detected no significant change in germ cell group observed in each seminiferous stage (Fig. 5H and I and data not shown) and proportion of stage VII to VIII tubules (Fig. 5J). Moreover, the numbers of Gata4-positive Sertoli cells (Fig. 5K to M) and Plzf-positive undifferentiated spermatogonia (Fig. 5N to P) were unchanged in these mice. To examine the role of Notch signaling in mouse spermatogonial stem cell maintenance, we analyzed 4-month-old *Pofut1$^{flox/-}$*; *Amh-cre* males. The older mutant testes were comparable to those of the control mice histologically (Fig. 5F and G), and the older males generated normal litter sizes (Table 1). These results indicate that Notch signaling in Sertoli cells is dispensable for normal differentiation of spermatogenic cells and for stem cell maintenance in the mouse testes during the periods we examined.

Inactivation of Notch signaling in mouse germ cells does not affect mouse spermatogenesis. Since Notch signaling is required for the maintenance of germ line stem cells in *C. elegans*, we examined the possibility that Notch signaling may be activated and function in mouse germ cells in a cell-autonomous manner. For this purpose, *Pofut1* cKO mice were crossed with *Nanos3-cre* mice which induce Cre in almost 100% of male germ cells from embryonic day 13.5 (E13.5) (38). In the 12-week-old *Pofut1$^{flox/-}$*;

