

Lgr4-mediated Wnt/ β -catenin signaling in peritubular myoid cells is essential for spermatogenesis

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SUMMARY

Peritubular myoid cells (PMCs) are myofibroblast-like cells that surround the seminiferous tubules and play essential roles in male fertility. How these cells modulate spermatogenesis and the signaling pathways that are involved are largely unknown. Here we report that *Lgr4* is selectively expressed in mouse PMCs in the testes, and loss of *Lgr4* leads to germ cells arresting at meiosis I and then undergoing apoptosis. In PMCs of *Lgr4* mutant mice, the expression of androgen receptor, alpha-smooth muscle actin and extracellular matrix proteins was dramatically reduced. Malfunctioning PMCs further affected Sertoli cell nuclear localization and functional protein expression in *Lgr4*^{-/-} mice. In addition, Wnt/ β -catenin signaling was activated in wild-type PMCs but attenuated in those of *Lgr4*^{-/-} mice. When Wnt/ β -catenin signaling was reactivated by crossing with *Apc*^{min/+} mice or by Gsk3 β inhibitor treatment, the *Lgr4* deficiency phenotype in testis was partially rescued. Together, these data demonstrate that *Lgr4* signaling through Wnt/ β -catenin regulates PMCs and is essential for spermatogenesis.

KEY WORDS: Wnt, Peritubular myoid cell, Spermatogenesis

INTRODUCTION

Peritubular myoid cells (PMCs) are a group of mesenchymal cells with a very flat morphology that express specific markers of both fibroblasts and smooth muscle cells (Holstein et al., 1996). These cells form the outer border of the seminiferous tubules and, in conjunction with Sertoli cells, are responsible for tubular contractility and sperm transport. The abundant expression of smooth muscle cell cytoskeletal proteins, including desmin, smooth muscle-specific myosin, and smooth muscle actin, facilitates the contractile ability of PMCs (Maekawa et al., 1996). One of the main functions of PMCs is the propulsion of testicular fluid containing spermatozoa towards the rete testis (Maekawa et al., 1996). The contraction of PMCs is tightly controlled by endothelin 1, Tgfb β , angiotensin II, and other hormones in endocrine, paracrine and autocrine fashion (Rossi et al., 2002; Santemma et al., 2001; Tung and Fritz, 1991).

PMCs not only serve as target structural cells of multiple hormones, but also secrete a number of substances to modulate the testicular microenvironment. By secretion of extracellular matrix (ECM) proteins, such as laminin, fibronectin, type I and IV collagens, and proteoglycans, PMCs form the basement membrane, which is implicated in the regulation of spermatogonial stem cells (SSCs) (Richardson et al., 1995; Shinohara et al., 1999). As mesenchymal cells, PMCs synthesize several secretory factors,

among which PModS (peritubular factors that modulate Sertoli cell function) have been reported to modulate the secretion of transferrin, inhibin and androgen-binding proteins by Sertoli cells (Verhoeven et al., 2000). Other PMC-derived factors, such as bFGF (Fgf2), Igf1 and several cytokines, share most of the PModS effects on Sertoli cells, suggesting the importance of paracrine regulation of Sertoli cells by PMCs.

These clues have suggested that PMCs are important for the regulation of male fertility, but little is known about the particular genes or pathways in PMCs that modulate spermatogenesis in genetic mouse models. It has been shown that specific deletion of androgen receptor (*Ar*) in PMCs impairs Sertoli cell function and results in azoospermia and male infertility, suggesting that androgen signaling in PMCs plays a pivotal role in the modulation of spermatogenesis (Welsh et al., 2009; Zhang et al., 2006). However, the other signaling pathways that regulate *Ar* in PMCs to modulate male fertility remain obscure.

Lgr4 (also known as *Gpr48*), is a former orphan receptor involved in various physiological functions. Our laboratory and others reported that loss of *Lgr4* results in reduced viability (Mazerbourg et al., 2004) and in developmental defects in multiple organs, including the kidney (Kato et al., 2006; Wang et al., 2011), eye (Weng et al., 2008), bone (Luo et al., 2009), blood (Song et al., 2008), male reproductive tract (Li et al., 2010; Mendive et al., 2006) and intestine (de Lau et al., 2011; Mustata et al., 2011). Recently, three groups independently reported that R-spondins, which are secreted Wnt signaling agonists, are the endogenous ligands for *Lgr4*, *Lgr5* and *Lgr6* to activate Wnt/ β -catenin and Wnt/planar cell polarity (PCP) signaling (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011). Wnts are a family of secreted morphogens involved in the regulation of a variety of developmental processes (Clevers, 2006). It is reported that *Wnt4* is required for the initial stages of Sertoli cell differentiation and maturation (Jeays-Ward et al., 2004), whereas *Wnt5a* regulates SSC self-renewal through β -catenin-independent mechanisms (Yeh et al., 2011). With a genetic mouse model expressing constitutively activated β -catenin in Sertoli cells, two groups demonstrated that hyperactivation of Wnt/ β -

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catenin signaling leads to male infertility due to germ cell loss (Boyer et al., 2008; Tanwar et al., 2010), but the specific role that Wnt/ β -catenin signaling plays in other cell types in spermatogenesis is yet to be determined. To our knowledge, no report has examined Wnt/ β -catenin activity in PMCs, and its role, if any, in PMC function.

Here we report that *Lgr4* is a PMC-specific gene in the postnatal mouse testis, and that a hypomorphic mutant of *Lgr4* results in defective germ cell differentiation during the first wave of spermatogenesis. In *Lgr4* mutant PMCs, the genes associated with contractile function and secretion of ECM, as well as Ar, were dramatically downregulated. Dysfunctional PMCs further affected Sertoli cell function. We also found that Wnt/ β -catenin signaling was activated in wild-type PMCs but attenuated in those of *Lgr4*^{-/-} mice. Furthermore, the activity of Wnt downstream signaling was restored in *Apc*^{min/+} and *Lgr4* double-mutant mice and in *Lgr4* mutant mice treated with a Gsk3 β inhibitor. Therefore, this study reveals a novel function of Wnt/ β -catenin signaling in PMCs to regulate spermatogenesis, and implicates *Lgr4*, the newly identified R-spondin receptor, in Wnt signaling activation in PMCs.

MATERIALS AND METHODS

Animals

The *Lgr4*^{-/-} mouse strain was generated as previously described (Weng et al., 2008). *Apc*^{min/+} was obtained from the National Resource Center of Mutant Mice (NRCMM) in Nanjing, China. All animal experiments conformed to the regulations drafted by the Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by East China Normal University Center for Animal Research.

β -galactosidase (*lacZ*) staining

Mouse testes were collected and fixed in ice-cold LacZ fixative buffer (2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 in PBS) for 2 hours at 4°C on a shaking platform. After washing in LacZ washing buffer (2 mM MgCl₂, 0.01% deoxycholate, 0.02% NP40 in PBS, pH 8.0) twice, testes were incubated in LacZ staining buffer (0.5 mg/ml X-gal dissolved in LacZ wash buffer) overnight at room temperature. Tissues were then fixed in 4% paraformaldehyde (PFA) and sectioned for histological analysis.

Testis transplantation

The method for testis transplantation is similar to that published previously (Honaramooz et al., 2002; Schlatt et al., 2003). Briefly, donor testes were dissected from neonatal pups, which were sacrificed by decapitation. Fifteen male pups of each genotype were used for this experiment. Testes were cut in half and inserted under the back skin of recipient immunodeficient NCr mice ($n \geq 8$). Castrated recipients receiving grafts were analyzed at 4 weeks and the testicular tissue was dissected from the skin, weighed and fixed in 4% PFA and then sectioned for histological analysis.

Immunofluorescence (IF) and immunohistochemistry (IHC)

Testes were collected and fixed in 4% PFA overnight, dehydrated in 70% ethanol, and embedded in paraffin. Sections (4 μ m) were deparaffinized in xylene and rehydrated in gradient alcohols. Endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol for 20 minutes and then washed in PBS. After antigen retrieval by boiling the slides in antigen retrieval buffer [10 mM sodium citrate buffer (pH 6.0); or 10 mM Tris base, 1 mM EDTA (pH 9.0)], the sections were blocked with 1% BSA and incubated with primary antibody overnight at 4°C. After washing in PBS, the sections were incubated with secondary antibody (10 minutes for IHC, 1 hour for IF), then sections were developed with DAB and counterstained with Hematoxylin (IHC) or DAPI (IF). For BrdU staining, a similar procedure was carried out with the addition of a 30-minute treatment with 2 M HCl after antigen retrieval. Antibody dilutions: β -gal (1:2000, Rockland), α -SMA (1:250, Abcam), laminin (1:1000, Abcam), desmin (1:200, Dako), fibronectin (1:500, Abcam), Tubb3 (1:1000, Abcam), Scp3 (1:2000, Santa Cruz), Wt1 (1:1000, Abcam), Plzf (1:500, Santa Cruz), Oct4 (1:1000, Santa Cruz), Ar (1:500, Santa Cruz), Gdnf (1:500, Santa Cruz) and β -catenin

(1:100, BD Transduction). To ensure reproducibility of results, testes from at least three animals at each age were used, and sections from *Lgr4*^{-/-} and wild-type littermates were processed in parallel on the same slide on at least three occasions.

