Does murine spermatogenesis require WNT signalling?
A lesson from Gpr177 conditional knockout mouse models

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Wingless-related MMTV integration site (WNT) proteins and several other components of the WNT signalling pathway are expressed in the murine testes. However, mice mutant for WNT signalling effector β-catenin using different Cre drivers have phenotypes that are inconsistent with each other. The complexity and overlapping expression of WNT signalling cascades have prevented researchers from dissecting their function in spermatogenesis. Depletion of the Gpr177 gene (the mouse orthologue of Drosophila Wntless), which is required for the secretion of various WNTs, makes it possible to genetically dissect the overall effect of WNTs in testis development. In this study, the Gpr177 gene was conditionally depleted in germ cells (Gpr177fl/fl, Mvh-Cre; Gpr177fl/fl, Stra8-Cre) and Sertoli cells (Gpr177fl/fl, Amh-Cre). No obvious defects in fertility and spermatogenesis were observed in these three Gpr177 conditional knockout (cKO) mice at 8 weeks. However, late-onset testicular atrophy and fertility decline in two germ cell-specific Gpr177 deletion mice were noted at 8 months. In contrast, we did not observe any abnormalities of spermatogenesis and fertility, even in 8-month-old Gpr177fl/fl, Amh-Cre mice. Elevation of reactive oxygen species (ROS) was detected in Gpr177 cKO germ cells and Sertoli cells and exhibited an age-dependent manner. However, significant increase in the activity of Caspase 3 was only observed in germ cells from 8-month-old germ cell-specific Gpr177 knockout mice. In conclusion, GPR177 in Sertoli cells had no apparent influence on spermatogenesis, whereas loss of GPR177 in germ cells disrupted spermatogenesis in an age-dependent manner via elevating ROS levels and triggering germ cell apoptosis.

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Retromer retrieves endocytosed WLS from endosomes and recycles it back to the trans-Golgi network for its further function in WNT secretion.34 Accordingly, the conditional knockout (cKO) mouse models of Gpr177, the mouse orthologue of Drosophila Wls, is more appropriate than other established models related to β-catenin for the study of the role of WNT signalling (both canonical and noncanonical) and WNTs.

Mice homozygous for Gpr177 (Gpr177⁻/⁻) die in the embryonic stage due to defects in body axis establishment.35 Carpenter et al.26 generated mice with a conditional null allele for Gpr177 (recombination of the loxP sites using Cre resulting in the removal of exon 1) and showed that Gpr177 is essential for the development of brain and pancreas. Fu et al.37 created a novel conditional Gpr177 knockout mouse line (loxP sites flanking exon 3) and observed the loss of Gpr177 in Wnt1-expressing cells causes mid/hindbrain and craniofacial defects, which resemble the double knockout of Wnt1 and Wnt3a as well as β-catenin deletion in the Wnt1-expressing cells. Zhu et al.38 generated a different Gpr177 cKO mouse line carrying an exon 3-floped allele and showed that Gpr177-mediated WNTs regulate early patterning along the three axes of the limb bud and also sustain cell proliferation and survival of distal limb mesenchyme. Subsequently, these Gpr177 cKO mouse lines have been utilised to investigate the roles of WNT signalling and WNTs in a variety of tissues, such as embryonic hair follicles, fungiform placodes and teeth.39–42

Because the Gpr177 mRNA level is expressed in mouse testis43 and the role of WNT signalling in spermatogenesis is still unclear, we generated and analysed germ cell-specific (Gpr177flox/flox, Mvh-Cre and Gpr177flox/flox, Stra8-Cre) and the Sertoli cell-specific (Gpr177flox/flox, Amh-Cre) Gpr177 cKO mice. We observed that selective loss of Gpr177 in germ cells or Sertoli cells blocks the secretion of cell-specific WNT ligands. Gpr177 in Sertoli cells has no apparent influence on spermatogenesis, whereas germ cell-specific Gpr177 deletion mice exhibit an age-dependent reproductive phenotype: fertile when young and subfertile when older. We further suggest that oxidative stress is involved in age-dependent spermatogenic damage of germ cell-specific Gpr177 deletion mice.

