Targeting the *Gdnf* Gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development

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Spermatogonial stem cells (SSCs) are a subpopulation of undifferentiated spermatogonia located in a niche at the base of the seminiferous epithelium delimited by Sertoli cells and peritubular myoid (PM) cells. SSCs self-renew or differentiate into spermatogonia that proliferate to give rise to spermatocytes and maintain spermatogenesis. Glial cell line-derived neurotrophic factor (GDNF) is essential for this process. Sertoli cells produce GDNF and other growth factors and are commonly thought to be responsible for regulating SSC development, but limited attention has been paid to the role of PM cells in this process. A conditional knockout (cKO) of the androgen receptor gene in PM cells resulted in male infertility. We found that testosterone (T) induces GDNF expression in mouse PM cells in vitro and neonatal spermatogonia (including SSCs) co-cultured with T-treated PM cells were able to colonize testes of germ cell-depleted mice after transplantation. This strongly suggested that T-regulated production of GDNF by PM cells is required for spermatogonial development, but PM cells might produce other factors in vitro that are responsible. In this study, we tested the hypothesis that production of GDNF by PM cells is essential for spermatogonial development by generating mice with a cKO of the *Gdnf* gene in PM cells. The cKO males sired up to two litters but became infertile due to collapse of spermatogenesis and loss of undifferentiated spermatogonia. These studies show for the first time, to our knowledge, that the production of GDNF by PM cells is essential for undifferentiated spermatogonial cell development in vivo.

spermatogonial stem cell | stem cell niche | male fertility | spermatogenesis | conditional gene targeting

The seminiferous epithelium is separated by tight junctions between Sertoli cells into a luminal compartment containing spermatocytes and spermatids and a basal compartment containing spermatogonial stem cells (SSCs) and spermatogonia. The basal compartment is bounded above and on the sides by Sertoli cells and below by the basement membrane of the seminiferous tubule and a layer of peritubular myoid (PM) cells. SSCs are thought to reside in a microenvironmental niche in the basal compartment, where extrinsic cues influence their decision to either self-renew or enter the pathway of spermatogonial development (1, 2). They are a minor fraction of the undifferentiated spermatogonia in the basal compartment. The other undifferentiated spermatogonia (progenitors) give rise to differentiating spermatogonia that proliferate mitotically to progress on a developmental pathway toward becoming spermatocytes (3, 4). Our current understanding of the progression of SSCs to differentiating spermatogonia comes mainly from cell kinetic studies, germ cell transplantation assays, and the use of molecular markers that identify different populations of spermatogonia.

The leading model for spermatogonial development specifies that when SSCs divide, they either self-renew by becoming two type A-single (A_s) spermatogonia or give rise to type A-paired (A_{pr}) spermatogonia connected by an intercellular bridge to become undifferentiated spermatogonia (5–7). The pairs continue to divide to form short chains of bridge-connected undifferentiated type A-aligned (A_{al}) spermatogonia, and these in turn divide to form longer chains of differentiating (type A_1, A_2, A_3, intermediate, and B) spermatogonia.

Although SSCs are single cells, not all A_s spermatogonia are likely to be SSCs. There are ~35,000 A_s spermatogonia in the testes of adult mice (8), but only about 3,000 of these have the ability to regenerate spermatogenesis when transplanted to germ cell-depleted testes (9). Although there are no generally accepted molecular markers specific for SSCs, potential candidates are inhibitor of DNA binding 4 (ID4) and paired box 7 (PAX7), which are expressed in minor subsets of A_s spermatogonia (10–12). However, it remains to be reported if ID4 and PAX7 are coexpressed in the same subset of A_s spermatogonia. SSCs also share molecular markers with undifferentiated spermatogonia, including *Nanos2*, *Gfra1*, *Zbtb16*, *Bcl6b*, and THY1(13–17). In addition, differentiating spermatogonia have characteristic molecular markers, including *Ngn3*, *Nanos3*, *Spo11*, and KIT (18–22). These molecular markers have been proven to be valuable tools for monitoring the presence or absence of different populations of spermatogonia.