RNA isolation and real-time PCR

Whole testes were removed from wild-type and *Lgr4*^{-/-} littermates. Before RNA extraction, each testis was weighed and homogenized with an electronic homogenizer. To allow specific mRNA levels to be normalized per testes and to monitor for the efficiency of RNA extraction, RNA degradation and the reverse transcription step, an external standard was used (Johnston et al., 2004; Wang et al., 2006) of *eGFP* mRNA produced by *in vitro* transcription, with 10 ng added to each testis at the start of the RNA extraction procedure. Total testicular RNA was obtained by tissue homogenization in TRIzol reagent (Takara), followed by RNA precipitation in isopropanol, resuspension in DEPC-treated water and verification as DNA free by PCR. cDNA was synthesized with the Superscript RNase H2 Reverse Transcriptase Kit (Takara) using 2.5 mM random hexamers (Takara). Real-time PCR was performed using the SYBR Green PCR Kit (Takara) on a Mx3005P thermal cycler (Stratagene).

Flow cytometry analysis

The method used for a monocellular suspension of a testicular cell preparation was similar to that of Zhang et al. (Zhang et al., 2006). Briefly, the tunica albuginea was removed, and the seminiferous tubules were minced in PBS to release the testicular cells. Then, the tissue was gently aspirated for 2 minutes and spun down at 800 g for 10 minutes. Cells were suspended in PBS, filtered through 100- μ m nylon mesh, and fixed in cold 70% ethanol overnight. Cells were washed twice with PBS and incubated in 500 μ l 0.2% pepsin for 10 minutes at 37°C. After centrifugation, the cells were stained with 25 μ g/ml propidium iodide (Sigma), followed by 40 μ g/ml RNase (Sigma) for 30 minutes at room temperature. DNA content was analyzed by a FACScan cell analyzer (BD Biosciences) equipped with Cellquest software (BD Biosciences). For the TUNEL assay, we used the Apo-Direct Kit (BD Biosciences) following the manufacturer's instructions. At least three mice of each genotype were used for each analysis.

BrdU incorporation and TUNEL assay

For BrdU staining, mice were injected intraperitoneally with a solution of BrdU (0.1 mg/g body weight) diluted in PBS. The TUNEL assay was performed on PFA-fixed paraffin-embedded sections according to the manufacturer's instructions using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (cat. #7100, Chemicon).

Primary PMC culture

PMCs were isolated from 3-week-old wild-type and *Lgr4*^{-/-} mice and plated in plastic culture dishes in MEM according to Fernández et al. (Fernández et al., 2008). The cells were treated with or without recombinant R-spondin 1 (cat. #50316-M08H, Sino Biological, China), and then the cells were fixed in 4% PFA for further analysis.

Statistical analysis

Statistical analyses were performed with Prism GraphPad software. Data are presented as mean \pm s.e. Student's *t*-test or one-way ANOVA was used for statistical comparison of means, with $P < 0.05$ defined as statistically significant. All results are presented from at least three independent experiments.

RESULTS

Lgr4 is selectively expressed in PMCs and modulates postnatal testis morphology and seminiferous epithelial development

Our previous studies and those of others have demonstrated that knockout of *Lgr4* (*Gpr48*) in mice results in male infertility mainly as a result of hypoplastic and poorly convoluted tracts of the efferent ducts and epididymis (Li et al., 2010; Mendive et al., 2006). However, it is clear that, in addition to the epididymis, *Lgr4* is strongly expressed in the mouse testes, implying a potential

testicular role of Lgr4 (Hoshii et al., 2007; Van Schoore et al., 2005). To explore the function of Lgr4 in spermatogenesis, we first confirmed its expression in mouse testes. Since there is no reliable antibody to detect endogenous Lgr4 expression, we took advantage of our *Lgr4* gene trap mouse strain in which a *lacZ* reporter gene is genomically inserted under the control of the native *Lgr4* promoter (Weng et al., 2008). A previous study indicated that Lgr4 is expressed on the surface of seminiferous tubules, but it is not clear by which cell type (Van Schoore et al., 2005). In *Lgr4*^{+/+} mice we found strong *lacZ* expression at the basement of the seminiferous tubules where PMCs reside (Fig. 1A). To confirm that Lgr4 is selectively expressed in PMCs, an immunofluorescence assay with an antibody against β -gal was performed. As shown in Fig. 1B, the β -gal-positive signal was only detected in PMCs, which were characterized by their flattened nuclei. To further determine whether Lgr4 is expressed in Sertoli cells, β -gal-stained testis sections were counterstained with an antibody against Wilms tumor 1 (Wt1), a widely used Sertoli cell marker (Welsh et al., 2009). β -gal-positive signals were not localized in Wt1-positive cells (Fig. 1C), indicating that Lgr4 is expressed selectively in PMCs and not in Sertoli cells.

Next, we examined whether loss of Lgr4 expression in PMCs could affect spermatogenesis. We examined the first wave of

spermatogenesis in prepubertal mice, as *Lgr4* knockout male mice exhibit a water reabsorption failure of the malformed epididymis that secondarily causes a dilated rete testis after puberty and eventually damages the germinal epithelium. In *Lgr4*^{-/-} mice, testicular weight was decreased compared with wild-type (WT) littermates beginning at 2 weeks of age (Fig. 1D). The average weight of *Lgr4* mutant testes was 54.8% and 35.7% of that of the WT mice at postnatal day (P) 14 and P21, respectively, suggesting that Lgr4 modulates prepubertal testes growth. No dramatic testicular phenotype was observed in *Lgr4*^{-/-} mice before P10 (data not shown), but from P14 onwards the diameter of the seminiferous tubules was reduced compared with WT males (Fig. 1E). In addition, the lumens of most seminiferous tubules did not appear until 4 weeks in *Lgr4*^{-/-} mice, but lumens were generally observed in 2-week-old WT mouse testes (Fig. 1E). A reduction in the total volume of germ cells was observed in *Lgr4* mutant seminiferous tubules (Table 1). Our previous data demonstrated that the serum follicle-stimulating hormone, luteinizing hormone and testosterone levels do not change in *Lgr4*^{-/-} compared with WT male mice (Li et al., 2010). Combined with our previous data, these results suggested that loss of Lgr4 impairs postnatal testicular development and that the phenotype is not caused by disruptions in hormonal regulation.

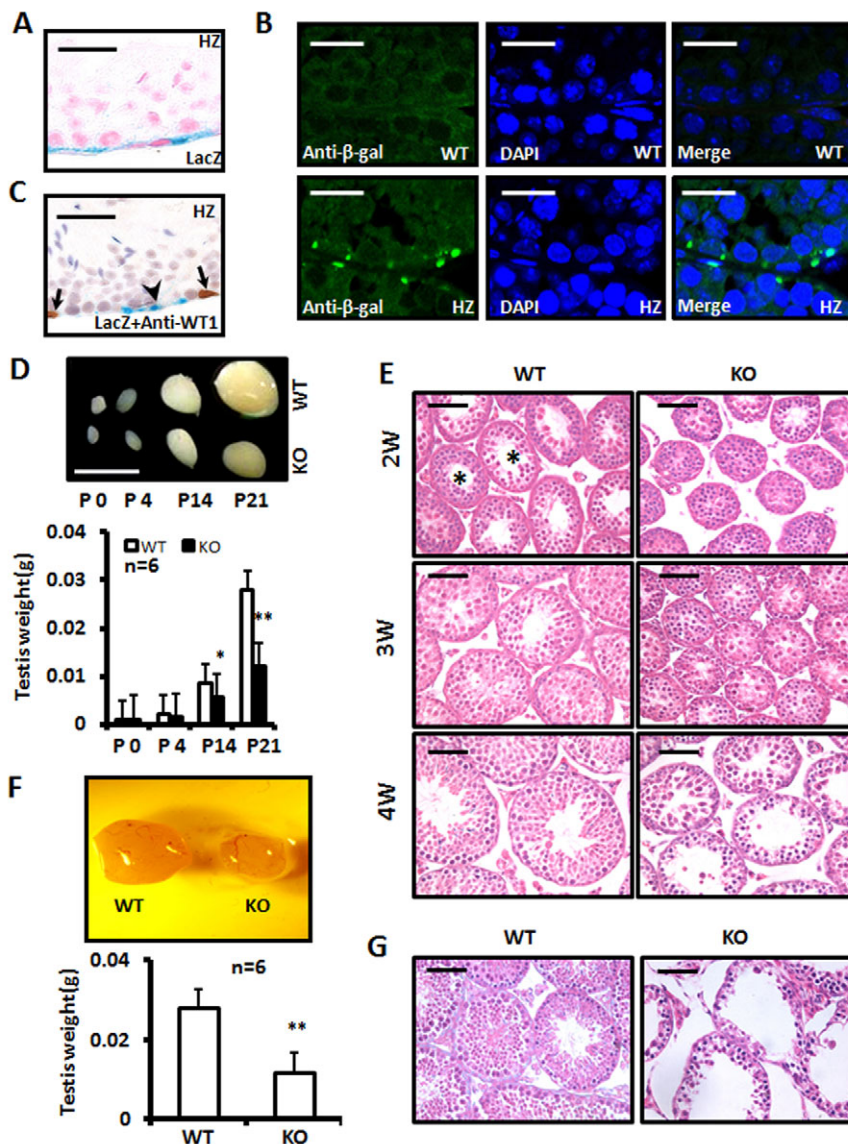


Fig. 1. Lgr4 deficiency impairs postnatal testis development.

