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The Luteinizing Hormone-Testosterone Pathway Regulates Mouse Spermatogonial Stem Cell Self-Renewal by Suppressing WNT5A Expression in Sertoli Cells

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SUMMARY

Spermatogenesis originates from self-renewal of spermatogonial stem cells (SSCs). Previous studies have reported conflicting roles of gonadotrophic pituitary hormones in SSC self-renewal. Here, we explored the role of hormonal regulation of SSCs using Fshb and Lhcgr knockout (KO) mice. Although follicle-stimulating hormone (FSH) is thought to promote self-renewal by glial cell line-derived neurotrophic factor (GDNF), no abnormalities were found in SSCs and their microenvironment. In contrast, SSCs were enriched in Lhcgr-deficient mice. Moreover, wild-type SSCs transplanted into Lhcgr-deficient mice showed enhanced self-renewal. Microarray analysis revealed that Lhcgr-deficient testes have enhanced WNTSA expression in Sertoli cells, which showed an immature phenotype. Since WNTSA was upregulated by anti-androgen treatment, testosterone produced by luteinizing hormone (LH) is required for Sertoli cell maturation. WNTSA promoted SSC activity both in vitro and in vivo. Therefore, FSH is not responsible for GDNF regulation, while LH negatively regulates SSC self-renewal by suppressing WNTSA via testosterone.

INTRODUCTION

Spermatogonial stem cells (SSCs) are the founder cell population of spermatogenesis (de Rooij and Russell, 2000; Meistrich and van Beek, 1993). Although their number in testes is very small (0.02%–0.03% of total germ cells), they are the only stem cells in the germline that have the unique ability to undergo self-renewal division to produce numerous progenitor cells. SSCs are thought to develop from gonocytes in the neonatal testes during the perinatal stage (Shinohara et al., 2001). SSCs rapidly expand their population size in adults. The SSC population size is determined based on the number of niches (Oatley et al., 2011), which likely develop through complex interactions among Sertoli cells, Leydig cells, peritubular myoid cells, and macrophages (DeFalco et al., 2015; Meng et al., 2000; Oatley et al., 2009; Spinnler et al., 2010). Sexual maturation is accompanied by dynamic changes in hormonal milieu, and development of these cell types is influenced by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted from the pituitary gland. However, little is known regarding the roles of these hormones in SSC and niche development.

SSC research was revolutionized by the development of spermatogonial transplantation in 1994 (Brinster and Zimmermann, 1994). This technique provided the first functional assay for SSCs and has been widely used to characterize SSCs. In initial attempts to improve the colonization efficiency of SSCs, suppression of hypothalamus signaling was found to confer beneficial effects on transplantation. Treatment of recipient animals with leuprolide, a gonadotropin-releasing hormone (GnRH) analog, resulted in enhanced colonization of donor cells. The donor cell colony count increased by approximately 2.7-fold, which was suggestive of enhanced homing of SSCs (Ogawa et al., 1998). Moreover, leuprolide appeared to enhance self-renewal division because the colonized area in leuprolide-treated recipients increased by 5.3-fold (Dobrinski et al., 2001). Prolonged administration of leuprolide suppresses the secretion of LH and FSH from the pituitary gland, which induces testosterone production by Leydig cells and stimulates the proliferation of Sertoli cells, respectively (Karashima et al., 1988). Although a GnRH analog is generally suppressive to spermatogenesis in wild-type (WT) mice, it protects SSCs from damage by chemical or radiation treatment (Meistrich, 1998). This suppressive effect of the gonadotrophic pituitary hormones on SSC self-renewal was confirmed in another study, which showed that SSCs underwent more extensive self-renewal divisions in hypophysectomized hosts (Kanatsu-Shinohara et al., 2004). Several possibilities, including the direct action of GnRH on germ cells, reduction of intratesticular testosterone, or increased kit ligand (KITL) expression, are believed to be involved. However, further studies are required (Ogawa et al., 1998). These studies performed...
Figure 1. Functional Analysis of SSCs in Fshb KO Mice
(A) Testis weight of 8-day-old and 6-week-old mice (n = 4 testes).
(B–D) Immunohistochemistry and quantification of indicated spermatogonia markers in Fshb KO adult mouse testes. At least 200 cells in four testes were counted.
(E) Real-time PCR analysis of busulfan-treated adult mouse testes (n = 7 experiments).
(F) Western blot analysis of busulfan-treated adult mouse testes.

