The Tyrosine Phosphatase SHP2 Regulates Sertoli Cell Junction Complexes

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The Tyrosine Phosphatase SHP2 Regulates Sertoli Cell Junction Complexes

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ABSTRACT

The blood-testis barrier (BTB) is a large junctional complex composed of tight junctions, adherens junctions, and gap junctions between adjacent Sertoli cells in the seminiferous tubules of the testis. Maintenance of the BTB as well as the controlled disruption and reformation of the barrier is essential for spermatogenesis and male fertility. Tyrosine phosphorylation of BTB proteins is known to regulate the integrity of adherens and tight junctions found at the BTB. SHP2 is a nonreceptor protein tyrosine phosphatase (PTP) and a key regulator of growth factor-mediated tyrosine kinase signaling pathways. We found that SHP2 is localized to Sertoli-Sertoli cell junctions in rat testis. The overexpression of a constitutive active SHP2 mutant, SHP2 Q79R, up-regulated the BTB disruptor ERK1/2 via Src kinase in primary rat Sertoli cells in culture. Furthermore, focal adhesion kinase (FAK), which also supports BTB integrity, was mislocalized of adherens N-cadherin, β-catenin, and ZO-1 away from the plasma membrane. These results suggest that SHP2 is a key regulator of BTB integrity and Sertoli cell support of spermatogenesis and fertility.

blood-testis barrier, spermatogenesis, tests

INTRODUCTION

The development of germ cells (spermatogenesis) and male fertility are dependent on somatic Sertoli cells that provide nourishment and mechanical support to germ cells. Sertoli cells also create the blood-testis barrier (BTB) that is required for fertility. The BTB is composed of tight junctions, specialized adherens junctions, and gap junctions that physically divide the seminiferous epithelium into the basal and apical compartments while creating a unique environment for germ cell development [1–3]. The BTB is a dynamic structure that must be periodically opened and then re-formed to allow preleptotene spermatocytes to pass from the basal compartment to the adluminal compartment of the seminiferous tubule. Thus, the timely opening and closing of the BTB is required to maintain spermatogenesis. Maintaining the BTB is also essential because it forms an immunological barrier that sequesters postmeiotic germ cell antigens from the immune system. When the BTB is dysfunctional, germ cell differentiation and development are arrested [4, 5]. The factors and mechanisms regulating the maintenance and periodic remodeling of the BTB are not fully understood. A number of components of the BTB and signals regulating BTB function have been investigated, and kinase-mediated signaling cascades have been found to be important for maintaining the BTB [6]. However, regulation of the BTB by phosphatases has received only limited study [7].

SHP2 is a nonreceptor tyrosine phosphatase containing two amino-terminal SH2 domains, a central phosphotyrosine phosphatase (PTP) domain, and C-terminal tyrosyl phosphorylation sites. In the absence of growth factors, SHP2 is autoinhibited due to interactions between the SH2 and PTP domains. On growth factor or cytokine stimulation, the SH2 domains of SHP2 interact with phosphorylated tyrosine residues on growth factor receptors [8]. Autoinhibition is then relieved, allowing the phosphorylation and activation of SHP2. Once activated, SHP2 stimulates the PI3K/AKT and Ras-MapK (ERK) pathways, but SHP2 can act as an activator or inhibitor of the JAK/STAT pathway [9–11]. Although best known as a cytoplasmic-plasma membrane localized signaling factor, SHP2 is also localized to the mitochondria, where it regulates cellular redox balance, and to the nucleus, where SHP2 regulates STAT transcription factors and telomerase reverse transcriptase (TERT) activity [12–14].

SHP2 is encoded by the Ptpn11 gene. In humans, missense mutations in Ptpn11 that decrease SHP2 activity result in LEOPARD syndrome that is characterized by heart, lung, ocular, growth, and genitalia abnormalities [15]. Disruption of the Ptpn11 gene in mice results in embryonic lethality [16]. Studies of conditional SHP2 knockout mice have implicated SHP2 in regulating metabolism, obesity, diabetes-insulin sensitivity, liver regeneration, retinal degeneration, cardiovascular protection, and stem cell-induced proliferation of bone marrow-derived mast cells [17–21].