A conditional knockout (cKO) of the androgen receptor (*Ar*) gene in PM cells resulted in progressive loss of spermatogonia beginning at postnatal d 21, leading to disorganization of the seminiferous epithelium and infertility (23). This strongly suggested that androgens regulate genes in PM cells whose products are essential for SSC maintenance. In other studies, mice heterozygous for a global mutation in the gene for glial cell-derived neurotrophic factor (*Gdnf*) had reduced stem cell reserves, whereas

Significance

Glial cell line-derived neurotrophic factor (GDNF) growth factor induces spermatogonial stem cells to proliferate in culture to produce progenitor spermatogonia. Sertoli cell GDNF was thought responsible for this in the testis. We showed that testosterone triggers GDNF secretion by peritubular myoid (PM) cells in culture and that undifferentiated spermatogonia (spermatogonial stem cells and progenitors) co-cultured with testosterone-treated PM cells and transplanted to sterile mice restored spermatogenesis. To determine if PM cell GDNF is essential to maintain the undifferentiated spermatogonial population, we made mice lacking the *Gdnf* gene in PM cells. The number of undifferentiated spermatogonia was severely depleted in 2-wk-old mice and adults were infertile. This is the first study, to our knowledge, to show that the undifferentiated spermatogonial pool cannot be maintained without GDNF from PM cells.

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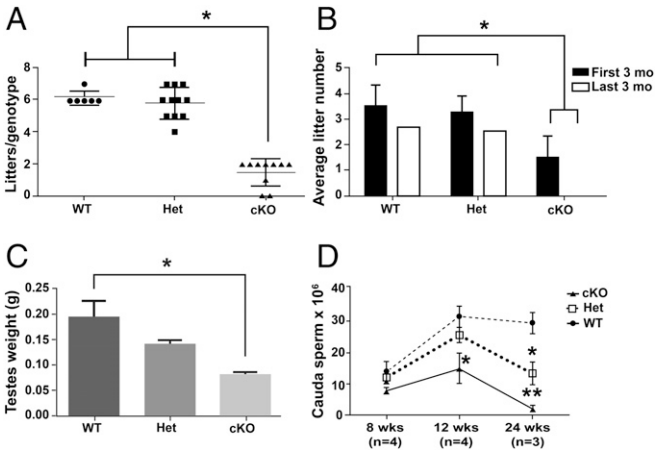

Fig. 1. Effects of targeted disruption of the *Gdnf* gene in PM cells on male reproductive function. (*A*) The number of litters sired by WT, Het, and cKO males in a 6 mo breeding study. (*B*) The average number of litters sired by WT, Het, and cKO males in the first and second 3-mo period of the breeding study. (*C*) The weights of testes from 8-wk-old WT, Het, and cKO mice. (*D*) The number of sperm released from the cauda epididymides of 8-, 12-, and 24-wk-old WT, Het, and cKO males. Each bar and data point represents the mean ± SEM. $*P < 0.05$, $**P < 0.01$.

mice with a transgene overexpressing GDNF experienced an overaccumulation of undifferentiated spermatogonia (24). GDNF also was reported to be critical for development of SSCs in vitro and their ability to restore spermatogenesis after transplantation to the testes of germ-cell-depleted mice (25, 26). This led us to hypothesize that regulation of GDNF production in PM cells by testosterone (T) is essential for SSC maintenance. In studies to test this hypothesis, we determined that PM cells isolated from adult mice and treated in vitro with T produce GDNF but not when untreated. We also found that SSCs from neonatal mice co-cultured with T-treated PM cells and transplanted to testes of germ cell-depleted mice restored spermatogenesis but not when they were co-cultured with untreated PM cells (10). These results supported the hypothesis but did not rule out the possibilities that PM cells or Sertoli cells produce other factors in vivo in addition to GDNF that are responsible for SSC self-renewal and differentiation. In these studies, we generated mice with a cKO of the *Gdnf* gene in PM cells to test the hypothesis that the production of GDNF by PM cells is essential for the in vivo development of undifferentiated spermatogonia.

Results

Disruption of the *Gdnf* Gene in PM Cells. We tested the hypothesis that the production of GDNF by PM cells in vivo was essential for development of undifferentiated spermatogonia by generating mice with a conditional deletion of one allele (Het) or both alleles (cKO) of the *Gdnf* gene in PM cells. This was done by crossing mice with exon 3 of the *Gdnf* gene flanked by LoxP sites with *Myh11*-cre,-EGFP transgenic mice (Fig. S1). The *Myh11*-cre was effective at disrupting the *Ar* gene in PM cells (23). We confirmed that MYH11 is present in PM cells by immunostaining (Fig. S1*E*, green) and that *Myh11*-cre,-EGFP is expressed in PM cells and not at detectable levels in GATA4-containing Sertoli cells (Fig. S1*F*, arrow heads). The efficacy of the *Myh11*-cre,-EGFP mice for targeting floxed genes in PM cells was verified by crossing them with (flox/stop/flox) ROSA-dtTomato mice and determining that Tomato protein is expressed in PM cells (Fig. S1*G*, red). Expression also was seen of MYH11 in blood vessels (Fig. S1*F*, red) and of dtTomato in Leydig cells (Fig. S1*G*, red), but GDNF was not expressed at detectable levels in those cells.

