Ectopic POU5F1 in the male germ lineage disrupts differentiation and spermatogenesis in mice

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Abstract

Expression levels of the pluripotency determinant, POU5F1, are tightly regulated to ensure appropriate differentiation during early embryogenesis. POU5F1 is also present in the spermatogonial stem cell/progenitor cell population in mice and it is downregulated as spermatogenesis progresses. To test if POU5F1 downregulation is required for SSCs to differentiate, we produced transgenic mice that ubiquitously express POU5F1 in Cre-expressing lineages. Using a Vasa-Cre driver to produce ectopic POU5F1 in all postnatal germ cells, we found that POU5F1 downregulation was necessary for spermatogonial expansion during the first wave of spermatogenesis and for the production of differentiated spermatogonia capable of undergoing meiosis. In contrast, undifferentiated spermatogonia were maintained throughout adulthood, consistent with a normal presence of POU5F1 in these cells. The results suggest that POU5F1 downregulation in differentiating spermatogonia is a necessary step for the progression of spermatogenesis. Further, the creation of a transgenic mouse model for conditional ectopic expression of POU5F1 may be a useful resource for studies of POU5F1 in other cell lineages, during tumorigenesis and cell fate reprogramming.

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Introduction

The generations are connected by an undulating cycle of developmental potency. Mammalian embryonic development starts with a zygote that is totipotent, continues with the formation of a pluripotent blastocyst and culminates with the formation of the germ lineage, which eventually produces unipotent gametes that may unite to repeat the cycle. Much of this cycle of developmental potency is influenced by the transcription factor POU5F1 (OCT4) (Scholer *et al.* 1990b). In the absence of POU5F1, homozygous null mutant zygotes undergo normal cleavage cell divisions, but then they fail to form a pluripotent inner cell mass (ICM) at the blastocyst stage and die (Nichols *et al.* 1998). While the cells in the ICM are initially viable, their normally pluripotent state is altered to a differentiated trophoblast fate. Since the ICM is the source of pluripotent embryonic stem cells, ES cells cannot be derived from *Pou5f1* mutants. Furthermore, by manipulating POU5F1 levels in established ES cells, differentiation and loss of pluripotency occur following only twofold alterations to POU5F1 levels (Niwa *et al.* 2000).

POU5F1 also functions later in embryonic development in primordial germ cells (PGCs). To overcome the peri-implantation lethality of *Pou5f1* knockout mice, *Pou5f1^{flox}* mice were generated

(Kehler *et al.* 2004). A Cre driver was used to delete *Pou5f1* in PGCs mid-gestation. In contrast to its function in the early embryo, in PGCs, the absence of POU5F1 led to apoptosis and impaired fertility. While some PGCs persisted, and in adult males some mature spermatozoa were even formed, the presence of the floxed allele in spermatozoa suggested that the remaining germ cells were the result of incomplete Cre excision (Kehler *et al.* 2004).

POU5F1 function was also examined in postnatal male germ cells *in vitro* (Dann *et al.* 2008, Wu *et al.* 2010). These experiments utilized RNA interference to knockdown POU5F1 in cultures containing spermatogonial stem cells (SSCs). One study transiently introduced small-interfering RNA into cells, but no effect on SSC self-renewal was seen (Wu *et al.* 2010). Another group used transgene-derived small hairpin RNA and showed a requirement for POU5F1 in proliferation and viability (Dann *et al.* 2008). They also showed that cultured SSCs with POU5F1 knockdown had reduced stem cell colonization ability following transplantation, suggesting a role for POU5F1 in SSC self-renewal. However, understanding the function of POU5F1 in the postnatal germ lineage in a true *in vivo* context remains unknown.

The expression pattern of POU5F1 throughout development parallels its function in maintenance of pluripotency and the germ lineage. In the early embryo, it is initially expressed equally in all blastomeres. Then, its expression in the outer cells decreases, as those cells form the trophectoderm and POU5F1 becomes restricted to the ICM by the blastocyst stage (Rosner *et al.* 1990, Scholer *et al.* 1990a, Palmieri *et al.* 1994). It is initially highly expressed throughout the ICM, but then becomes restricted once again during mid-gestation and disappears from all cells except the PGCs (Rosner *et al.* 1990, Pesce *et al.* 1998). POU5F1 is initially strongly expressed in the PGCs of the embryo and its expression is downregulated before the onset of meiosis in both sexes (Pesce *et al.* 1998). In females, downregulation of POU5F1 mRNA and protein occurs at embryonic day 14.5 (Pesce *et al.* 1998). In males, however, meiosis begins postnatally and is preceded by a complex series of proliferation, migration and differentiation events. The timing of POU5F1 downregulation in the male germ lineage is not clear from the published reports and clarifying the timing of POU5F1 downregulation is relevant to understanding its role during these male-specific events.

During late embryogenesis in males, PGCs undergo a transient mitotic arrest before forming gonocytes, the term for male germ cells present at birth in rodents. A few days after birth, gonocytes exit mitotic quiescence, migrate from the lumen to the basal membrane of the seminiferous tubules and then a subset of cells initiates the first wave of spermatogenesis, while another subset generates the first pool of SSCs. Steady-state adult spermatogenesis depends on the self-renewal of SSCs, but the first wave of meiotic cells is thought to originate directly from differentiated spermatogonia, not SSCs (Yoshida *et al.* 2006). Subsequent waves of spermatogenesis originate from SSCs that proliferate and form transit amplifying progenitor cells and ultimately fully differentiated spermatogonia. The SSCs and transit amplifying progenitor cells are collectively called 'undifferentiated spermatogonia,' and following further proliferation and the onset of KIT expression, the cells are called 'differentiated spermatogonia' (Schrans-Stassen *et al.* 1999). Ultimately, the differentiated spermatogonia undergo meiosis and spermiogenesis, forming spermatozoa.

There is consensus that POU5F1 protein is present in gonocytes and in undifferentiated spermatogonia (Pesce *et al.* 1998, Tadokoro *et al.* 2002, Filipponi *et al.* 2007). POU5F1 may be limited to only a small subset of the undifferentiated spermatogonia known as A_s and A_{pr} (A_{single} and A_{pair}), some of which include the true SSCs (Tadokoro *et al.* 2002, Filipponi *et al.* 2007). However, a few contradictory reports have shown that POU5F1 is present in a broader group of spermatogonia that includes A_{al} ($A_{aligned}$) and differentiated spermatogonia (Kehler *et al.* 2004, Hofmann *et al.* 2005,

Tokuda *et al.* 2007, Ketkar & Reddy 2012). Nonetheless, POU5F1 has often been described as a marker for SSCs.