(A) Lgr4 expression in testes was detected in PMCs. X-gal staining was performed on testes sections from 4-week-old *Lgr4* heterozygous (HZ) mice; sections were counterstained with Nuclear Fast Red. (B) Immunofluorescence (IF) was performed with an antibody against β -gal on testes sections from 4-week-old wild-type (WT) and *Lgr4* heterozygous mice. Nuclei were stained with DAPI. (C) X-gal-stained (blue) testes sections were counterstained with an antibody against the Sertoli cell marker Wt1 (brown). Arrowhead, PMC; arrows, Sertoli cell. (D) Reduced testis size in *Lgr4*^{-/-} mice (KO) compared with WT littermates on the indicated postnatal days. The average weight of the testes in WT and *Lgr4*^{-/-} mice is presented beneath. (E) Testis histology of WT and *Lgr4*^{-/-} mice at 2, 3 or 4 weeks (W) of age. Hematoxylin and Eosin (H&E) staining showing that the lumen (asterisks) of seminiferous tubules appeared in 2-week-old WT mice but not in *Lgr4*^{-/-} littermates. (F,G) Evaluation of neonatal WT and *Lgr4*^{-/-} testes grafted under the skin of nude mice. The testes of P0 mice of the indicated genotype were grafted as described in Materials and methods. (F) The gross morphology of typical transplants and the weight of the testes transplants from *Lgr4*^{-/-} and WT donors. (G) The histology of H&E-stained transplants. Error bars indicate mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test). Scale bars: 25 μ m in A-C; 5 mm in D; 50 μ m in E,G.

Table 1. Germ cell composition of 3-week-old WT and *Lgr4* knockout mice

Genotype	Spermatogonia	Spermatocyte
WT	0.5±0.2	0.5±0.2
<i>Lgr4</i> ^{-/-}	0.4±0.6	0.8±0.1 (44%)**

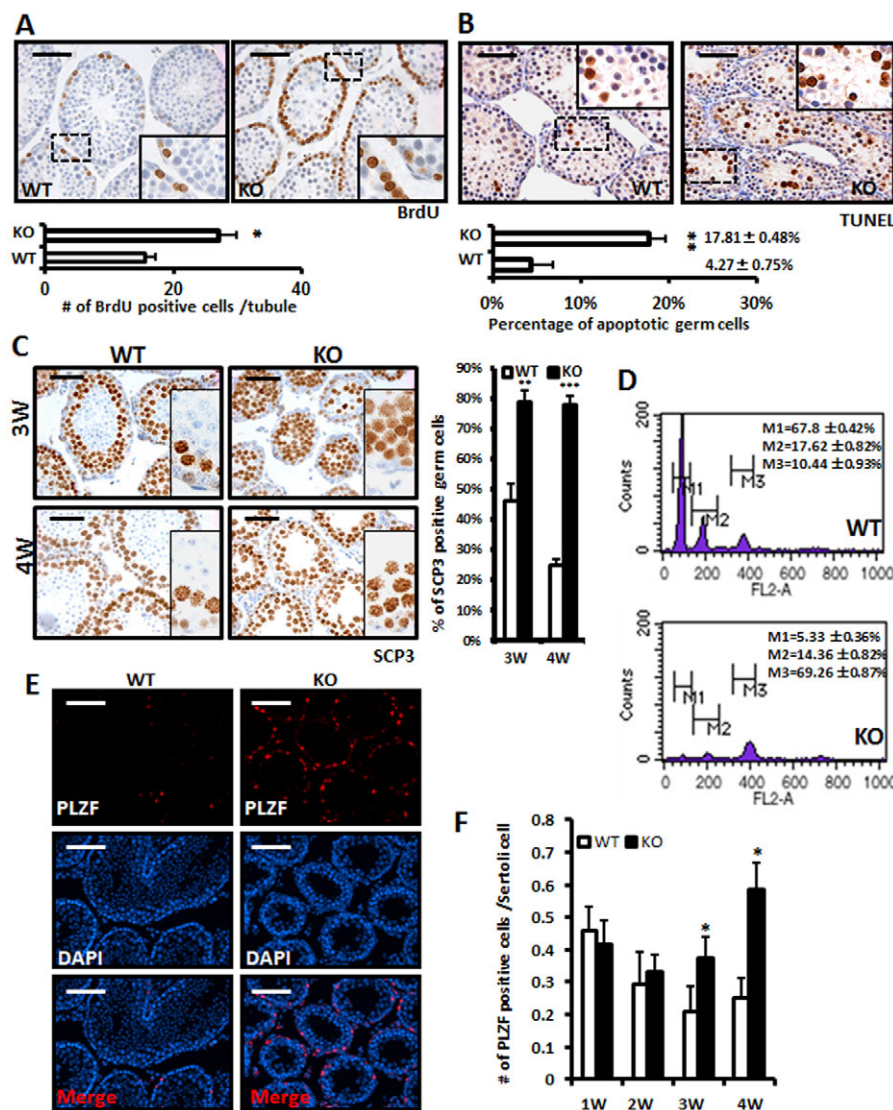
Data are expressed as average nuclear volume, indicative of total germ cell volume per testis. Values are mean ± s.e.m. for five mice. *Lgr4* mutant values as a percentage of WT are indicated in parentheses. ***P*<0.01 versus WT. Spermatid values are not available.

To test whether the defects described above were caused directly by the deficiency of *Lgr4* in the testis or were secondary effects due to reproductive tract malformation we further employed a testes grafting model (Honaramooz et al., 2002). The testes from neonatal WT and *Lgr4*^{-/-} littermates were dissected out and implanted under the dorsal skin of nude mice. Four weeks after grafting, the implants were harvested and analyzed. Consistently, both the volume and the weight of implanted *Lgr4* mutant testes were dramatically reduced (Fig. 1F). As shown in Fig. 1G, the germ cell number and the seminiferous epithelial thickness were dramatically decreased in *Lgr4* mutant testis implants compared with those of WT transplants,

suggesting that *Lgr4* directly modulates postnatal testis morphology and seminiferous epithelial development.

Reduced testes size in *Lgr4*-deficient mice is associated with increased germ cell apoptosis

We speculated that the reduced size of the *Lgr4* mutant testis and the histological morphology defects could be due to decreased cell proliferation and/or increased apoptosis. First, we examined cell proliferation in P28 testes of WT and *Lgr4* mutant mice. At this time point, the Sertoli cells have stopped dividing, efficiently eliminating the interference that these somatic cells might otherwise have on the analysis of germ cell replication (Vergouwen et al., 1991). Two hours after BrdU injection into WT and *Lgr4*^{-/-} mice, the testes were harvested and sectioned. Immunostaining was performed to detect incorporation of BrdU into proliferating cells. To our surprise, compared with WT, the number of BrdU-positive cells was slightly increased in *Lgr4*^{-/-} testes, suggesting that in *Lgr4*-deficient males the proliferation of spermatogonia was activated (Fig. 2A). Moreover, the slightly increased proliferation in *Lgr4*-deficient germ cells was confirmed by immunostaining against proliferating cell nuclear antigen (Pcna), a marker of proliferation that labels cells in the late G1 and S phases of the cell cycle (data not shown).

**Fig. 2. Depletion of *Lgr4* leads to inhibition of germ cell differentiation during the first wave of spermatogenesis.**

(A) BrdU incorporation assays showed that proliferation of germ cells was elevated in *Lgr4*^{-/-} mice at 4 weeks of age. Testes from three mice of each genotype were analyzed. The number of BrdU-positive germ cells was obtained from more than 40 tubules/section and 20 sections/testis. (B) A significant increase of apoptotic germ cells was detected in *Lgr4*^{-/-} seminiferous tubules in 3-week-old mice by TUNEL assays using immunohistochemistry (IHC) or flow cytometry. The boxed regions are magnified in the insets. (C) IHC analysis of testes of 3- and 4-week-old WT and *Lgr4*^{-/-} mice. Scp3 antibody was used and nuclei were counterstained with Hematoxylin. Testes from three mice of each genotype were analyzed. The percentage of Scp3-positive germ cells among total germ cells for each genotype is shown to the right. (D) Flow cytometry analyses of germ cell DNA content of testes from WT and *Lgr4*^{-/-} mice at 4 weeks of age. M1, M2 and M3 represent haploid (1n), diploid (2n) and tetraploid (4n) cells. Note the dramatic decrease of haploid germ cells and significant increase of tetraploid cells in *Lgr4*^{-/-} mice. (E,F) An increase in the number of undifferentiated spermatogonia was detected in *Lgr4*^{-/-} mice. (E) Undifferentiated spermatogonia were labeled with antibody against Plzf in 4-week-old WT and *Lgr4*^{-/-} littermates. Nuclei were stained with DAPI. Statistical analysis showed that the ratio of Plzf-positive cells to Sertoli cells was dramatically increased in *Lgr4*^{-/-} males at 3 or 4 weeks of age (F). Testes from three mice of each genotype were analyzed. The statistical data were obtained from more than 40 tubules/section and 20 sections/testis. Error bars represent the mean ± s.e.m. **P*<0.05, ***P*<0.01, WT versus *Lgr4* knockout mice. (Student's *t*-test). Scale bars: 50 μm.

Next, we performed TUNEL labeling to examine whether more cells undergo apoptosis in *Lgr4*^{-/-} than WT testes. Numerous germ cells undergoing apoptotic cell death were observed in each seminiferous tubule in *Lgr4*-deficient mice, whereas in WT mice few apoptotic cells were observed in individual tubules and the number of tubules containing apoptotic cells was much reduced compared with the *Lgr4*^{-/-} testes (Fig. 2B). Detection of TUNEL-positive cells by flow cytometry also confirmed the increase in apoptotic cell number in *Lgr4*^{-/-} testes (Fig. 2B). The dramatic increase in apoptotic germ cells was further confirmed by immunofluorescence with an antibody against cleaved caspase 3 (supplementary material Fig. S1). These results indicate that in *Lgr4*-deficient testes many germ cells undergo apoptotic cell death and are subsequently eliminated.