Effects of cKO on Fertility and Testis. The role of GDNF production by PM cells was examined first by determining the effect of the *Gdnf* cKO on male fertility. Eight-week-old wild-type (WT), Het, and cKO males were mated continuously for 6 mo with one WT female each, and the numbers of litters sired by WT males (6.17 ± 0.71) and Het males (5.82 ± 0.98) were not significantly different, whereas the number of litters sired by cKO males was significantly lower (1.5 ± 0.85) (Fig. 1*A*). The WT and Het males sired 4–7 litters and were fertile throughout the mating trial, whereas the cKO males sired 0–2 litters and were fertile only during the first 3 mo (Fig. 1*B*), suggesting an age-dependent loss of fertility in cKO males. However, the sizes of the litters sired by WT (6.54 ± 0.48), Het (7.43 ± 0.52), and cKO (5.8 ± 1.05) males were not significantly different.

To identify possible underlying causes for the loss of fertility in cKO males, other male reproductive system parameters were examined. At 8 wk, testis weights were significantly lower in cKO than in Het and WT males (Fig. 1*C*), but there were no differences in body weights (Fig. S1*D*). In addition, the sperm numbers in the cauda epididymis of 8-, 12-, and 24-wk-old WT, Het, and cKO males were determined (Fig. 1*D*). There were no significant differences at 8 wk, but by 12 wk sperm numbers were significantly lower in cKO males than in WT and Het males. By 24 wk, the sperm numbers were dramatically lower for cKO males and significantly lower for Het males than for WT males. These results led us to hypothesize that the loss of fertility with age was due to disruption of spermatogonial development.

To test this hypothesis, sections of testes from 1- to 12-wk-old WT, Het, and cKO males were immunostained for ZBTB16 (Table S1), a marker for undifferentiated spermatogonia (15). Differences were not apparent at 1 wk (Fig. S2*A*), but at 2 wk some seminiferous tubules in cKO males lacked spermatogonia (Fig. S2*B*). This increased at 4 wk (Fig. S2*C*) and was more pronounced at 8 wk (Fig. S2*D*). By 12 wk, some tubules in Het mice lacked undifferentiated spermatogonia, and spermatogenesis was severely defective in most tubules in cKO mice (Fig. S2*E*). In addition, sections of testes from 2-wk-old WT, Het, and cKO mice were immunostained with antibodies to GATA4, a marker for Sertoli cells, and to KI67 (Table S1), a marker for cell proliferation (27–29). All tubules in WT and Het mice contained KI67-labeled

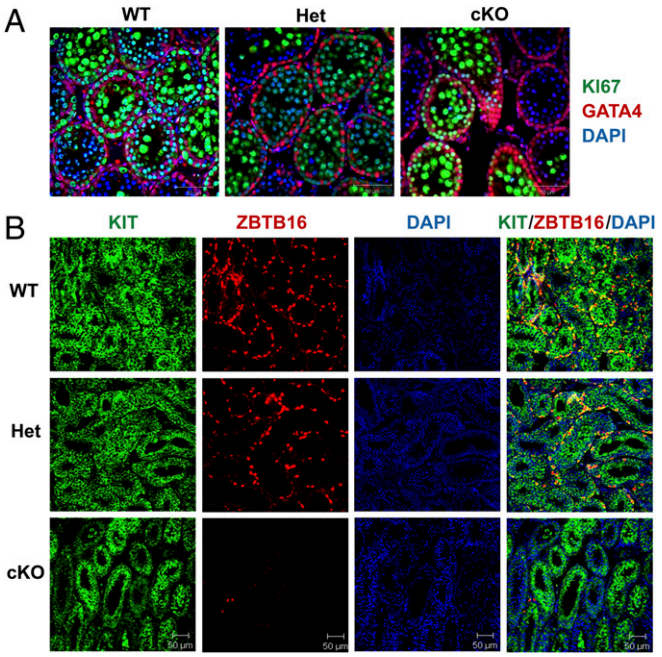

Fig. 2. Scanning confocal microscopy images of sections of testes from 2-wk-old WT, Het, and cKO mice immunostained for markers of spermatogonia, Sertoli cells, and cellular proliferation. (*A*) Sections immunostained for markers of Sertoli cells (GATA4) and for cellular proliferation (KI67). (*B*) Sections immunostained for markers of differentiating spermatogonia (KIT) and for SSCs and undifferentiated spermatogonia (ZBTB16), counterstained for DNA (DAPI).