Pou5f1 gene expression in mice has also been studied by analyzing expression of reporter transgenes. The well-studied GOF18 PE transgene contains 18 kb of genomic DNA with GFP (including stop codon and SV40 polyadenylation site) inserted at the start codon of *Pou5f1*, and with an epiblast-specific 986 bp 'proximal enhancer' removed (Yeom *et al.* 1996). Multiple laboratories have made transgenic mice with GOF18 Δ PE, and one line ('OG2') is publicly available and widely utilized (Szabo *et al.* 2002). There is consensus that GOF18 Δ PE (herein, 'Oct4-GFP') mice express GFP in PGCs and neonatal spermatogonia, but only some have seen GFP in adult spermatogonia (Ohbo *et al.* 2003, Garcia & Hofmann 2012, Azizi *et al.* 2016). Interestingly, Oct4-GFP expression has been reported in KIT+ differentiated cells and in long chains of spermatogonia, suggesting that GFP from the transgene may be more widely expressed than endogenous POU5F1 (Ohbo *et al.* 2003).

The goal of our study was to clarify the expression pattern of OCT4 during spermatogenesis in pups vs adults, and to determine the relevance of POU5F1 regulation for neonatal and adult spermatogenesis. We found that POU5F1 is widely expressed in undifferentiated spermatogonia in pups and continues to be present in rare undifferentiated spermatogonia of the adult. By creating transgenic mice with ectopic POU5F1 expression that persists in the germ lineage, we found that POU5F1 downregulation is important for spermatogonia to progress through differentiation into meiosis during the first wave of spermatogenesis. Interestingly, undifferentiated spermatogonia were maintained throughout adulthood and sometimes produced a complete round of spermatogenesis, suggesting that gonocytes transitioned to establish an initial pool of functional stem cells that were subsequently maintained through self-renewal. However, the majority of tubules in adults lacked differentiated cells, similar to the situation during the first wave, demonstrating the importance of POU5F1 downregulation for spermatogenesis in pup and adult stages.

Materials and methods

Mice

All procedures involving mice were approved by the Bloomington Institutional Animal Care and Use Committee. cOct4 transgenic mice were generated and maintained on a pure C57BL/6 background. cOct4 mice are available from The Jackson Laboratory as Stock No. 029084. Oct4-GFP (B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J), Stra8-Cre (Tg(Stra8-icre)1Reb/J) and Vasa-Cre (FVB-Tg(Ddx4-cre)1Dcas/J) breeder pairs were from The Jackson Laboratory and maintained by breeding with wild-type mice of equivalent background. Certain indicated

experiments used Oct4-GFP heterozygotes, which were produced by breeding with C3H or C57Bl6.

For fertility testing, three cOct4;Vasa-Cre (from lines 53 and 18) and three control (WT or Vasa-Cre) 2–3-month-old males were paired with wild-type females. Control males were paired with one female for 3 weeks and each sired two litters. cOct4;Vasa-Cre males were paired with females for 2.5–6.5 weeks and were checked daily for the presence of a vaginal plug. Each cOct4;Vasa-Cre male was kept in a breeding test until at least two plugs were noted for each of at least two females. No litters were sired by any of the cOct4;Vasa-Cre males tested for the duration of the study.

GS cell culture and proteasome inhibitor treatment

GS cells (cell line DGC3) from a DBA/2 mouse were derived and cultured as described (Heim *et al.* 2011). MG132 (Calbiochem) was reconstituted in ethanol at 10.5 mM and stored at -80°C for no more than 1 month. MG132 was diluted in culture medium just before use and treatment times ranged from 0 to 4 h as indicated.

Immunostaining of whole tubules

Whole tubule immunostaining was performed as published with modifications (Gassei & Orwig 2013). Testes or separated seminiferous tubules were rocked in 4% (v/v) paraformaldehyde or 10% (v/v) neutral buffered formalin at 4°C overnight. Tubules were washed with PBS (phosphate-buffered saline) for 15 min twice, treated with mild dehydration solution (PBS with 10% (v/v) methanol and 0.1% (v/v) TritonX-100) at 4°C for 1 h and blocked with PBSMT (PBS with 1xRoche blocking solution (Roche) and 0.5% (v/v) TritonX-100) at 4°C overnight. Tubules were incubated with primary antibody diluted in PMSMT (rabbit anti-POU5F1 1:100, mouse anti-GFP 1:500, rabbit anti-SALL4 1:800 or goat anti-GFRA1 1:50) at 4°C overnight. Antibody details are in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. Tubules were washed in PBT (PBS with 0.1% (v/v) TritonX-100) six times for 15 min. Secondary antibodies were diluted in PBT with 1 $\mu\text{g}/\text{mL}$ DAPI and applied for 1 h followed by six 15 min washes in PBT. Tubules were mounted on glass slides in Vectashield or Fluoro-Gel mounting medium. Images were obtained using a Nikon NiE upright microscope equipped with a Hamamatsu Orca-Flash 2.8 sCMOS camera or with a Nikon Eclipse TiS inverted microscope equipped with a Retiga 2000R Fast 1394 camera. Images were acquired with Q-capture Pro software, and Image J was used for pseudocoloring and to create overlays of colors.

Immunostaining of sectioned testes

Testes were dissected and fixed in 4% (v/v) paraformaldehyde for cryosections or in formalin for histology at 4°C for 2 h to overnight (depending on tissue size). Sections stained with hematoxylin/eosin were prepared from tissue embedded in paraffin using standard procedures. For immunostaining, tissue was saturated with increasing concentrations of sucrose and embedded in O.C.T. Compound (Fisher Healthcare,

Houston, TX, USA). Ten micron sections were cut and stored at -80°C before staining. Cryosections were rehydrated with PBS, blocked for 15 min in 0.1 M glycine, permeabilized in PBT for 5 min and blocked for 30 min in 1% (w/v) bovine serum albumin (Jackson ImmunoResearch) diluted in PBT. For GFRA1 immunostaining, PBT was replaced with PBS, and for GFRA1/STRA8 costaining, permeabilization was with PBS containing 10% (v/v) methanol. Primary antibodies and dilutions used were: rabbit anti-DAZL (1:1000), rabbit anti-POU5F1 (1:200), rabbit anti-SALL4 (1:500), rabbit anti-STRA8 (1:200), rabbit anticleaved caspase 3 (1:500), rabbit anti-CREM (1:200), rat anti-Tra98 (1:500), goat anti-GFRA1 (1:50) or mouse anti-GFP (1:800). Antibody details are in [Supplementary Table 1](#). Primary antibodies were applied overnight at 4°C . Appropriate secondary antibodies conjugated with Alexa-594, Alexa-488 or Cy3 were diluted (1:500) in PBT (or PBS for GFRA1 staining) with 3.6e-6 mol/L DAPI and applied for 1 h at room temperature or 4°C overnight. Secondary antibodies were from Jackson ImmunoResearch, Life Technologies and Cell Signaling Technology. Where not specified, incubations/washes were at room temperature. For a negative control, the primary antibody was omitted. Images were obtained as described above.