(legend continued on next page)
more than a decade ago established the beneficial effects of suppressing gonadotropic pituitary hormones in promoting SSC self-renewal.

In 2000, glial cell line-derived neurotrophic factor (GDNF) was identified as an SSC self-renewal factor (Meng et al., 2000). GDNF is a member of the transforming growth factor β superfamily, which is expressed in Sertoli cells. Transgenic mice overexpressing Gdnf showed clumps of undifferentiated spermatogonia in seminiferous tubules, while Gdnf heterozygous knockout (KO) mice underwent depletion of spermatogonia, resulting in male infertility. These results suggest that the amount of GDNF influences the fate of SSCs. The fact that GnRH analog treatment increased the length of the colony suggests that GDNF is induced by suppression of the gonadotropic pituitary hormones. However, subsequent studies showed that GDNF expression in Sertoli cells is positively regulated by FSH (Tadokoro et al., 2002). In that study, the proliferation of undifferentiated spermatogonia was significantly reduced when FSH was depleted using a gonadotropin-releasing hormone antagonist (Nal-Glu). These testes showed reduced GDNF expression. Moreover, FSH (but not testosterone) increased GDNF expression in testis cell culture. Regulation of GDNF expression by FSH was also supported by another in vivo study that showed increases in Gdnf mRNA levels in testes of immature mice that had been treated with FSH (Ding et al., 2011). However, this FSH-mediated regulation of GDNF was not confirmed in a testis cell-culture system that can maintain SSCs for the long term without FSH (Kanatsu-Shinohara et al., 2012).

In addition to FSH-mediated regulation, more recent studies suggest the involvement of testosterone in GDNF expression. Although GDNF was thought to be expressed in Sertoli cells, it has been shown that GDNF is expressed in peritubular myoid cells in both mouse and human testes (Chen et al., 2014; Spinnler et al., 2010). Testosterone induced GDNF expression at the mRNA and protein levels in peritubular cells in vitro (Chen et al., 2014). THY1-expressing mouse spermatogonia, which are thought to be enriched for SSCs, produced more colonies by testosterone treatment when they were cultured with peritubular myoid cells. Males that lacked Gdnf in peritubular cells were initially fertile but lost undifferentiated spermatogonia over the long term (Chen et al., 2016). Thus, conflicting reports exist on the role of the gonadotropic pituitary hormones in SSC regulation, and our current understanding is apparently incomplete.

In this study, we examined the impact of hormonal signaling on SSC self-renewal using follicle-stimulating hormone β (Fshb; FSH β subunit) and luteinizing hormone/choriogonadotropin receptor (Lhcgr) KO mice. FSH acts on Sertoli cells within seminiferous tubules, while LHCGR are detected only on the testosterone-producing Leydig cells located between the tubules. Fshb KO mice are fertile but have smaller testes with reduced Sertoli and germ cell numbers (Kumar et al., 1997). Lhcgr KO mice have undescended testes and are infertile (Lei et al., 2001; Zhang et al., 2001). SSC activities of immature and mature testes of these mutant mice were determined based on spermatogonial transplantation into WT mice. We also examined the effect of mutant testicular microenvironments on SSC homing and self-renewal division by serial transplantation. Microarray analysis revealed that Wnt5a is involved in SSC self-renewal by hormonal signaling.

RESULTS

Phenotypic and Functional Analysis of Spermatogonia in Fshb KO Mice

Because FSH has been implicated in the regulation of GDNF expression, we first used Fshb KO mice to examine the effect of this gene on SSCs (Kumar et al., 1997). Testis weight was significantly lower in both pup and adult Fshb KO mice than in the control at each stage (Figure 1A) (p = 0.0073 for pup; p = 0.0059 for adult), suggestive of abnormalities in differentiation. Immunohistochemical analysis of adult testes showed no significant changes in the number of cells expressing glial cell line-derived neurotrophic factor family receptor α1 (GFRA1; a marker for A single, A paired, and A aligned spermatogonia) (Figure 1B). However, the number of cells expressing cadherin 1 (CDH1; a marker for undifferentiated spermatogonia) or Kit oncogene (KIT; a marker for differentiating spermatogonia) was significantly decreased (Figures 1C and 1D) (p < 0.0001 for CDH1; p = 0.0037 for KIT), suggesting that FSH may play a role in spermatogonia differentiation. We also examined the expression of several molecules involved in spermatogonia proliferation/fate in busulfan-treated testes based on real-time PCR. Although neuregulin 1 (Nrg1) was expressed more strongly in Fshb KO mice (Figure 1E) (p = 0.0017), western blot analysis showed no changes in NRG1 expression (Figure 1F). Neither GDNF nor fibroblast growth factor 2 (FGF2) showed significant changes by western blotting.