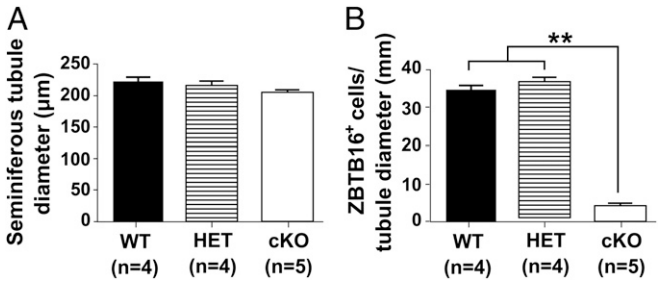

Fig. 3. Tubule diameters and numbers of undifferentiated spermatogonia in WT, Het, and cKO mice at 2 wk. (*A*) Seminiferous tubule cross-section diameter (μm). (*B*) The number of ZBTB16-positive cells per tubule diameter (mm). Each bar represents the mean ± SEM. ANOVA analysis with Tukey test. $^{**}P < 0.01$. (Scale bar: 50 μm.)

germ cells, whereas some tubules in cKO mice lacked KI67-labeled germ cells (Fig. 2*A*). In addition, sections of testes from 2-wk-old mice were co-immunostained with antibodies to ZBTB16 and to KIT (Table S1), a marker for differentiated spermatogonia (22). There were little apparent differences in the KIT staining in WT, Het, and cKO mice (Fig. 2*B*), but the number of ZBTB16-positive germ cells per tubule cross-section was dramatically lower in cKO testes compared with the HET or WT testes (Figs. 2*B* and 3*B*). There were no differences in the diameter of seminiferous tubules of WT, Het, and cKO mice (Fig. 3*A*). Similar results were seen in sections immunostained for HSPA2 (Table S1), a marker for meiotic and postmeiotic spermatogenic cells (30, 31). An occasional cKO tubule cross-section showed a lack of HSPA2-positive germ cells at 2 wk (Fig. 4*A*), more tubules lacked HSPA2-positive germ cells at 4 and 8 wk (Fig. 4 *B* and *C*), and most tubules lacked HSPA2-positive germ cells (Sertoli cell only) at 12 wk (Fig. 4*D*). Possible interpretations of these results were that the low number was either due to apoptosis of spermatogonia or to a failure to repopulate the spermatogonial pool in cKO testes.

TUNEL labeling and flow cytometry were used to test these possibilities. There were no significant differences in the frequency of TUNEL-labeled apoptotic cells per tubule cross-section in testes of 2-wk-old WT, Het, and cKO mice (Fig. S3 *A* and *B*). The gated area for analyzing apoptosis of undifferentiated spermatogonia by flow cytometry was set using previously reported criteria (10, 32) and captured 6.44 ± 1.23% of cells in WT and 3.37 ± 0.56% of cells in cKO samples (Fig. S3*C*). When an antibody to Annexin V (ANXA5) (Table S1) (33) was used to label the early apoptotic cell population (Q4), differences were not significant between WT (0.06 ± 0.09%) and cKO (0.08 ± 0.1%) testes (Fig. S3 *C* and *D*). Similar results were seen for the late apoptotic cells population (Q2) of WT (0.06 ± 0.09%) and cKO mice (0.1 ± 0.08%). These results suggest that the reduction of differentiated spermatogonia was not due to apoptosis but rather the result of a preceding reduced population of undifferentiated spermatogonia.

Effects of cKO on *Gdnf* mRNA and GDNF Protein Expression. The previous findings suggested that a lack of GDNF production by PM cells in cKO mice disrupts developmental progression of undifferentiated spermatogonia. To examine this possibility, we first used quantitative PCR (qPCR) to compare *Gdnf* (Table S2) mRNA levels in testes from 8-wk-old WT, Het, and cKO mice. The *Gdnf* steady-state levels were significant lower in cKO mice compared with the levels in WT and Het mice (Fig. 5*A*). To determine if this was due to a reduction in Sertoli cell numbers, sections were immunostained for SOX9 (Fig. S4*A*), a marker for Sertoli cells (34). When SOX9-positive cells were counted and normalized to the total number of tubules per section, no significant differences in Sertoli cell numbers were found between WT, Het, and cKO mice (Fig. S4*B*). In addition, ELISAs were used to measure the GDNF protein concentration in testes of WT, Het, and cKO mice. When the GDNF ELISA results were normalized to the ELISA levels for inhibin beta-B (INHBB), an indicator of Sertoli cell numbers and function (35), the GDNF levels in cKO testes were found to be reduced by about 40% compared with WT testes (Fig. 5*B*). Also, when sections of testes from 12-wk-old mice were immunostained, the GDNF signal seemed similar in Sertoli cells in WT, Het, and cKO testes; appeared to be reduced in PM cells in Het testes compared with WT testes; and were not detectable in PM cells in cKO testes (Fig. S4*D*). Furthermore, seminal vesicle weights were not significantly different between WT, Het, and cKO mice, implying that their androgen levels were comparable (Fig. S4*C*). These results suggested that production of GDNF by Sertoli cells and of androgens by Leydig cells were not disrupted in cKO mice and that decreases in *Gdnf* mRNA and GDNF protein levels in the testis were the direct result of disruption of the *Gdnf* gene in PM cells.