Results

GPR177 expression in mouse testes. The findings of a previous study suggest that Gpr177 mRNA is expressed ubiquitously.43 In this study, we observed that GPR177 was expressed in many mouse tissues, including the spleen, lung, kidney, thymus, stomach, brain and testes, using western blot analysis (Figure 1a). Furthermore, the protein level of GPR177 in W/ W* recipient testes did not obviously differ between embryonic day (E) 15.5 and postnatal days (PND) 3, 7, 14, 21, 28 and 56 (Figure 1b). To evaluate GPR177 expression in different seminiferous cells, we assessed the GPR177 protein level in W/ W* testes (lacking endogenous germ cells), W/ W* recipient testes after SSC transplantation, freshly isolated Sertoli cells, germ cells and interstitial cells from adult mouse testes. GPR177 was highly expressed in germ cells and including SSCs, which mediate the proliferation but not the maintenance of undifferentiated spermatogonia.

Collectively, previous studies have suggested that WNT signalling and WNTs play multiple roles in spermatogenesis, but there are still the following problems: (1) the conclusions about the function of WNT/β-catenin in germ cells are inconsistent with each other;26,28,29 (2) the overlapping expression pattern of the various WNTs and their functional redundancy obscure the true consequences of removing individual Wnt genes; and (3) β-catenin does play a central role in the canonical WNT pathway, but it also serves as a membrane protein of the cell junction complex.30

WNTs are secreted as glycoproteins from WNT-producing cells into the extracellular milieu.31 In 2006, Bänziger et al.32 and Bartscherer et al.33 identified a novel WNT pathway component, Wntless (WLS), and showed that it is responsible for the secretion of WNT proteins from signalling cells. Loss of WLS function has no effect on other signalling pathways, but it appears to impede all of the WNT signals.32,33

Figure 1 GPR177 protein level and localisation in mouse testes. (a) Expression of GPR177 in mouse tissues, including spleen, lung, kidney, thymus, stomach, brain and testes by western blot. (b) Expression of GPR177 in testes at different developmental stages, including E15.5, PND 3, 7, 14, 21, 28 and 56. (c) Protein level of GPR177 in W/ W* testes, W/ W* recipient testes after SSC transplantation, and isolated Sertoli, germ and interstitial cells. β-Tubulin served as a protein loading control in (a–c). (d) The cellular localisation of GPR177 in sections of testes from E15.5 and PND56 mice was displayed by immunofluorescent staining. The nuclei are counterstained with DAPI (blue). The images shown were representative results of experiments that were repeated three times using samples from different sets of testes, which yielded similar results. Scale bars, 50 μm.

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Sertoli cells, with little expression in interstitial cells (Figure 1c). The expression level of GPR177 protein was higher in $W/W^v$ recipient testes after SSC transplantation than in $W/W^v$ testes (Figure 1c). Immunofluorescence staining in E15.5 and PND56 testes further showed that GPR177 was visibly present in several testicular cell types, including germ cells and Sertoli cells (Figure 1d).

Efficient and specific disruption of Gpr177. To assess the cell type-specific function of GPR177 during spermatogenesis, we generated mice in which the Gpr177 gene was specifically disrupted in germ cells using Mvh-Cre (Figure 2a) or Stra8-Cre (Figure 2b) and Sertoli cells using Amh-Cre (Figure 2c). Genotyping of the mice was performed by PCR using specific primers to distinguish wild-type (+) (~100 bp) and floxP alleles (~200 bp) or different Cre bands (Mvh-Cre: 240 bp; Stra8-Cre: 326 bp and Amh-Cre: ~100 bp). (d-f) qRT-PCR analysis showing the conditional loss of Gpr177 mRNA in total testis extracts, germ cell or Sertoli cell extracts of three Gpr177 cKO mice. Gapdh served as the internal control gene. The data are expressed as the mean ± S.E.M. *$P<0.05$, **$P<0.01$