Deletion of *Lgr4* in mice inhibits germ cell differentiation

Spermatogenesis is a cyclic process involving the differentiation of spermatogonial stem cells, meiotic cell division and the formation of haploid spermatids. Haploid germ cells were very rare in *Lgr4* mutant testes (Fig. 1C), and we therefore speculated that spermatogenesis was impaired in *Lgr4*-deficient mice. Immunohistology was performed with an antibody against synaptonemal complex protein (Scp3; Sycp3 – Mouse Genome Informatics), which is a marker for all primary spermatocytes (Yuan et al., 2000). As shown in Fig. 2C, the percentage of Scp3-positive germ cells was increased 2- to 3-fold compared with WT littermates at the indicated ages. Numerous Scp3-negative cells were present in the inner layer of the seminiferous epithelium in the WT mouse, whereas in *Lgr4* mutant mice few Scp3-negative germ cells were observed, suggesting that most of the *Lgr4*^{-/-} germ cells were arrested in meiosis I.

Next, we used flow cytometry to analyze the relative distribution of germ cell populations in the testes of *Lgr4*^{-/-} and WT mice. Three main histogram peaks of DNA content were detected, which corresponded to haploid (M1: spermatids and spermatozoa), diploid (M2: spermatogonia, preleptotene primary spermatocytes and secondary spermatocytes) and tetraploid (M3: spermatogonia, leptotene, zygotene, pachytene and diplotene primary spermatocytes) cells. *Lgr4* mutant mice showed a dramatic reduction in haploid cells and a remarkable increase in cells with tetraploid DNA content (Fig. 2D). These results suggest that *Lgr4* regulates spermatogenesis during the first meiosis.

Considering that germ cell differentiation was inhibited in *Lgr4*^{-/-} mice, we postulated that SSC homeostasis might also be affected in *Lgr4*^{-/-} testes. Immunofluorescence analysis was performed with an antibody against Plzf (Zbtb16 – Mouse Genome Informatics), a marker for undifferentiated spermatogonia including SSCs and early progenitor cells. In 4-week-old WT testes, a small number of Plzf-positive undifferentiated spermatogonia were scattered at the base of the seminiferous tubules, but in *Lgr4*^{-/-} testes the number of undifferentiated spermatogonia was dramatically increased (Fig. 2E). To determine when the homeostasis of spermatogonia was affected in *Lgr4* mutant mice, we examined Plzf-positive germ cell number relative to the number of Sertoli cells [which was determined to be comparable in both genotypes (supplementary material Fig. S2)] at different time points during development. Similar relative numbers of undifferentiated spermatogonia/Sertoli cells were observed in both WT and *Lgr4*^{-/-} mice at 1-2 weeks of age, whereas a significant difference was observed in 3- to 4-week-old mice (Fig. 2F). The increase in undifferentiated spermatogonia in *Lgr4* mutant testes was also confirmed by immunohistology with an antibody against Oct4 (Pou5f1 – Mouse Genome Informatics), another widely used marker for SSCs (supplementary material Fig.

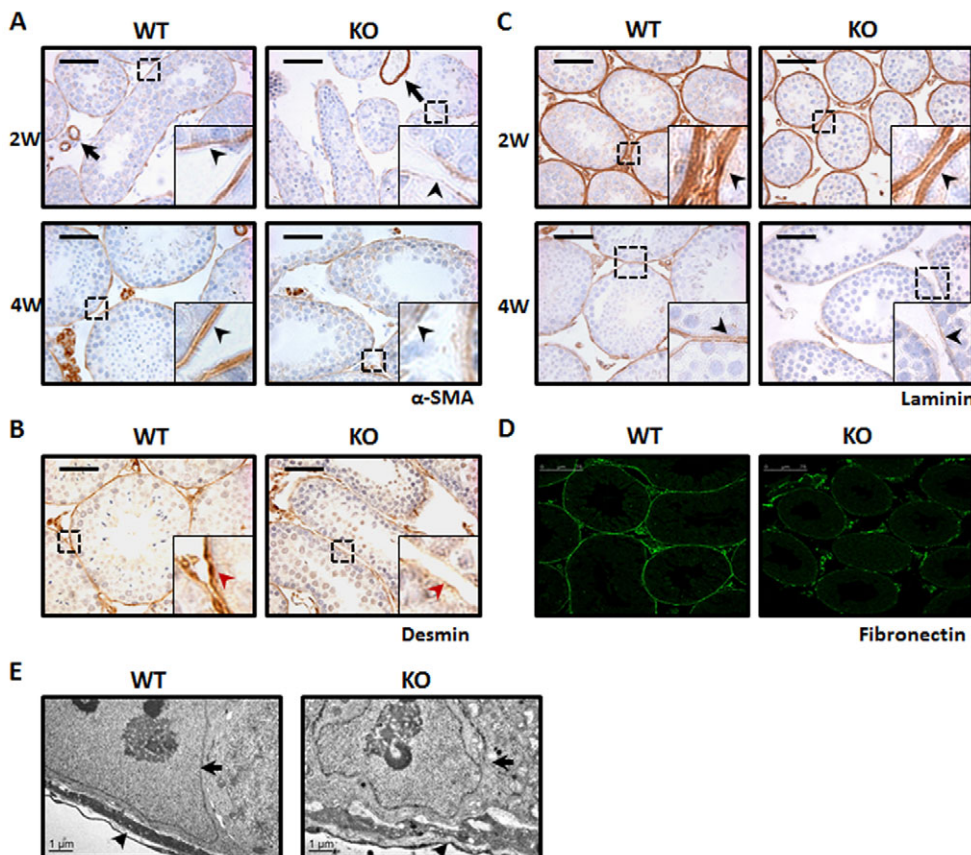


Fig. 3. Depletion of *Lgr4* impairs the expression of marker genes for contraction and adhesion. (A,B) The expression of muscle contractile proteins was decreased in PMCs of *Lgr4*^{-/-} mice. IHCs were performed with antibodies against alpha-smooth muscle actin (α-SMA) (A) and desmin (B) on testes sections from WT and mutant littermates. Expression of α-SMA in PMCs was dramatically reduced (arrowheads) whereas expression was unaffected in blood vessels (arrows). (C,D) Secretion of extracellular matrix (ECM) was downregulated in testes of *Lgr4*^{-/-} mice. Antibodies against laminin (C) and fibronectin (D) were used for IHC and IF assays in WT and mutants. Note that the ECM formed a defined ring at the basement membrane of seminiferous tubules in WT testes, but was impaired in *Lgr4*^{-/-} tubules. Arrowheads indicate the laminin level at the basement membrane of tubules. (E) Transmission electron micrograph showing the structure of the seminiferous tubule basement membrane in 4-week-old WT and *Lgr4*^{-/-} mouse testes. Arrowheads, PMCs; arrows, Sertoli cells. Scale bars: 50 μm in A-D; 1 μm in E.

S3). These data suggested that spermatogenesis was attenuated in *Lgr4* mutant mice, but that the defect caused by *Lgr4* deficiency was indirect as *Lgr4* was specifically expressed in PMCs.

Lgr4 modulates PMC functions

To explore the direct function of *Lgr4* in mouse testes, we examined the characteristics of PMCs in detail. Two major functions of PMCs are contraction for spermatozoa transport and basement membrane production to maintain normal seminiferous tubule morphology. As shown in Fig. 3A,B, expression of the smooth muscle proteins α -SMA and desmin was significantly reduced in *Lgr4*^{-/-} PMCs, whereas no change in their expression was observed in the smooth muscle cells surrounding the blood vessels.

PMCs secrete a number of ECM components, such as laminin, fibronectin and collagen I, IV and XVIII, which contribute to the integrity of the seminiferous tubule basement membrane (Albrecht, 2009; Maekawa et al., 1996). We found that the laminin-labeled ECM was substantially thinner in *Lgr4*^{-/-} than in WT testis, and the

disruption of ECM was progressive and became more apparent at 4 weeks of age (Fig. 3C). Moreover, the expression of fibronectin was also significantly decreased in *Lgr4*^{-/-} testis, with a less well defined 'ring' in the basement membrane compared with that in WT littermates (Fig. 3D). In addition, we performed transmission electron microscopy to examine the structure of the seminiferous tubule basement membrane. As shown in Fig. 3E, compared with WT the *Lgr4*^{-/-} testis ECM was disorganized and PMCs constantly detached from Sertoli cells. These data suggest that *Lgr4* plays an important role in modulating contraction-associated gene expression and ECM secretion in PMCs.