cOct4 transgene construction

The cOct4 plasmid was derived from pLLU2G (Addgene #21620, Cambridge, MA, USA), which contains the human Ubiquitin C (Ubc) promoter that we and others have shown previously to be active in a variety of cell types including germ cells (Schorpp *et al.* 1996, Dann *et al.* 2006). We removed sequences of pLLU2G that were irrelevant to this study by digestion, Klenow fill-in and relegation (SmaI/XbaI to remove U6 and LoxP and EcoRI/PvuII to remove LoxP and WPRE). A T2A/furin ribosomal skip sequence was amplified with TD462/TD498 primers ([Supplementary Table 2](#)) and inserted by traditional molecular cloning 5' of eGFP (Yang *et al.* 2008, Fanslow *et al.* 2014). The *Pou5f1* coding sequence was amplified from mouse ES cell cDNA and sequence verified upon insertion into a unique AgeI site 5' to T2A/furin. A BGH polyA-addition site was PCR amplified from pCDNA4/myc-His (Invitrogen) with primers TD598/TD599, and isothermal ligation was used to insert the sequence 3' of eGFP (Gibson *et al.* 2010a,b). The LoxP-flanked stop cassette, which contained stop codons and a triplicate repeat of SV40 polyA-addition sites, was amplified from Ai2 plasmid (Addgene #22796) using TD595/TD596 primers and was cloned into a NheI site immediately 3' of the Ubc promoter by traditional molecular cloning (Madisen *et al.* 2010). Enzymes for plasmid construction were from New England Biolabs (Ipswich, MA, USA), and primers synthesized at Integrated DNA Technologies (Coralville, IA, USA). Critical regions of the plasmid including *Pou5f1*, T2A, eGFP and LoxP sites were confirmed by Sanger sequencing. The plasmid design was validated by cotransfecting HEK293 cells with the indicated plasmids using Lipofectamine2000 following manufacturer's guidelines (Thermo Fisher) and lysing cells 3 days after transfection. Ngn3-Cre (DM#259) was from Addgene and Ubc-Cre was constructed in our lab (Gu *et al.* 2002).

cOct4 transgenic mouse production and genotyping

The cOct4 transgene fragment was prepared for injection by *AccI*/*PvuII* digestion and gel purification. The linearized transgene was injected into pronuclei of C57BL/6 embryos using standard procedures at the UT Southwestern Medical Center Transgenic Core facility (Dallas, TX, USA). Fifty-five candidate mice were screened by PCR and 11 founders were obtained, five of which could be bred and transmitted the transgene to the next generation. Genotyping was performed on ear, tail or neonatal toe-derived DNA using standard PCR and gel analysis procedures. Alternatively, the quick-release reagent from Phire Animal Tissue Direct PCR kit (Thermo Fisher) in combination with quantitative PCR analysis with Maxima Sybr (Thermo Fisher) on a 480 LightCycler (Roche) was employed for routine genotyping, using wild-type genomic DNA as a comparison. [Supplementary Table 2](#) lists the primers used, and primers for *Stra8*-Cre and *Vasa*-Cre genotyping were described previously ([Gallardo et al. 2007](#), [Sadate-Ngatchou et al. 2008](#)). Transnetyx genotyping service was also used.

For PCR analysis of genomic DNA from cauda epididymal-derived swimming spermatozoa, DNA extraction was performed using a published method ([Griffin 2013](#)). In brief, lysis was conducted by adding extraction buffer (4.2 M guanidine thiocyanate, 100 mM NaCl, 1% Sarkosyl, 150 mM DTT and 200 µg/mL Proteinase K) and incubating at 56°C for 2 h and then the DNA was precipitated with isopropanol and rehydrated. A transgene-specific primer pair (TD535/YZ2) that flanked the floxed stop cassette was used to detect the unexcised vs excised versions of the transgene by amplification using Herculase II polymerase (Agilent), and products were analyzed with gel electrophoresis.

Cell dissociation and sorting

Testes were dissected and rinsed in DMEM/F-12 medium, the tunica albuginea was removed and seminiferous tubules were separated with forceps. After rinsing twice in DMEM/F-12, tubules were digested in 1 mg/mL Collagenase Type IV (Sigma) with 7 mg/mL DNase for 15 min at 37°C. After washing twice in Hanks' balanced salt solution, tubules were incubated in 0.25% trypsin/EDTA with 7 mg/mL DNase I (Sigma) for 20 min at 37°C. The digestion was stopped by adding approximately one-tenth volume fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and mixing vigorously. Cell samples were strained with a 40 micron strainer and then spun and resuspended in PBS with 2% (w/v) FBS. Dissociated cells were incubated for 25 min with antibodies or isotype controls diluted in PBS with 2% (w/v) FBS. [Supplementary Table 1](#) lists antibodies used. Sorting was performed on an Aria II (BD Bioscience, San Jose, CA, USA).

Immunoblotting

Cells or tissue were washed in PBS before lysis. For sorted cells, equal numbers (50,000–100,000) of cells were resuspended in sample buffer ('SB,' Laemmli buffer with 0.1 M DTT) and boiled at 100°C for 5 min. Testes were lysed in SB using a FastPrep-24 instrument (MP Biochemicals, Santa Ana, CA, USA) with Matrix D beads for 20–60 s at 4 m/s.

Amounts of beads and SB were scaled according to the weight of tissue lysed. Lysates were electrophoresed through a 10% SDS-PAGE gel and transferred onto PVDF membrane using standard procedures. Following blocking in 5% milk with PTw (PBS+10% Tween 20), the membrane was incubated in mouse anti-POU5F1 (1:250), washed in PTw, incubated in goat anti-mouse IgG-HRP secondary and washed in PTw. Supersignal West Femto (Thermo Fisher) was used for detection. Blots were stripped using standard procedures and reprobed with anti-tubulin to verify even loading.

Statistical analysis

Mean and standard deviation or standard error mean, as indicated, were calculated with sample sizes described in figure legends. The *t*-test function in Excel (Microsoft) was used to calculate *P*-values based on assumptions of unpaired data with equal variance.