GPR177 is responsible for secretion of WNT proteins. WLS/GPR177 is required for the secretion of WNT proteins from signalling cells. We postulated that selective loss of Gpr177 in germ cells and Sertoli cells would block the secretion of germ cell-specific and Sertoli cell-specific WNT proteins, respectively. Thus, extracellular secretion of WNT proteins, as examined by enzyme-linked immunosorbent assay (ELISA), was detected in supernatants of germ cells or Sertoli cells from control and Gpr177 cKO testes. Using qRT-PCR, we observed that Wnt3, Wnt3a and Wnt7a were predominantly expressed in germ cells (Supplementary Figures S1a and b), while Sertoli cells mainly expressed several Wnts, including Wnt4, Wnt6 and Wnt11 (Supplementary Figures S1a and c), which is consistent with previous studies.10,29 We observed that secreted protein levels of WNT3, WNT3A and WNT7A were significantly decreased in culture supernatants of germ cells from two germ cell-specific Gpr177 knockout testes, but not from Gpr177flox/flox.
Amh-Cre testes (Figures 3a–c). In addition, loss of Gpr177 in Sertoli cells inhibits extracellular secretion of WNT4, WNT6 and WNT11 by Sertoli cells (Figures 3d–f).

Normal spermatogenesis in Gpr177 cKO mice at 8 weeks.
After successfully generating two germ cell-specific Gpr177 knockout mouse models, we investigated the role of GPR177 in germ cells. In 8-week-old cKO mice, there were no overt abnormalities in testis weight (127 ± 2 mg in wild-type mice and 110 ± 7 mg in Gpr177^fl/fl, Mvh-Cre, Gpr177^fl/fl, Stra8-Cre and Gpr177^fl/fl, Amh-Cre testes) (Figure 4a), pregnancy rate (95% in wild-type mice and 99% in Gpr177^fl/fl, Mvh-Cre mice; 95% in wild-type mice and 95% in Gpr177^fl/fl, Stra8-Cre mice) (Figure 4b) and litter size (11.6 ± 0.2 in wild-type mice and 10.7 ± 0.3 in Gpr177^fl/fl, Mvh-Cre mice; 11.8 ± 0.2 in wild-type mice and 11.4 ± 0.3 in Gpr177^fl/fl, Stra8-Cre mice) (Figure 4c). Furthermore, Gpr177^fl/fl, Mvh-Cre testes (Figures 5d–f), Gpr177^fl/fl, Stra8-Cre testes (Figures 5j–l) and their respective littermate control testes (Figures 5a–c, g–i) exhibited typical seminiferous tubule morphology with all stages of spermatogonia to spermatozoa at 8 weeks, indicating that spermatogenesis was normal in germ cell-specific Gpr177 cKO mice. The same conclusion was drawn from the immunofluorescence results of staining germ cell-specific marker MVH in the testes and spermatozoa-specific marker AQP3 in the cauda epididymis of Gpr177^fl/fl, Mvh-Cre males (Supplementary Figures S2b and h) and Gpr177^fl/fl, Stra8-Cre males (Supplementary Figures S2d and j) at 8 weeks.