Lgr4 is required for the maintenance of Sertoli cell function

Since ECM and other substances produced by PMCs are crucial for functional Sertoli cells (Maekawa et al., 1996; Thompson et al., 1995), we next explored whether the morphology and function of Sertoli cells are altered in *Lgr4*-deficient mice. We used Wt1 as a

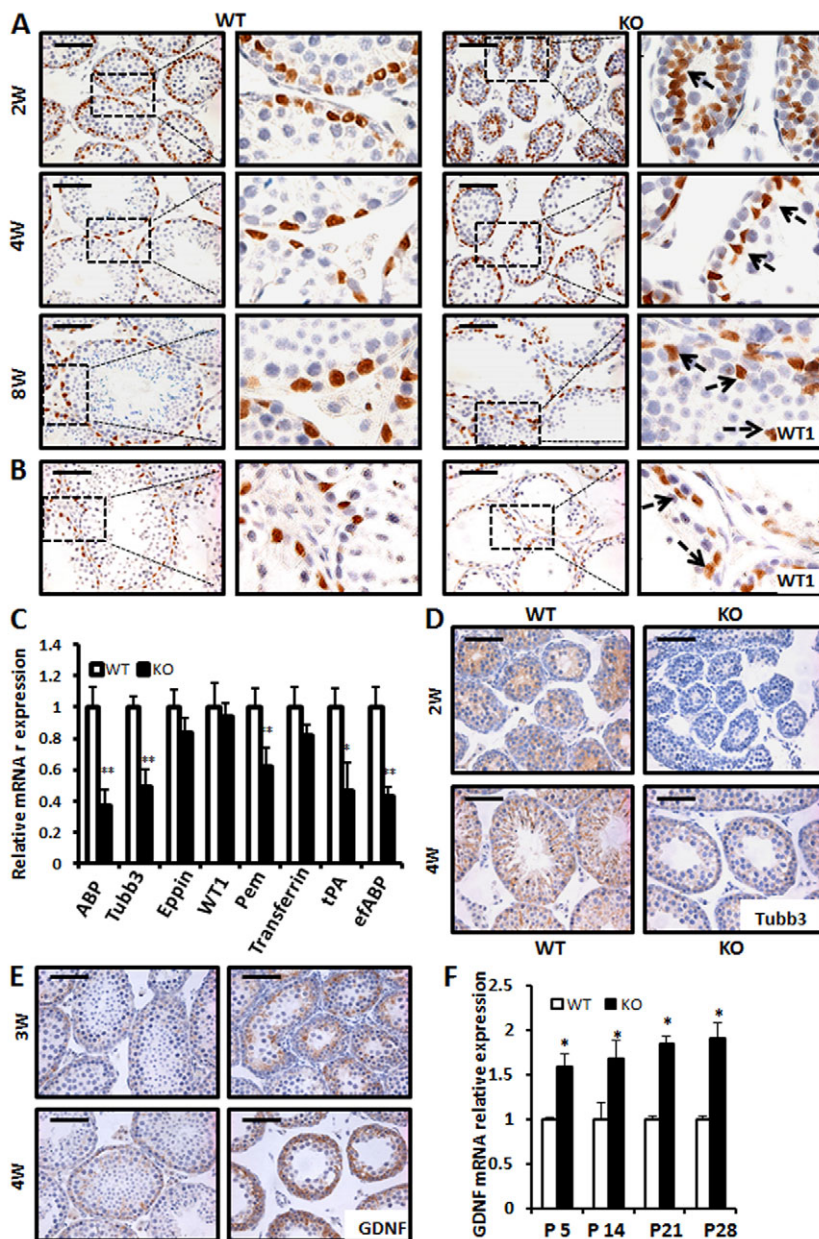


Fig. 4. Ablation of *Lgr4* leads to defective Sertoli cell nuclear localization and function.

(A) Disorganization of Sertoli cells in *Lgr4*^{-/-} testes at the indicated ages. Sertoli cells were stained with Wt1 antibody. The nuclei of Sertoli cells were located at the base of the seminiferous tubules in WT mice, but were located abnormally in *Lgr4*^{-/-} testes (arrows). (B) Neonatal WT and *Lgr4*^{-/-} testes were grafted under the skin of nude mice for 4 weeks. The transplants were sectioned and stained with Wt1 antibody; mislocated Sertoli cell nuclei are indicated (arrows). (C) Expression of Sertoli cell genes was examined by real-time PCR in WT and *Lgr4*^{-/-} 4-week-old male mice relative to external control *eGFP*. (D) *Tubb3* expression in Sertoli cells of *Lgr4*^{-/-} mice was dramatically downregulated at 2 and 4 weeks of age compared with WT littermates. (E,F) Expression of *Gdnf* was impaired in *Lgr4*^{-/-} testes. (E) Testis sections from 4-week-old WT and *Lgr4*^{-/-} mice were subjected to IHC with antibody against *Gdnf*. (F) Relative expression of *Gdnf* examined by real-time PCR in WT and *Lgr4*^{-/-} testes at the indicated ages. Error bars indicate the mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ (one-way ANOVA). Scale bars: 50 μ m.

Sertoli cell marker. The Sertoli cell number was not significantly altered in *Lgr4*^{-/-} mice (supplementary material Fig. S2), but nuclear location was obviously affected. As shown in Fig. 4A, in a number of *Lgr4*^{-/-} seminiferous tubules, the Sertoli cell nuclei were located in the middle of the tubules instead of at the base, as seen in WT littermates. In accordance with this result, disordered Sertoli cell nuclei were observed in *Lgr4*^{-/-} but not WT transplanted testes (Fig. 4B).

The expression of Sertoli cell functional genes, such as *Abp* (*Scgb1b27*), *Tubb3*, *Pem* (*Rhox5*), transferrin, *tPA* (*Plat*) and eFABP (*Fabp5*), was significantly decreased in *Lgr4*^{-/-} mice (Fig. 4C,D). It is well established that Sertoli cells construct the niche for SSCs (Oatley et al., 2011). Since SSC number was increased in *Lgr4* mutant mice (Fig. 2E,F), we examined whether the expression of Sertoli cell-produced niche factors was altered. *Gdnf* is one of the most important Sertoli cell-secreted niche factors that modulate the balance between self-renewal and differentiation (Meng et al., 2000). The expression of *Gdnf* was detected by IHC. The level of *Gdnf* protein was higher in *Lgr4*^{-/-} than in WT testis (Fig. 4E). Moreover, an increase in the *Gdnf* mRNA level in *Lgr4*^{-/-} testis was confirmed in real-time PCR assays (Fig. 4F). These data demonstrate that loss of expression of *Lgr4* in PMCs results in impaired function of Sertoli cells, suggesting that *Lgr4* is important for PMC-Sertoli cell interactions necessary for the maintenance of Sertoli cell functions.

Downregulation of androgen receptor expression in *Lgr4*^{-/-} PMCs

In *Lgr4* mutant mice the localization of Sertoli cell nuclei was altered, as was the expression of certain Sertoli cell-specific genes, and a similar phenotype has been reported in a PMC-specific *Ar* knockout mouse strain (Welsh et al., 2009; Zhang et al., 2006). We speculated that in *Lgr4* mutant PMCs the expression of *Ar* was impaired. In keeping with PMCs and Sertoli cells being androgen target cell types, we observed *Ar* protein in their nuclei in WT testes (Fig. 5A). The level of *Ar* protein was comparable in Sertoli cells in both genotypes, although the localization of Sertoli cell nuclei was altered in the *Lgr4*-deficient mice. However, the level of *Ar* protein was dramatically decreased in *Lgr4* mutant PMCs (Fig. 5A, both at 3 weeks and 4 weeks). Similar results were obtained in testicular transplants (Fig. 5B). These results suggest that *Lgr4*

deficiency downregulates *Ar* expression in PMCs, but not in Sertoli cells, resulting in the malfunction of Sertoli cells in the seminiferous epithelium.

Lgr4 modulates Wnt/β-catenin signaling in PMCs

In *Lgr4* mutant PMCs, the expression of *Ar*, α-SMA and numerous components of the ECM (e.g. fibronectin, laminin, collagen) was downregulated, and the majority of these genes, especially those encoding *Ar*, α-SMA and fibronectin, have been reported as downstream targets of Wnt/β-catenin signaling (Carthy et al., 2012; De Langhe et al., 2005; Gradl et al., 1999; Hlubek et al., 2001; Yang et al., 2002; Yang et al., 2006). We speculated that Wnt/β-catenin signaling is functioning in PMCs and that the activity of this pathway is impaired in *Lgr4* mutant PMCs, as *Lgr4* has recently been demonstrated to be one of the receptors for R-spondins, which amplify Wnt signaling (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011; Hao et al., 2012). Immunostaining with an antibody against β-catenin was performed to evaluate the activity of Wnt signaling, as β-catenin localizes in nuclei upon canonical Wnt signaling pathway activation. In WT testes, β-catenin was often detected in the nuclei of PMCs, but the number of PMCs with nuclear β-catenin was dramatically decreased in *Lgr4* mutant testes (Fig. 6A,B). These data suggest that *Lgr4* deficiency attenuates the activation of Wnt/β-catenin signaling in PMCs.

As R-spondins have recently been identified as ligands for *Lgr4*, we used a primary cell culture system to test whether R-spondin 1/*Lgr4* functions in PMCs. WT and *Lgr4* mutant seminiferous tubules were dissected and testicular cells were cultured. After being challenged with or without recombinant R-spondin 1, the cells were stained with specific antibodies. α-SMA was used as the marker for PMCs. Upon R-spondin 1 administration, the number of PMCs with nuclear β-catenin was dramatically increased in WT cultures but not in *Lgr4* mutant cultures (Fig. 6C,D). These data imply that *Lgr4* modulates R-spondin 1-mediated activation of Wnt/β-catenin signaling in PMCs.