Results

POU5F1 expression in pup and adult spermatogonia

While it is known that *POU5F1* is expressed in undifferentiated spermatogonia, to our knowledge, a direct quantitative comparison of expression during neonatal and adult spermatogenesis has not been reported. Using Oct4-GFP mice, we sorted undifferentiated spermatogonia (THY1+/GFP+) and differentiated spermatogonia (THY1–/GFP+). The equivalent cell populations from pup (dpp 13) and adult testes were compared. We found that *POU5F1* could only be detected in the THY1+ population of cells ([Fig. 1A](#) and [B](#)) and that *POU5F1* levels within this sorted cell population were lower in adult than in pups ([Fig. 1B](#)). Consistent with the immunoblotting, by immunostaining whole seminiferous tubules, we observed dim nuclear staining of endogenous *POU5F1* in cells that coexpressed Oct4-GFP ([Fig. 1D–K](#)). Endogenous *POU5F1* could be detected in both pups and adults, but the *POU5F1*+ cells were very rare in adults ([Fig. 1H–K](#)). *POU5F1* was seen in individual, paired and sometimes chains of spermatogonia. Similar results were obtained by immunostaining sections of tubules ([Fig. 1L–S](#)). For unknown reasons, *POU5F1*+ cells were more readily detected in the *Oct4-GFP* reporter mouse strain compared with other strains (data not shown). Our results confirmed published data showing that *POU5F1* is present in undifferentiated spermatogonia, and is typically present in a small subset of A_s and A_{pr} undifferentiated spermatogonia. Furthermore, the rarity of detectable *POU5F1* in adults suggested that the fraction of spermatogonia with *POU5F1* is lower in adults compared with pups.

Immunoblotting and immunostaining approaches both showed that the Oct4-GFP reporter was expressed in a broader group of cells including long chains of spermatogonia where endogenous *POU5F1* could

Figure 1 POU5F1 protein expression in Oct4-GFP pup and adult testes. (A) Dot plot showing cells sorted from heterozygous Oct4-GFP pup (dpp 13) testes and the percentage of parent gate cells for each subpopulation. Parent gates (not shown) include forward and side scatter to select singleton, viable cells. Percentages of the corresponding populations from adult Oct4-GFP mouse are shown in parenthesis. Isotype controls were used to define background fluorescence for the CD90 (THY1) antibody (not shown). (B) POU5F1 (top) or TUBULIN (bottom) immunoblotting of indicated sorted cell fractions. (C) POU5F1 (top) or TUBULIN (bottom) immunoblotting of cultured spermatogonial stem/progenitor GS cells treated for 0, 2 or 4 h with 30 μM MG132. Size standards (kDa) are indicated. (D–S) Immunostaining of whole tubules (D–K) or cryosections (L–S) from dpp 11 (D–G), dpp 13 (L–O) or adult (H–K and P–S) Oct4-GFP heterozygous mice. (D, H, L and P) DAPI (DNA), (E, I, M and Q) GFP immunostaining, (F, J, N and R) POU5F1 immunostaining or (G, K, O and S) merge of GFP and POU5F1. Arrows point to cells with GFP and without endogenous POU5F1 and arrowheads point to cells with both GFP and POU5F1. Bar, 50 μm.

not be detected. One possible explanation was that POU5F1 protein is regulated by posttranslational mechanisms, while the GFP reporter reflects only *Pou5f1* promoter activity but not posttranscriptional/translational regulation. To test whether POU5F1 may be regulated by proteasome-mediated degradation, we used cultured spermatogonia ('GS' cells), which have been shown previously to express POU5F1 heterogeneously, likely reflecting the mixed population of SSCs and partly differentiated transit amplification populations that are present (Kanatsu-Shinohara *et al.* 2003, Dann *et al.* 2008). Treatment with the proteasome inhibitor MG132 led to stabilization of POU5F1, indicating that POU5F1 is subject to downregulation by the proteasome and consistent with the possibility that it may be directly regulated by the destabilizing modification, ubiquitin (Fig. 1C).

Generation of transgenic mice for conditional ectopic expression of POU5F1

Given the known importance of POU5F1 levels in controlling cell fate during early embryogenesis, we hypothesized that downregulation of POU5F1 could be important for the progression of spermatogenesis.

Figure 2 cOct4 transgene design and validation in cultured cells. (A) cOCT4 transgene with Ubiquitin C promoter, LoxP sites (triangles), 3x repeated SV40 polyA stop cassette, *Pou5f1* coding sequence, Myc tag ('m'), T2A ribosomal skip sequence and GFP coding sequence. Following excision by Cre, transcription and translation, it is expected that the POU5F1 protein contains a c-terminal Myc epitope tag and T2A peptide ('POU5F1*') while GFP is unmodified. (B) POU5F1 (top) or MYC (bottom) immunoblotting of HEK293 cells transfected with the indicated plasmids. Size standards (kDa) are indicated.

To test this idea, we created a transgene ('cOct4') designed to ubiquitously express POU5F1 and GFP following Cre-mediated excision of a stop cassette flanked by LoxP sites (Fig. 2A). Transient transfections of the transgene with and without Cre recombinase into cultured cells validated that the transgene produced exogenous POU5F1 and GFP in the presence of Cre (Fig. 2B and data not shown).

Transgenic mice were obtained by DNA injection into pronuclei of C57BL/6 embryos, and several transgenic lines were established for analysis. We initially used a Stra8-Cre driver, which was reported to produce Cre in postnatal spermatogonia and spermatocytes (Sadate-Ngatchou *et al.* 2008). Testes from cOct4;Stra8-Cre mice and sibling controls were analyzed from 1- and 2-month-old mice. Transgene-derived POU5F1 could be detected by immunoblotting of testicular lysates from multiple lines (Fig. 3A and B); occasionally, transgene expression was also observed in cOct4 mice without Stra8-Cre, albeit at lower levels than the doubly transgenic mice, suggesting that there was a low level of expression that presumably occurred by read-through

Figure 3 Transgene expression in cOct4;Stra8-Cre mice. (A and B) POU5F1 immunoblotting of testicular lysates from dpp 31 cOct4 or cOct4;Stra8-Cre mice of line 53 (A) or dpp 66 mice of line 21 (B). Cultured spermatogonial GS cells from wild-type mice or from mice containing cOct4 were used as a comparison. Size standards (kDa) are indicated and the arrows point to endogenous ('e') and transgenic ('t') bands. (C-F) Immunostaining to detect GFP in testes from cOct4 and cOct4;Stra8-Cre dpp 31 mice of line 18. DAPI (DNA) staining (C and E), GFP immunostaining (D and F). Bar, 50 μm.