To test whether Sertoli cell-specific Gpr177 deletion causes defects in fertility, we bred Gpr177^fl/fl, Amh-Cre males with wild-type females. The testis weight (126 ± 2 mg in wild-type mice and 124 ± 3 mg in Gpr177^fl/fl, Amh-Cre mice), pregnancy rate (100% in wild-type mice and 95% in Gpr177^fl/fl, Amh-Cre mice) and number of pups per litter (11.9 ± 0.2 in wild-type mice and 11.7 ± 0.3 in Gpr177^fl/fl, Amh-Cre mice) were not significantly different between Gpr177^fl/fl, Amh-Cre males and their respective littermate control males at 8 weeks (Figures 4a–c), indicating normal fertility of Gpr177^fl/fl, Amh-Cre male mice. Furthermore, histological and immunofluorescence analysis
Figure 4  Eight-week-old Gpr177 cKO male mice were fertile. (a) Testis weights were examined. The data are expressed as the mean ± S.E.M. (b) The pregnancy rate was calculated as the ratio of the number of pregnant females to the number of successfully mating females. (c) When calculating the average litter size, only the females that generated pups were included. The data are expressed as the mean ± S.E.M.

Figure 5  Testis and cauda epididymis morphology of 8-week-old Gpr177 cKO mice. (a–r) Testicular and cauda epididymal sections stained with H&E. No overt morphological abnormalities were observed in Gpr177fl/fl, Mvh-Cre (d–f), Gpr177fl/fl, Stra8-Cre (j–l) and Gpr177fl/fl, Amh-Cre (p–r) mice relative to their respective littermate controls (a–c, g–i, m–o). The images shown are representative results of experiments that were repeated three times using samples from different sets of testes, which yielded similar results. Scale bars in the first and third lines, 50 μm. Scale bars in the second line, 100 μm.
of Gpr177flx/flx, Amh-Cre testes did not identify any major structural defects in testis morphology and spermatogenesis (Figures 5p–r and Supplementary Figures S2f and I).

Late-onset testicular atrophy and fertility decline in germ cell-specific Gpr177 deletion mice. The spermatogenesis and fertility of Gpr177flx/flx, Mvh-Cre and Gpr177flx/flx, Stra8-Cre mice were indistinguishable from those of control mice at 8 weeks (above, Figures 4 and 5). However, these two germ cell-specific Gpr177 knockout mice developed age-dependent testicular atrophy. By 8 months of age, the average weight of a Gpr177flx/flx, Mvh-Cre testis was approximately 59% of that of a control testis (124 ± 2 mg in wild-type mice and 73 ± 4 mg in Gpr177flx/flx, Mvh-Cre mice) (P < 0.05), while the average weight of a Gpr177flx/flx, Stra8-Cre testis was approximately 62% of that of a control testis (122 ± 1 mg in wild-type mice and 75 ± 3 mg in Gpr177flx/flx, Stra8-Cre mice) (P < 0.05) (Figure 6a). Testicular atrophy in the cKO mice was accompanied by a significant decline in pregnancy rate (80% in control mice and 30% in Gpr177flx/flx, Mvh-Cre mice; 85% in wild-type mice and 40% in Gpr177flx/flx, Stra8-Cre mice) (Figure 6b) and litter size (10.3 ± 0.2% in wild-type mice and 7.6 ± 0.3% in Gpr177flx/flx, Mvh-Cre mice; 9.8 ± 0.3% in wild-type mice and 7.0 ± 0.3% in Gpr177flx/flx, Stra8-Cre mice) (Figure 6c) at 8 months. Observation of the mating behaviour of Gpr177 cKO males showed that they copulated with females at a rate comparable to control males, which indicated that behavioural factors were not the cause of the reduced fertility. H&E staining examination of the seminiferous epithelium of Gpr177 cKO mice revealed some histological abnormalities. The most obvious of these abnormalities was epithelial vacuolisation in some tubules, which were devoid of spermatocytes and spermatids and were evident in 8-month-old Gpr177 cKO mice (Figure 7a). As the mouse age increased, the percentage of tubules with abnormal spermatogenesis significantly increased in Gpr177flx/flx, Mvh-Cre males (6.2 ± 0.8% at 7 months, 15.5 ± 0.4% at 8 months, 16.9 ± 0.4% at 9 months and 24.2 ± 0.6% at 10 months) and Gpr177flx/flx, Stra8-Cre males (3.2 ± 0.4% at 7 months, 11.1 ± 0.6% at 8 months, 13.8 ± 0.8% at 9 months and 20.3 ± 0.8 at 10 months), compared with their controls (0% at 7 months, 2.5 ± 0.2% at 8 months, 2.1 ± 0.2% at 9 months and 3.6 ± 0.2% at 10 months) (Figure 7b). In contrast, we did not observe obviously abnormal spermatogenesis or fertility decline even in aged (7- to 10-month-old) Gpr177flx/flx, Amh-Cre mice (Figures 6 and 7).

