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The Transition from Stem Cell to Progenitor Spermatogonia and Male Fertility Requires the SHP2 Protein Tyrosine Phosphatase

PAWAN PURI,[a] BART T. PHILLIPS,[a] HITOMI SUZUKI,[a] KYLE E. ORWIG,[a] ALEKSANDAR RAJKOVIC,[a] PHILIP E. LAPINSKI,[b] PHILIP D. KING,[b] GEN–SHENG FENG,[c] WILLIAM H. WALKER[a]

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[a]Department of Obstetrics, Gynecology and Reproductive Sciences, Center for Research in Reproductive Physiology, Magee Women's Research Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [b]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA; [c]Department of Pathology and Division of Biological Sciences, University of California, San Diego School of Medicine, San Diego, La Jolla, California, USA

Correspondence: William H. Walker, Ph.D., Department of Obstetrics, Gynecology and Reproductive Sciences, Magee Women's Research Institute, University of Pittsburgh, 204 Craft Avenue, Room B305, Pittsburgh, Pennsylvania 15261, USA. Telephone: 412-641-7672; Fax: 412-641-7676; e-mail: walkerw@pitt.edu

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ABSTRACT

SHP2 is a widely expressed protein tyrosine phosphatase required for signal transduction from multiple cell surface receptors. Gain and loss of function SHP2 mutations in humans are known to cause Noonan and LEOPARD syndromes, respectively, that are characterized by numerous pathological conditions including male infertility. Using conditional gene targeting in the mouse, we found that SHP2 is required for maintaining spermatogonial stem cells (SSCs) and the production of germ cells required for male fertility. After deleting SHP2, spermatogenesis was halted at the initial step during which transit-amplifying undifferentiated spermatogonia are produced from SSCs. In the absence of SHP2, proliferation of SSCs and undifferentiated spermatogonia was inhibited, thus germ cells cannot be replenished and SSCs cannot undergo renewal. However, germ cells beyond the undifferentiated spermatogonia stage of development at the time of SHP2 knockout were able to complete their maturation to become sperm. In cultures of SSCs and their progeny, inhibition of SHP2 activity reduced growth factor-mediated intracellular signaling that regulates SSC proliferation and cell fate. Inhibition of SHP2 also decreased the number of SSCs present in culture and caused SSCs to detach from supporting cells. Injection of mice with an SHP2 inhibitor blocked the production of germ cells from SSCs. Together, our studies show that SHP2 is essential for SSCs to maintain fertility and indicates that the pathogenesis of infertility in humans with SHP2 mutations is due to compromised SSC functions that block spermatogenesis. STEM CELLS *2014;32:741–753*

INTRODUCTION

Male fertility is maintained by the highly productive process of spermatogenesis that in men generates more than 100 million sperm every day. In mice, spermatogenesis initiates 3 days after birth with gonocyte germ cell precursors giving rise to two types of cells: spermatogonial stem cells (SSCs) or differentiated spermatogonia [1, 2]. SSCs are rare in adults (0.03% of all adult mouse germ cells) and are present as single cells that continuously self-renew and produce undifferentiated spermatogonia that function as transit-amplifying progenitor cells [3]. Undifferentiated spermatogonia transition to become differentiated spermatogonia that undergo additional mitotic divisions resulting in cells committed to meiosis and sperm development. The only exception to this well-ordered process is the first wave of spermatogenesis that consists of a shortened developmental program in which a population of gonocytes produce differentiated type A2 spermatogonia 3–4 days after birth without passing through the SSC and undifferentiated spermatogonia stages of development [1, 4]. After completion of the first wave of spermatogenesis, all germ cells are replenished from SSCs.

Growth factors including glial cell-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF) produced by Sertoli cells support the renewal and proliferation of SSCs [5–7], but the intracellular factors and signaling pathways that regulate SSC fate decisions are not well-characterized. A candidate mediator of GDNF and bFGF signaling in SSCs is the widely expressed SHP2 protein tyrosine phosphatase encoded by the *Ptpn11* gene. SHP2 mediates intracellular signaling after stimulation with GDNF and bFGF in nontesticular cell types [8, 9]. Upon growth factor or cytokine stimulation, SHP2 is recruited to growth factor receptors [10]. SHP2 is then capable of regulating signaling cascades that are known to decide SSC fate. In other cell types, SHP2 stimulates the PI3K/AKT and Ras-MAPK (ERK) pathways but SHP2 can activate or inhibit the JAK/STAT pathway [11–13].

Mutations that constitutively activate or inhibit SHP2 activity result in human pathologies. Missense mutations in *Ptpn11* result in LEOPARD syndrome that is characterized by heart, lung, ocular, growth, and genitalia abnormalities [14]. In mice, disruption of the *Ptpn11* gene results in embryonic lethality [15]. Constitutive activation of SHP2 can result in juvenile leukemias [16, 17] and the juvenile development disorder Noonan Syndrome that includes facial dysmorphia, congenital heart defects, short stature, and male infertility [17]. Noonan syndrome is also the most common human autosomal dominant genetic disease that results in infertility [18]. We found that expression of a constitutively active SHP2 mutant in cultured rat Sertoli cells causes the mislocalization of proteins required to maintain the blood-testis barrier (BTB) [19]. Thus, SHP2 is a potential regulator of essential processes in both Sertoli cells and SSCs that are required for male fertility.

To date, the consequences of eliminating SHP2 activity in the testis have not been investigated. In this study, we used two complimentary SHP2 knockout mouse models to determine that SHP2 is required to maintain spermatogenesis and male fertility. In the absence of SHP2, spermatogenesis is blocked at the initial step due to defects in the proliferation and survival of SSCs such that undifferentiated spermatogonia cannot be produced. Also, global knockout of SHP2 in adult mice causes defects in the orientation and migration of developing spermatids. In addition, we find that inhibition of SHP2 activity causes detachment of SSCs from supporting cells, decreases the number of SSCs present in culture, and disrupts intracellular signaling pathways required for the self-renewal and proliferation of SSCs.

Materials and Methods

Transgenic Mice

Animals used in these studies were maintained and euthanized according to the principles and procedures described in the NIH Guide for the Care and Use of Laboratory Animals. These studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Previously described *Ptpn11*$^{fl/fl}$ mice and ubiquitin-promoter-driven *ert2-cre* transgenic mice [20, 21] were mated to generate *Ptpn11*$^{fl/fl}$ *ert2-cre* mice. To create *Ptpn11*$^{\Delta/\Delta}$ mice, adult (6–8 weeks old) or pubertal (8 days old) *Ptpn11*$^{fl/fl}$ *ert2-cre* mice were injected twice with tamoxifen (200 μg/g b.wt. in corn oil) on 2 consecutive days as described [22] and sacrificed 6–63 days later. Wild-type littermates were similarly injected with tamoxifen. To generate GCSHP2KO mice, *Ptpn11*$^{fl/fl}$ mice were mated with *Vasa-cre* (*Ddx4-cre*) mice [23] and resulting *Ptpn11*$^{wt/\Delta}$ *Vasa-cre*$^{+/-}$ males were mated to *Ptpn11*$^{fl/fl}$ *Vasa-cre*$^{-/-}$ females to obtain *Ptpn11*$^{\Delta/\Delta}$ *Vasa-cre*$^{+/-}$ mice that lack Ptpn11 exon 4 specifically in germ cells.

Genotyping

Generation and genotypic identification of *Ptpn11*$^{fl/fl}$ and *ert2-cre* transgenic mice are described elsewhere [20, 21]. The primer pairs used in PCR assays to determine the presence of floxed *Ptpn11*, recombined *Ptpn11*, ert2-cre, and *Vasa-Cre* are listed in Supporting Information Table S1.

Reagents and Antibodies

FGF, GDNF, NSC-87877, and tamoxifen were obtained from BD Biosciences (San Jose, CA, www.bdbiosciences.com), PeproTech (Rocky Hill, NJ, www.preprotech.com), Tocris Bioscience (Bristol, U.K.), and Sigma (St. Louis, MO, www.sigmaaldrich.com), respectively. Antisera sources, applications, and the dilutions used are summarized in Supporting Information Table S2.