Effects of cKO on Genes Involved in Spermatogonial Development. We determined if the cKO of *Gdnf* in PM cells alters the expression of certain other genes involved in SSC development. FGF2 is expressed by Sertoli cells and was reported to enhance the ability of GDNF to maintain SSC proliferation in vitro (26, 32, 36, 37). However, no significant differences were detected by ELISA in FGF2 protein levels in testes of 8-wk-old WT, Het, and cKO mice (Fig. 5*C*) or in *Fgf2* (Table S3) mRNA levels in testes of 1- and 4-wk-old WT, Het, and cKO mice (Fig. S5 *A* and *B*). The steady-state transcript levels for other growth factors (Table S3) indicated to be beneficial to SSC self-renewal in vitro, including *Lif* (produced by Leydig cells) (38, 39) and *Csf1* (produced by Leydig cells and PM cells) (40), were not significantly different in testes of 1- and 4-wk-old WT and cKO mice (Fig. S5 *A* and *B*).

We also evaluated the expression of genes in testes of 1-wk-old WT, Het, and cKO mice for markers of SSCs and undifferentiated spermatogonia (Tables S2 and S3), including *Bcl6b* (16), *Zbtb16* (15, 41), and *Nanos2* (42), and for markers of differentiating spermatogonia, including *Ngn3* (18, 43), *Kit* (21), *Nanos3* (44), *Spo11* (45), and *Dnmt3l* (46). The expression levels of markers of undifferentiated spermatogonia were significantly lower in cKO mice than in WT or Het mice (Fig. 6), whereas the markers for differentiated spermatogonia were significantly lower in WT than in cKO mice (Fig. 7). This suggests that most spermatogonia in the

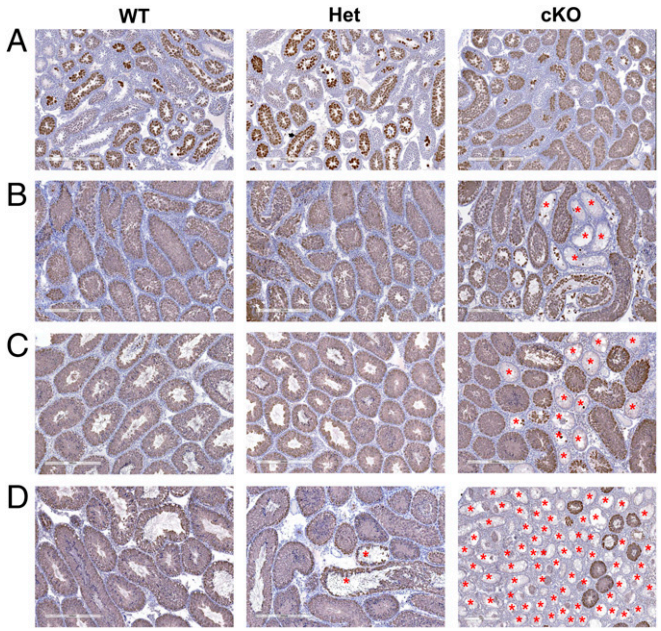

Fig. 4. Sections of testes from WT, Het, and cKO mice immunostained for HSPA2, a marker for meiotic and postmeiotic spermatogenic cells. (*A*) Two-week-old mice. (*B*) Four-week-old mice. (*C*) Eight-week-old mice. (*D*) Twelve-wk-old mice. (Scale bar: 300 μm.)