Oxidative stress is involved in age-dependent spermatogenic damage. Given that elevation of reactive oxygen species (ROS) impairs spermatogenesis in an age-dependent manner, we examined the status of ROS and
apoptosis in different cell types from control and Gpr177 cKO testes at both 8 weeks and 8 months. As shown in Figure 8a, a significant increase of ROS level was observed in germ cells from Gpr177flox/flox, Mvh-Cre and Gpr177flox/flox, Stra8-Cre testes at 8 weeks, compared with germ cells from control and Gpr177flox/flox, Amh-Cre testes at the same age. Selective loss of Gpr177 in Sertoli cells promoted a significant increase of ROS level in Sertoli cells at 8 weeks (Figure 8b). These data suggest that blocking WNT secretion by Gpr177 cKO causes increase of oxidative stress in corresponding cells from adult (8-week-old) testes. Furthermore, elevation of ROS exhibited an age-dependent manner. We observed that the ROS level was significantly increased in germ cells (Figure 8c) and Sertoli cells (Figure 8d) from 8-month-old Gpr177 cKO testes, compared with cells from 8-week-old Gpr177 cKO testes. The activity of Caspase 3 in germ cells (Figure 8e) and Sertoli cells (Figure 8f) was similar between control and Gpr177 cKO testes at 8 weeks. However, selective loss of Gpr177 in germ cells caused significant increase in the activity of Caspase 3 at 8 months relative to 8 weeks (Figure 8g). In contrast, the activity of Caspase 3 in Sertoli cells from 8-month-old Gpr177flox/flox, Amh-Cre testes was not statistically different from Gpr177-deficient Sertoli cells at 8 weeks (Figure 8h).

Discussion

Cell–cell communication via WNT signalling represents a fundamental means by which animal development and homoeostasis are controlled. Components of the cellular machinery responsible for transducing WNT signals from the cell surface to the nucleus, which is mainly mediated by β-catenin, have been identified in receiving cells, but the identification of components associated with the events occurring in WNT-secreting cells is incomplete (reviewed by Logan and Nusse1). Importantly, recent studies have identified a novel WNT pathway component, Wntless (WLS), that promotes WNTs secretion from WNT-producing cells into the extracellular milieu.32,33 WLS is evolutionarily and functionally conserved; seven-pass membrane protein, intriguingly, acts exclusively in WNT signal-sending cells. Accordingly, Gpr177 (mouse orthologue of Drosophila Wls) cKO mice are excellent models to study the role of WNT signalling (both canonical and noncanonical) and total WNTs.