Preparation of Whole Cell Extracts and Immunoblot Analysis

To prepare testis whole cell lysates (WCE), testes were decapsulated and homogenized in enhanced lysis buffer (250 mM NaCl, 0.1% Nonidet P40, 50 mM Hepes, pH 7.0, 5 mM EDTA, and 0.5 mM dithiothreitol) with a protease inhibitor cocktail, rocked for 15 minutes at 4°C, and then pelleted (12,000*g* 15 minutes) to remove cell debris. WCE were fractionated by 10% SDS-PAGE and transferred to Immobilon-P, PVDF membranes (Millipore Corp., Bedford, MA, www.millipore.com), blocked with 5% non-fat dry milk in Tris-buffered saline (25 mM TRIS-HCl, pH 7.4, 0.15 M NaCl, containing 0.1% Tween 20), and incubated overnight at 4°C with the appropriate primary antibody. The blots were washed, incubated with species-specific secondary antibodies, and visualized with chemiluminescent HRP substrate (Millipore Corp.).

Germ Line Stem Cultures

Germ line stem (GS) cell cultures were isolated from 6-day-old DBA/2 mice as described previously [24]. Briefly, a testicular cell suspension was isolated by enzymatic digestion with DNase and trypsin and filtered through a 40 μm mesh to remove cell clumps. Germ cells were then enriched through a 30% percoll gradient and subjected to magnetic activated cell sorting to select Thy1+ cells to enrich for SSCs. The cells were washed in mouse SSC serum-free medium (mSFM) and plated onto SIM mouse embryo-derived thioguanine- and ouabain-resistant (STO) fibroblast cell line feeder cells in mSFM supplemented with 20 ng/ml recombinant human GDNF and 20 ng/ml human recombinant basic FGF (bFGF). GS cultures were subcultured at 1:2–1:3 ratios onto fresh STO feeders every 7 days and were used for experiments between 1 and 3 months after establishment. GS cultures were starved of bFGF and GDNF overnight and incubated in the absence and presence of NSC-87877 for 6–8 hours. SSCs were detached from feeders and bFGF or GDNF was added for 10 minutes. Detached SSCs were then pelleted (600*g*, 7 minutes) and lysed in Laemmli sample buffer.

Immunocytochemistry and Immunofluorescence

Immunostaining of testis tissue was performed on paraffin-embedded sections (5 μm) from paraformaldehyde (4%, o/n) or Bouin's fixed adult rat testis as previously described [19]. Testis sections were deparaffinized in xylene and rehydrated. The sections were subjected to antigen retrieval in citrate buffer (10 mm citrate, pH 6.0, containing 0.1% Tween 20) at 95°C for 30 minutes and then at room temperature for 30 minutes. The sections were washed two times for 5 minutes in phosphate buffered saline and blocked with goat, donkey, or horse serum. The testis sections were then incubated 12–24 hours with nonimmune serum or antiserum as detailed in Supporting Information Table S2. For colorimetric assays, anti-rabbit or anti-mouse biotinylated secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories,

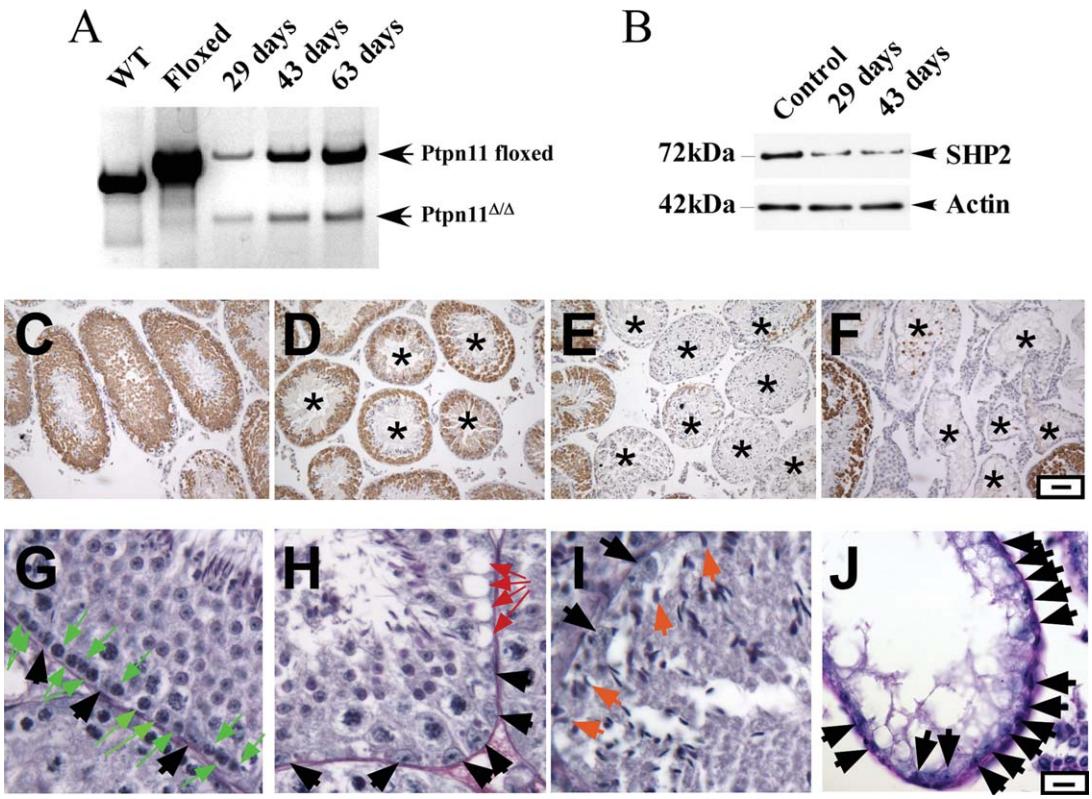

Figure 1. Induced global knockout of SHP2 blocks spermatogenesis. **(A):** PCR analysis of testis genomic DNA using primers flanking exon 4 of the gene encoding SHP2. The amplified products are shown for reactions using genomic DNA isolated from the testes of WT mice, floxed *Ptpn11* and $Ptpn11^{\Delta/\Delta}$ mice 29, 43, and 63 days after tamoxifen treatment. **(B):** Western immunoblot analysis of SHP2 and actin protein expression in whole cell extracts from testes isolated from control and $Ptpn11^{\Delta/\Delta}$ mice 29 and 43 days after tamoxifen treatment. **(C–F):** Testis tissue sections from control mice (C), and $Ptpn11^{\Delta/\Delta}$ mice 29 (D), 43 (E), and 63 (F) days after tamoxifen treatment were probed with antisera against VASA (brown stain). Nuclei are stained blue with hematoxylin. Seminiferous tubule cross-sections showing disrupted spermatogenesis as measured by two or fewer layers of germ cells are marked with an asterisk (*). **(G–J):** Testis tissue sections from control mice (G) and $Ptpn11^{\Delta/\Delta}$ mice 29 (H), 43 (I), and 63 (J) days after tamoxifen treatment were stained with periodic acid Schiffs-hematoxylin (PAS-H). Sertoli cell nuclei are marked with black arrowheads. Spermatogonia and preleptotene spermatocytes on the basement membrane are marked with green arrows. Vacuoles in regions lacking germ cells are noted with red arrows. Mislocalized elongated spermatids are marked with orange arrows. C–F: Bar = 100 μm, G–J: Bar = 20 μm. Abbreviation: WT, wild type.

Burlingame, CA, www.vectorlabs.com) was added, and bound antibodies were detected using DAB staining colorimetric reagent and counterstained with hematoxylin. For immunofluorescence studies, Cy3 and ALexa488-conjugated secondary antisera were added, nuclei were stained with 4′6′-diamidino-2-phenylindole, and immunostaining was detected using a Nikon Provis II fluorescence microscope or confocal microscope. A charge-coupled video camera system was used to capture images. All files were digitally processed with Photoshop (Adobe Systems, Inc., San Jose, CA, www.adobe.com).

TUNEL Assay, Cell Counting, and LIVE/DEAD Staining

Paraformaldehyde-fixed testis sections were evaluated using a TUNEL assay kit (Roche). All immunostained cells and tubules from an entire testis section were counted and the number of stained cells per tubule was determined. GS cell viability was determined using the LIVE/DEAD viability assay kit for mammalian cells (Life Technologies, Carlsbad, CA, www.lifetechnologies.com).

Statistical Analysis

Immunoreactive signals from Western blot films were scanned with an Epson 1600 Expressions scanner using Epson Scan software. Results were analyzed by ANOVA with Newman-Keuls PLSD or unpaired *t* test at a 5% significance level using Prism 4.3 (GraphPad Software, LA Jolla, CA, www.graphpad.com).