Mutations that disrupt the autoinhibitory conformation of SHP2 result in constitutive activation of the phosphatase and can cause juvenile leukemias [22]. Thus, SHP2 is a proto-oncogene [22, 23]. Constitutive active SHP2 mutations also result in the juvenile development disorder Noonan syndrome, which includes facial dysmorphia, congenital heart defects, short stature, and male infertility [23, 24]. Cryptorchidism was thought to be the main cause of male infertility in Noonan syndrome patients. However, there is evidence that Noonan syndrome patients with normal testicular descent have degenerating spermatocytes and immature Sertoli cells that produce low levels of inhibin. These findings suggest that misregulation of SHP2-mediated signaling causes fertility defects independent of cryptorchidism [25–27].
Animal Care and Use

In this study, we show that SHP2 is a critical regulator of Sertoli cell junctional complexes. SHP2 expression and function have not been characterized in Sertoli cells or other testis cells. However, in other cell types, SHP2 regulates cell adhesion proteins that in Sertoli cells are needed to maintain the BTB and the attachment of elongated spermatids [28]. There is further support for SHP2 as a candidate regulator of the BTB because SHP2 activates ERK and the activation of ERK is associated with disruption of BTB [29]. SHP2 also interacts with and deactivates focal adhesion kinase (FAK) [30, 31], which is required to maintain tight junctions in Sertoli cells [32]. Inappropriate activation of ERK as occurs in Noonan syndrome patients disrupts tight junctions and adherence junctions in other cell types [33–37]. Furthermore, in Sertoli cells, alterations in the phosphorylation status of transmembrane and adaptor proteins can cause junction-associated proteins to be relocalized to the cytoplasm or diffuse through the plasma membrane away from the junction [28, 38, 39]. In this study, we show that SHP2 is a critical regulator of Sertoli cell junctional complexes.

Table 1. Antibodies employed, applications, and dilutions.

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Isolation of Primary Sertoli Cells

Sertoli cells were isolated from 20-day-old Sprague-Dawley rats and cultured in serum-free media as described previously [42]. Sertoli cells at this age have completed their proliferation period and are no longer dividing in vivo [43–45]. Briefly, decapsulated testes were digested with collagenase (0.5 mg/ml, 33°C, 12 min) in enriched Krebs-Ringer bicarbonate buffer followed by three washes in enriched Krebs-Ringer bicarbonate medium to isolate seminiferous tubules. Tubules were digested with trypsin (0.5 mg/ml, 33°C, 12 min). An equal volume of Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum was added to the Sertoli cells, which were then pelleted (100 x g, 5 min) and resuspended in serum-free medium containing 50% DMEM, 50% Ham’s F-12, 5 mg/ml insulin, 5 mg/ml transferrin, 10 mg/ml epidermal growth factor, 1 mM sodium pyruvate, 200 units/ml penicillin, and 200 mg/ml streptomycin. Sertoli cells were cultured on Matrigel (BD Bioscience, Franklin Lakes, NJ) coated dishes (32°C, 5% CO2) or Matrigel coated coverslips in dishes. The cells were washed with PBS on Day 2 and cultured further in serum-free media. The cultures were found to be routinely >95% pure as determined by phase microscopy and alkaline phosphatase staining.

Preparation of Whole Cell Extracts, Membrane-Associated Protein Extracts, Western Blots, and Immunoprecipitations

To prepare testis whole cell lysates, testes isolated from 5- to 60-day-old rats were decapsulated and homogenized in enhanced lysis buffer (ELB; 250 mM NaCl, 0.1% NP40, 50 mM Heps, pH 7.0, 5 mM EDTA, 0.5 mM dithiothreitol) with a protease inhibitor cocktail, rocked for 15 min at 4°C, and then pelleted (12,000 x g 15 min) to remove cell debris. To prepare whole cell lysates of Sertoli cells, the cells were cultured for 3 days and then incubated in the absence and presence of hormones and/or signaling pathway regulators for 10 min. In some cases, cells were pretreated for 4 h with NSC-87877 or 2 h with PP2, PD98059, or wortmannin. To prepare whole cell extracts for direct analysis by Western immunoblot, cells were washed once with PBS and then lysed on the plates by using boiling Laemmli sample buffer to minimize phosphatase activity. Cell lysates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody. The antigen-antibody complex was visualized with Millipore Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA). Protein concentrations were not performed prior to SDS-PAGE, but equal loading of samples was confirmed with control antisera, including those against actin and nonphosphorylated proteins.