(G) Macroscopic appearance of recipient testes.

(H) Colony counts (n = 18 testes for pup, n = 16 testes for adult; three experiments). Counterstain: Hoechst 33342 (B–D). Scale bars represent 25 μm (B–D) and 1 mm (G). Error bars indicate SEM. Asterisks indicate statistical significance. See also Tables S2 and S3.
Although these results indicate that undifferentiated spermatogonia are not influenced by the absence of FSH signaling, SSCs are defined by their function and comprise a small number among undifferentiated spermatogonia. Therefore, the effects on SSCs could not be determined based on morphology alone. To clarify this point, we performed spermatogonial transplantation using pup and adult testes and examined their SSC activity. Fshb KO mice were crossed with green mice to introduce a donor cell marker. Testis cells from pup and adult mice were transplanted into congenitally infertile WBB6F1-W/W° mice (W mice) to determine the SSC activity.

Analyses of recipient mice at 2 months post transplantation revealed that comparable numbers of germ cell colonies were generated from Fshb KO and WT testes regardless of age (Figure 1G). The numbers of colonies from Fshb KO and WT pup testis cells were 6.3 and 7.3 per 10⁵ cells, respectively (n = 18). Likewise, the numbers of colonies from Fshb KO and WT adult testis cells were 2.7 and 2.3 per 10⁵ cells, respectively (n = 16) (Figure 1H). Histological analysis of recipient testes also showed similar levels of colonization from both Fshb KO and control mice regardless of the donor age (data not shown). These results suggest that the absence of FSH signaling does not influence the development of SSCs in postnatal testis, although immunohistochemical analysis is suggestive of abnormalities in differentiation of committed spermatogonia.

### Phenotypic and Functional Analysis of Spermatogonia in Lhcgr KO Mice

In the second set of experiments, we used Lhcgr KO mice and examined their SSC activity (Lei et al., 2001). Although the testis weight of Lhcgr KO pups was comparable with that of control mice, it was significantly reduced in adults (Figure 2A) (p = 0.00013), suggesting that Lhcgr deficiency caused testis abnormalities distinct from those observed in Fshb mice. Consistent with this hypothesis, immunohistochemical analysis of adult testes revealed that Lhcgr KO mice have more GFRA1-expressing spermatogonia than controls (Figure 2B) (p < 0.0001). However, the number of CDH1+ and KIT+ spermatogonia was reduced in these mice (Figures 2C and 2D) (p < 0.0001 for CDH1; p < 0.0001 for KIT), suggesting that the total undifferentiated spermatogonia population and differentiating spermatogonia population are smaller than those in WT mice. Moreover, although meiotic cells were found, no elongated spermatids developed in these mice (Figure 2E), which was consistent with our previous study (Lei et al., 2004). Because GFRA1 is thought to be expressed in SSCs (Kanatsu-Shinohara and Shinohara, 2013), these results suggest that Lhcgr deficiency is accompanied by increases in SSCs and decreases in differentiating spermatogonia. To explore the mechanism underlying this observation, we performed real-time PCR and examined busulfan-treated mouse testes that do not have germ cells. Although testosterone is thought to increase GDNF expression in testis (Chen et al., 2016), our analyses revealed significantly increased expression of several candidate mRNAs, including those of Gdnf, Fgf2, and Nrg1, in Lhcgr KO mice (Figure 2F) (p < 0.0001 for Gdnf; p < 0.0001 for Fgf2; p < 0.0001 for Nrg1). This occurred despite the significantly reduced production of testosterone (Figure 2G) (p = 0.0023). However, we did not observe an increase in the protein level based on western blot analysis (Figure 2H).

We used pup and adult testes and quantified the numbers of SSCs based on spermatogonial transplantation. These transplantation experiments showed a comparable number of colonies between testis cells prepared from immature Lhcgr KO and WT mice (Figure 2I). The number of colonies generated by 10⁵ Lhcgr KO and control pup testis cells were 16.9 and 15.1, respectively (n = 14) (Figure 2J). However, as expected from the immunohistochemical staining, significantly more colonies were found in adult Lhcgr KO mice. The number of colonies generated by 10⁵ Lhcgr KO and control adult testis cells was 3.0 and 1.1, respectively (n = 18), showing approximately 2.7-fold SSC enrichment in Lhcgr KO mice (p = 0.0071). Transplantation experiments also rescued the abnormal spermatogenesis of Lhcgr KO Mice.