Results

The Induced Deletion of SHP2 in Adult Mice Blocks Spermatogenesis

To define the function of SHP2 in spermatogenesis, we first eliminated SHP2 expression in adult $Ptpn11^{fl/fl}$*ert2-cre* mice. These mice express floxed alleles of Ptpn11 and a tamoxifen-inducible Cre recombinase driven by a ubiquitin promoter. Administration of tamoxifen to these mice causes the excision of exon 4 at the *Ptpn11* locus, creating a null mutant allele for SHP2 [21, 22]. Adult ([mt]60 days old) $Ptpn11^{f//fl}$*ert2-cre* and $Ptpn11^{fl/fl}$ (control) mice were treated with tamoxifen to ablate SHP2 expression and sacrificed 29, 43, and 63 days later. PCR analysis of genomic DNA isolated from the testis of the tamoxifen-treated $Ptpn11^{fl/fl}$*ert2-cre* mice (hereafter $Ptpn11^{\Delta/\Delta}$ mice) and control mice confirmed the Cre-mediated excision of *Ptpn11* exon 4 in the testis (Fig. 1A). Immunoblot analysis showed that the protein levels of SHP2

in animals treated with tamoxifen were decreased (Fig. 1B). The PCR and immunoblot analyses also indicated that the excision of exon 4 did not occur in all cells and that some SHP2 protein expression was retained in the *Ptpn11*$^{\Delta/\Delta}$ mouse testis.

Testes weights of *Ptpn11*$^{\Delta/\Delta}$ mice 29 and 43 days after tamoxifen treatment were reduced by 36% and 52%, respectively. In contrast to the intact spermatogenesis observed in controls (Fig. 1C), spermatogenesis was disrupted in 33% of tubule cross-sections from *Ptpn11*$^{\Delta/\Delta}$ mice 29 days after tamoxifen treatment as defined by the presence of two or fewer layers of germ cells stained with Vasa antiserum that identifies all germ cells (Fig. 1D). After 43 and 63 days, 43% and 27% of tubule cross-sections showed disrupted spermatogenesis, respectively (Fig. 1E, 1F). The mosaic nature of the gene knockout is commonly seen in tamoxifen-induced knockout mouse models [25] and varying efficiencies of gene ablation using the *ert2-cre* to knockout SHP2 have been shown for various tissues [22]. The presence of tubules with intact spermatogenesis also indicates that the observed disrupted spermatogenesis did not result from nonspecific actions of tamoxifen, damage to Leydig cells, or some insult to the hypothalamic-pituitary-testis endocrine axis.

SHP2 Ablation Blocks Spermatogenesis Prior to Spermatogonia Development

The disruption of spermatogenesis in *Ptpn11*$^{\Delta/\Delta}$ mice led us to examine the germ cell complement of *Ptpn11*$^{\Delta/\Delta}$ testes in greater detail. In cross-sections from control mice, spermatogonia, preleptotene spermatocytes, and Sertoli cell nuclei are present along the basement membrane (Fig. 1G). In contrast, cross-sections from disrupted tubules in *Ptpn11*$^{\Delta/\Delta}$ mice 29 days after tamoxifen treatment had only Sertoli cell nuclei along the basement membrane as spermatogonia and preleptotene spermatocytes were absent (Figs. 1H, 3). Leptotene and zygotene spermatocytes also were absent from some disrupted tubules and numerous vacuoles were found in locations in which premeiotic and meiotic germ cells were missing. Pachytene spermatocytes as well as round and elongated spermatids were present 29 days after tamoxifen treatment.

At 43 days after knockout, all germ cells were absent except for elongated spermatids (Fig. 1I). The elongated spermatids were distributed randomly with many having their acrosomes misoriented away from the Sertoli cell nuclei suggesting that the attachment of the elongated spermatids to the supporting Sertoli cells was misregulated. The mislocalization of elongated spermatids has been shown to result in phagocytosis of the germ cells by Sertoli cells [26]. At 63 days after tamoxifen treatment, tubules with disrupted spermatogenesis lacked all germ cells and contained only Sertoli cells (Fig. 1J). *Ptpn11*$^{\Delta/\Delta}$ male mice retained normal mating behavior, but fertility was reduced as matings of at least 6 weeks produced litters at a 50% rate (5/10), whereas control mice treated with tamoxifen produced litters at a rate of 82% (14/17). Fertility of the *Ptpn11*$^{\Delta/\Delta}$ mice is likely maintained due to the presence of tubules in which SHP2 expression was retained and spermatogenesis was not disrupted.

SHP2 Is Expressed in SSCs and Undifferentiated Spermatogonia

Immunohistochemical studies of adult mouse testis sections showed that SHP2 was expressed in Sertoli cell nuclei as well as in Sertoli cytoplasm at the basement membrane, the BTB, and between germ cells (Fig. 2A). SHP2 also was expressed in spermatogonia on the basement membrane but expression was low or undetectable in spermatocytes and spermatids (Fig. 2A, Supporting Information Fig. S1). To determine whether SHP2 is expressed in SSCs and undifferentiated spermatogonia in the testis, we assessed SHP2 coexpression with the promyelocytic leukemia zinc finger (PLZF)-transcription factor (Zbtb16), a marker for these cell types [27, 28]. In testes sections from postnatal day 15 (P15) wild-type mice that have a high proportion of undifferentiated spermatogonia cells, we found that SHP2 was coexpressed in positive cells and present in the Sertoli cell cytoplasm surrounding germ cells (Fig. 2B). We also found that SHP2 was coexpressed with the more rare PLZF-positive cells in testis cross-sections from adult wild-type mice (Fig. 2C). Further studies were performed in primary cultures of mouse GS cells. These cultures consist of SSCs as well as undifferentiated spermatogonia derived from SSCs that are supported by feeder cells [7, 29]. SHP2 was coexpressed with all PLZF-positive cells in the GS cell culture indicating that SHP2 is expressed in SSCs and undifferentiated spermatogonia (Fig. 2D). There also were some SHP2-positive cells that did not express PLZF. This pattern of SHP2 expression is consistent with the hypothesis that SHP2 regulates SSC fate as well as the production of undifferentiated spermatogonia.

SHP2 Is Required to Replenish Germ Cells

To identify the first stages of germ cell development affected by SHP2 knockout, testis cross-sections from *Ptpn11*$^{\Delta/\Delta}$ mice were probed with antisera against SOHLH1 or PLZF. These studies showed that PLZF- and SOHLH1-positive cells were not detected in disrupted tubules 29 or 43 days after SHP2 knockout and no germ cells were detected at 63 days (Fig. 3). The staining of GATA4-positive Sertoli cells did not change in the disrupted tubules. The lack of SOHLH1- and PLZF-positive cells detected in disrupted tubules after tamoxifen treatment suggests that SSCs are depleted or unable to proliferate in the absence of SHP2.

Examination of testis cross-sections from P15 *Ptpn11*$^{\Delta/\Delta}$ mice that were treated with tamoxifen on P8 and P9 revealed an increase in PLZF-positive cells lacking SHP2 due to the ablation of SHP2 expression. These SHP2-deficient/PLZF-positive cells were clustered in specific tubule cross-sections, whereas PLZF-positive/SHP2 negative cells were less frequent and more evenly distributed in cross-sections from control mice (Supporting Information Fig. S2). These data are consistent with the mosaic pattern of spermatogenesis disruption in adult *Ptpn11*$^{\Delta/\Delta}$ mice originating in pups with SHP2 deficient SSCs and undifferentiated spermatogonia localized in specific seminiferous tubules.

The Production of Germ Cell-Specific SHP2 Knockout Mice

Studies of the *Ptpn11*$^{\Delta/\Delta}$ mice indicated that SHP2 is required to maintain the production of germ cells. However, the global