For immunoprecipitations, Sertoli cells were lysed in ELB buffer and a cocktail of protease and phosphatase inhibitors, sonicated for 10 sec, and...
subjected to centrifugation (12,000 × g 15 min). The supernatants (5–20 μg) were added to 500 μl of ELB buffer and incubated with antibodies followed by incubation with protein G sepharose. Immunobound material was eluted by boiling in 2× Laemmli sample buffer for 5 min and fractionated on 10% SDS-PAGE gels. Detection of antigen-bound antibody was carried out by Western analysis as described above.

**Immunocytochemistry**

Immunostaining of testis tissue was performed on paraffin-embedded sections (5 μm) from paraformaldehyde fixed (4%, o/n) adult rat testis. Testis sections were deparaffinized in xylene, rehydrated, and then permeabilized for 1 min in cold 100% methanol. The sections were microwaved on high power for 20 min in citrate buffer (10 mm citrate, 30 mm NaCl, pH 5.5) and then left undisturbed at room temperature for 20 min. The sections were washed two times for 5 min in PBS and blocked for 12 h in normal goat serum, 0.5% BSA, and 0.15% glycine at 4°C. Cultured cells were fixed in 4% paraformaldehyde for 5 min, permeabilized for 1 min in ice-cold 100% MeOH, and dried completely followed by blocking with normal goat serum, 0.5% BSA, and 0.15% glycine. The testis tissue or cultured cells were then incubated 12–24 h with preimmune serum or rabbit polyclonal antisera directed against SHP2 at a dilution of 1:2,000. Anti-rabbit biotinylated secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) was added, and bound antibodies were detected as described by the kit instructions using DAB staining, chromogenic reagent. Slides were washed in H2O and counterstained with hematoxylin. A charged coupled device video camera system was used to capture images of stained cells or tubule cross sections.

**Transepithelial Resistance Assays of Tight Junctions**

Sertoli cells isolated from 20-day-old rats were plated onto bicameral units (Millipore Corp, Bedford, MA) coated with Matrigel (BD Bioscience) in 12-well plates at 1.5 × 10⁶ cells/1 cm² bicameral unit in serum-free media as previously described [7]. Media was replaced every 24 h. The quality of tight junction formation was assessed by assaying transepithelial resistance (TER) across the Sertoli cell monolayer using a Millicell electrical resistance system (EMB Millipore) as described [7]. Observations were made using duplicate cultures from three different isolations of cultured Sertoli cells.

**Immunofluorescence**

For confocal studies, rat testes were fixed and sections subjected to antigen retrieval, permeabilized, and blocked as described for the immunocytochemistry studies. The sections were probed with rabbit SHP2 and mouse vimentin antiserum followed by incubation with Cy3 and Alexa 488 fluorescently tagged secondary antisera and stained with 4',6-diamidino-2-phenylindole (DAPI). Primary Sertoli cells were cultured in 35-mm² plates containing coverslips in serum-free media as described. Three days after initiating the cultures, the cells were infected with AdSHP2 Q79R or AdGFP. After a further 3 days, the cells were washed twice in ice-cold PBS and fixed with 2% paraformaldehyde in saline for 20 min, washed three times with PBS, blocked with goat serum, and then incubated with a rabbit antiserum directed against SHP2, N-cadherin, ZO-1, or β-catenin or mouse antiserum against β-actin. The cells were washed and then incubated with Cy3 tagged fluorescent secondary goat anti-rabbit antiserum or Cy3 tagged goat anti-mouse antiserum and then stained with DAPI. Images were obtained on a Nikon Provis II (Nikon, Melville, NY) or confocal microscope. All image files were digitally processed for presentation in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

**Statistical Analysis**

Immunoreactive signals from western blot films were scanned with an Epson 1600 Expressions scanner using Epson Scan software. For Western blots, the mean ± SEM relative signal intensities were determined for at least three independent experiments. Results were analyzed by analysis of variance with Newman-Keuls PLSD or t-test at a 5% significance level utilizing GraphPad Prism 4.3 (GraphPad Software, San Diego, CA).