**Figure 2. Functional Analysis of SSCs in Lhcgr KO Mice**

(A) Testis weight of 8-day-old and 6-week-old mice (n = 4 testes).

(B–D) Immunohistochemistry and quantification of indicated spermatogonia markers in Lhcgr KO adult mouse testes. At least 200 cells in four testes were counted.

(E) Immunohistochemistry of meiotic (SYCP3) and haploid (peanut agglutinin, PNA) cell markers.

(F) Real-time PCR analysis of busulfan-treated adult mouse testes (n = 8 testes). PCR was performed in triplicate.

(G) Intratesticular testosterone levels in untreated adult Lhcgr KO mice (n = 7 testes).

(H) Western blot analysis and quantification of GDNF, FGF2, and NRG1 in busulfan-treated adult mouse testes (n = 6 testes).

(I) Macroscopic appearance of recipient testes.

(J) Colony counts (n = 14 testes for pup, n = 18 testes for adult; three experiments).

(K) Histology of donor and recipient testes. Arrowheads indicate elongated spermatids.

Counterstain: Hoechst 33342 (B–E). Scale bars represent 25 µm (B–E and K), and 1 mm (I). Error bars indicate SEM. Asterisks indicate statistical significance. See also Tables S2 and S3.
KO germ cells. Although germ cell differentiation was arrested at the round spermatid stage in Lhcgr KO donor testis, elongated spermatids were found after transplantation in recipient testes (Figure 2K). These results indicate that SSCs are enriched in Lhcgr KO testes and suggest that arrested spermatogenic differentiation is caused by abnormalities in the testicular microenvironment.

Evaluation of the Testicular Microenvironment by Serial Transplantation

Although the results in the preceding section showed 2.7-fold enrichment of SSCs in Lhcgr KO testes, it remains unclear whether the enrichment was due to the reduced number of post-meiotic germ cells in these mice or increased self-renewal factor secretion. To explore these possibilities, we performed spermatogonial transplantation into the mutant microenvironment to examine the impact of the testicular microenvironment on donor SSC self-renewal.

When the recipients were analyzed 2 months after transplantation, we did not observe significant changes in the number of colonies in both Fshb and Lhcgr KO mouse recipient testes. The numbers of colonies generated by 10^5 donor cells were 6.3 ± 1.0 and 5.1 ± 0.8 in Fshb KO and control recipient mice, respectively (n = 18 for Fshb KO; n = 20 for control) (Figures 3B and 3C). Likewise, the numbers of colonies generated by 10^5 donor cells were 4.9 ± 0.9 and 3.6 ± 0.4 in Lhcgr KO and control recipient mice, respectively (n = 18 for Lhcgr KO; n = 19 for control) (Figures 3D–3F); however, the differences in these numbers were not statistically significant. The lack of effects in Fshb and Lhcgr KO mice contrasted with previous studies using...
GnRH analogs or hypophysectomized mice, which showed increased colony formation.

Although the results indicated no apparent changes in colony numbers in both mutants, the increased number of GFRA1+ spermatogonia and enrichment of SSCs in Lhcgr KO mice were suggestive of enhanced SSC self-renewal in Lhcgr KO mouse testes. Therefore, we performed serial transplantation to explore whether the degree of self-renewal is improved in Lhcgr KO mouse recipient testes by dissociating the recipient testes and transplanting into secondary recipients using W mice. Assuming that each colony is derived from single SSCs, and that 10% of the injected cells colonize in the secondary recipients (Kanatsu-Shinohara et al., 2006; Nagano et al., 1999), the ratio of SSC number between the two time points was 20.5 ± 4.9 and 10.6 ± 3.5 in Lhcgr KO and control recipients, respectively (n = 15 for Lhcgr KO; n = 14 for control) (Figure 3G).

The difference between the Lhcgr KO and control recipients was statistically significant (p = 0.028, one-tailed t test). These results suggested that SSCs that had been transplanted in Lhcgr KO mice underwent more extensive self-renewal divisions.