of the floxed stop cassette (Supplementary Fig. 1 and data not shown). Surprisingly, we did not observe any abnormalities in seminiferous tubule cross sections in cOct4;Stra8-Cre mice (Fig. 3E and data not shown). One possible explanation was that transgene (GFP) expression appeared to be limited to a small subset of tubules, and it was only visible in haploid spermatids but not in spermatogonia (Fig. 3F). Altogether, our analysis of the cOct4;Stra8-Cre mice showed that Cre excision activated the transgene and produced ectopic POU5F1 in testes, but excision may have been inefficient and occurred in later spermatogenic stages than expected. Evidently, the amount of ectopic POU5F1 that was produced was insufficient to lead to a phenotype and/or the presence of POU5F1 in postmeiotic germ cells was inconsequential (see 'Discussion' section).

cOct4;Vasa-Cre mice have a severe disruption in spermatogenesis

Next, we used a Vasa-Cre driver to examine the effect of ectopic POU5F1 during neonatal spermatogenesis. Vasa-Cre male mice were reported to produce Cre in the germ lineage starting during late embryogenesis in gonocytes (Gallardo *et al.* 2007). In contrast to cOct4;Stra8-Cre mice, the cOct4;Vasa-Cre mice exhibited a distinctive defect in spermatogenesis. The testes of adult mice were visibly smaller in cOct4;Vasa-Cre mice for two independent lines (descendants from founders 53 and 18; Fig. 4A and Supplementary Fig. 2). Histological examination of seminiferous tubule cross sections from adult mice showed a dramatic and heterogeneous defect. Many seminiferous tubules contained only spermatogonia and Sertoli cells (Fig. 4E and Supplementary Fig. 2). Occasional tubules contained the complete progression of germ cells from spermatogonia to spermatozoa and others contained no germ cells (Supplementary Fig. 2). Interestingly, the phenotype of seminiferous tubules from a third line of mice (descendants of founder 21) had qualitatively the same array of defects as in line 53 and line 18; however, quantitatively, the phenotype was far less severe with most tubules containing apparently normal spermatogenesis (Supplementary Fig. 2). Analysis of epididymal cross sections revealed similar heterogeneity, with almost no spermatozoa evident in the caput of cOct4;Vasa-Cre mice from lines 18 or 53, and a higher frequency of caput cross sections containing spermatozoa in mice from line 21 (Fig. 4F and Supplementary Fig. 2). Male cOct4;Vasa-Cre mice from lines 18 and 53 were subjected to breeding tests and failed to sire any progeny (Supplementary Fig. 2).

Immunoblotting confirmed the presence of transgene-derived POU5F1 in testicular lysates (Fig. 4B); it should be noted that the samples used in the immunoblotting experiment were total testicular lysates that differed widely in their composition of germ cell numbers and cell types, precluding conclusions about relative levels

Figure 4 Phenotypic analysis of adult cOct4;Vasa-Cre testes. (A) Testes of 2-month-old cOct4;Vasa-Cre mice (line 53 and 18) and sibling controls. (B) POU5F1 (top) and TUBULIN (bottom) immunoblotting of testicular lysates from 5-month-old mice of lines 53, 18 and 21 cOct4;Vasa-Cre and wild type. ES cells were used as a positive control. Size standards (kDa) are indicated and the arrows point to endogenous ('e') and transgenic ('t') bands. (C–F) Hematoxylin/eosin staining of wild type (C and D) and cOct4;Vasa-Cre (E and F) 2-month testes (C and E) and caput epididymis (D and F). Bar, 50 μ m.

between the samples. PCR analysis of spermatozoa recovered from the caput epididymis of cOct4;Vasa-Cre mice from line 21 confirmed the presence of the Cre-excised version of the transgene (Supplementary Fig. 3). We chose to use lines 18 and 53 for additional analysis because these lines had the most severe germ cell loss phenotype.

SSCs self-renew to sustain spermatogenesis in the long term in cOct4;Vasa-Cre mice, but germ cell differentiation is disrupted

Immunostaining was used to further define the extent of germ cell loss in adult cOct4;Vasa-Cre mice. TRA98 was used to detect all germ cells, SALL4 and GFRA1 to detect undifferentiated spermatogonia and CREM- τ to detect meiotic cells (Foulkes *et al.* 1992, Tanaka *et al.* 1997, Gassei & Orwig 2013). Most tubule cross sections contained at least one TRA98-positive cell and the pattern and number of germ cells present were variable. Interestingly, we frequently (~35% of 509 tubules in 13 mice aged 2–12 months) saw

Figure 5 Immunostaining of adult cOct4;Vasa-Cre testes. (A, B, C, D, E and F) SALL4 (green) and TRA98 (red) coimmunostaining to detect undifferentiated spermatogonia in wild-type (A–C) and cOct4;Vasa-Cre (D–F) 2-month mice. (G) Mean percentage of tubule cross sections with no germ cells (SCO), only SALL4+ germ cells ('SALL4+ only'), partial germ cell recovery and full germ cell recovery in 2 month ($n=6$ mice), 5 month ($n=3$ mice) and 10–12-month ($n=4$ mice) cOct4;Vasa-Cre. 240, 109 and 160 tubules were scored for 2, 5 and 10–12 months respectively. (H) Mean percentage of tubule cross sections with no CREM staining ('No CREM'), some CREM staining but abnormal pattern ('CREM+/Partial') or CREM staining in a normal pattern including nuclei/DAPI with morphology of elongating spermatids ('CREM+/Full'). 1652, 387 and 548 tubules were scored for 2, 5 and 10–12-month mice respectively. (I and J) CREM (haploid spermatids) and TRA98 coimmunostaining in cross sections from wild-type (I) and cOct4;Vasa-Cre (J) 2-month mice. Images shown are a merge of CREM (green), TRA98 (red) and DAPI (blue). (K and L) SALL4 and GFRA1 co-immunostaining in whole seminiferous tubules from wild-type (K) and cOct4;Vasa-Cre (L) 2-month mice. Images shown are a merge of SALL4 (green), GFRA1 (red) and DAPI (blue). Arrows point to GFRA1+ spermatogonia. Bar, 50 μ m.

tubule cross sections where 100% of the TRA98 cells costained with SALL4. Therefore, in these tubules, undifferentiated spermatogonia were the sole remaining germ cell type (Fig. 5E–G). Consistent with the idea that a block occurred during spermatogonial differentiation, most tubules (~70% of 2587 tubules in 13 mice aged 2–12 months) also lacked CREM-tau staining (Fig. 5H).