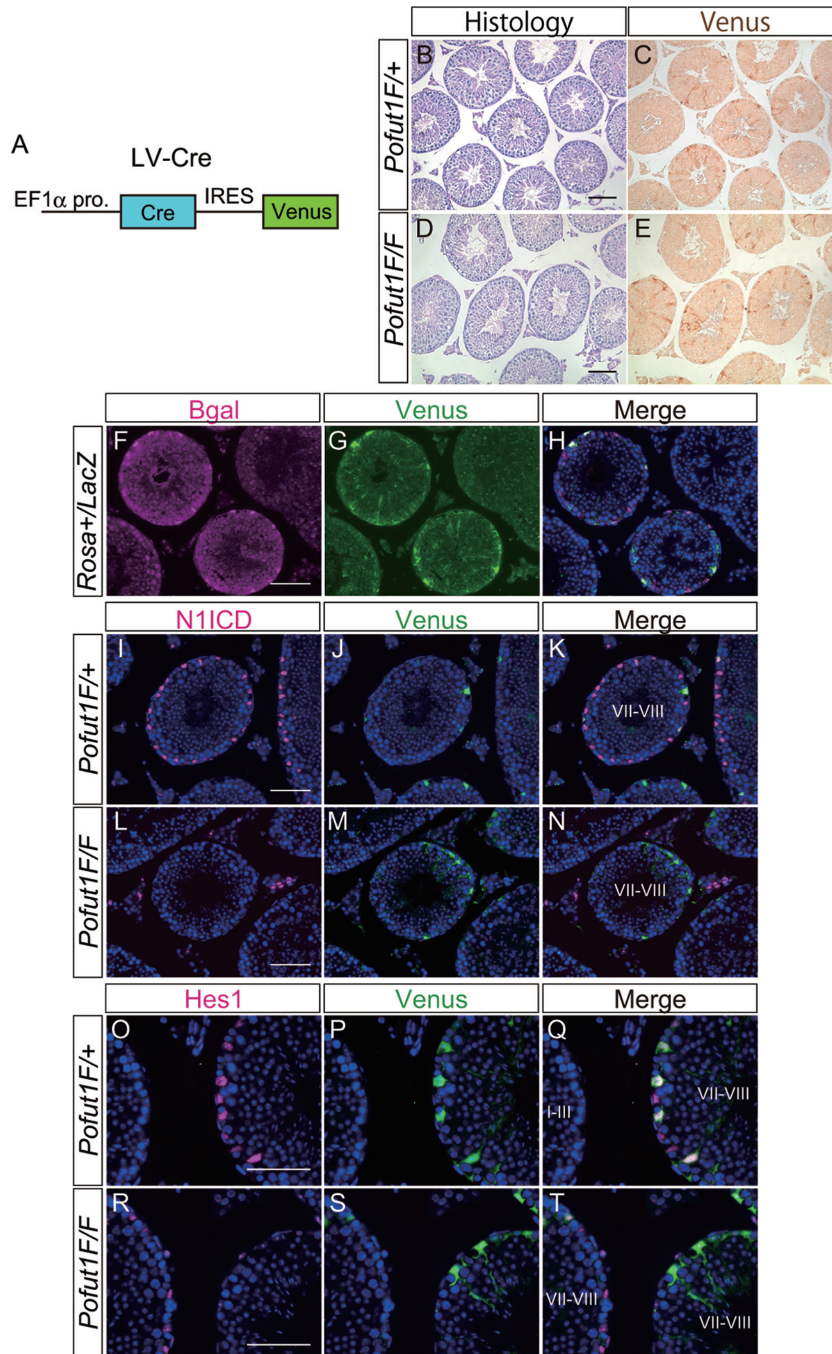

FIG 3 Inactivation of Notch signaling in mouse Sertoli cells induced by using lentivirus. (A) Construction of LV-Cre. Cre and the following IRES-Venus are under the control of EF1α promoter (pro). (B to E) Histological sections stained with PAS and hematoxylin (B and D) and their serial sections stained with anti-GFP antibody (C and E) in adult testes from *Pofut1*$^{flox/+}$ (*Pofut1*$^{F/+}$) or *Pofut1*$^{flox/flox}$ (*Pofut1*$^{F/F}$) mice injected with LV-Cre. (F to H) Immunostaining of β-galactosidase (β-Gal) and Venus and their merged signal in a testis from a 6-week-old *Rosa-LacZ* reporter mouse injected with LV-Cre. (I to N) Immunostaining of N1ICD and Venus and their merged signal in adult testes from *Pofut1*$^{flox/+}$ or *Pofut1*$^{flox/flox}$ mice injected with LV-Cre. (O to T) Immunostaining of Hes1 and Venus and their merged signal in adult testes from *Pofut1*$^{flox/+}$ or *Pofut1*$^{flox/flox}$ mice injected with LV-Cre. Nuclei were stained with DAPI (blue). Scale bar, 80 μm.

Nanos3-cre mice, the external morphology of the testes (Fig. 6A), the histology of the seminiferous tubules (Fig. 6B and C), and the number of sperm in epididymides (Table 1) were normal. Furthermore, when 8-week-old *Pofut1*$^{flox/-}$; *Nanos3-cre* male mice were crossed with wild-type female mice, fecundity was again similar to that of the control mice (Table 1), and all the pups derived from three different fathers were heterozygous for the *Pofut1* allele (Fig. 6D and data not shown), indicating that both alleles of *Pofut1* were inactivated in male sperm and that rare stem cells with an intact *Pofut1* locus did not have an advantage. These results indi-