**RESULTS**

**SHP2 Expression in the Testis**

Because SHP2 expression has not been characterized previously in the testis, we first studied the developmental expression of SHP2 in postnatal testes. Western blot analysis of whole cell extracts isolated from rats identified a single immunoreactive protein band at 72 kDa corresponding to SHP2 throughout development. Overall SHP2 expression levels in the testis were relatively constant from 5 to 60 days after birth except for a decrease in signal at 60 days (Fig. 1A). In contrast, the levels of the germ cell-specific PP1γ2 protein [40] increased between 16 and 21 days after birth and remained elevated due to the expansion of germ cells that occur during this developmental stage [46].

To determine whether SHP2 expression changes during Sertoli cell development, SHP2 levels were assayed in cultured Sertoli cells isolated from rats 5–20 days after birth. These studies demonstrated that SHP2 was expressed in Sertoli cells and that SHP2 levels remained constant during Sertoli cell development (Fig. 1B). Immunohistochemical analysis of testis sections from an adult rat revealed that SHP2 was expressed in Sertoli cell nuclei, the Sertoli cytoplasm between germ cells extending to the tubule lumen, and that SHP2 was specifically concentrated near the basement membrane in the vicinity of the BTB (Fig. 1, C and D). SHP2 was expressed in spermatogonia, but less SHP2 immunoreactivity was detected in the more mature spermatocytes and spermatids. The region of the Sertoli cell surrounding elongated spermatids also displayed intense SHP2 immunostaining, raising the possibility that SHP2 regulates the attachment of elongated spermatids to Sertoli cells.

Further analysis of SHP2 immunoreactivity using confocal fluorescent microscopy confirmed that SHP2 was expressed in Sertoli cell nuclei as well as Sertoli cytoplasm, including a line of SHP2 immunoreactivity on the apical side of preleptotene spermatocytes and spermatogonia consistent with the position of the BTB (Fig. 1E). SHP2 immunoreactivity in the region of the BTB did not colocalize with vimentin, an intermediate filament protein that is not associated with tight junctions of the BTB. In the Sertoli cell cytoplasm extending to the lumen, SHP2 did colocalize with vimentin, which is known to be expressed in the cytoplasmic extensions of Sertoli cells [47]. Analysis of the expression of SHP2 in Sertoli cells cultured from 20-day-old rats confirmed that SHP2 was expressed in most Sertoli cell nuclei, throughout the cytoplasm, and at the periphery of the cell at cell attachment sites (Fig. 1F).

**SHP2 Is Activated by Hepatocyte Growth Factor**

Hepatocyte growth factor (HGF), is also known as scatter factor because it disrupts cell-cell adhesion and causes contiguous epithelial cells to disperse [48]. HGF activates SHP2 in other cell types [49] and modulates Sertoli cell tight junctions that are required to maintain the BTB [50], but it is not known whether HGF activates SHP2 in Sertoli cells. To determine whether HGF regulated SHP2 activity, cultured Sertoli cells were stimulated with HGF for 15 min and SHP2 activity was assessed by assaying the extent of phosphorylation at Tyr-542. HGF was found to increase SHP2 and ERK phosphorylation (Fig. 2A). The activation of ERK by HGF was found to be dependent on SHP2 because the SHP2 selective inhibitor NSC-87877 [51] blocked HGF-dependent ERK phosphorylation (Fig. 2B).