Mice mutant for β-catenin using other Cre drivers have phenotypes that are inconsistent with each other. Germ cell-specific β-catenin knockout mediated by Stra8-Cre, which is expressed in differentiating spermatogonia at the onset of differentiation, did not cause a detectable phenotype.26 However, spermatid-specific β-catenin knockout mediated by Prm1-Cre has been shown to cause impaired fertility as a result of reduced sperm counts.26 Kerr et al.44 reported disrupted spermatogenesis in both loss- and gain-of-WNT signalling function experiments using AhCre. In a recent article, Takase et al.45 reported that WNT6 secreted by Sertoli cells activates WNT/β-catenin signalling and mediates the proliferation of undifferentiated spermatogonia. These researchers demonstrated that undifferentiated spermatogonia are WNT-responsive cells by taking advantage of genetic lineage tracing using the WNT target gene Axin2. We hypothesise that undifferentiated spermatogonia are not the only WNT-responsive cell population within the testes, because previous studies have demonstrated the expression and conditional deletion β-catenin in meiotic and post-meiotic germ cells.26,28 Furthermore, the Axin2-LacZ reporter line used in the current study revealed different WNT-responsive cells than the Tcf/Lef-LacZ mouse reporter line.7 Conditional deletion of β-catenin in AXIN2-expressing cells upon tamoxifen injection reduced the proliferation of undifferentiated spermatogonia. Thus, the authors suggested that WNT/β-catenin signalling promotes stem cell proliferation. However, we hypothesise that the reduced proliferation could also be due to impaired cadherin-mediated adherens junctions rather than disrupted WNT/β-catenin signalling, based on the following evidence. First, although β-catenin plays a central role in the canonical WNT pathway, it also serves as a membrane protein in the cell junction complex.45,46
Second, we found in current study that conditional deletion of the Gpr177 gene in Sertoli cells using Amh-Cre (blocking WNTs secretion from Sertoli cells) had no effect on spermatogenesis and male fertility. Additionally, the evidence that Sertoli cells secrete WNT6 as a paracrine signal for undifferentiated spermatogonia is insufficient. In addition to Sertoli cells, other somatic cell types contribute to the SSC niche, including Leydig cells, peritubular myoid cells, and peritubular macrophages. Thus, WNT-producing cells are not limited to Sertoli cells. Wnt6 was shown to be specifically expressed in Sertoli cells, particularly in the basal compartment. However, using qRT-PCR, we observed that Sertoli cells express several Wnts, including, but not limited to, Wnt6 (Supplementary Figure S1). The generation of Sertoli cell-specific Wnt6 knockouts would be helpful to confirm this conclusion. Thus, these data are insufficient to draw the conclusion that Sertoli cells secrete WNT6 to activate canonical WNT signalling in undifferentiated spermatogonia.

Our study demonstrates that GPR177 in Sertoli cells has no apparent influence on spermatogenesis, whereas germ cell-specific Gpr177 deletion mice exhibit an age-dependent reproductive phenotype: fertile when young and subfertile when older. We suggest that accumulated WNTs do harm to germ cells and oxidative stress and apoptosis are involved in age-dependent spermatogenic damage of germ cell-specific Gpr177 deletion mice. ROS has a dual role in reproductive systems, both beneficial and harmful depending on their nature and concentration as well as location and length of exposure. The decline in male fertility with aging is associated with increasing oxidative damage in the male reproductive system. Mice lacking nuclear factor-erythroid 2-related factor 2 (Nrf2), or superoxide dismutase 1 (Sod1), or inner mitochondrial membrane peptidase 2-like (Immp2l) develop impaired spermatogenesis in an age-dependent manner. In contrast, SSCs depleted of ROS stop proliferating, while enhanced self-renewal is observed when ROS levels are increased. Thus, ROS levels need to be tightly controlled in germ cells. In this study, we suggest that elevation of ROS level in germ cells triggers apoptotic signalling to disrupt spermatogenesis in aged (8-month-old) Gpr177-deficient germ cells. The reason why young (8-week-old) germ cell-specific Gpr177 knockout mice (germ cells also endure oxidative stress) exhibit normal spermatogenesis is still unclear. One of the main reasons is that ROS level in 8-week-old Gpr177-deficient germ cells was significantly lower than that in 8-month-old mutant germ cells (Figure 8c). Compared with germ cells, Sertoli cells seem to...
endure oxidative stress to a certain extent, because we did not observe significant apoptosis in even 8-month-old Gpr177-deficient Sertoli cells (Figures 8d and h).