Based on SALL4 and TRA98 immunostaining, we categorized the patterns visible in tubule cross sections as having no TRA98+ germ cells ('Sertoli cell only' or SCO), only SALL4+/TRA98+ germ cells ('SALL4 only'), partially recovered spermatogenesis (some TRA98+/SALL4+ cells, but in an abnormal pattern) or fully recovered spermatogenesis (normal pattern). Comparison of 2-month-old mice with older mice revealed similar frequencies of tubules with only SALL4+ germ cells (Fig. 5G). The continued presence of undifferentiated spermatogonia, including mice older than 10 months, suggested that SSC self-renewal occurred in cOct4;Vasa-Cre. Further supporting this conclusion, GFRA1, another marker of undifferentiated spermatogonia and SSCs, persisted throughout adulthood (Figs 5L and 6C–F) (Hofmann *et al.* 2005, Nakagawa *et al.* 2010).

While the majority of tubules lacked meiotic cells, there was a significant number of tubules that appeared to contain normal spermatogenesis. The presence of some tubules with CREM+ cells and elongating spermatids ('CREM+/Full') may have been caused by insufficient

overexpression of POU5F1, and/or overriding regulatory mechanisms that downregulate POU5F1 or work redundantly to block differentiation/spermatogenesis (Fig. 5H–J). Nonetheless, spermatogonial differentiation and germ cell maturation were greatly disrupted in the majority of tubules, showing that POU5F1 downregulation is an important step for the progression of spermatogenesis.

The germ cell population begins to be reduced in neonates around dpp 4

Given the severe lack of germ cells in adult cOct4;Vasa-Cre mice, we sought to identify the earliest point in spermatogenesis that was affected. Histological analysis revealed an overt phenotype beginning at dpp 13. While spermatocytes were evident in controls at dpp 13, cOct4;Vasa-Cre mice

Figure 6 GFRA1 immunostaining in cOct4;Vasa-Cre. (A–F) GFRA1 immunostaining of whole seminiferous tubules from dpp 13 (A and B), 2 month (C and D) and 10 month (E and F) in wild type (A, C and E) and cOct4;Vasa-Cre (B, D and F). Bar, 50 μ m.

Figure 7 Phenotypic analysis of neonatal cOct4;Vasa-Cre testes. Hematoxylin/eosin staining of dpp 0 (A and B), dpp 6 (C and D), dpp 13 (E and F) and dpp 31 (G and H) testes from control (A, C, E and G) and cOct4; Vasa-Cre (B, D, F and H) mice. Wild-type sibling controls are shown except in (C), which is a cOct4 sibling control. Bar, 50 μ m.

Figure 8 Germ cell loss in neonates of cOct4;Vasa-Cre. TRA98 immunostaining to detect germ cells in seminiferous tubule cross sections at dpp 0 (A and B), dpp 4 (D–F) and dpp 6 (G–I). Images shown are a merge of DAPI (blue) and TRA98 (red) and are from wild-type or Vasa-Cre controls (A, D and G) and cOct4;Vasa-Cre (B, E and H) mice. (C, F and I) Histograms show the mean and standard error mean of the number of TRA98+ cells present per tubule cross section for 80 wild-type tubules (2 mice) and 73 cOct4;Vasa-Cre tubules (2 mice) at dpp 0, for 74 wild-type tubules (2 mice) and 234 cOct4;Vasa-Cre tubules (5 mice) at dpp 4, and for 144 wild-type tubules (3 mice) and 320 cOct4;Vasa-Cre tubules (6 mice) at dpp 6. Quantitative analysis was restricted to circular (transverse) tubule cross sections. *P*-values are shown; Bar, 50 μ m.

had a scarcity of meiotic cells (Fig. 7E–F). Testes from dpp 6 appeared generally normal, with spermatogonia being present at the basal membrane as expected (Fig. 7C–D). However, following immunostaining with pan-germ cell markers, it became apparent that there were fewer germ cells in cOct4;Vasa-Cre mice at dpp 6 and dpp 4 (Fig. 8C–F and Supplementary Fig. 4). In contrast, the number of gonocytes at dpp 0 was similar in cOct4;Vasa-Cre compared with controls (Fig. 8A and B). Our analysis showed that the first point in postnatal development when downregulation of POU5F1 is critical is during the establishment of the first spermatogonia from gonocytes.

Gonocytes undergo an abnormal transition to spermatogonia during the initiation of the first wave of spermatogenesis

Several important events for the establishment of spermatogenesis occur during dpp 4 to dpp 6. Some gonocytes are transitioning to form the first pool of SSCs. Concurrently, other gonocytes are thought to directly differentiate. Recently, Niedenberger and coworkers proposed a model in which GFRA1 marks the first SSCs, while GFRA1-negative cells represent the differentiated spermatogonia that constitute the first wave of spermatogenesis (Niedenberger *et al.* 2015). Given the normal presence of endogenous POU5F1 in gonocytes and undifferentiated spermatogonia, we predicted that the SSC pool would be established normally despite the presence of cOCT4;Vasa-Cre. To assess whether the establishment of the first SSC pool was affected, we performed GFRA1 double staining

with a pan-germ cell marker (TRA98 and SALL4 in neonates) to determine the fraction of total germ cells with GFRA1. By assessing the fraction, rather than the absolute number of GFRA1+ cells, we accounted for the observation that in cOct4;Vasa-Cre mice, the total germ cell population was reduced. Consistent with our prediction, while the total number of germ cells was smaller, the fraction that was GFRA1+ was not significantly different between cOct4;Vasa-Cre and GFRA1+ tended to even be higher in cOct4;Vasa-Cre (Fig. 9A–G). Similar GFRA1+ staining results were obtained using whole tubule immunostaining approaches (Supplementary Fig. 4). Then, we tested whether the reduction in total germ cell number could be explained by a block in differentiation in cOct4;Vasa-Cre mice. Differentiation can be visualized by the onset of STRA8 expression (Zhou *et al.* 2008, Niedenberger *et al.* 2015). While STRA8+ cells were clearly less frequent in cOCT4;Vasa-Cre, and the fraction of STRA8+ germ cells tended to be lower, the difference was not significantly significant (Fig. 9H–N). In wild-type mice, GFRA1 and STRA8 immunostaining are generally expected to be in mutually exclusive subsets of spermatogonial populations – namely, the undifferentiated and differentiated spermatogonia respectively (Niedenberger *et al.* 2015). However, in cOct4;Vasa-Cre mice, at dpp 6, we frequently observed GFRA1+ cells that were also STRA8+ (Fig. 9O–U). The presence of STRA8 and GFRA1 in the same cells likely indicates abnormalities in the differentiation program and may explain the inability to detect a clear alteration in the fractions of germ cells comprising the undifferentiated and differentiated spermatogonia.