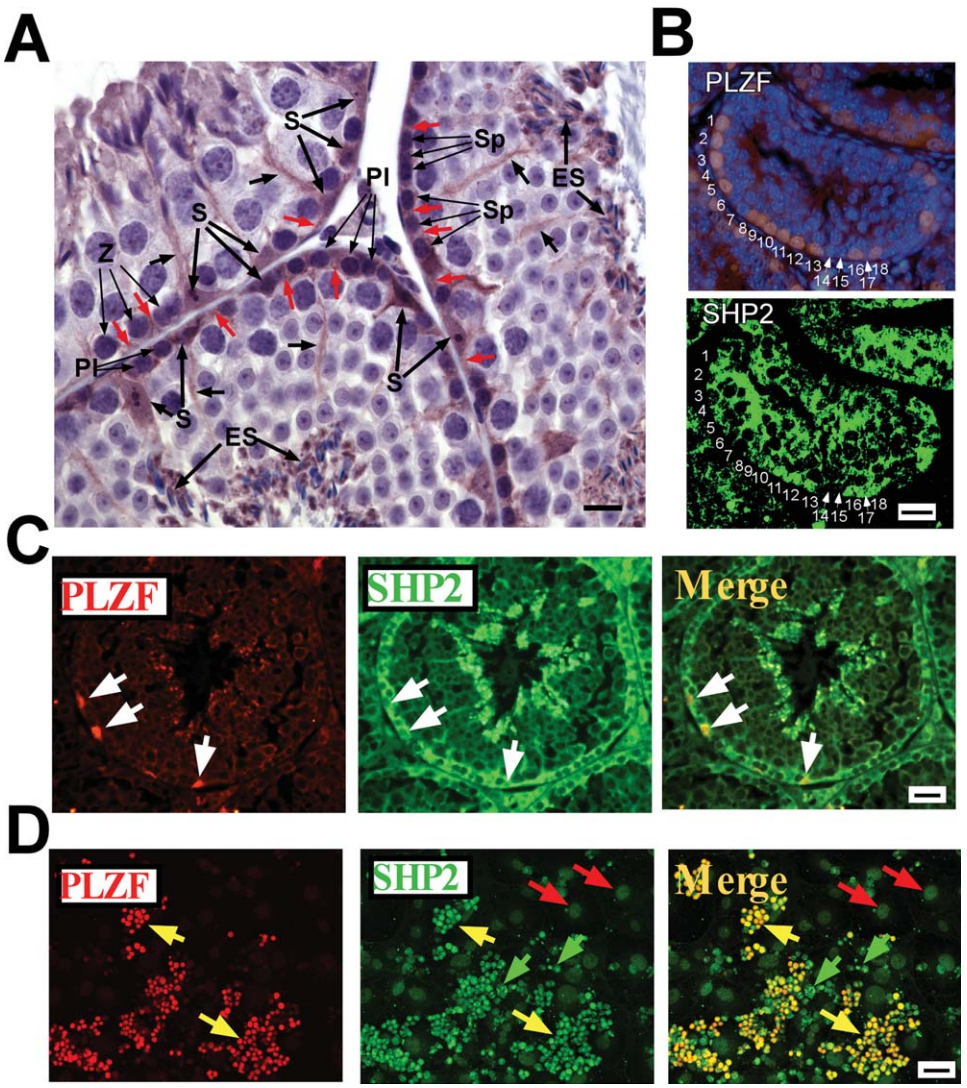

Figure 2. SHP2 is expressed in spermatogonial stem cells and undifferentiated spermatogonia. **(A):** Adult wild-type mouse testis sections were probed with antiserum against SHP2 and stained with hematoxylin (blue). The SHP2 immunostaining (brown) was localized to Sertoli (S) nuclei and Sertoli cell cytoplasm between germ cells (black arrowheads) and around ES as well as along the basement membrane in a region consistent with the blood-testis barrier (red arrows) that did not extend beyond the apical side of spermatogonia (Sp) or preleptotene spermatocytes (Pl). Bar = 20 μm. **(B):** P15 wild-type mice were probed with antisera against PLZF (top, red) or SHP2 (bottom, green). Nuclei were stained blue with 4′6′-diamidino-2-phenylindole. Cells numbered 8–13, 16, and 18 were PLZF and SHP2 positive. Bar = 100 μm. **(C):** Images of adult testis cross-sections are shown after probing with antisera against PLZF and SHP2 and the merging of the signals. Arrows mark spermatogonia that express PLZF and SHP2. Bar = 20 μm. **(D):** Images of GS cells probed with PLZF and SHP2 and the merged signals. Yellow arrows mark clusters of GS cells that coexpress PLZF and SHP2. Green arrows mark GS cells that express only SHP2. Red arrows mark larger nuclei of feeder cells in the background that express SHP2. Bar = 20 μm. Abbreviation: ES, elongated spermatids.

knockout of SHP2 did not determine whether germ cell replenishment and cell attachment defects in the absence of SHP2 were due to SHP2 actions in SSCs or in Sertoli cells. To identify the functions of SHP2 specific to germ cells, $Ptpn11^{fl/fl}$ mice were mated with mice expressing Cre recombinase driven by the *Vasa* (*Ddx4*) promoter, which causes Cre to be expressed specifically in germ cells beginning on fetal day 15 [23]. Genotypic analysis of progeny identified germ cell-specific SHP2 knockout (GCSHP2KO) mice having *Ptpn11* genes with exon 4 deleted ($Ptpn11^{\Delta/\Delta}$) in germ cells (Fig. 4A). Immunoblot analysis showed that SHP2 levels decreased in GCSHP2KO mice 4 and 8 weeks after birth (Fig. 4B).

The weights of GCSHP2KO testes 3, 4, and 8 weeks after birth were reduced 56%, 46%, and 80%, respectively, in comparison to control littermates (Fig. 4C). Diameters of the seminiferous tubules from 3-, 4-, and 8-week-old GCSHP2 mice were reduced by 24%, 39%, and 51%, respectively (Fig. 4D). In contrast to the mosaic of disrupted tubules observed in the $Ptpn11^{\Delta/\Delta}$ mice, low magnification analysis of testis sections from GCSHP2KO mice revealed a progressive loss of germ cells in nearly all seminiferous tubules (Fig. 4E). By 8 weeks after birth, GCSHP2KO mice totally lacked germ cells in all tubule cross-sections. These data indicate that SHP2 expression in germ cells is essential to maintain the germline.

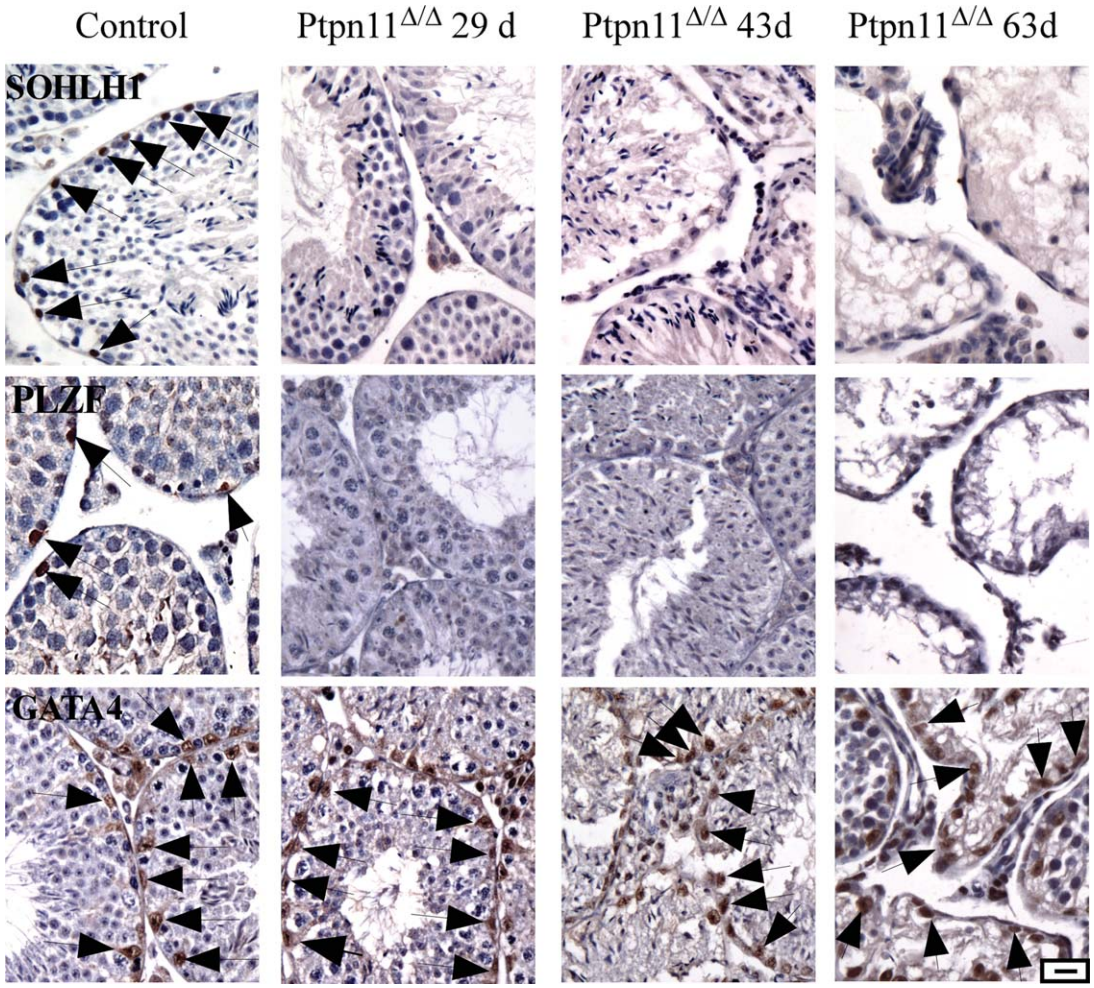

Figure 3. Undifferentiated and differentiated spermatogonia are not present after broad knockout of SHP2. Testis tissues from control mice and *Ptpn11*$^{\Delta/\Delta}$ mice 29, 43, and 63 days after tamoxifen treatment were probed with antisera against PLZF, SOHLH1, and GATA4. Representative immunopositive staining (brown) is noted with arrows. Bar = 20 μm.