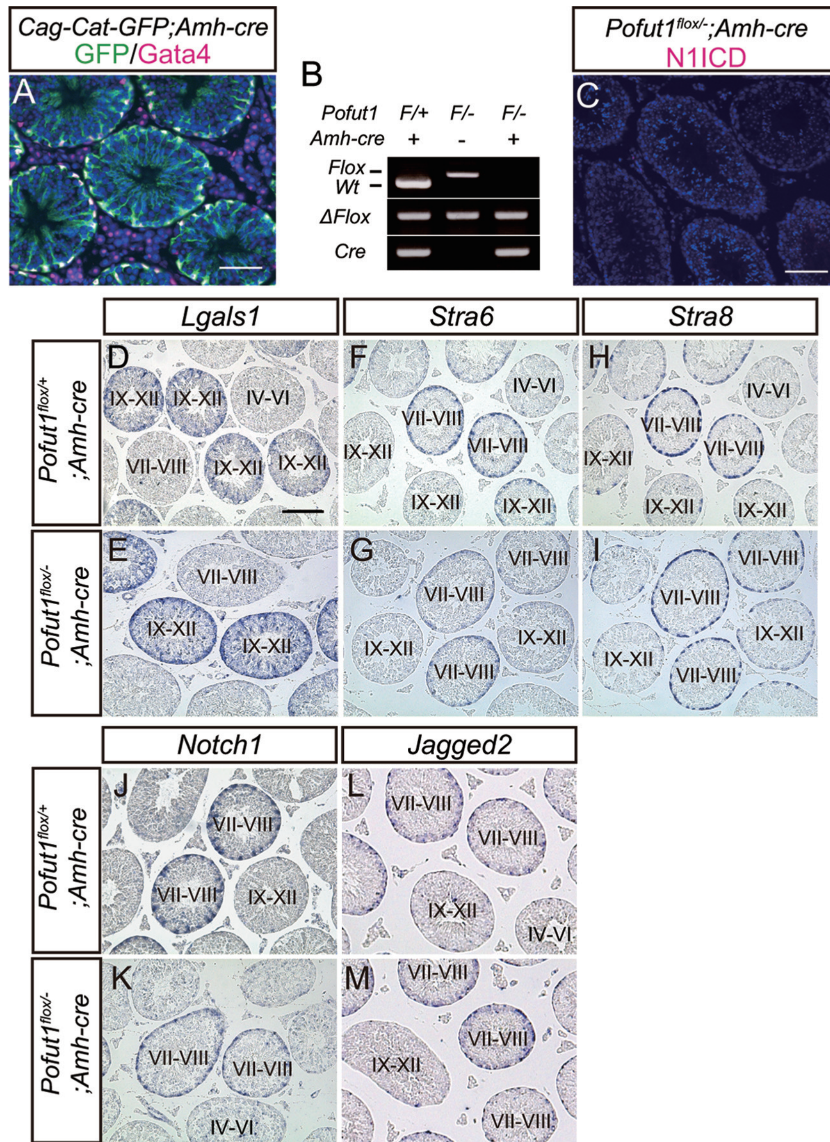

FIG 4 Inactivation of Notch signaling in mouse Sertoli cells by *Amh-cre* and expression pattern of stage-dependent genes in *Pofut1*$^{flox/-}$; *Amh-cre* mice. (A) Immunostaining of GFP (green) and Gata4 (magenta) in a testis obtained from an adult *Cag-Cat-GFP*; *Amh-cre* mouse. (B) Genotyping PCR of cultured Sertoli cells isolated from the indicated mice. (C) Immunostaining of N1ICD (magenta) in a *Pofut1*$^{flox/-}$; *Amh-cre* testis. (D to M) Expression of *Lgals1*, *Stra6*, *Stra8*, *Notch1*, and *Jagged2* in *Pofut1*$^{flox/+}$; *Amh-cre* (D, F, H, J, and L) and *Pofut1*$^{flox/-}$; *Amh-cre* (E, G, I, K, and M) mice detected by *in situ* hybridization. Seminiferous epithelial stages were determined by staining of serial sections with PAS and hematoxylin. Scale bar, 80 μm. Wt, wild type.

cate that Notch signaling in germ cells is also dispensable for mouse spermatogenesis.

TABLE 1 Fertility of mutant mice

Genotype	Sperm count (10^6)	Litter size (no. of pups) of mutant mice mated at: 8 wk	4 mo
Pofut1$^{flox/+}$; *Amh-cre*	19.0 ± 6.3	11.1 ± 3.0	11.3 ± 3.1
Pofut1$^{flox/-}$; *Amh-cre*	20.0 ± 3.6	12.0 ± 3.0	10.0 ± 1.3
Pofur1$^{flox/+}$; *Nanos3-cre*	15.6 ± 5.0	11.2 ± 4.4	ND[a]
Pofut1$^{flox/-}$; *Nanos3-cre*	18.7 ± 4.4	10.9 ± 2.7	ND

[a] ND, not determined.