Increased Expression of Wingless-type MMTV Integration Site Family Member 5A in Lhcgr KO Mice

Because GDNF protein levels did not change in Lhcgr KO mice, we considered it likely that other genes are involved in enhanced SSC self-renewal. To explore this possibility, we used busulfan-treated Lhcgr KO and control WT mice and examined their gene-expression patterns using microarray analysis. Comparison between Lhcgr KO and control mice showed increased expression of 101 genes in Lhcgr KO mice, while 385 genes were downregulated (>2-fold change) (Table S1). Of these, we searched for cytokines or secreted molecules implicated in cell proliferation and identified several candidate molecules, including wingless-type MMTV integration site family member 5A (Wnt5a), delta-like 1 homolog (Dlk1), secreted frizzled-related protein 1 (Sfrp1), and secreted frizzled-related protein 4 (Sfrp4), which were upregulated in Lhcgr KO mice.

The increased expression of these candidate genes was also confirmed based on real-time PCR, which showed increased expression of Wnt5a, Dlk1, Sfrp1, and Sfrp4 by 7.7-, 35.1-, 6.8-, and 12.1-fold, respectively (Figure 4A) (p < 0.0001).

To examine the effect of these genes on SSC self-renewal, we used germline stem cells (GSCs), cultured spermatogonia with enriched SSC activity (Kanatsu-Shinohara et al., 2003). Candidate genes were transfected into NIH3T3 cells using a lentivirus vector, and GSCs were cultured for 7 days on these virus-infected feeder cells. Although proliferation of GSCs on NIH3T3 cells transfected with Dlk1, Sfrp1, or Sfrp4 did not show significant differences from controls, those on Wnt5a-transfected cells showed increased recovery (Figures 4B and 4C) (p = 0.018 for amplification; p < 0.0001 for...
expression). Cell recovery after trypsin digestion was 1.2-fold greater on Wnt5a-secreting cells. Although the increase was very minor, the difference was statistically significant. These results suggested that Wnt5a promotes GSC proliferation.

Since Wnt5a appears to play a role in the promotion of SSC self-renewal in Lhcgr KO mice, we examined the expression of WNT5A in vivo by immunohistochemistry. WNT5A was barely expressed in busulfan-treated control testis, while it was strongly detected in Lhcgr KO mice (Figure 5A). As expected from real-time PCR, Lhcgr KO mice showed stronger WNT5A expression based on western blotting (Figure 5B). Because androgens are produced by LH stimulation from Leydig cells, we examined whether androgens are involved in Wnt5a expression. Busulfan-treated mice were exposed to flutamide, a synthetic anti-androgen that acts by binding to an androgen receptor (Tohda et al., 2001). When their testes were recovered 2 weeks after treatment, increased expression was demonstrated by both real-time PCR and western blotting (Figures 5C and 5D) (p = 0.031). These results suggest that androgens mediate the suppression of WNT5A expression from Sertoli cells.

Although Wnt5a expression was very weak in WT adult testes, we noted that this gene was expressed more strongly in pup testes (Figure 5E) (p < 0.0001). Because this result suggests that Sertoli cells in Lhcgr KO mice exhibit an immature phenotype, we examined the expression of other immature Sertoli cell markers. As expected, anti-Müllerian hormone (AMH) and podoplanin (PDPN) were upregulated in Lhcgr KO testes (Figures 5F and 5G) (p < 0.0001 for Amh; p = 0.0017 for Krt18; p = 0.028 for Pdpn). On the other hand, adult Sertoli cell markers, such as GATA binding protein 1 (GATA1) or androgen receptor (AR), were only weakly expressed in Lhcgr KO testes. However, this does not necessarily support that Sertoli cells are completely immature because keratin 18 (KRT18), another marker of immature Sertoli cells, did not show apparent changes, and we could not identify cells stained with proliferating cell nuclear antigen or antigen identified by monoclonal antibody Ki67 (MKI67). These findings suggest that Sertoli cells in Lhcgr KO mice are mitotically quiescent.