Reactivation of Wnt/β-catenin signaling partially rescues the defects in *Lgr4*^{-/-} testis

The above data suggested that *Lgr4* mediates R-spondin 1 activation of Wnt/β-catenin signaling in PMCs, but whether the phenotype

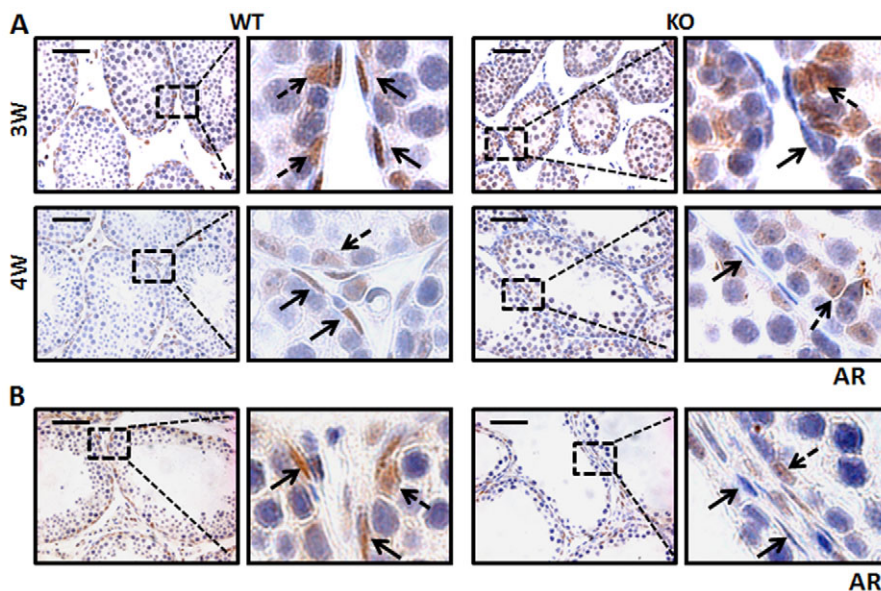


Fig. 5. Selective decrease in androgen receptor expression in *Lgr4*^{-/-} PMCs.

(A) Androgen receptor (*Ar*) expression was reduced in *Lgr4*^{-/-} PMCs (solid arrows) but not in Sertoli cells (dashed arrows) compared with WT controls at 3 and 4 weeks of age. (B) *Ar* expression was decreased in PMCs of transplanted *Lgr4*^{-/-} testes compared with WT transplants. Note that the expression of *Ar* was not affected in Sertoli cells (dashed arrows). Scale bars: 50 μm.

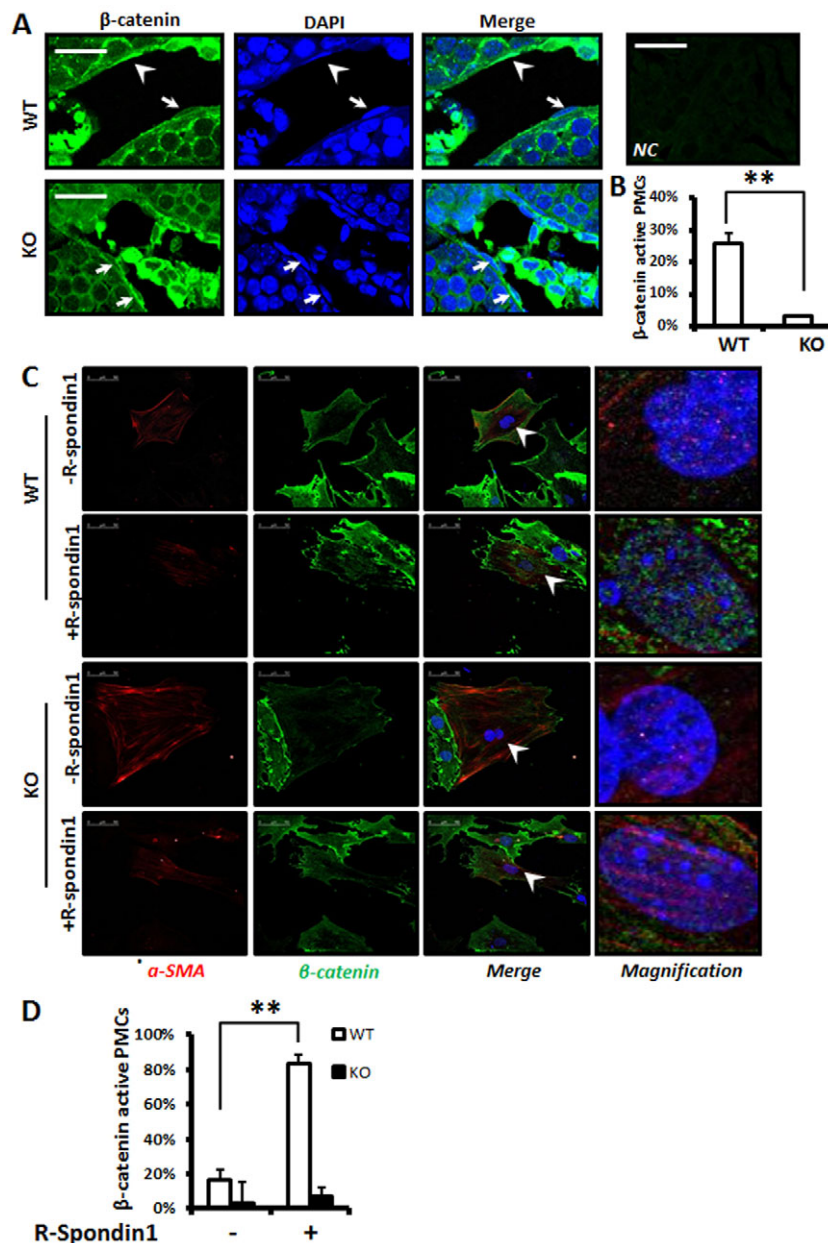


Fig. 6. *Lgr4* is crucial for Wnt/ β -catenin signaling activation in PMCs. (A,B) Wnt/ β -catenin signaling was activated in some WT PMCs but few *Lgr4*^{-/-} PMCs. (A) IF staining was performed with a β -catenin antibody. Arrowheads indicate nuclear β -catenin indicating activation of Wnt signaling in PMCs; arrows indicate Wnt/ β -catenin inactive PMCs. Purified mouse IgG1 was used as an isotype control for primary antibody (NC, top right). (B) Quantitation of Wnt/ β -catenin signaling activation in PMCs. (C,D) The *Lgr4* ligand R-spondin 1 activates β -catenin in WT but not *Lgr4*^{-/-} PMCs. (C) Primary cultured PMCs from 3- to 4-week-old WT and *Lgr4*^{-/-} mice were challenged with or without R-spondin 1, then the cells were subjected to IF with a β -catenin antibody and an antibody against α -SMA to indicate PMCs. (D) Quantitation of the effect of R-spondin 1 on Wnt/ β -catenin signaling activation in PMCs. Error bars indicate the mean \pm s.e.m. ** $P < 0.01$ (Student's *t*-test). Scale bars: 50 μ m.

observed in *Lgr4* mutant testes was due to the attenuation of Wnt signaling in PMCs was still obscure. We generated *Lgr4*^{-/-};*Apc*^{min/+} mice and examined the effects of *Apc* mutation in restoring the defects caused by *Lgr4* deletion. *Apc*^{min/+} mice contain a germline mutation in the *Apc* gene that spontaneously activates β -catenin (Lefebvre et al., 1998). We found that the number of PMCs with nuclear β -catenin was significantly increased in *Lgr4*^{-/-};*Apc*^{min/+} testes (Fig. 7A), suggesting that mutation of *Apc* partially restored the activation of Wnt/ β -catenin signaling in the double-mutant mice. Furthermore, we treated *Lgr4*^{-/-} mice with SB216763, a Gsk3 β inhibitor, and also found that Wnt/ β -catenin signaling was reactivated in PMCs (Fig. 7A).

With these two strategies to reactivate Wnt signaling in *Lgr4* mutant mice, we examined the expression of specific genes in order to explore whether the phenotype caused by *Lgr4* deletion could be restored. As shown in Fig. 7B, the expression of fibronectin was increased and the ECM formed continuous rings surrounding the seminiferous tubules in the testes of *Lgr4*^{-/-};*Apc*^{min/+} and SB216763-

treated *Lgr4*^{-/-} mice. In addition, the expression of α -SMA in PMCs was increased in double mutants and in Gsk3 β inhibitor-treated mice (Fig. 7B, α -SMA). We also found that, in the PMCs of Wnt/ β -catenin reactivated *Lgr4* mutant mice, the expression of Ar was restored and most of the Sertoli cell nuclei localized at the basement membrane layer of the seminiferous tubules (Fig. 7B, Ar). Not only were the PMC-specific genes restored upon Wnt/ β -catenin reactivation in *Lgr4*-deficient mice, but the differentiation of germ cells was also promoted. In SB216763-treated *Lgr4* mutant testes, Scp3-negative germ cells appeared in the inner layer of the seminiferous epithelium, suggesting that upon Wnt/ β -catenin reactivation many primary spermatogonia went through the differentiation process. In addition, seminiferous tubule diameter and lumen size were increased in SB216763-treated *Lgr4* mutant mice compared with untreated controls (Fig. 7C). These data demonstrate that reactivation of Wnt/ β -catenin signaling partially rescues the phenotype of *Lgr4* mutant mice, suggesting that *Lgr4*-mediated Wnt signaling plays an important role in PMCs.