Notably, two recent studies suggest that mammalian spermatooza respond to WNT signals released from the epididymis and WNT signalling controls sperm maturation independent of β-catenin. It is a novel way of WNT signalling in regulating spermatogenesis. We suggest that generation of cKO mice in which Gpr177 was specifically knocked out in epididymal cells (using Rnase10-Cre or Lcn5-Cre) could provide further evidence whether GPR177-mediated WNT secretion from epididymal cells act as an epididymal sperm maturation signal.

Material and Methods

Mice. All animal works were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the Institute of Zoology (IZ), Chinese Academy of Sciences (CAS). All the mice were maintained in a C57BL/6;129/SvEv mixed background. Gpr177fl/fl mice homozygous for a flox allele of Gpr177 (The Jackson Laboratory, Bar Harbor, ME, USA, stock no. 012888), Mvh-Cre (The Jackson Laboratory, stock no. 006954), Stra8-Cre (The Jackson Laboratory, stock no. 008208) and Amh-Cre (The Jackson Laboratory, stock no. 007915) mice were used in the present study and were described previously.37,38,59 W/W’ mice were introduced from The Jackson Laboratory (stock no. 006933) and SSC transplantation was performed as described previously.60

Fertility rate. Two- and eight-month-old Gpr177fl/fl, Mvh-Cre, Gpr177fl/fl, Stra8-Cre and Gpr177fl/fl, Amh-Cre males and their respective littermate controls were separately housed with wild-type C57BL/6 females (ratio = 1:2) for 3 months. The pregnancy rate (no. of litter/mating) and litter size (no. of pup/litter) was recorded in each group. All mice were housed under controlled photoperiod conditions and supplied with food and ddH2O ad libitum.

Isolation of Sertoli, interstitial and spermatogenic cells. The method to isolate cells from the testes of adult mice was modified slightly based on previous reports. Briefly, tubules without tunica albuginea were incubated in 1 mg/ml BSA containing 1 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and under shaking (100 r.p.m.) for 20 min in a 37°C water bath. Tubules were collected by centrifugation at 40 × g, and the crude cell suspension was filtered through a 200-mesh nylon membrane. The cell suspension was separated in a discontinuous Percoll (Pharmacia, Shanghai, China) gradient of 30, 40, 50 and 60% at 800 × g for 20 min (A gradient fraction mainly containing interstitial cells between 50 and 80% layers (1.067–1.077 g/ml) was collected, washed once in culture medium and then plated in DMEM/F12. For germ cell and Sertoli cell isolation, precipitated seminiferous tubules mentioned above were dissociated and incubated with 1 mg/ml collagenase IV, 1 mg/ml hyaluronidase, 1 mg/ml trypsin and 0.5 mg/ml DNase I (Sigma-Aldrich) in DMEM/F12 medium for 15 min at 37°C in a shaker. Dispersed cells were centrifuged at 500 × g for 5 min at 4°C and washed twice. Then, cell suspension was cultured in DMEM/F12 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS. This technique called panning for 20 min. A gradient fraction mainly containing interstitial cells between 50 and 60% interface were collected. Cells were cultured at 34°C in a mixture of 5% CO2:95% air for 18 h.

Histological examination and immunofluorescence. The control and Gpr177 cKO male mice were killed via cervical dislocation and the testes were immediately fixed in Bouin’s solution for hematoxylin and eosin (H&E) staining or in 4% formaldehyde (PFA) in PBS for immunofluorescence, as previously described. In brief, tissue sections were deparaffinised and rehydrated, followed by antigen retrieval in 10 mM sodium citrate buffer. The sections were blocked using a blocking buffer (donkey serum, 0.3% Triton X-100 in PBS) and incubated with primary antibodies against GPR177 (1:200; Santa Cruz, St. Louis, MO, USA, sc-133635), MVH (1:200; Abcam, Cambridge, UK, ab13840) or AQP3 (1:400; kind gift from Dr. Qi Chen) overnight at 4°C. Sections were washed and incubated with FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h and counterstained with DAPI (1:1000; Sigma-Aldrich) to identify the nuclei.