SHP2 Is Required for the Production of Undifferentiated Spermatogonia

Immunohistochemistry studies performed on testes from GCSHP2KO mice 3 weeks after birth revealed that spermatocytes and spermatids were present, but Vasa-positive cells were not detected on the basement membrane indicating that spermatogonia and preleptotene spermatocytes were absent (Fig. 5, top row). By 4 weeks after birth, germ cells in the GCSHP2KO mice had progressed to become round spermatids and elongated spermatids but less mature germ cells from spermatogonia to pachytene spermatocytes were absent. By 8 weeks, no Vasa-positive germ cells were present. Probing with SOHLH1 antisera (Fig. 5, second row) or PLZF antisera (Fig. 5, third row) identified differentiated and undifferentiated spermatogonia, respectively, on the basement membrane of control mouse testis but these cells were absent 3, 4, and 8 weeks after birth in GCSHP2KO mice. The inability to replenish germ cells in GCSHP2KO mice indicates SHP2 is essential for the survival or proliferation of SSCs. In contrast, staining of Sertoli cells with antiserum against GATA4 was similar in control and GCSHP2KO testes at all developmental ages (Fig. 5, bottom row).

The presence of spermatocytes and spermatids in GCSHP2KO mice 3 and 4 weeks after birth demonstrates that the elimination of SHP2 specifically from germ cells did not interfere with the production of differentiated spermatogonia from gonocytes in the first wave of spermatogenesis. Also, the progression of spermatogenesis after the differentiated spermatogonia stage of development was not affected. These results confirm that knockout of SHP2 expression does not inhibit the differentiation or proliferation of spermatogonia or the developmental transition to spermatocytes.

SHP2 Is Required for the Proliferation and Survival of SSCs

In testis sections from control mice 5 days after birth that contain a high proportion of SSCs and undifferentiated spermatogonia, SHP2 and PLZF immunostaining were colocalized in SSCs and undifferentiated spermatogonia plus SHP2 was present in the Sertoli cell cytoplasm surrounding germ cells. In GCSHP2KO mice, SHP2 immunostaining was retained in the Sertoli cell cytoplasm but SHP2 colocalization with PLZF was rare indicating that SHP2 expression was extinguished in nearly all SSCs and undifferentiated spermatogonia (Supporting Information Fig. S3).

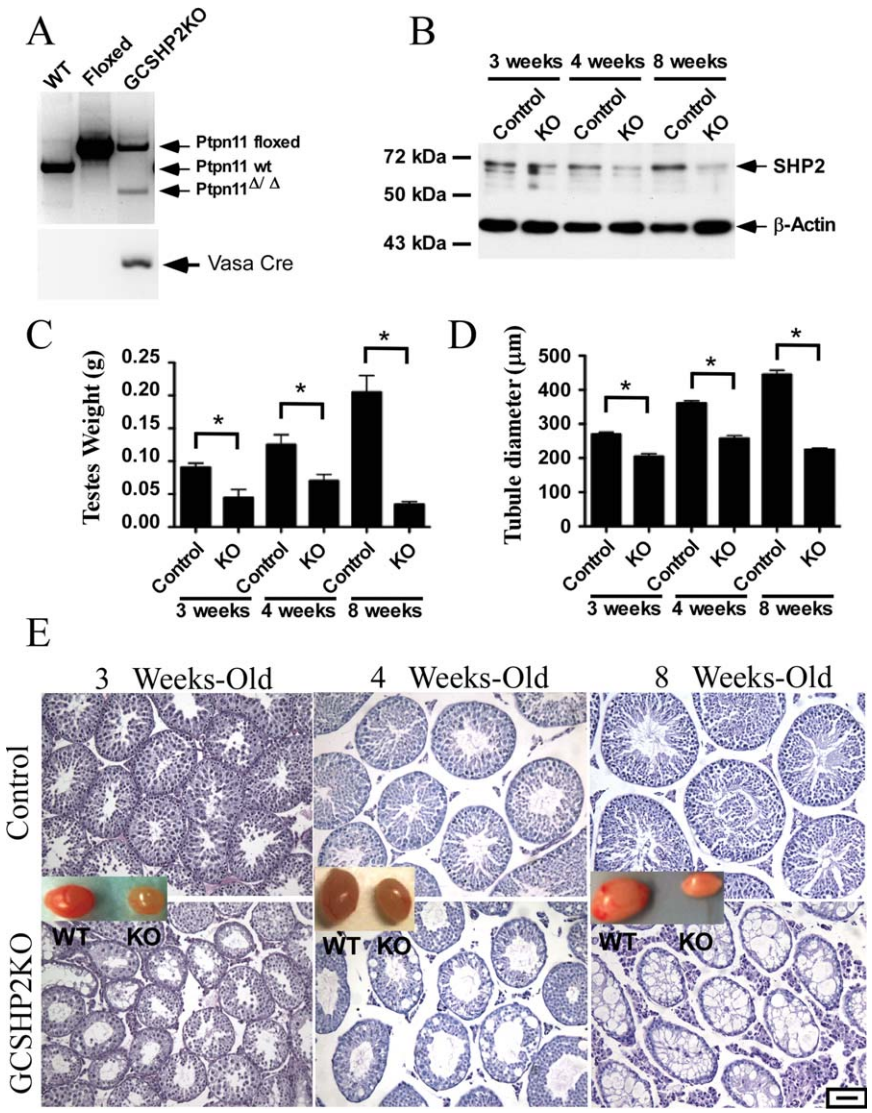

Figure 4. Ablation of SHP2 expression in germ cells results in the loss of all germ cells. **(A):** PCR analysis of the region encompassing exon 4 of Ptpn11 in testis tissue from WT, floxed *Ptpn11* (floxed), and GCSHP2KO mice. Analysis of the presence of Cre in the VasaCre transgene is shown below. **(B):** Western immunoblot analysis of whole testis extracts from control and GCSHP2KO (KO) mice probed with antisera against SHP2 or β-actin. **(C, D):** Mean ($\pm$SEM) weights of pairs of testes (C) and seminiferous tubule diameters (D) from 3-, 4-, and 8-week-old control and KO mice are shown. Age matched pairs marked with asterisks (*) differ significantly (p [lt] .05), For (C) $n = 2$ except 8 week-old KO = 5. For (D) at least 10 cross-sections were measured for each condition. **(E):** Testis tissue from 3-, 4-, and 8-week-old control and GCSHP2KO mice stained with PAS-H. Bar = 100 μm. Insets show the relative sizes of the testes isolated from wild-type and GCSHP2KO mice. Abbreviations: KO, knockout; WT, wild type.

To determine whether SSCs and undifferentiated spermatogonia were absent in GCSHP2KO mice because the knockout of SHP2 caused the loss of gonocyte precursor cells, testis sections from P2 GCSHP2KO mice were probed with antisera against Vasa. These studies indicated that the distribution and number of gonocytes were similar in control and GCSHP2KO mice (Fig. 6A, 6D). Thus, the lack of SHP2 does not cause the loss of germ cell precursors and SHP2 must be required at a later stage in germ cell development.

Gonocytes reenter the cell cycle on P3 and give rise to SSCs from P3.5 to P5 [1, 2]. We compared the numbers of cells per tubule for SSCs and any undifferentiated spermatogonia that were present in control and GCSHP2KO mice at P5 by probing testis tissue sections with PLZF antiserum. As expected, the proliferation of gonocytes, SSCs, and undifferentiated spermatogonia in control mice increased the number of PLZF-positive cells at P5 over that of the number of gonocytes (Vasa-positive cells) at P2 (Fig. 6B, 6D). However, PLZF-positive cells in GCSHP2KO mice were reduced to 44% of littermate controls. In control mice, the number of PLZF-positive cells per tubule was not altered significantly from P5 to P7. However, PLZF immunostained cells in GCSHP2KO testes were further reduced to 39% of control levels at P7 (Fig. 6C). These results indicate there is a reduction in the number SSCs and/or undifferentiated spermatogonia in the absence of SHP2. In contrast, GATA4 staining of Sertoli cells was similar for control and GCSHP2KO mice at each developmental time point (Fig. 6A–6C).