Because immunohistochemical staining was suggestive of an immature phenotype, we further examined the effect of Lhcgr deficiency on the blood-testis barrier (BTB), which is found only in mature testes. Although claudin 11 (CLDN11) expression was clearly observed between Sertoli cells (Figure 5H), injection of biotin into the interstitium of untreated mature Lhcgr KO mouse testes resulted in leakage into the adluminal compartment, whereas such leakage was not observed in control WT mice (Figure 5I). These phenotypic and functional analyses suggest that Sertoli

Figure 5. Immaturity of Sertoli Cells in Lhcgr KO Mice
(A) Immunohistochemistry of WNT5A expression in busulfan-treated Lhcgr KO mice.
(B) Western blot analysis of busulfan-treated Lhcgr KO mouse testes.
(C and D) Real-time PCR analysis (C; n = 4 experiments) and western blot (D) of busulfan-treated WT testes after flutamide administration. Testes were recovered 2 weeks after the flutamide treatment.
(E) Real-time PCR analysis of Wnt5a expression in 7-day-old and 6-week-old WT testes (n = 4 experiments).
(F and G) Real-time PCR (F; n = 8 experiments) and western blot analyses (G) of Sertoli cell markers in busulfan-treated Lhcgr KO mouse testes.
(H) Immunohistochemistry of CLDN11 expression in Lhcgr KO testis.
(I) Functional assessment of BTB in Lhcgr KO mouse testes. Lhcgr KO mice were injected interstitially with biotin (red). Counterstain: Hoechst 33,342. Scale bars represent 25 μm (A, H, and I). Error bars indicate SEM. Asterisks indicate statistical significance. See also Tables S2 and S3.
cells in Lhcgr KO mice are phenotypically immature and have a defective BTB.

**Enhanced Self-Renewal Division by Wnt5a**

To confirm whether WNT5A promotes SSC self-renewal, we performed spermatogonial transplantation. This was because only 1%–2% of GSCs showed SSC activity, and it was possible that increased proliferation was due to enhanced progenitor cell proliferation (Kanatsu-Shinohara and Shinohara, 2013). We transplanted cells before and after culturing on NIH3T3 cells. Analysis of recipients showed that the number of SSCs was greater when GSCs on Wnt5a-expressing NIH3T3 cells were transplanted (Figures 4D and 4E). The number of colonies generated by GSCs on Wnt5a-expressing cells and control cells was 318.8 and 250.6 per $10^5$ cells, respectively ($n = 16$ for Wnt5a; $n = 17$ for control). There was no significant difference in the number of colonies. However, while SSCs on control NIH3T3 cells increased by 4.3 ± 0.4-fold ($n = 16$), those on Wnt5a-expressing cells increased by 6.7 ± 1.0-fold ($n = 17$), showing a 1.5-fold increase by Wnt5a stimulation ($p = 0.032$), suggesting that the effect of Wnt5a is promoting proliferation, and not the rescue of apoptosis. These results confirm the beneficial effect of WNT5A on SSC self-renewal in vitro.

Because in vitro culture experiments showed increased self-renewal division of SSCs by Wnt5a, we examined the impact of Wnt5a in vivo. We transfected Wnt5a in Sertoli cells of busulfan-treated mice and examined the effect of this transfection on colony number after spermatogonial transplantation (Figure 6A). We microinjected a lentivirus-expressing Wnt5a into the seminiferous tubules 7 days after transplantation (Figure 6B). Empty vector was transfected or control mice were 6.1 ± 0.9 and 5.2 ± 0.8 per $10^5$ cells, respectively ($n = 8$ for Wnt5a-treated; $n = 10$ for control) (Figures 6C and 6D). Although more colonies were observed in Wnt5a-transfected hosts, the difference was not statistically significant. These results suggest that Wnt5a expression does not have a significant effect on SSC migration into germline niches.

In the next set of experiments we microinjected Wnt5a-expressing lentivirus into testes of untreated adult green mice (Figure 6E). Testis cells were collected 2 months after Wnt5a OE (Figure 6F) ($p = 0.0013$), and dissociated testis cells were transplanted into the seminiferous tubules of busulfan-treated mice. The numbers of colonies generated by Wnt5a-transfected and control donor cells were 2.2 ± 0.4 and 1.0 ± 0.3 per $10^5$ cells, respectively ($n = 18$ for Wnt5a-treated; $n = 19$ for control) (Figures 6G and 6H) ($p = 0.009$), suggesting that Wnt5a has a beneficial effect on SSC self-renewal in vivo. Taken together, these results suggest that LH suppresses SSC self-renewal by downregulating Wnt5a via androgens (Figure 6I).

**DISCUSSION**

Regulation of SSC self-renewal by gonadotropic hormones has been suggested since the early days of spermatogonial transplantation experiments. Several studies based on GnRH analog administration have demonstrated the involvement of the gonadotropic pituitary hormone in SSC regulation (Dobrinski et al., 2001; Ogawa et al., 1998), which was confirmed by another study using hypophysectomized animals (Kanatsu-Shinohara et al., 2004). However, the mechanism underlying this phenomenon has remained unclear. Moreover, controversy exists regarding the regulation of GDNF expression (Chen and Liu, 2016). In this study, we explored this question using Fshb and Lhcgr KO mice and dissected the role of gonadotropic pituitary hormones in SSC regulation.