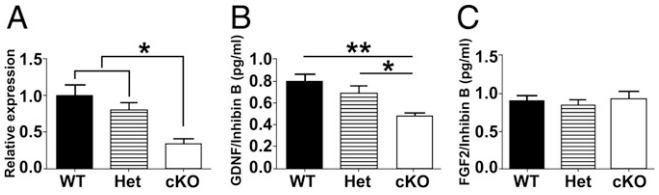

Fig. 5. The *Gdnf* mRNA and GDNF and FGF2 protein levels in testes of 8-wk-old mice. (*A*) The *Gdnf* mRNA levels in WT, Het, and cKO mice relative to *Sox9* levels. (*B*) The GDNF protein levels in WT, Het, and cKO mice relative to INHBB levels. (*C*) The FGF2 protein levels in WT, Het, and cKO mice relative to INHBB levels. ANOVA analysis with Tukey test. $*P < 0.05$, $**P < 0.01$.

testes of 1-wk-old cKO mice are differentiating spermatogonia. In addition, we determined the steady-state levels of expression of genes in 4-wk-old WT, Het, and cKO mice for markers of undifferentiated spermatogonia (*Bcl6b*, *Gfra1*, *Zbtb16*, and *Ret*) and differentiating spermatogonia (*Spo11*, *Nanos3*, *Kit*, and *Dnmt3l*). The markers for undifferentiated spermatogonia were significantly lower at 4 wk in cKO mice than in WT mice (Fig. S6 *A–D*), whereas there were no significant differences for most markers for differentiated spermatogonia in cKO and WT mice (Fig. S6 *E–H*). The exception was a higher level of expression of *Spo11* in cKO than in WT mice, indicating a higher spermatocyte-to-spermatogonia ratio at 4 wk in cKO than in WT or Het mice. These results suggest that the initial fertility in a few cKO males and subsequent collapse in spermatogenesis in all cKO males are preceded by a decline in the level of undifferentiated spermatogonia but not in the level of differentiated spermatogonia in testes of 1-wk-old cKO mice or of spermatocytes in 4-wk-old cKO mice.

Lack of Effects of cKO on Undifferentiated Spermatogonia. A defining characteristic of SSCs is their ability to establish colonies of undifferentiated spermatogonia and to reestablish spermatogenesis when transplanted to testes of mice depleted of germ cells. To determine if the cKO had a direct effect on the development potential of SSCs, testicular cells from neonatal Het and cKO mice carrying the *Myh11*-cre,-EGFP transgene were transplanted into the testes of germ cell-depleted recipient mice. When testes of recipient mice were examined 11 wk later, colonies of GFP-expressing spermatogenic cells derived from Het and cKO donors were seen (Fig. S7*A*). Sections of testes receiving germ cells from Het and cKO donor mice immunostained with an antibody to GFP contained elongated spermatids expressing *Myh11*-cre,-EGFP (Fig. S7*B*), indicating that the SSCs from Het and cKO mice were able to restore spermatogenesis in germ cell-depleted testes and that neonatal SSCs do not require GDNF from PM cells to acquire their developmental potential.

Discussion

We generated mice with a cKO in the *Gdnf* gene in PM cells to test the hypothesis that GDNF produced by PM cells is essential for development of undifferentiated spermatogonia. The cKO resulted in a loss of fertility, with cKO males siring 0–2 litters during the first 3 mo and no litters during the second half of a 6-mo mating study. There was a significant reduction by 8 wk in testis weight in cKO males compared with WT males due to a collapse of spermatogenesis, with most seminiferous tubules lacking ZBTB16-positive undifferentiated spermatogonia and HSPA2-positive spermatocytes and spermatids by 12 wk of age. This resulted in a significant reduction in the number of sperm in the cauda epididymis of 12-wk-old cKO males and a paucity of sperm in the cauda epididymis of 24-wk-old cKO males. These results supported the hypothesis and encouraged further studies to determine the underlying cause of the infertility.

The first wave of spermatogenesis in the mouse starts 2–3 d after birth with gonocytes dividing and giving rise to SSCs and undifferentiated spermatogonia, continues with the expansion of the undifferentiated spermatogonial population, and progresses to undifferentiated spermatogonia giving rise to differentiating spermatogonia beginning at 6 d of age (47, 48). SSCs share molecular markers with undifferentiated spermatogonia (13–17), and these markers were expressed at significantly higher levels at 1 wk after birth in the testes of WT mice than in cKO mice. However, molecular markers for differentiated spermatogonia (18–22) were present at significantly higher levels at 1 wk after birth in cKO mice than in WT mice. In addition, germ cells containing the ZBTB16 marker for SSCs and undifferentiated spermatogonia (15) were detected by immunohistochemistry in 2-wk-old WT mice, but rarely in cKO males, and some of their tubules lack proliferating germ cells. We interpret these results to indicate that SSCs and undifferentiated spermatogonia generate differentiated spermatogonia in the first wave of spermatogenesis in both WT and cKO mice but that the undifferentiated spermatogonial pool is not replenished in cKO mice. In addition, markers for differentiating spermatogonia and spermatocytes were expressed at comparable levels in testes of 4-wk-old WT and cKO mice, suggesting that spermatogonial and spermatocyte differentiation proceeds on schedule during the first wave of spermatogenesis in cKO mice. These results provide strong evidence that GDNF produced by PM cells is required to replenish the undifferentiated spermatogonial pool but not for generating the first wave of spermatogonial differentiation or for the further development of spermatogenic cells.