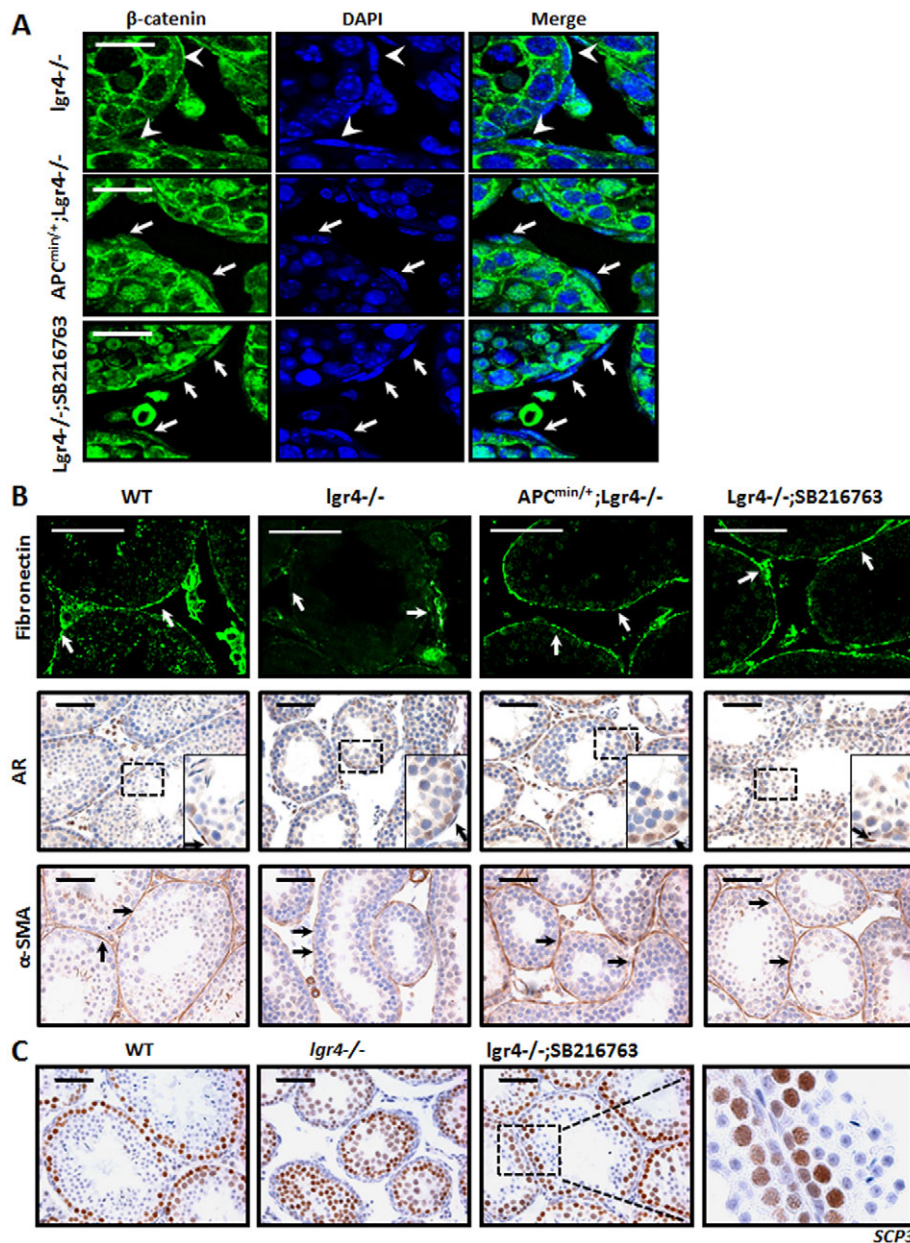


Fig. 7. Forced activation of Wnt/ β -catenin signaling in $Lgr4^{-/-}$ mice partially rescues PMC defects. (A) β -catenin was activated in PMCs of $Lgr4^{-/-};ApC^{min/+}$ mice and in $Lgr4^{-/-}$ mice after Gsk3 β -specific inhibitor (SB216763) treatment. IF with β -catenin antibody and nuclei counterstained with DAPI. PMCs with nuclear β -catenin are indicated by arrows, whereas PMCs with cytoplasmic β -catenin are indicated with arrowheads. (B) Forced activation of Wnt/ β -catenin signaling in $Lgr4^{-/-}$ mice increased the expression of Wnt target genes that play essential roles in PMCs. IHC and IF were performed against testis sections of the indicated genotypes using antibodies against fibronectin, Ar or α -SMA. Arrows indicate positive signals typical for each antibody. (C) The differentiation of germ cells was also promoted in SB216763-treated $Lgr4$ mutant testis. Differentiated germ cells (Scp3 negative) appeared in the inner layer of the seminiferous epithelium in SB216763-treated $Lgr4^{-/-}$ mice. Scale bars: 25 μ m in A; 50 μ m in B,C.

DISCUSSION

Four groups, including us, have shown that *Lgr4* is essential for male fertility by modulation of epididymal epithelial development and function (Hoshii et al., 2007; Li et al., 2010; Mendive et al., 2006), but the distinct roles of *Lgr4* in spermatogenesis have not been studied. Here we explored the physiological function of *Lgr4* in testes and found that *Lgr4* is specifically expressed in PMCs and modulates spermatogenesis. Disruption of *Lgr4* in the testes attenuated PMC Wnt/ β -catenin signaling and led to impaired germ cell differentiation and maturation.

Previous studies have shown that the degeneration of the seminiferous epithelium in *Lgr4* mutants is partially due to hypoplasia of efferent ducts and epididymis and to fluid circulation blockage, which occurred after 4 weeks of age in these tubules (Li et al., 2010; Mendive et al., 2006). To elucidate whether *Lgr4* plays a direct role in testes, we focused on the first 4 weeks of postnatal development and performed testes transplant experiments. In early

postnatal stages, both the diameter and the lumen size of the seminiferous tubule in *Lgr4* mutant testes were significantly reduced (Fig. 1C). In addition, the growth of testes as well as germ cell differentiation were dramatically affected in *Lgr4* mutant transplants (Fig. 1D,E). These data showed that the decreased spermatogenesis is due to direct *Lgr4* action in the testes, rather than being a side effect of the blockage of male reproductive ducts.

In mouse testis, PMCs are viewed as structural cells with the ability to contract (Maekawa et al., 1996; Rossi et al., 2002). In addition, PMCs secrete key basement membrane components. Although we did not provide direct evidence that *Lgr4* regulates PMC contraction, the expression of contractile ability-associated genes, such as those encoding α -SMA and desmin, was selectively downregulated in *Lgr4^{-/-}* PMCs, suggesting that deletion of *Lgr4* might affect PMC contractility and the transport of spermatozoa through seminiferous tubules. Whether PMCs have physiological functions beyond the two major roles remains obscure. SSCs reside

in the niche formed by somatic cells in the basal compartments (surrounded by Sertoli cells and PMCs) of the seminiferous tubules. Sertoli cells are widely studied niche cells that stimulate either self-renewal or differentiation of the SSCs through secretion of growth factors such as Gdnf and SCF (Kitl – Mouse Genome Informatics) (Blume-Jensen et al., 2000; Meng et al., 2000; Ohta et al., 2000). It is believed that Gdnf is mainly produced in Sertoli cells, but it is also reported that Gdnf is constitutively produced by PMCs and may contribute to the SSC niche (Spinnler et al., 2010). It is difficult to demonstrate which cell type is more important for the elevation of the Gdnf level in *Lgr4*^{-/-} mice, as Sertoli cells are also affected indirectly through mesenchymal-epithelial cell interactions. However, it is clearly demonstrated that *Lgr4* is specifically expressed in PMCs, and disrupting the gene resulted in an increase of undifferentiated spermatogonia and in an attenuation of germ cell differentiation. This strongly suggested that *Lgr4* signaling in PMCs also plays a role in regulating SSC differentiation and self-renewal through either direct or indirect mechanisms and provided genetic evidence that PMCs might also contribute to the SSC niche.

PMCs regulate Sertoli cell function through the production of a paracrine protein, termed PModS, which modulates Sertoli cell function including the secretion of key factors (Anthony et al., 1991; Norton and Skinner, 1989). However, PModS is as yet unidentified, with undefined functions and mechanisms. With PMC-specific *Ar* knockout mouse models, researchers have demonstrated the importance of androgen-driven mesenchymal-epithelial cell interactions for the regulation of spermatogenesis (Welsh et al., 2009; Zhang et al., 2006). In this study, we reported that *Lgr4* signaling is a second pathway in PMCs to modulate Sertoli cell function during spermatogenesis. Similar phenotypes were observed in PMC-specific *Ar* mutant mice, and most of the genes downregulated in *Ar* mutant mice were also observed in *Lgr4*-deficient animals. Although we do not have direct evidence to show how *Lgr4* signaling regulates the expression of *Ar* in PMCs, a previous publication reported that Wnt signaling directly regulates *Ar* expression through three functional LEF1/TCF binding elements within the *Ar* promoter in prostate cancer cells (Yang et al., 2006). Since *Lgr4* is the newly identified receptor for Wnt activator R-spondins, we hypothesize that *Lgr4* regulates *Ar* expression through Wnt signaling. In *Lgr4* mutant mice upon Wnt/ β -catenin reactivation (*Lgr4*^{-/-}; *Apc*^{min/+} double-mutant strain and Gsk3 β inhibitor-treated *Lgr4*^{-/-} mice), *Ar* expression is restored in *Lgr4* mutant PMCs, further indicating that *Lgr4* is an upstream regulator of *Ar* expression. We do not consider *Lgr4* regulation of *Ar* expression to be PMC specific. Our previous study and unpublished data have shown that *Ar* expression is also reduced in *Lgr4* mutant epididymal epithelium (Li et al., 2010). Epididymal-specific deletion of *Ar* resulted in epithelial hypoplasia and hypotrophy and led to male infertility, similar to the phenotype observed in adult *Lgr4* mutant mice (Krutskikh et al., 2011; Mendive et al., 2006).