Analysis of Fshb KO mice showed that SSCs in these animals are apparently normal. Immunohistochemical staining indicated that the numbers of GFRα1+ spermatogonia and undifferentiated spermatogonia were not significantly different from those in WT mice. Because a positive relationship between FSH receptor and GDNF expression was reported (Ding et al., 2011; Tadokoro et al., 2002), we expected that SSCs would be significantly reduced in Fshb KO mice. However, Fshb KO mice contained a normal number of SSCs, suggesting that the lack of FSH signaling does not influence SSCs. These results were in contrast to previous studies that showed the beneficial effects of FSH on GDNF expression (Ding et al., 2011; Tadokoro et al., 2002). At least two explanations may explain this difference. The first is the method of FSH suppression. Although GnRH treatment was shown to increase GDNF expression in vivo via FSH suppression (Tadokoro et al., 2002), this treatment also decreases LH levels, which were not investigated in the previous report because of the lack of effect of testosterone on GDNF expression in vitro. The second is the experimental method; several studies showing increased GDNF expression were based on testis cell culture. However, FSH receptor expression levels may have changed because only 1%–2% of GSCs showed SSC activity, and it was possible that increased proliferation was due to enhanced progenitor cell proliferation (Kanatsu-Shinohara and Shinohara, 2013). We transplanted cells before and after culturing on NIH3T3 cells. Analysis of recipients showed that the number of SSCs was greater when GSCs on Wnt5a-expressing NIH3T3 cells were transplanted (Figures 4D and 4E). The number of colonies generated by GSCs on Wnt5a-expressing cells and control cells was 318.8 and 250.6 per $10^5$ cells, respectively ($n = 16$ for Wnt5a; $n = 17$ for control). There was no significant difference in the number of colonies. However, while SSCs on control NIH3T3 cells increased by 4.3 ± 0.4-fold ($n = 16$), those on Wnt5a-expressing cells increased by 6.7 ± 1.0-fold ($n = 17$), showing a 1.5-fold increase by Wnt5a stimulation ($p = 0.032$), suggesting that the effect of Wnt5a is promoting proliferation, and not the rescue of apoptosis. These results confirm the beneficial effect of WNT5A on SSC self-renewal in vitro.

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concentration of testosterone is very low (Zhang et al., 2001). Because it has been suggested that Leydig cells contribute to SSC niches and a recent study showed positive regulation of GDNF by testosterone (Chen et al., 2014; Oatley et al., 2009), we expected that SSCs in these mice might be decreased due to the lack of adult-type Leydig cells. However, we found an increased number not only of GFRA1-expressing spermatogonia but also SSCs. While these results suggest that SSC activity is not compromised by the lack of mature Leydig cells or testosterone, it remains
possible that the enrichment of SSCs was due to the absence of differentiating germ cells.

This hypothesis was tested by serial transplantation experiments, in which WT SSCs were transplanted into germ cell-deficient Lhcgr KO microenvironment. Although we did not observe an increase in colony number when Lhcgr KO mice were used as recipients, the number of secondary colonies increased in secondary recipients. Because an increased colony number in secondary recipients suggests enhanced self-renewal division in primary recipients, our results suggest that the absence of LH does not influence SSC homing but increases self-renewal divisions. Although this result demonstrated that LH suppresses proliferation of SSCs, it did not agree with our previous study that showed both increased colony numbers and self-renewal divisions in hypophysectomized adult recipients (Kanatsu-Shinohara et al., 2004). These results suggest that increased colonization and enhancement of SSC self-renewal are two separate phenomena, the latter of which is caused by the suppression of LH.

