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Short-type PB-cadherin promotes survival of gonocytes and activates JAK-STAT signalling

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Abstract

Neonatal development of the rat testis involves a number of critical events including re-entry of gonocytes into the cell cycle and eventual loss of many of these cells and their progeny via apoptosis. Since surviving gonocytes give rise to subsequent generations of germ cells, regulation of their fate is critical for adult testicular function. Here, we have identified a role for short-type PB-cadherin (STPB-C) in promoting survival of gonocytes in neonatal rats and we have linked its expression to the JAK-STAT signaling pathway. These findings were obtained with varied approaches including use of transgenic rats overexpressing STPB-C which were studied with protein microarrays and other techniques, direct examination of germ cell apoptosis and survival in gonocyte–Sertoli cell co-cultures, and direct study of the JAK-STAT pathway in these models and in L cells transfected with STPB-C. These data provide new information on the regulation of gonocyte fate and exciting new evidence supporting a link between the JAK-STAT pathway and cadherin-based cell–cell interactions.

Keywords: Neonatal gonocytes; STPB-C; Cadherin; Survival; JAK-STAT pathway

Introduction

Perinatal development of testes in male rodents is critical in establishing spermatogenesis and thus in insuring fertility of the adult animal. At birth, the germ cell population is comprised exclusively of gonocytes, some of which give rise to all subsequent generations of germ cells. Gonocytes re-enter the cell cycle on post-natal day 3 and assume their proper position at the basal lamina during the subsequent several days (McGuinness and Orth, 1992). The ensuing proliferative activity among the gonocytes and early spermatogonia is accompanied by a substantial level of apoptosis (Rodriguez et al., 1997), during which a critical degree of culling occurs to insure a germ cell population of appropriate size. Regulation of apoptosis among these germ cells is thought to involve regulation of various, well-recognized genes such as Bax and Bcl-2 variants (Knudson et al., 1995; Furuchi et al., 1996). However, it remains unclear how expression of these and other genes is modified so that gonocytes which are destined to give rise to spermatogonia avoid apoptosis and survive.

Survival of gonocytes is likely, at least in part, to depend on maintenance of proper associations between these cells and Sertoli cells. Many events that occur throughout development require critical germ cell–Sertoli cell interactions such as maintenance of physical attachment between the cells, metabolic support of germ cells by Sertoli cells, and expression of various factors by one or both cells (Griswold, 1995; Orth et al., 2000). In our earlier work, we identified a novel adhesion molecule in the testis, short-type PB-cadherin (STPB-C), and found that it has a specific spatial and temporal expression pattern (Wu et al., 2003). It is highly expressed by gonocytes and Sertoli cells of neonates, with STPB-C mRNA levels maximal during the first post-natal week and declining by day 10. Subsequently, STPB-C remains at low levels throughout adulthood. This pattern of expression suggests a potential role for this cadherin in post-natal development of the testis. Interestingly, unlike most cadherins, STPB-C lacks an intracellular catenin-binding

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domain, indicating that any associated signaling pathway does not involve interaction with cytoskeletal elements and that its function may extend beyond that of classical adhesion molecules. To date, no information beyond our previous findings is available regarding this unusual testicular cadherin and its potential function.

Our goals in the current study were first, to determine whether the level of STPB-C affects gonocyte numbers and, potentially, their survival in neonates and second, to identify some of the subcellular mechanisms involved in STPB-C function. To this end, we used varied approaches, including developing and studying transgenic rats overexpressing STPB-C, assessing the effect of its blockade in primary co-cultures from testes of newborn rats, and examining the effect of transfecting cadherin-negative L cells with STPB-C. Our findings support a role for this cadherin in suppressing gonocyte apoptosis, suggesting that STPB-C may be important in determining the fate of these cells. In addition, findings from other experiments identified a link between expression of STPB-C and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway. We identified JAK2 and/or SH2B as STPB-C binding partners and demonstrated that STPB-C induces phosphorylation of JAK2 and STAT3. Thus, STPB-C appears to promote survival of germ cells in neonates and acts via the JAK-STAT pathway. Linking STPB-C to the JAK-STAT pathway is a novel observation previously unreported for any member of the cadherin family.

Materials and methods

Constructs

A 2.1 kb fragment containing the total open reading frame of short-type PB-cadherin (STPB-C) and a Kozak sequence (Genscript Corp., Edison, NJ) was cloned into pCMV-script vector (Stratagene, La Jolla, CA) (pCMV-STPB-C) for transfection studies, or the fragment was cloned into the proprietary StealthGene vector for transgenesis (StealthGene-STPB-C; Tosk, Inc., Santa Cruz, CA).