**SHP2 Regulates MAP Kinase and FAK Signaling in Sertoli Cells**

Gain-of-function mutations in SHP2 that cause Noonan syndrome are associated with male infertility and Sertoli cell dysfunction. To determine whether similar overactivation of SHP2 alters the activity of kinases that are essential for Sertoli...
cell-mediated support of spermatogenesis, an adenovirus expressing a constitutive active SHP2 (AdSHP2 Q79R) [41] was introduced into cultured Sertoli cells. Western blot analysis of whole cell extracts determined that SHP2 Q79R increased ERK phosphorylation fourfold (Fig. 3A). Addition of the Src kinase inhibitor PP2 decreased ERK phosphorylation induced by SHP2 Q79R by 68% (Fig. 3B). In contrast, wortmannin, an inhibitor of PI3 kinase, did not affect ERK phosphorylation, whereas the MAP kinase inhibitor PD98059 abolished ERK phosphorylation as expected. These data indicate that much of SHP2 signaling to the MAP kinase cascade is dependent on the activation of Src kinases by SHP2.

SHP2 has not been found to directly interact with Src kinases, but SHP2 has been shown to dephosphorylate FAK in other cell types [30, 31, 54]. Adenoviral-mediated expression of SHP2 Q79R in Sertoli cells decreased FAK phosphorylation on the activating Tyr 397 residue by 80% in comparison to cells infected with a control adenovirus (Fig. 4A). Immunoprecipitation studies determined that SHP2 and FAK interact directly (Fig. 4B). These data suggest that SHP2 directly dephosphorylates FAK in Sertoli cells.

**FIG. 1.** SHP2 is expressed in Sertoli cells and early stage germ cells in the testis. A) Whole testis extracts from rats 5, 16, 21, 29, and 60 days postpartum (dpp) were subjected to Western blot analysis with antisera against SHP2, PP1′2, or actin. B) Whole cell extracts from cultured Sertoli cells isolated from rats 5, 11, 15, and 20 dpp were analyzed by Western blot using antisera against SHP2 or actin. Adult rat testis sections were probed with secondary antibody only (C) or SHP2 antisera (D) and stained with hematoxylin. SHP2 staining (brown) was localized to Sertoli (S) nuclei as well as Sertoli cell cytoplasm between germ cells (black arrowheads) and around elongated spermatids (ES). SHP2 immunostaining was also present along the basement membrane in the region of the blood-testis barrier (red arrows) that did not extend beyond the apical side of spermatogonia (Sp) and preleptotene spermatocytes (Pl). F) Confocal fluorescence microscopy analysis of SHP2 (red) and vimentin (green) shows that SHP2 is localized to Sertoli nuclei (S) and Sertoli cytoplasm between germ cells (white arrowheads). SHP2 was also detected (red arrows) just to the apical side of preleptotene spermatocytes (Pl) and spermatogonia (Sp). SHP2 staining around elongated spermatocytes (ES) is identified by arrows. Nuclei are stained blue with DAPI. F) Sertoli cells cultured from 20-day-old Sertoli cells were probed with SHP2 antiserum, and immunolocalization was performed using immunofluorescence microscopy. SHP2 localized to the cell periphery at cell-cell contact points is denoted by arrows. Original magnification ×60 (C, D, and F), ×100 (E).
The integrity of the BTB is dependent in large part on the formation of tight and adherens junctions between Sertoli cells. To determine whether SHP2 regulates the integrity of tight and adherens junctions formed by Sertoli cells, the cells were cultured as confluent monolayers on bicameral units, and TER was assessed as previously described [7, 55–60]. Infection of cultured Sertoli cells with AdSHP 2Q79R decreased TER by 38%. Stimulation with HGF for 72 h reduced TER levels by 22% (Fig. 4C). These data suggest that continuous stimulation with HGF or extended overactivation of SHP2 inhibits tight junction formation.