Our studies strongly suggest that Wnt5a upregulation is responsible for increased SSC activity in Lhcgr KO mice. The beneficial effects of Wnt5a were reported in a recent study (Yeh et al., 2011) in which Wnt5a increased the survival of SSCs in vitro. Although this study suggested that suppression of apoptosis is the primary role of Wnt5a, a net increase in the SSC number by Wnt5a was not demonstrated. Moreover, Wnt5a expression was examined only by measuring Wnt5a mRNA levels via in situ hybridization, and the role of Wnt5a in vivo remains unclear. In contrast, we showed that Wnt5a increases the number of SSCs both in vitro and in vivo. Because the effect of Wnt5a OE on SSC increase was modest compared with Lhcgr KO mice, it remains possible that other factors are involved. While we cannot exclude the possibility that WNT5A may not have been overexpressed in all cells, these results strongly suggest that Wnt5a is responsible, at least in part, for enhanced SSC self-renewal in Lhcgr KO mice. Because WNT5A is expressed more strongly in pup testes, it may be one of the factors that contribute to the rapid increase in SSC numbers during perinatal tests development (Shinohara et al., 2001). In fact, the plasma concentration of LH in prepubertal mice is relatively low until 7–8 days of age (Michael et al., 1980), which may induce WNT5A expression. The lack of LH appears to partially induce an immature phenotype in Sertoli cells because AMH immunoreactivity also increased in mature Lhcgr KO mice. Therefore, LH is not only required for Leydig cell maturation but is also important for suppressing the immature phenotype in Sertoli cells, although this effect of LH on Sertoli cells is probably indirect and most likely involves testosterone.

At least two important questions arise from the current study. First, how GDNF is regulated remains unclear. Although it was recently proposed that androgens regulate GDNF expression in peritubular cells (Chen et al., 2014, 2016), the effect of testosterone was not examined in vivo in these studies and we did not observe significant changes in GDNF expression in Lhcgr KO mice. We also failed to induce changes in GDNF levels by flutamide treatment, which suggests that GDNF is not regulated by androgens. However, caution is necessary in the latter experiment because it remains possible that flutamide treatment may induce testosterone production via an LH increase in normal mice. Identification of positive and negative regulators of GDNF expression and understanding how they are coordinated GDNF expression are urgent problems in understanding niche function. Second, it is important to identify the signal that enhances SSC colonization in GnRH-treated recipients or hypophysectomized animals. Although increases in colony numbers were observed in GnRH-treated and hypophysectomized mice, respectively (Dobrinski et al., 2001; Kanatsu-Shinohara et al., 2004; Ogawa et al., 1998), we did not observe an apparent increase in the number of colonies in Lhcgr KO recipients. Therefore, pituitary signals other than LH are likely responsible for the enhanced colonization of donor cells. In this context it should be noted here that, although GnRH antagonist treatment before the transplantation improved colonization, the same treatment did not enhance colonization when GnRH was administered at the time of transplantation (Dobrinski et al., 2001). It is believed that the major effect of GnRH antagonist is primarily on the homing step (Wang et al., 2010). Such differences between our current results and previous studies may be due to Lhcgr KO mice having cryptorchid testes, which might have influenced homing efficiency. Understanding the mechanism underlying the difference between GnRH treatment and Lhcgr KO mice may lead to the development of improved techniques to increase spermatogonial transplantation efficiency.

Although it is believed that gonadotropin pituitary hormones regulate GDNF expression in the testes, our study showed that neither FSH nor LH regulates GDNF expression. Instead, we found that increased WNT5A by LH suppression is responsible for enhanced SSC self-renewal. This suppression of WNT5A was likely mediated by androgens because flutamide also increased WNT5A expression. We also suggest that enhancement of colonization and increased self-renewal divisions are two distinct phenomena regulated by gonadotropic pituitary hormones. Considering that Sertoli and Leydig cell division is influenced by gonadotropic pituitary hormones, it is not surprising that complex regulations and interactions exist between the testis and pituitary gland. Future studies should explore these interactions, which not only increase our understanding of interorgan interactions to regulate
SSCs, but may provide efficient methods for improving spermatogonial transplantation, manipulating the amount of sperm production, and developing male contraceptives.

EXPERIMENTAL PROCEDURES

Animals

Fshb KO mice were purchased from the Jackson Laboratory (B6; 129S7-Fshb<sup>tm1Zuk/J>). Lhgr KO mice were generated previously (Lei et al., 2001). The genotypes of the mice were examined by PCR with the primers listed in Table S2. We also used the transgenic mouse line CS7BL/6 Tg14(act-EGFP)OsbY01 (designated green; a gift from Dr. M. Okabe, Osaka University) to introduce the Egfp transgene as a donor cell marker. Dissociated testis cells were transplanted into W mice (Japan SLC) to quantify SSCs in KO mouse testes by donor spermatogonial stem cells after transplantation in mice. Tissue Cell 33, 200–207.