DISCUSSION

We report that Notch signaling is cyclically activated to regulate stage-dependent expression of Hes1 in mouse Sertoli cells during stages VII to VIII of spermatogenesis. Specifically, we found that Sertoli cells express *Notch1* receptor in a stage-dependent manner that coincides with the stage-dependent expression of *Jagged2* ligand in the adjacent germ cells. We addressed the question of how the cyclical expression of the *Jagged2* and *Notch1* is regulated and, more importantly, whether it is functionally important for spermatogenesis. It has been reported that *Notch1* expression is regulated through feedback regulation of Notch signaling itself (47); however, Sertoli cell-specific *Pofut1* deletion did not affect expression of either *Notch1* or *Jagged2*, suggesting that cyclical activation

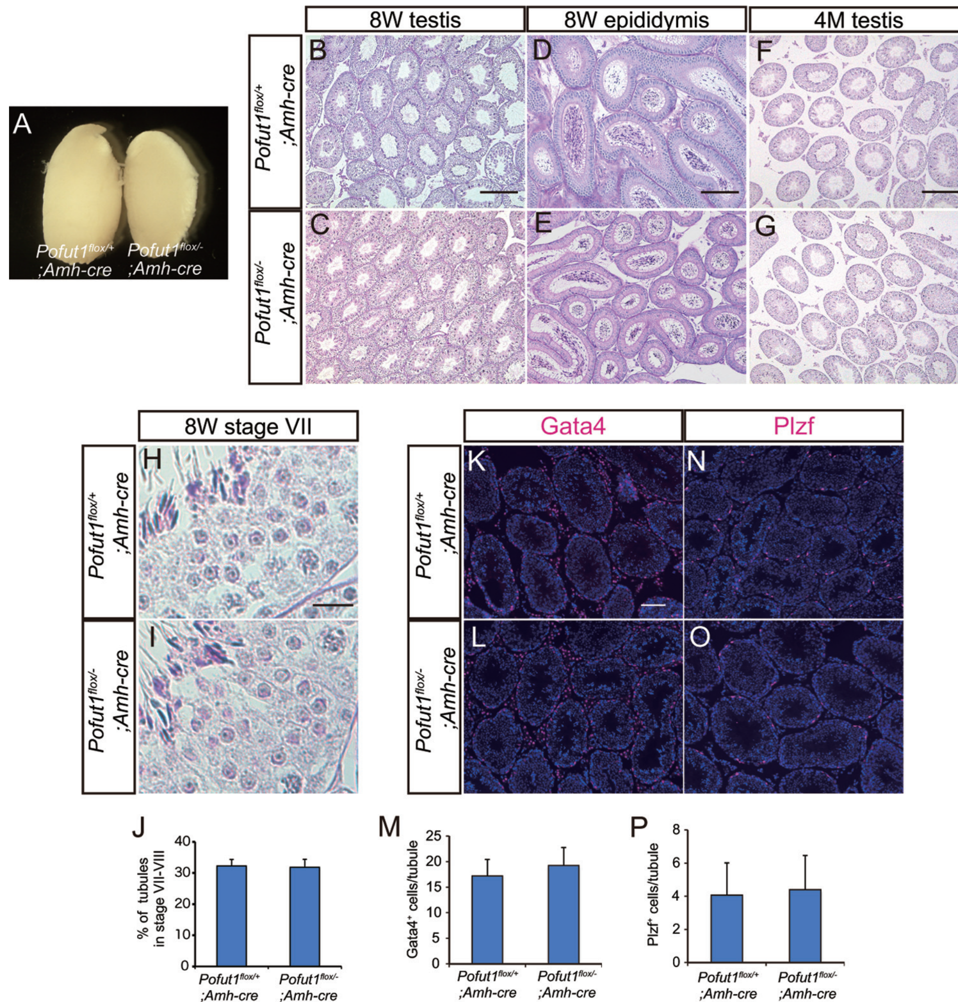

FIG 5 Spermatogenesis in *Pofut1*$^{flox/-}$; *Amh-cre* mice. (A) Testes dissected from *Pofut1*$^{flox/+}$; *Amh-cre* (left) and *Pofut1*$^{flox/-}$; *Amh-cre* mice (right). (B to G) Histological sections of 8-week-old (8W) testes and epididymides and from 4-month-old (4M) testes obtained from *Pofut1*$^{flox/+}$; *Amh-cre* and *Pofut1*$^{flox/-}$; *Amh-cre* mice. (H and I) High-magnification image of spermatogenic cells in stage VII tubules obtained from *Pofut1*$^{flox/+}$; *Amh-cre* and *Pofut1*$^{flox/-}$; *Amh-cre* mice. (J) Proportion of stage VII to VIII tubules. (K to M) Distributions of Gata4-positive Sertoli cells (magenta) in *Pofut1*$^{flox/+}$; *Amh-cre* (K) and *Pofut1*$^{flox/-}$; *Amh-cre* (L) mice. The average numbers of Gata4-positive Sertoli cells in a seminiferous tubule are indicated in panel M. (N to P) Distributions of Plzf-positive spermatogonia (magenta) in *Pofut1*$^{flox/+}$; *Amh-cre* (N) and *Pofut1*$^{flox/-}$; *Amh-cre* (O) mice. The average numbers of Plzf-positive spermatogonia in a seminiferous tubule are indicated in panel P. Nuclei were stained with DAPI (blue). Error bars, standard deviations. Scale bars, 200 μm (B, D, and F), 20 μm (H), and 80 μm (K).