To determine whether SHP2 regulates the expression of BTB components, Western blot analysis was performed after adenoviral-mediated expression of SHP2 Q79R or the control GFP protein. These studies indicated that SHP2 Q79R did not alter the expression of the transmembrane proteins N-cadherin, occludin, and JAM-A; the adaptor proteins α-catenin, β-catenin, and ZO-1; or the cytoskeletal protein β-actin (Fig. 5, A–C). However, expression of SHP2 Q79R did alter the localization of the adherens protein N-cadherin and the adaptor proteins ZO-1 and β-catenin. Specifically, after infection of a control adenovirus expressing GFP, the immunofluorescent signals for the proteins were localized predominantly to the periphery of Sertoli cells at adhesion sites associated with the plasma membrane. In contrast, after expression of SHP2 Q79R, the N-cadherin, ZO-1, and β-catenin immunostaining localized at the plasma membrane was decreased (Fig. 6, A–F). Immunofluorescence analysis of β-actin in Sertoli cells also revealed that expression of SHP2 Q79R resulted in disruption of the actin cytoskeleton (Fig. 6, G and H). Further communoprecipitation studies revealed that N-cadherin and β-catenin interaction was decreased after overexpression of SHP2 Q79R in cultured Sertoli cells (Fig. 6I). These studies suggest that SHP2 regulates the localization and interactions of BTB-associated proteins as well as the formation of filamentous actin stress fibers.

**DISCUSSION**

In this study, we describe the expression, functional significance and potential mechanisms by which SHP2 regulates Sertoli cell adhesion. SHP2 was found to be expressed at a constant level in rat Sertoli cells throughout development, and overall SHP2 levels in the testis do not change from 5 to 29 days after birth. There was a decrease in SHP2 levels between 29 and 60 days after birth that may reflect dilution of the SHP2 signal by the increase in the number of germ cells that occurs with development. In vivo, we found that SHP2 was expressed in the seminiferous tubules near the basement membrane where the BTB is localized, at sites of Sertoli-cell-elongated spermatid attachment, as well as in the Sertoli cell cytoplasm and nucleus. SHP2 expression also was found to be present in germ cells prior to the preleptotene stage of development, whereas more mature germ cells expressed low levels of SHP2. The strong immunostaining of the Sertoli cell regions associated with the BTB and regions surrounding elongated spermatids supports the hypothesis that SHP2 regulates the maintenance of the BTB and spermatid-Sertoli cell adhesion. SHP2 is a strong candidate for regulating the adherens proteins required to maintain spermatid-Sertoli attachment because SHP2 activates ERK and Src, which in turn phosphorylate proteins required to build and sever the connections between the two cell types [61]. Interestingly, the colorimetric staining of testis sections revealed SHP2 immunostaining near the BTB as a zone extending from the basement membrane to the apical side of spermatogonia and preleptotene spermatocytes, whereas confocal microscopy analysis delineated a more fine line of SHP2 immunoreactivity in the BTB region only on the apical side of spermatogonia and preleptotene spermatocytes. This line of
SHP2 immunoreactivity is reminiscent of that observed previously for the ZO-1, N-cadherin, and Arp3 proteins associated with the BTB [62]. It is not yet known whether the more diffuse SHP2 immunoreactivity detected by the colorimetric protocol indicates that SHP2 functionality extends from the basement membrane to the BTB or whether the confocal approach provides a higher-resolution result.

HGF stimulation increased SHP2 activity in cultured Sertoli cells. Previously, HGF was shown to activate SHP2 in myoblasts, satellite cells, vascular cells, and pancreatic acinar cells [63–65]. HGF interacting with its receptor c-met results in the recruitment of Gab1, which then recruits and causes the activation of SHP2 [49]. Interestingly, HGF levels in seminiferous tubules in vivo are low except for a peak during the stages in which the BTB is remodeled to allow the passage of preleptotene spermatocytes through the barrier [66]. Furthermore, HGF was found to modulate the integrity of Sertoli cell tight junctions that are required for the BTB [50]. Although HGF is known to be produced by peritubular myoid cells and Sertoli cells [67], the source of the stage-specific increases in HGF expression is not yet known. Nevertheless, the temporal control of HGF levels in the testis may limit the
time span of SHP2 activation that is required for the brief opening and re-formation of the BTB. SHP2 also is stimulated by other BTB regulators, including the cytokine TNF-α [68–70] and beta 1 integrin [71]. TNF-α is produced in a stage-specific fashion by germ cells prior to BTB remodeling [69, 72] and causes the BTB to be transiently disrupted [73]. Beta 1 integrin, when activated, causes the disassembly of adherens junctions that are essential for maintaining the BTB [74]. Together, these findings raise the possibility that SHP2 is a central transducer of signals in Sertoli cells that regulate BTB integrity.