TUNEL assays revealed that apoptosis generally increased in seminiferous tubules during the P2 to P7 developmental

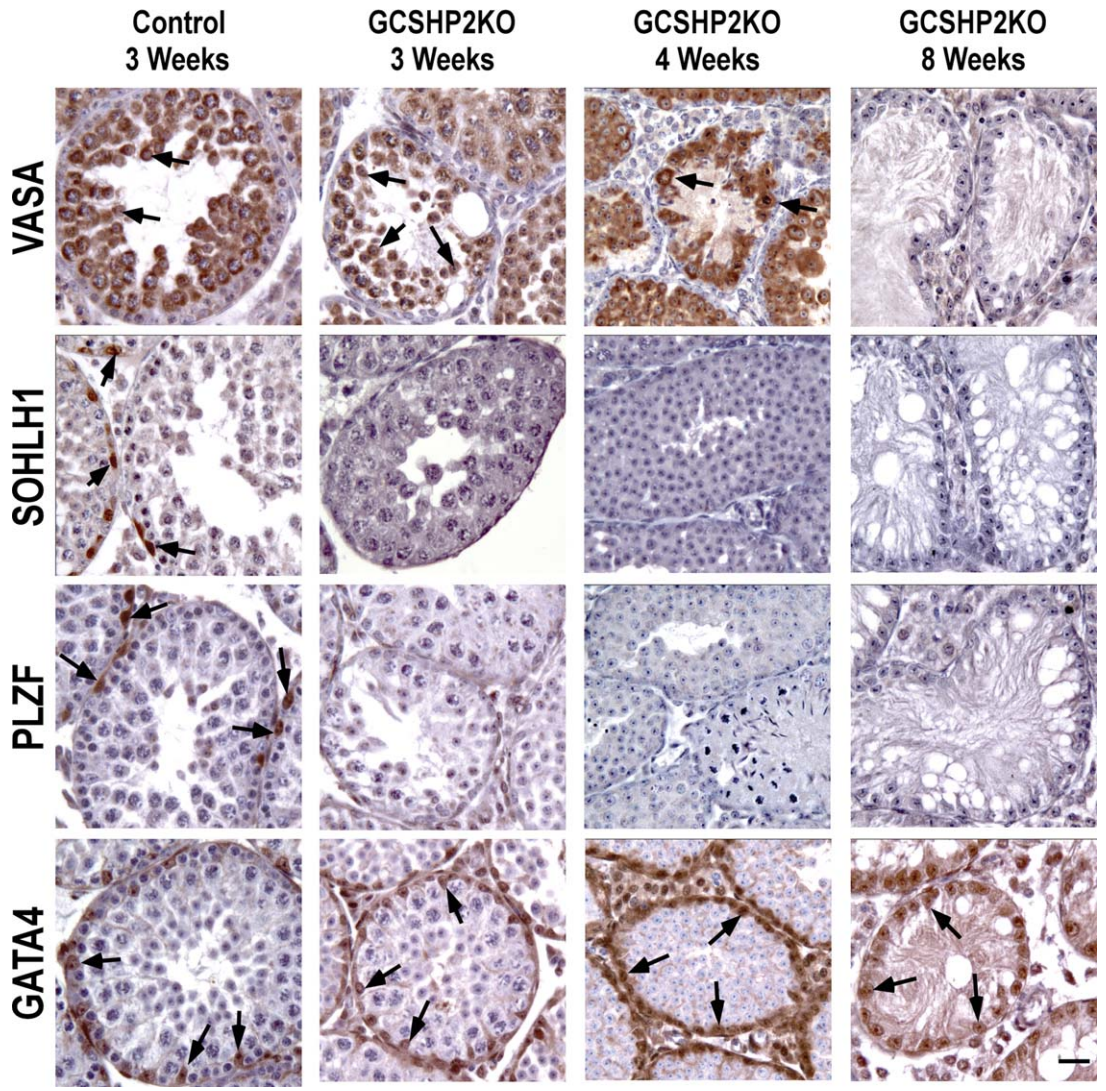

Figure 5. Undifferentiated and differentiated spermatogonia are not produced by GCSHP2 mice. Testis tissue sections from 3-week-old control and 3-, 4-, and 8-week old GCSHP2KO mice were probed with antisera against Vasa, SOHLH1, PLZF, and GATA4. For Vasa staining, arrows mark the presence of stained spermatogonia on the basement membrane (control testes only) and more mature germ cells in the adluminal compartment in control and GCSHP2 testes. For SOLHLH1 and PLZF staining, arrows mark differentiated and undifferentiated spermatogonia, respectively, present only in control testes. For GATA4 staining, arrows mark Sertoli cell nuclei in all conditions. Bar = 20 μm.

time course, but the overall frequency of apoptosis was relatively low ([lt] 0.6 TUNEL-positive cells/tubule) (Fig. 6E). At P5 and P7, the number of apoptotic cells per tubule for GCSHP2KO and control mice were similar. However, because the number of PLZF-positive cells in GCSHP2 mice was reduced to 44% or 39% of controls, these results suggest that the remaining cells undergo apoptosis more frequently than control mice. This idea was confirmed by further analysis showing that the percentage of PLZF-positive cells in GCSHP2KO mice that were also positive for the cleaved caspase-3 marker of apoptosis was elevated by 2.5- and 2.7-fold at P5 and P7, respectively (Fig. 6F, Supporting Information Fig. S4A). As suggested by the TUNEL results, the frequency of apoptotic PLZF-positive cells was low in GCSHP2KO mice ([lt] 2%) and did not reconcile with the 56%–61% decrease in PLZF-positive cells observed in GCSHP2KO mice. In contrast, proliferation of PLZF-positive cells as assayed by Ki-67 immunostaining was reduced by 52% in GCSHP2 mice at P5. Two days later (P7) the number of proliferating PLZF-positive cells in control mice was 35% lower, but in GCSHP2KO mice there was a further 33% decrease in PLZF-positive cells compared to control littermates (Fig. 6G, Supporting Information Fig. S4B). Together, these data suggest that the decrease in PLZF-positive cells in GCSHP2KO mice is predominately due to the failure of SSCs to proliferate, although apoptosis may contribute to the elimination of SSCs and undifferentiated spermatogonia (Fig. 6H).

SHP2 Supports the Attachment of SSCs to Supporting Cells, SSC Proliferation, and Growth Factor Signaling in Culture

Further studies were performed to determine whether SHP2 mediated the intracellular signals originating from bFGF and GDNF growth factors that are required to maintain the