is independent of Notch signaling. The cyclical expression patterns are thus regulated by another signaling pathway(s), for example, RA signaling. RA signaling has been shown to be important for the progression of germ cell differentiation as well as the regulation of cycling in Sertoli cells. It was suggested that activation of RA signaling is required for the transition from stage VII to stage VIII spermatogonia (42, 43). In the *Pofut1* cKO mice, the expression levels of two RA downstream target genes, *Stra6* and *Stra8*, were unchanged, suggesting that RA signaling in the mutant testis was unaffected. These findings indicate that RA signaling could work upstream from, or independent of, *Jagged2* and *Notch1* expression. Another possibility is that the spermatogenic cycle is robust, is independent of any perturbation in a single signaling axis, and utilizes species-specific mechanisms to ensure robustness. This idea is supported by the observation that when rat germ cells are transplanted to a mouse testis, spermatogenesis proceeds with the cycle characteristics of a rat (7). However, Sertoli cell-specific RA receptor α (RARα) deletion, a major RA receptor expressed in Sertoli cells, showed disruption of the stage-dependent expression of many genes without affecting the cycle of germ cells (44). This suggests that Notch signaling might be activated in the Sertoli cells by adjacent germ cells, which leads, in turn, to the induction of *Hes1* in a stage-dependent manner. It remains to be tested if this is true and if other signaling pathways could play a role in the coordination between Sertoli and germ cell cycles.

To understand the function of Notch1 receptor in Sertoli cells, we performed conditional KO experiments of Pofut1 in Sertoli cells. Since the conventional *Pofut1* KO results in embryonic lethality by E10, similar to other global Notch mutants (e.g., the *Presenilin1* and *-2* double KO [6] and *RBP-Jk* KO [27]), it is believed that a *Pofut1* deletion leads to inactivation of Notch signaling mediated by all Notch receptors. We found that Sertoli cell-