The SHP2 Q79R mutation and other mutations within the SH2 domain result in the constitutive activation of SHP2 and Noonan syndrome in humans. Transgenic mice bearing the SHP2 Q79R mutation show up-regulation of the MAPK pathway and recapitulate many classical symptoms of Noonan syndrome [41, 75]. We determined that, in Sertoli cells, SHP2 Q79R increases the phosphorylation of ERK via Src kinase. We also observed that constitutive activation of SHP2 in Sertoli cells decreased the levels of phosphorylated paxillin in Sertoli cells. Previous studies have shown that SHP2 can act through paxillin to reverse inhibitory tyrosine phosphorylation of the pool of Src that is complexed with paxillin. Specifically, SHP2 dephosphorylation of paxillin results in the dissociation of the negative regulator C-terminal Src kinase (Csk) that normally phosphorylates and inhibits Src in the paxillin-Src complex [52, 53]. We were unable to detect any changes in the phosphorylation of Src in the whole cell extracts of Sertoli cells expressing constitutive active SHP2 (data not shown). The inability to detect increased p-Src in our whole cell extracts may be explained by the limiting of activated p-Src to the pool of Src associated with paxillin. Thus, the compartmentalized increases in p-Src levels were not detectable above the basal levels of the remaining Src phosphorylation in Sertoli cells. It is also possible that SHP2 may act via other known mechanisms to activate MAP kinase pathway. Specifically, SHP2 could dephosphorylate and inactivate inhibitors of RAS and the MAP kinase pathway, including the RasGAP and sprouty proteins [76].

Expression of constitutively active SHP2 also resulted in reduced tight junction integrity and mislocalization of adaptor proteins required for Sertoli-Sertoli adhesion. One mechanism by which SHP2 may regulate junctional and adhesion proteins is via FAK. Recent studies have established the role of FAK as a regulator of BTB and the blood-brain barrier [32, 77]. RNA interference-mediated knockdown of FAK in Sertoli cells resulted in the disruption of tight junction and adherens junction integrity [32]. In Sertoli cells, FAK complexes with the tight junction protein occludin and the adaptor protein ZO-1 [78]. Down-regulation of FAK signaling results in disruption of interaction between ZO-1 and occludin [77]. Our studies indicate that SHP2 interacts with FAK and decreases phosphorylation at Tyr397 in Sertoli cells, which is associated with decreased FAK activity [79]. FAK phosphorylated at Tyr397 also is localized to specialized junctions between Sertoli and germ cells called ectoplasmic specializations (ES).
Thus, the SHP2 found to localize to Sertoli-elongated spermatid adhesion sites may regulate FAK at the ES. The decreased TER levels of cultured Sertoli cells observed after expression of SHP2 Q79R indicates that tight junction connections that contribute to the BTB in vivo are decreased after overactivation of SHP2. HGF stimulation of Sertoli cells, which we found increased SHP2 activity and elevated ERK phosphorylation to levels similar to that observed after SHP2 Q79R expression, also significantly decreased TER levels in cultured Sertoli cells. The HGF-mediated decrease in TER levels is consistent with a previous report showing that HGF modulates tight junction dynamics in Sertoli cells by influencing the levels, localization, and assembly of occludin at tight junctions as well as actin organization in the tight junction region [66]. Although these studies indicate that HGF can disrupt Sertoli cell tight junctions, further work will be required to determine why HGF is not as effective as SHP2 Q79R expression in decreasing tight junction integrity.