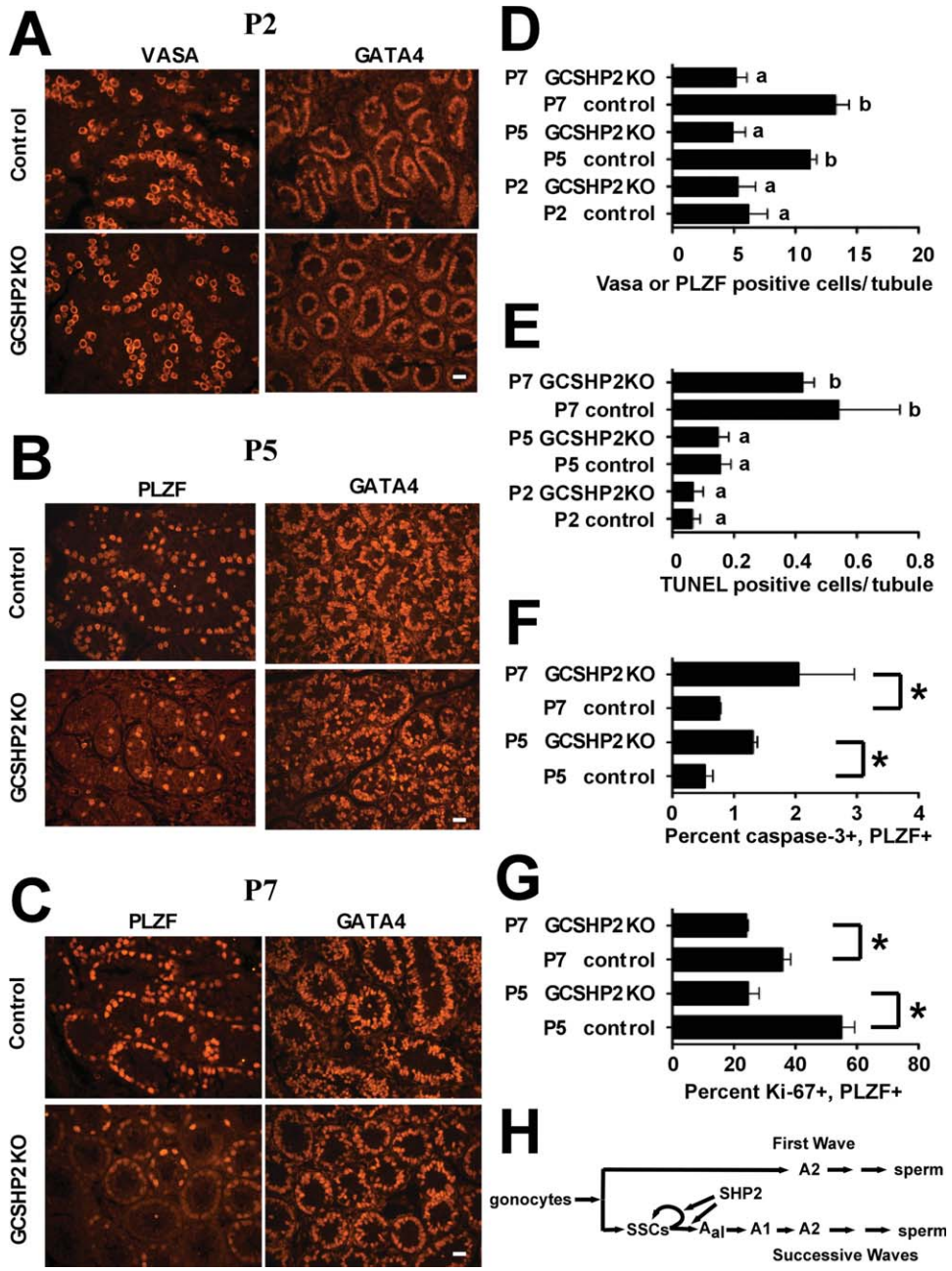

Figure 6. The number of SSCs and undifferentiated spermatogonia are decreased 5 and 7 days after birth in GCSHP2KO mice. **(A):** Testis tissue sections from P2 control and GCSHP2KO mice were probed with antisera against Vasa or GATA4. Bar = 20 μm. **(B, C):** Testis sections from P5 and P7 control and GCSHP2KO mice were probed with PLZF or GATA4 antisera. Bar = 20 μm. **(D):** Quantitation of Vasa-positive (P2, $n = 2$) and PLZF-positive (P5, $n = 4$ and P7, $n = 2$) cells per tubule for control and GCSHP2KO testis sections. **(E):** Quantitation of TUNEL-positive cells per tubule for P2 ($n = 2$), P5 ($n = 2$), and P7 ($n = 3$) control and GCSHP2KO testis sections. **(F):** Testis sections from P5 and P7 control and GCSHP2KO mice were probed with antisera against PLZF and the apoptosis marker cleaved caspase-3. The percentage of PLZF-positive cells that also express cleaved caspase-3 was quantified from entire tissue sections (259–1,453 PLZF-positive cells/section, $n = 3$). **(G):** Testis sections from P5 and P7 control and GCSHP2KO mice were probed with antisera against PLZF and the proliferation marker Ki-67. The percentage of PLZF-positive cells that also express Ki-67 was quantified as in panel G from 386 to 1,107 PLZF-positive cells/section. Statistical analysis of P5 control versus P5 GCSHP2KO as well as P7 control versus P7 GCSHP2KO comparisons in panels (F) and (G) was evaluated by unpaired *t* test. Asterisks denote pairs in which the means are statistically significant ($n = 3$). **(H):** Schematic summary of spermatogenesis including gonocytes producing differentiated A2 spermatogonia in the first wave. After conversion of gonocytes to SSCs, successive waves of spermatogenesis require SSC proliferation for self-renewal and production of A aligned (A_{al}) undifferentiated spermatogonia that transition into A1 differentiated spermatogonia and eventually to sperm. Values with different lower case letters differ significantly (p [lt] .05). Abbreviation: SSC, spermatogonial stem cell.

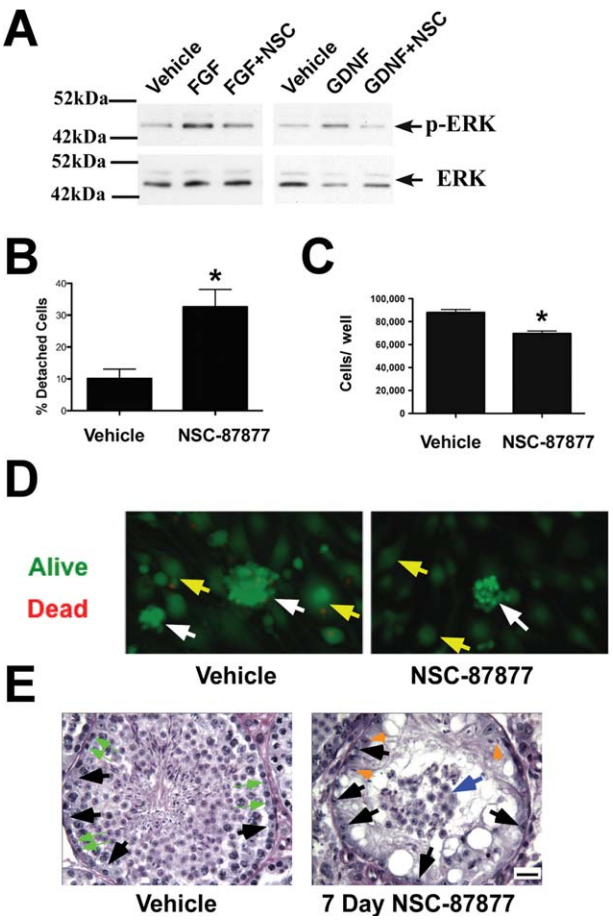

Figure 7. Inhibition of SHP2 blocks GDNF and bFGF signaling, causes spermatogonial stem cells (SSCs) to detach from their niche, decreases the number of SSCs in culture, and disrupts spermatogenesis. **(A):** Germ line stem (GS) cultures were stimulated for 10 minutes with bFGF (20 ng/ml) or GDNF (20 ng/ml) with or without 5 hours pretreatment with NSC-87877 (NSC, 50 μM). Immunoblot analysis of p-ERK and ERK levels after the treatments is shown. Data shown is representative of two (GDNF) and three (FGF) replicate cultures **(B)** GS cultures were treated for 5 days with vehicle or NSC-87877 (50 μM). The percentage of cells detached from feeder cells was determined ($n = 3$; *, p [lt] .05). **(C):** GS cells treated with vehicle or NSC (50 μM) as in (B) were isolated from feeder cells, dispersed, and counted ($n = 3$; *, p [lt] .05). **(D):** Clumps of GS cells (white arrows) and feeder cells (yellow arrows) treated as in (D) were assayed for live (green) and dead (red) cells. Magnification is ×20. **(E):** Testis sections from wild-type mice isolated 15 days after injection with vehicle, or NSC-87877 (10 mg/kg) for 7 days were stained with PAS-H. Green arrows: spermatogonia; black arrows: Sertoli cell nuclei; Orange arrows: mislocalized elongated spermatids; Blue arrow: a cluster of prematurely detached germ cells. Abbreviations: FGF, fibroblast growth factor; GDNF, glial cell-derived neurotrophic factor; NSC, NSC-87877.

survival, and proliferation of SSCs in GS cell cultures and in vivo [5, 7, 30–32]. We focused on SHP2 regulation of ERK kinase that supports the proliferation and renewal of SSCs [33, 34]. GS cell cultures were isolated from their feeder cells and treated with bFGF or GDNF for 10 minutes in the absence and presence of the SHP2 selective inhibitor NSC-87877. Addition of bFGF or GDNF increased the phosphorylation (activation) of ERK as expected (Fig. 7A), but pretreatment with NSC-87877 inhibited the bFGF and GDNF-mediated phosphorylation of ERK. These data suggest that SHP2 activity is required to transmit signals within SSCs that are essential for proliferation and survival. Addition of NSC-87877 to the GS cell cultures for 5 days resulted in a threefold increase in the number of GS cells detached from feeder cells (Fig. 7B) suggesting that SHP2 may be required for GS cells to attach to their niche.

A 5-day treatment with NSC-87877 caused the number of GS cells to be reduced by 22% (Fig. 7C). Cell death in the GS cultures was similarly low in the absence and presence of NSC-87877 (Fig. 7D). The magnitude of the NSC-87877-mediated decrease in GS cells is consistent with the inability of the less abundant SSCs to proliferate. However, we cannot rule out the possibility that NSC-87877 specifically mediated the death of SSCs that make up a small portion of the GS cell population. Injection (ip) of NSC-87877 (10 mg/kg) into adult mice daily for 7 days followed by analysis 15 days later revealed that NSC-87877 caused disruption of spermatogenesis similar to that observed for Ptpn11$^{\Delta/\Delta}$ mice including the absence of spermatogonia and most mature germ cells except for a few mislocalized elongated spermatids (Fig. 7E). The germ cells that were present were detached from supporting Sertoli cells and localized to the tubule lumen. These results provide further evidence that SHP2 activity is required for SSCs to replenish germ cells.