Earlier studies provided evidence that SSCs were sensitive to changes in the level of GDNF in the testis. Mice with a global targeted disruption of one allele of the *Gdnf* gene (global Het) were fertile but showed depletion of stem cell reserves, whereas transgenic mice with global overexpression of GDNF showed accumulation of undifferentiated spermatogonia (24). Disruption of both alleles (global KO) of the genes for GDNF or for either the RET or GDNFR1 subunits of the GDNF receptor resulted in disruption of renal and neuronal development and early postnatal death (49–52). In addition, 30–35% of *Gdnf* global Het mice had renal defects (49, 53). In vitro studies since have demonstrated that GDNF regulates expression of genes in SSCs that influence their self-renewal and differentiation (1, 3, 54). Because GDNF has a critical role in multiple developmental processes in addition to spermatogenesis, it remained uncertain whether the perturbations of SSC development in vivo were due to direct effects on SSCs or indirect effects of total GDNF levels. In the present study with PM cell *Gdnf* Het and cKO males, the following was observed: (*i*) a modest reduction in testis weight in Het males at 8 wk and a collapse in spermatogenesis in some tubules in Het males at 12 wk; (*ii*) a significant reduction in the number of sperm in the cauda epididymis in 12- and 24-wk-old Het males; (*iii*) a significant reduction in *Gdnf* mRNA and GDNF protein levels in the testes of Het and cKO mice, with protein levels being reduced about 20% in Het mice and 40% in cKO mice; (*iv*) a lack of GDNF detectable by confocal microscopy in PM cells of cKO mice; (*v*) an absence of obvious changes in GDNF levels in Sertoli cells of Het or cKO mice; and (*vi*) SSCs from neonatal Het and cKO males were able to repopulate the testes of germ cell-depleted mice. In addition, seminal vesicle weights and Sertoli cell numbers were comparable in WT and cKO mice, suggesting intact endocrine function. This confirms and extends the earlier findings that GDNF levels influence SSC development and demonstrates that GDNF levels must be kept within a relatively narrow range in

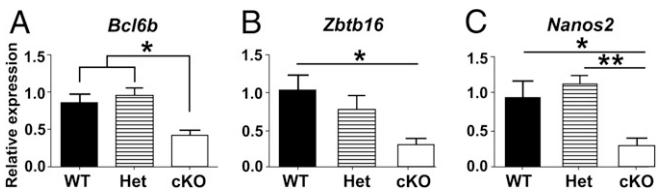

Fig. 6. The mRNA levels for markers of undifferentiated spermatogonia in testes of WT, Het, and cKO 1-wk-old mice relative to *Sox9* levels. (*A*) *Bcl6b*. (*B*) *Zbtb16*. (*C*) *Nanos2*. ANOVA analysis with Tukey test. $*P < 0.05$, $**P < 0.01$.

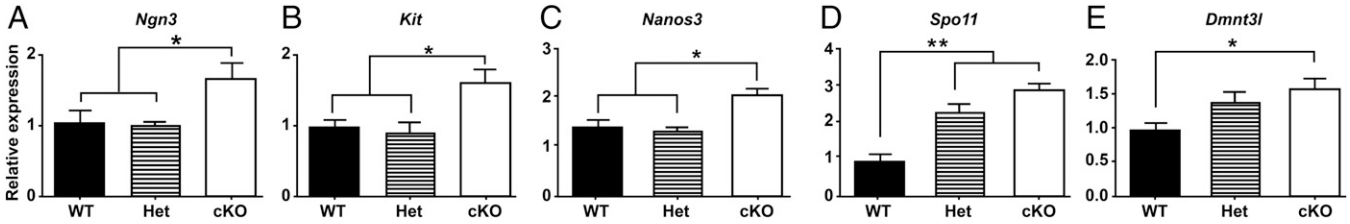

Fig. 7. The mRNA levels for markers of differentiated spermatogonia in testes of WT, Het, and cKO 1-wk-old mice relative to *Sox9* levels. (*A*) *Ngn3*. (*B*) *Kit*. (*C*) *Nanos3*. (*D*) *Spo11*. (*E*) *Dnmt3l*. ANOVA analysis with Tukey test. $*P < 0.05$.

the testis for SSC maintenance. A novel finding of the present study is that the production of GDNF by PM cells is essential for ensuring that optimal GDNF levels exist for repopulating the undifferentiated spermatogonial pool.