Constitutive activation of SHP2 in Sertoli cells caused the mislocalization of adherens and tight junction adaptor proteins. These results are consistent with previously published studies in which misregulation of SHP2 results in disruption of junctional complexes in endothelial cells and fibroblasts [28, 39, 81, 82]. SHP2-mediated disruption of Sertoli cells tight junction and adherens junction integrity could occur by multiple mechanisms initiated after altering the activities of the MAP kinase pathway and Src kinase. Overstimulation of Src and MAP kinase components such as Ras, Raf, and MEK results in disruption of adherens and tight junction integrity [37, 83, 84]. Altering the phosphorylation status of adhesion

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FIG. 5. Constitutively active SHP2 does not alter the expression of transmembrane, adaptor, or cytoskeletal proteins involved in cellular adhesion. Cultured Sertoli cells isolated from 20-day-old rats were infected with AdGFP or AdSHP2 Q79R adenoviruses, and 3 days later Western blots were performed using antisera against transmembrane proteins (A), adaptor proteins (B), or cytoskeletal proteins (C) as noted. Quantitation of the relative levels of expression for each protein in the presence of AdGFP or AdSHP2 Q79R is shown to the right. *Significant difference from the AdGFP control (P < 0.05, n = 4).
Ad SHP2 Q79R

FIG. 6. Constitutive activation of SHP2 results in mislocalization of ZO-1, β-catenin, and N-cadherin away from the plasma membrane and disruption of the actin cytoskeleton. Sertoli cells cultured from 20-day-old rats were infected with AdGFP or AdSHP2 Q79R adenovirus. Three days after infection, the cultures were fixed and subjected to immunofluorescence analysis (red staining) with antisera against ZO-1 (A and B), β-catenin (C and D), N-cadherin (E and F), or actin (β-actin; G and H). Arrows denote immunostaining at cell-cell contact points (A, C, and E) or actin filaments (G, I). Whole cell extracts from cultured Sertoli cells were immunoprecipitated (IP) with antisera against β-catenin and then immunoblotted with antisera against β-catenin or N-cadherin. Original magnification ×60 (A–H).

SHP2 acts upstream of Src, ERK, FAK, and Rho kinases, it is possible that all of these signaling pathways contribute to SHP2-mediated disruption of Sertoli cell junctions. However, more research is needed to determine the relative contribution of each pathway.

SHP2 Q79R also disrupted the Sertoli cell actin cytoskeleton and decreased polymerized actin filament formation. The disruption of the actin cytoskeleton removes the anchor required for maintaining the BTB and Sertoli-germ cell adhesion. Our findings are consistent with the observations made by other groups reporting that up-regulation of SHP2 activity in epithelial cells and fibroblasts results in fewer actin filaments [39, 81]. SHP2 is a well-established regulator of actin stress fibers via its ability to modulate activity of Rho kinases [81, 87]. Inhibition of SHP2 activity decreases RhoA activity and increases stress fiber formation [28, 81]. Thus, constitutively active SHP2 may disrupt the actin cytoskeleton in Sertoli cells by directly inhibiting Rho kinase. It is also possible that SHP2 may act via ERK kinase to regulate the actin cytoskeleton, as constitutive activation of K-ras and B-Raf via ERK also inhibits Rho activation, resulting in the disruption of actin cytoskeleton [88, 89].

Our studies indicate that SHP2 is a critical regulator of Sertoli cell junction complexes and the integrity of the BTB. Thus, maintaining the correct levels of SHP2 activity in Sertoli cells is essential for spermatogenesis and male fertility. Our observations also provide important information on the pathogenesis of male infertility in Noonan syndrome patients having constitutively active SHP2 mutations. Cryptorchidism was considered to be the main cause of male infertility in Noonan syndrome patients. However, recent studies have shown that Noonan syndrome patients with normal descent of testes have impaired Sertoli cell function [25]. It is likely that the descended testes of Noonan syndrome patients will have disrupted spermatogenesis due to the lack of BTB integrity, although additional effects of SHP2 overactivation may disrupt other Sertoli cell and germ cell functions.

In summary, SHP2 acts in Sertoli cells to regulate spermatogenesis and male fertility by controlling the localization of Sertoli-Sertoli adhesion proteins. SHP2 may also contribute to the regulation of Sertoli-germ cell adhesion. Further studies are required to determine whether SHP2 also acts in developing germ cells to maintain spermatogenesis.

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