Discussion

Using mouse models in which SHP2 expression is eliminated in all cells or only in germ cells, we found that SHP2 is essential to complete the initial step of spermatogenesis, the production of undifferentiated spermatogonia from SSCs. In the absence of SHP2, undifferentiated spermatogonia are not present but germ cells beyond this stage of development are capable of completing the process of spermatogenesis.

The inability to produce undifferentiated spermatogonia is not due to the death of gonocyte precursors of SSCs because the elimination of SHP2 specifically in germ cells did not alter the number of gonocytes present 2 days after birth. We cannot rule out the possibility that SHP2 contributes to the transition of gonocytes into SSCs between P2 and P5. However, ablation of SHP2 in adult *Ptpn11*$^{\Delta/\Delta}$ mice prevents the replenishment of undifferentiated spermatogonia well after all gonocytes have been converted to A2 spermatogonia or SSCs indicating that the lack of undifferentiated spermatogonia must be due to a defect in SSCs.

In the GCSHP2KO mouse, production of undifferentiated spermatogonia from SSCs was inhibited as evidenced by the 56% and 61% decrease in PLZF-positive cells on P5 and P7, respectively, and the absence of all spermatogonia production prior to 3 weeks after birth. This decrease in the SSC and undifferentiated spermatogonia population coincided with a 55% and 33% decrease in proliferating PLZF-positive cells in GCSHP2KO mice at P5 and P7. These data support the hypothesis that germ cells cannot be replenished and self-renewal of SSCs cannot occur due to a block in the proliferation of SSCs in the absence of SHP2. Similar effects have been described in SHP2-deficient neuronal stem cells [35]. Apoptosis may contribute to the decrease in SSCs and undifferentiated spermatogonia as the frequency of apoptosis increases by two- to

threefold in GCSHP2KO mice. It is possible that the increase in apoptosis may reflect a clearing of SSCs and undifferentiated spermatogonia in response to their inability to proliferate. However, the overall low percentage of PLZF-positive cells undergoing apoptosis would not appear to be a major determinant of the loss of PLZF-positive cells.

The relatively small but significant reduction (22%) in the number of GS cells in culture after 5 days of treatment with the SHP2 inhibitor NSC-87877 is consistent with blocking the proliferation of the more rare SSCs ([lt]10% of the heterogeneous culture [36]) that are needed to produce undifferentiated spermatogonia. The smaller magnitude of decrease for cultured GS cells compared to PLZF-positive cells in vivo after the loss of SHP2 activity also is consistent with the slower proliferation of GS cells (5.6-day doubling time) [7] versus that of undifferentiated spermatogonia in vivo (30–70 hours) [37, 38]. The absence of spermatogonia 15 days after injection of mice with NSC-87877 further supports the hypothesis that SHP2 is required for SSCs to replenish germ cells. Inhibition of SHP2 produced a dramatic disruption of spermatogenesis similar to that observed after knockout of SHP2 in all cells in the *Ptpn11*$^{\Delta/\Delta}$ mice including the detachment of meiotic and postmeiotic germ cells as well as mislocalization of elongated spermatids. These data suggest that in addition to maintaining SSCs, SHP2 performs functions in Sertoli cells (and perhaps other cells) required for germ cell attachment and the support of spermatogenesis. The NSC-87877-mediated elimination of SSCs and differentiated germ cells also suggests inhibitors of SHP2 or SHP2 targets, if delivered specifically to the testis, could be used to permanently sterilize animals as a mechanism to humanely solve challenges of animal overpopulation.

NSC-87877-mediated inhibition of SHP2 caused a threefold increase in the number of GS cells that were detached from their feeder cells. This result raises the possibility that SHP2 is required to maintain SSCs in their niche and that release from the niche may contribute to decreased survivability of SSCs or remove the SSCs from the signals required to produce undifferentiated spermatogonia. It is not known yet whether SSC detachment is caused by the inhibition of SHP2 in SSCs or their supporting cells.

Ptpn11$^{\Delta/\Delta}$ mice and mice lacking the SHP2-regulated ETV5 gene are similar in that both display misorientation of elongated spermatids [39] suggesting that SHP2 is part of a signaling pathway required to regulate germ cell attachment. The localization of SHP2 immunoreactivity in Sertoli cells to regions surrounding elongating spermatids is consistent with our observations that SHP2 is a regulator of Sertoli-germ cell adhesion. Previously, we found that constitutive activation of SHP2 disrupts Sertoli-Sertoli cell junctional complexes by mislocalization of adherens junction proteins β-catenin and N-cadherin and disruption of the actin cytoskeleton [19].

In the GCSHP2KO mice 3 and 4 weeks after birth, pachytene spermatocytes and spermatids were present indicating that the first wave of spermatogenesis occurs after knockout of SHP2. The production of the first wave provides further evidence that gonocytes in GCSHP2KO mice are able to produce differentiated spermatids 3–5 days after birth and confirms that the lack of SHP2 expression only affects the survival of germ cells prior to the differentiated spermatogonia stage of development. The transient presence of pachytene spermatocytes and spermatids in *Ptpn11*$^{\Delta/\Delta}$ mice also showed that germ cells that were beyond the undifferentiated spermatogonia stage of development at the time of SHP2 knockout were able to continue their maturation in a "final wave" of spermatogenesis.

The necessity for SHP2 to replenish germ cells from SSCs is reminiscent of SHP2 functions in other stem cells. Deletion of the SHP2 gene in hematopoietic stem cells disrupts their quiescent state, and self-renewal while increasing apoptosis resulting in a severe reduction in mature blood cells [40, 41]. Similarly, the survival of trophoblast stem cells is dependent upon SHP2 [42]; whereas SHP2 deletion in neuronal stem cells impairs their proliferation and differentiation resulting in lethality [35].

The progressive loss of germ cells and the resulting Sertoli cell only phenotype that was found after elimination of SHP2 expression has been reported in mice engineered to lack expression of SSC markers such as PLZF, ETV5, FOXO1, and Sin3a [39, 43–45]. Mice lacking a single allele of GDNF show a similar phenotype [5] and GDNF is known to activate SHP2 in other cells [8, 46]. bFGF that acts in concert with GDNF to maintain the pool of SSCs also mediates its signaling through SHP2 in a variety of cell types [9, 34, 47]. Additionally, the ETV5 and FOXO1 transcription factors that are required to maintain the SSC pool are downstream targets of SHP2 [11, 44, 46]. The more dramatic phenotype of the SHP2 deficient mouse models may be due to SHP2 being a mediator of both GDNF and bFGF signaling as well as a regulator of ETV5 and FOXO1 required for SSC self-renewal and/or survival. Thus, it is possible that SHP2 is a central nexus and rheostat for intracellular signaling pathways in SSCs that are essential for the fine-tuning of signals required for the production of undifferentiated spermatogonia. Supporting this hypothesis is our finding that inhibition of SHP2 activity by NSC-87877 in cultured GS cells decreased bFGF- and GDNF-mediated activation of ERK. In addition, recent analysis of mutation frequencies in sperm from Noonnan syndrome patients suggests that SHP2 gain of function mutations in SSCs provides a competitive advantage for the production of sperm [48]. Further studies will be required to determine whether SSC fate is determined by SHP2 regulation of ETV5, FOXO1, or other targets in response to GDNF and bFGF signaling.

Conclusion

The expression of SHP2 is critical for maintaining the male germline and fertility. SHP2 mediates GDNF and FGF signals needed for the maintenance of SSCs and the production of undifferentiated spermatogonia. In the absence of SHP2, SSCs are not able to proliferate and germ cells cannot be replenished. Our findings may be applied to develop therapies for SHP2 defects that result in infertility. Specifically, Noonan and LEOPARD patients having mutations that misregulate SHP2 expression would be expected to have compromised SSC and Sertoli cell function leading to disruption of spermatogenesis.

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Author contributions

P.P.: conception and design, data collection, analysis, interpretation, manuscript writing, and financial support; B.P., K.O., A.R., P.L., P.K., and G.-S.F.: provision of study material; S.H.: provision of study material and data collection; W.H.W: conception and design, data collection, analysis, interpretation, manuscript writing, financial support, and final approval of manuscript.

Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

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