Although these studies show the importance of GDNF production by PM cells in vivo, previous in vitro studies suggested that other factors might be required as well. Colony stimulating factor 1 (CSF1) is produced by Leydig and PM cells and enhanced the self-renewal of SSCs in vitro in the presence of GDNF (40), as did fibroblast growth factor 2 (FGF2) (26, 37, 55), whose transcripts were identified in Sertoli cells (56, 57). Sertoli cells are recognized to have a key role in forming the niche and in determining the number of niches and SSCs in the testis (58). In addition, an androgen-stimulated factor produced by rat PM cells, referred to as peritubular factor that modulates Sertoli cell function (PModS), positively influenced production of transferrin (TRF), androgen binding protein (ABP, SCGB1B27), inhibin (59), and other proteins by Sertoli cells (60, 61). The activity was contained in 56–59-kDa fractions of PM cell-conditioned medium, but its molecular identity remains unknown. It also was found that PM cells produced neuregulin, Sertoli cells expressed receptors for neuregulin, and recombinant neuregulin stimulated TRF and ABP production by Sertoli cells (62). Furthermore, PM cells secrete IGF1 and FGF2, and these also were able to stimulate TRF and ABP production by Sertoli cells (62). However, these studies were performed in vitro, and GDNF is the only factor shown so far to influence SSCs in vivo. Although FGF2 was reported to regulate *Gdnf* expression levels in Sertoli cells (63), changes in FGF2 protein or *Fgf2* mRNA levels were not seen in *Gdnf* cKO testes. Disruption of the *Ar* gene in Sertoli cells resulted in infertility due to arrest of spermatogenesis in the meiotic (64, 65) or postmeiotic phases (66), suggesting that androgen-regulated processes in Sertoli cells are not essential for spermatogonia. Nevertheless, Sertoli cells are likely to be key players in the network of cell–cell interactions and in the production by neighboring cells of factors that define and regulate the microenvironment of the SSC niche. The generation of mice with a cKO of genes for GDNF or other factors in Sertoli cells or in other cells in the testis will be needed to determine their roles in regulating SSC development in vivo.

Previous studies demonstrated that (*i*) cKO of the *Ar* gene in PM cells resulted in infertility (23), (*ii*) androgen response elements (AREs) were present in the promoter region of the *Gdnf* gene in mice (67), (*iii*) GDNF was essential for SSC maintenance in vitro (1, 9, 26), and (*iv*) PM cells isolated from human testes produced GDNF (68). We recently reported that (*i*) PM cells in adult mice produce GDNF, (*ii*) isolated PM cells from adult mice produce GDNF in response to T treatment, and (*iii*) SSCs co-cultured with T-treated PM cells are significantly more effective at repopulating the testes of germ cell-depleted mice following transplantation than SSCs co-cultured with untreated PM cells (10). The results of our previous and present studies have led to the identification of a model for the direct endocrine role in the regulation of spermatogonial cell development, one in which T acts through PM cells to modulate the level of GDNF in the testis niche to optimize renewal of the undifferentiated spermatogonial pool. This represents a novel and significant new paradigm for the extrinsic regulation of spermatogonial development. It remains to be determined if GDNF acts in vivo to influence the SSC self-renewal/differentiation switch, to regulate the proliferation of undifferentiated spermatogonia, or to modulate a combination of these processes.

Materials and Methods

Mice with a floxed *Gdnf* allele [$Gdnf^{f/+}$;B6.129S1(Cg)-$Gdnf^{tm1.1Neas}$/J, JAX stock no. 014097] and transgenic mice expressing cre recombinase driven by the *Myh11* promoter [B6.Cg-Tg(*Myh11*-cre,-EGFP)2Mik/J, JAX stock no. 007742] were used. All animal procedures were approved in advance by the National Institute of Environmental Health Sciences Animal Care and Use Committee. Further details on generating the PM cell *Gdnf* cKO mice and the procedures used in these studies are described in *SI Materials and Methods*.

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