Figure 9 Fractions of undifferentiated and differentiated spermatogonia during first wave of spermatogenesis. (A, B, C, D, E and F) GFRA1 (green) and TRA98 (red) coimmunostaining to detect undifferentiated spermatogonia in wild type (A–C) and cOct4;Vasa-Cre (D–F). (G) Percentage of TRA98+ cells costained with GFRA1. Histogram shows mean and standard deviation for three wild-type mice (1130 TRA98+ cells) and three cOct4; Vasa-Cre mice (752 TRA98+ cells). (H, I, J, K, L and M) STRA8 (green) and TRA98 (red) coimmunostaining to detect differentiated spermatogonia in wild type (H–J) and cOct4;Vasa-Cre (K–M). (N) Percentage of TRA98+ cells costained with STRA8. Histogram shows mean and standard deviation for four wild-type mice (2627 TRA98+ cells) and five cOct4;Vasa-Cre mice (1711 TRA98+ cells). (O, P, Q, R, S and T) GFRA1 (green) and STRA8 (red) coimmunostaining in wild type (O–Q) and cOct4;Vasa-Cre (R–T). Arrows point to abnormally large nuclei. (U) Percentage of stained cells (either GFRA1+ or STRA8+) having costaining of GFRA1 and STRA8. Histogram shows mean and standard deviation for two wild-type mice (590 stained cells) and two cOct4; Vasa-Cre mice (403 stained cells). *P*-values are shown. Bar, 50 μ m.

cOct4;Vasa-Cre leads to anomalies in spermatogonial survival, cell size and chain morphology

Along with having fewer spermatogonia in cOct4;Vasa-Cre neonatal mice, we noticed several other anomalies. We observed a slight increase in apoptosis at dpp 4 (Supplementary Fig. 5); however, the amount of apoptosis was unlikely to fully explain the dramatic reduction in germ cell numbers visible at dpp 6. We also frequently observed abnormally large nuclei at dpp 6, perhaps indicative of a cell cycle regulation defect that led to endoreduplication (Fig. 9R, S arrows and Supplementary Fig. 5). At dpp 13, cOct4;Vasa-Cre mice appeared to have an increase in the chain length and density of GFRA1+ cells, also suggestive of a defect in cell cycle regulation in the GFRA1+ population (Fig. 6B). Unusually long chains of GFRA1+ spermatogonia were also observed in adult mice (Fig. 6C–F). Altogether, these results suggest that spermatogonia in cOct4;Vasa-Cre proliferate abnormally and fail to appropriately make the transition to a differentiated state during the first wave of spermatogenesis.

Discussion

While POU5F1 is a well-known regulator of the pluripotent state, its function in the germ lineage has been less clear. Here, we focused on the postnatal expression and function of POU5F1. We found that POU5F1 expression in adults was downregulated compared with pups and that regulation of its levels in spermatogonia was proteasome dependent. Additionally, we showed that the regulation of POU5F1 expression was critical during the transition of gonocytes to differentiated spermatogonia and ultimately for the generation of meiotic germ cells. Substantial reductions in germ cells were observed by dpp 6, although interestingly the GFRA1+ undifferentiated spermatogonia population was largely spared. Consistent with the presence of self-renewing stem cells, a population of undifferentiated spermatogonia was maintained into late adulthood (up to 12 months), and some of these SSCs escaped a differentiation block, leading to spermatogenic recovery in a few tubules. Nonetheless, a block in differentiation continued to be a major phenotype in adults, suggesting that the presence of ectopic POU5F1 during steady-state spermatogenesis had an inhibitory effect on differentiation, similar to its effect during the first wave of differentiation in neonates.

Using a variety of approaches, including quantitative comparisons of isolated cell populations, we showed that endogenous POU5F1 is detectable in both adult and neonatal undifferentiated spermatogonia. While we could detect low levels of POU5F1 in adult THY1+ cells by immunoblotting, and rare POU5F1+ cells in adults by two immunostaining approaches, POU5F1 was less abundant in adults compared with pups. Our data

support the published data showing that POU5F1 is frequently but not exclusively present in A_s and A_{pr} cells and not observed in long chains of differentiated spermatogonia (Filipponi *et al.* 2007). In contrast, GFP expression in the widely used Oct4-GFP reporter mouse strain is present in differentiated spermatogonia in pups and adults. Hence, cells with endogenous POU5F1 are far less frequent than GFP+ cells in Oct4-GFP mice, which exhibit GFP in A_s through A_{dif} ($A_{differentiated}$) stages of spermatogonia (Ohbo *et al.* 2003, Garcia & Hofmann 2012, Azizi *et al.* 2016). Immunoblotting of sorted cells confirmed that KIT+ differentiated spermatogonia express Oct4-GFP but not endogenous POU5F1 (Y Zheng and CT Dann, unpublished data). Altogether, the data support the notion that endogenous POU5F1 is present in a limited number of undifferentiated spermatogonia and that its overall expression diminishes over time from pup to adulthood.

The number of proteins like POU5F1 whose expression is limited to a subset of predominantly A_s and A_{pr} undifferentiated spermatogonia is growing and includes GFRA1, ID4 and PAX7 (Nakagawa *et al.* 2010, Oatley *et al.* 2011, Grasso *et al.* 2012, Aloisio *et al.* 2014). Whether individual cells expressing POU5F1 are the particular undifferentiated spermatogonia with stem cell activity is a challenging question. However, using transgenic reporter approaches and/or immunostaining and cell sorting, it was shown that populations of cells expressing Oct4-GFP or the other aforementioned proteins are enriched in stem cells (Ohbo *et al.* 2003, Ebata *et al.* 2005, Aloisio *et al.* 2014). Testing the relevance of each of these proteins for stem cell function is yet another question with significance but greatly difficult to answer because of the widespread roles each of these proteins plays during development.