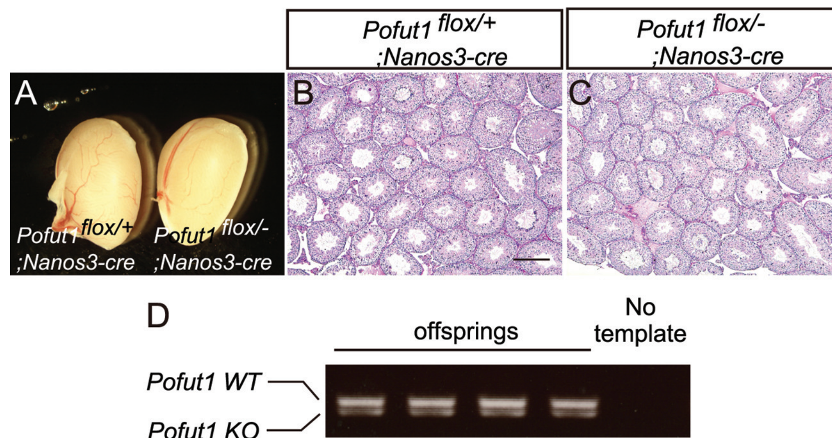

FIG 6 Inactivation of Notch signaling in germ cells. (A) Testes dissected from *Pofut1*$^{flox/+}$; *Nanos3-cre* and *Pofut1*$^{flox/-}$; *Nanos3-cre* mice. (B and C) Histological sections prepared from *Pofut1*$^{flox/+}$; *Nanos3-cre* and *Pofut1*$^{flox/-}$; *Nanos3-cre* mice. (D) PCR genotyping of the offspring from a female wild-type mouse crossed with a male *Pofut1*$^{flox/-}$; *Nanos3-cre* mouse. The DNA was amplified by specific primer pairs for wild-type (WT) and knockout (KO) alleles. Scale bar, 200 μm.

specific *Pofut1* deletion did not produced any abnormality in spermatogenesis. Consistent with this report, another group reported that hyperactivation and inactivation of Notch signaling in somatic cells of mouse embryonic testes affected the development of Leydig cells but not Sertoli cells (39). Collectively, our results indicate that Notch signaling is dispensable for normal Sertoli cell development and mouse spermatogenesis. To our knowledge, this is the first report about a tissue in which Notch signaling is activated but is dispensable for its normal function. Hes1 expression was activated in a stage-dependent manner in Sertoli cells after birth. Hes1 expression is known to be regulated through other signaling pathways such as fibroblast growth factor (FGF) and transforming growth factor β (TGF-β) (24, 48), and therefore our current study cannot rule out the possibility that Hes1 in Sertoli cells play an important role during mouse spermatogenesis.

In *Drosophila* testis, Notch signaling was reported to be activated in the precursor of hub cells and to be important for development of hub cells (14). This is in striking contrast to our result that Notch signaling is dispensable for the function and development of Sertoli cells although activation of Notch signaling in niche cells is similar. In *C. elegans*, Notch signaling is activated in the germ line stem cells and prevents them from entering meiosis. Other groups have previously reported by immunostaining that the protein expression of Notch receptors occurs in spermatogonia, spermatocytes, and round spermatids in mammalian testes (5, 8, 45). We examined the possible involvement of Notch signaling in germ cell functions using *Pofut1*$^{flox/-}$; *Nanos3-cre* mice. These mutant mice retained normal spermatogenesis and fertility over a long period, suggesting that Notch signaling in the germ cells is also dispensable for spermatogenesis in the mouse. Altogether, these differences suggest that mammals have changed the strategy to maintain spermatogenesis and evolved another signaling pathway(s) to compensate the function of Notch signaling.

Our results here demonstrate that Notch signaling is involved in the establishment of the stage-dependent expression of Hes1 in Sertoli cells and provide an insight into how the cyclicity of germ cells and Sertoli cells is coordinated. Our genetic approach revealed, however, that Notch signaling is dispensable for Sertoli cell function and germ cell maintenance and for mouse spermatogenesis. This is distinctly different from findings in *Drosophila* and *C. elegans*, indicating that not all aspects of germ cell development are evolutionarily conserved.

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