In conclusion, our results demonstrate that *Lgr4* plays an indispensable role in PMCs to modulate spermatogenesis. Using differentiation strategies, we also demonstrated for the first time that Wnt/ β -catenin is not only activated but also directly modulated by *Lgr4* in PMCs. Our observations provide deeper insights into the functions and the regulatory molecular mechanisms of this particular cell type. Since *Lgr4* is a seven-transmembrane receptor that is accessible to small molecules, the identification of an *Lgr4* antagonist might have potential clinical value for contraception.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.093641/-/DC1>

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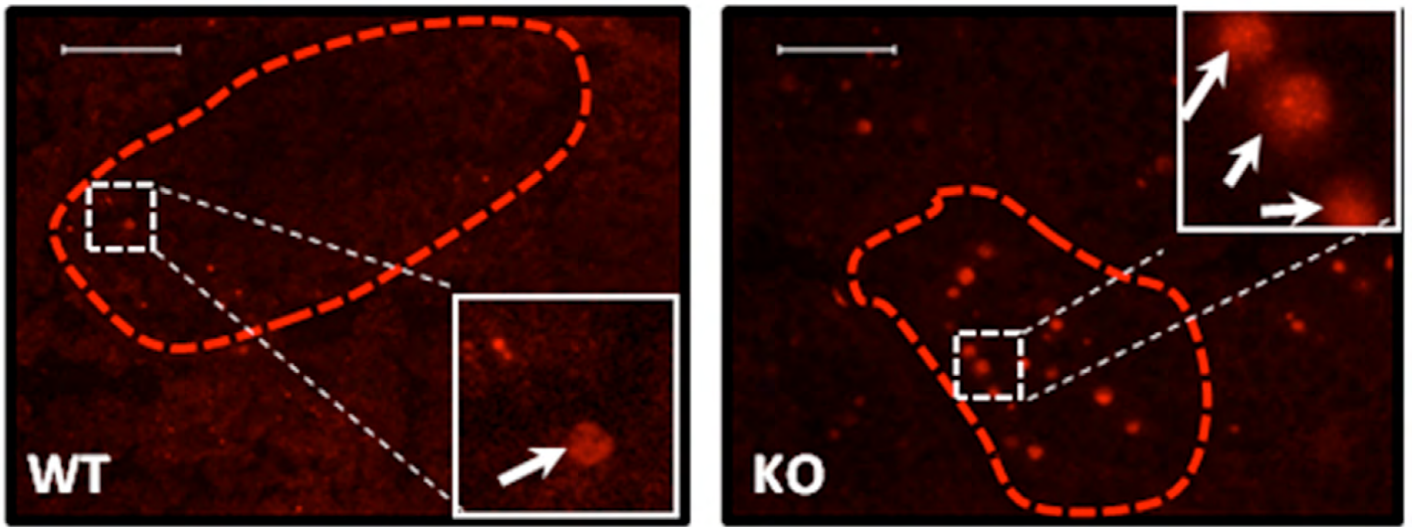


Fig. S1. Germ cell apoptosis analysis by immunofluorescence staining with antibody against cleaved-caspase 3. Testes of 3-week-old *Lgr4* mutant mice and WT littermates were prepared and subjected to immunofluorescence staining with cleaved caspase 3 antibody. Red dashed circle indicates the boundary of one seminiferous tubule. Arrows indicate cleaved caspase 3-positive germ cells. Scale bars: 50 μ m.

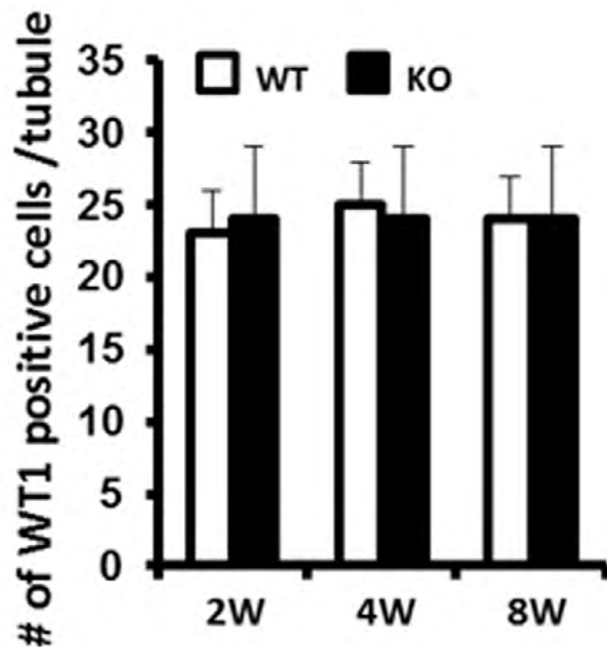


Fig. S2. Sertoli cell number is not significantly altered in *Lgr4*^{-/-} mice. Immunostaining for the Sertoli cell marker Wt1 was performed on testes sections of WT and *Lgr4* mutant mice and the number of Wt1-positive cells was counted. The mean value is shown with standard error. Testes from three mice of each genotype were analyzed for each stage. The number of Wt1-positive cells was obtained from more than 40 tubules/section and 20 sections/testis.

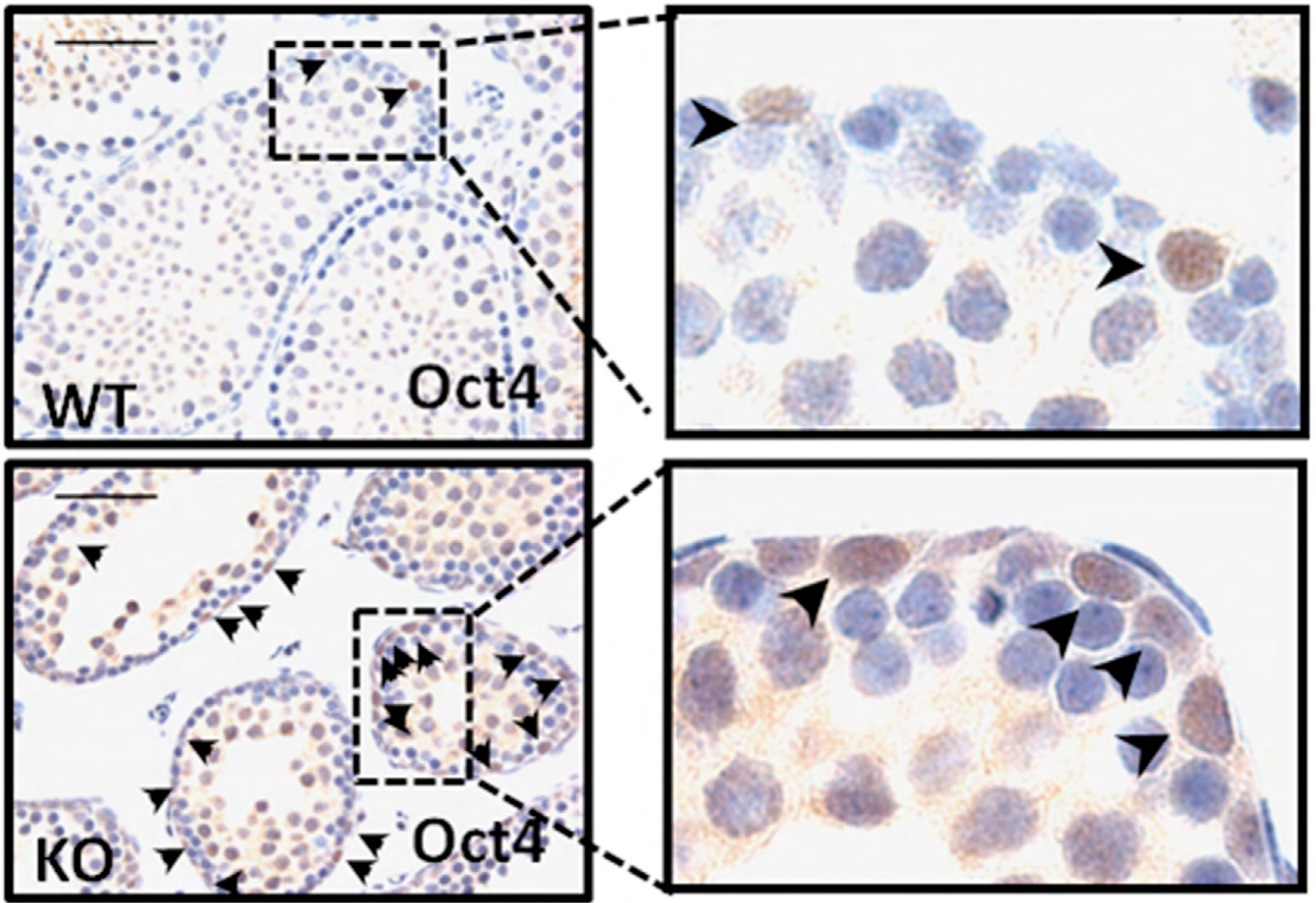


Fig. S3. Undifferentiated spermatogonia cell number in 3-week-old *Lgr4* mutant mice and WT littermates. IHC was performed with antibody against Oct4. Arrows indicate Oct4-positive germ cells. Scale bar: 50 μ m.