Using an shRNA knockdown approach, we showed previously that *Pou5f1* is required for SSC maintenance *in vitro*. In this study, we used an overexpression approach to test the function of POU5F1 in spermatogenesis *in vivo*. We used Stra8-Cre and Vasa-Cre drivers to remove the stop cassette from the transgene in the postnatal germ lineage, thereby forcing the expression of POU5F1 in all germ cells derived from cells having Cre-mediated excision. While Cre expression from the male germ cell-specific driver, Stra8-Cre, was originally reported to start at dpp 3 and continue through spermatocytes, recent reports suggest that excision may only begin to occur in slightly differentiated spermatogonia (Sadate-Ngatchou *et al.* 2008, Hobbs *et al.* 2015). Additionally, Bao and coworkers showed that incomplete excision occurs in the presence of two floxed alleles, but not one allele, leading to mosaicism (Bao *et al.* 2013). In cOct4;Stra8-Cre testes, in our study, we observed transgene expression (GFP) in spermatids in only a small subset of seminiferous tubule cross sections and there was no apparent phenotype. The reason for mosaic transgene expression in cOct4;Stra8-Cre testes

may be a consequence of incomplete excision related to transgene copy number, variable Ubc promoter activity or weak GFP expression inherent in the use of T2A for producing two proteins from a single promoter. Still, the presence of some exogenous POU5F1 (detected by immunoblotting) combined with the GFP expression data suggest that the presence of ectopic POU5F1 in haploid spermatids in cOct4;Stra8-Cre mice may be inconsequential. In contrast, the testes of cOct4;Vasa-Cre mice exhibited a striking phenotype. Our contrasting results when using the Stra8-Cre driver compared with the Vasa-Cre driver is similar to the experience recently reported by Hobbs and coworkers, who were also using the Stra8-Cre and Vasa-Cre drivers to study the effect of gene perturbation on spermatogonial stem/progenitor cells (Hobbs *et al.* 2015).

Vasa-Cre first leads to excision in males in the late embryonic germ cells and leads to 95% efficient excision by birth (Gallardo *et al.* 2007). While the number and appearance of gonocytes in cOct4;Vasa-Cre mice at dpp 0 were normal, we could discern a modest decrease in the number of germ cells already at dpp 4 and a substantial reduction by dpp 6. A key event that occurs during this stage is that some gonocytes develop into the first round of differentiated spermatogonia. Given that POU5F1 is normally present in undifferentiated spermatogonia, we predicted that forced expression of POU5F1 would not affect undifferentiated spermatogonia but instead may block differentiation. One possibility to explain the lower number of germ cells at dpp 6 following forced expression of POU5F1 is that the differentiation step is blocked. However, cOct4;Vasa-Cre mice still turned on the differentiation markers STRA8 (Fig. 9) and KIT (Y Zheng and CT Dann, unpublished data). We were unable to quantify a major shift in the portions of germ cells from a differentiated (STRA8+) to undifferentiated (GFRA1+) state. The data may have been confounded by the presence of abnormal GFRA1+/STRA8+ spermatogonia, which could be categorized as neither undifferentiated nor differentiated. Nonetheless, the persistent expansion of GFRA1+ cells into adulthood and the continued presence of SALL4+ and GFRA1+ undifferentiated spermatogonia up to 12 months strongly imply that the stem cell population is not affected while major perturbations to differentiation prevail.

Another interesting aspect of the phenotype of cOct4;Vasa-Cre adults is that in some tubules, there is a significant spermatogenic recovery. This is particularly true in one of the three lines analyzed (line 21). We ruled out the possibility that 'leaky spermatogenesis' was a consequence of incomplete Cre excision (Supplementary Fig. 3 and data not shown) using PCR to show that sperm contained the excised version of the transgene. It is likely that the three lines represent an allelic series with higher (lines 53 and 18) and lower (line 21) expression of the transgene. A likely explanation for the leaky phenotype is that there are redundant mechanisms for regulating

differentiation and/or downregulating POU5F1, and the amount of POU5F1 protein produced in cOct4;Vasa-Cre germ cells was insufficient to lead to a complete differentiation block.

POU5F1 expression, regulation and function have been studied extensively in contexts such as ES cells and tumor cells. For instance, in ES cells, POU5F1 protein levels are ultimately determined by a combination of transcriptional control and posttranslational modifications including phosphorylation, sumoylation and ubiquitination (Ramakrishna *et al.* 2014). How POU5F1 levels are controlled in the postnatal and adult male germline remains unclear, but the presence of endogenous POU5F1 in fewer cells than the Oct4-GFP transcriptional reporter suggests that posttranscriptional and/or posttranslational mechanisms are likely to be involved in regulating POU5F1 during spermatogenesis. One explanation for the difficulty in detecting either endogenous or transgene-derived POU5F1 by immunostaining of testes may be related to the labile nature of the POU5F1 protein. Indeed, our data show that POU5F1 levels in cultured SSCs and testicular cells can be boosted by treatment with the proteasome inhibitor, MG132, similar to what has been shown in ES cells by others (Xu *et al.* 2004). POU5F1 may be ubiquitinated and quickly degraded in germ cells. Why POU5F1 could be detected by immunostaining in Oct4-GFP testes more readily than in other strains remains unclear. Further experimentation is necessary to overcome the issue of reliable POU5F1 detection by immunostaining and to dissect the pathways involved in regulating POU5F1 downregulation in spermatogenesis.

Another aspect of POU5F1 function that is of great interest is its potential role in tumorigenesis. POU5F1 is often aberrantly overexpressed in germ cell tumors, and in an ES cell-derived tumor model, the dose of POU5F1 was correlated with potential for malignancy (Gidekel *et al.* 2003). In a transgenic mouse model with a doxycycline-dependent expression system, ectopic POU5F1 led to dysplasia in epithelial tissues (Hochedlinger *et al.* 2005). However, in this model, it was not possible to address the role of ectopic POU5F1 in the germ lineage because doxycycline exposure did not result in POU5F1 overexpression in the testes, presumably because the blood–testes barrier prevented exposure of germ cells to doxycycline. Somewhat surprisingly, germ cell tumors were not observed in four cOct4;Vasa-Cre mice analyzed to date (10+ months); however, future studies may still be warranted (data not shown).

In summary, our results reveal the importance of POU5F1 regulation during neonatal and adult spermatogenesis. Just as POU5F1 levels must be strictly regulated during early embryogenesis for proper fate determination, the timing and levels of POU5F1 must be strictly controlled to allow the appropriate differentiation of SSCs into differentiated spermatogonia

capable of undergoing meiosis. Finally, the creation of a transgenic mouse for conditional ectopic expression of POU5F1 may be a useful resource for studying the effect of POU5F1 regulation in other lineages during development or in pathological situations such as tumorigenesis or in studies of reprogramming to pluripotency.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0140>.

Declaration of interest

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

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