Quantitative (q)RT-PCR. RNA was extracted using Trizol (Invitrogen, Dallas, TX, USA) according to the manufacturer’s protocol. RNA samples were subjected to reverse transcription using a PrimeScript RT Reagent Kit (Takara, Dalian, China). The reactions were run in triplicate in three independent experiments. The CT values for the samples were normalised to the corresponding Gapdh CT values, and relative expression levels were calculated using the ΔΔCT method. The primer pair for Gpr177: forward (5’-TGAGGAACGATCTGAGGCTCC-3’) and reverse (5’-GC AGACACAGCCCAAGTTGATA-3’). The primer pair for Gpdh forward (5’-AGGTGCG GTGGGAACCAGGAT-3’) and reverse (5’-TGAGACCATGATGTGGA-3’).

Western blot. Western blot analysis was performed as described previously. Briefly, proteins were electrophoresed under reducing conditions in 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were blocked in 5% BSA and incubated overnight at 4°C with the primary antibody, followed by incubation with the secondary antibody (anti-rabbit Dye 800CW; LI-COR, St. Louis, MO, USA) for 1 h at room temperature. The specific signals and the corresponding band intensities were evaluated using Odyssey Infrared Imaging system (Odyssey, Berlin, Germany). The protein level was normalised and plotted against β-tubulin. The following antibodies were used in this study: rabbit anti-GPR177 (1:800; Santa Cruz, sc-133635) and rabbit anti-β-tubulin (1:3000; Abcam, 6046).

ELISA. The germ cells and Sertoli cells were isolated from control and Gpr177 cKO testes and cultured in DMEM/F12 with 10% serum as described above. Supernatants were collected and used for ELISA analysis for quantification of secreted WNT3 (CSB-EL026135MO), WNT4 (CSB-EL026137MO), WNT6 (CSB-EL026140MO), WNT7A (CSB-EL026141MO), WNT7B (CSB-EL026136MO), WNT10B (CSB-EL026131MO) and WNT11 (CSB-EL026135MO) using the manufacturer's instructions.

ROS assays. The generation of ROS in germ cells and Sertoli cells from control and Gpr177 cKO testes was measured using 5, and 6-chloromethyl-2,7-dichlorofluoroscein diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA, USA) at a concentration of 5 mM for 20 min. This ester diffuses into cells where it is cleaved and trapped inside the cells asDCFH. DCFH is oxidised by ROS to 2’,7’-dichlorofluorescein, which can be easily detected by its strong fluorescence. After washing the cells three times with PBS, the conversion of DCFH to dichlorofluorescein (DCF; green fluorescence) was measured using a flow cytometer (BD FACs Calibur; BD Pharmingen, St. Louis, MO, USA). Data were expressed as the percentage of the fluorescent cells.

Analysis of Caspase 3 activity. Caspase 3 activity was determined using PE active caspase 3 apoptosis kit (BD Pharmingen). Germ cells or Sertoli cells from control and Gpr177 cKO testes were resuspended in 0.5 ml Cytofix/Cytoperm solution for 20 min on ice and then incubated in 100 μl of Perm/Wash buffer containing 20 μl Caspase 3 antibody for 30 min at room temperature. Each sample was then added with 400 μl Perm/Wash buffer, and Caspase 3 activity signals were analysed by flow cytometry (BD FACS Calibur; BD Pharmingen).

Statistical analysis. Protein and mRNA levels, testis weights, ROS levels, Caspase 3 activity, fertility rate, litter size and percentage of tubules with abnormal spermatogenesis between control and Gpr177 cKO mice were analysed by using the Student’s t-test. Results are presented as mean±SEM. Statistical significance was set at *P<0.05; **P<0.01.

Conflict of Interest

The authors declare no conflict of interest.

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