Generation of STPB-C transgenic rats

All animal work was carried out under the National Institutes of Health guidelines and all animals were Sprague–Dawley rats (Charles River Breeding Labs, Kingston, RI). Mosaic founders of transgenic rats (Tgs) were produced by Tosk, Inc. (Santa Cruz, CA) using a proprietary process involving combining the STPB-C gene with the Tosk "StealthGene" vector. In general, mosaic founders were produced at the Tosk facility by injecting STPB-C-StealthGene vector into adult rats, where 20–40% of cells are randomly transformed with 1–3 copies of the transgene/cell, including some germ cells (per Tosk, Inc.). Since transposase is targeted for degradation, all StealthGene integrations are stabilized. Founder rats were mated to wild-type animals and heterozygotic transgenic progeny were identified by Tosk, Inc. via Southern blotting. These transgenic progeny were mated in our facility, where we characterized the offspring by confirming the presence and stability of the transgene, as follows. First, real-time PCR was carried out to identify Tg lines with different StealthGene vector copy numbers. Next, we used Southern blotting to confirm the resulting transgenic lines and to quantify copy number for transgenic STPB-C. Finally, Western analysis was used to confirm overproduction of STPB-C protein in the transgenics. These analyses are described below.

Real-time PCR

Genomic DNA was extracted from rat tails with the DNeasy Tissue kit according to the manufacturer's instructions (QIAGEN, Valencia, CA), and the real-time PCR procedure was carried out using a SmartCycler (Cepheid, Sunnyvale, CA). The primers used for amplification of the StealthGene vector were provided by Tosk, Inc. and were: sense, 5′-GTGGGAGCAGAGCCTTGGGTGCAG-3′; antisense, 5′-CTAGGTACGGCATCTGCGTTGAGTCG-3′. Real-time PCR was carried out in a total volume of 20 μl, each reaction containing 2 μl LightCycler-DNA Master SYBR Green 1 (Roche, Indianapolis, IN), 250 ng of template DNA, 4 mM $Mgcl_2$, and 0.5 μM of each primer. The standard curves generated by this procedure contained the equivalent of 0.0625–1 μg DNA input/tube. The real-time PCR program consisted of an initial hold at 95°C for 120 s to denature the genomic double-stranded DNA, followed by 40 cycles of 15 s at 95°C and 20 s at 69°C.

Southern blotting

Genomic DNA was isolated from rat tails as described above and digested with *Bcl*I and *Hin*cII. Samples were loaded onto 0.7% agarose gel along with molecular weight markers and run at 35 V for 10 h. These samples were then depurinated in 0.2 M HCl for 10 min, denatured in 0.5 M NaCl/0.5 M NaOH for 45 min, and neutralized in 1.5 M NaCl/1.0 M Tris–Cl (pH 7.4) for 15 min. After neutralization, DNAs were transferred by capillary action to a nylon membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). Hybridization was carried out with a probe homologous to exon 6 of the STPB-C gene. Prehybridization, hybridization, and subsequent washings were performed as described previously and copy numbers were quantified on resulting blots with densitometry (Wu et al., 2003).

Gonocyte–Sertoli cell co-cultures and anti-STPB-C treatment

Gonocytes–Sertoli cell co-cultures were prepared as previously described (Orth and Boehm, 1990). Briefly, Sertoli cells and gonocytes were isolated from testes of 2-

day-old pups (Sprague–Dawley rats) by sequential digestion with collagenase/hyaluronidase and collagenase. After incubating cell aggregates in cell dissociation buffer (Life Technologies, Inc., Gaithersburg, MD), the resulting single cell suspension was plated on growth-factor-depleted Matrigel (Collaborative Research, Waltham, MA) in either 35 mm plastic pertri dishes or eight-chamber culture slides (Lab-Tek-Nunc, Naperville, IL). Gonocyte–Sertoli cell co-cultures were maintained in hormone- and serum-free Eagle's D-valine MEM (Sigma, St Louis, MO) with supplements in a 5% CO_2 atmosphere at 37°C for up to 32 h. One day after plating, the co-cultures were exposed to anti-STPB-C (Wu et al., 2003) (5 ng/μl, Resgen, Huntsville, AL) or preimmune serum (negative control) in the presence of Chariot Reagent (Activemotif, Carlsbad, CA) to facilitate penetration of the antisera into the cells. Additional cultures were exposed to β-galactosidase under similar conditions as a positive control for cell penetration and to provide a measure of penetration efficiency (68%) after staining for the enzyme. For Western blot analysis, cells were scraped from the dishes at 15 min–32 h after treatment, rinsed and frozen, followed by isolation of protein as described below. For morphology, co-cultures in chamber culture slides (3 slides/group) were observed with phase-contrast optics 1 day post-treatment, and then fixed. For each slide, the number of gonocytes adherent to the confluent Sertoli cell monolayer was counted under 60× magnification in each of 30–50 contiguous visual fields, using an eyepiece grid and beginning in the upper left corner of each slide. The totals were expressed as the mean number of gonocytes/field and the Student's *t* test was applied to the data.

Antibody microarrays

We used antibody microarray analysis to identify potential candidate proteins that might be over- or under-expressed in Tg vs. wild-type rats. For this, testes obtained from transgenic rat pups (4 days old) or age-matched controls were decapsulated, snap-frozen in liquid nitrogen, and stored at −80°C until extraction of protein. Total protein from these testes was extracted and antibody microarray analysis was used with a BD Clontech Ab Microarray kit following the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA). Protein concentrations were determined using a BCA protein Assay Reagent Kit (Pierce, Rockford, IL). Protein was labeled using Cy3 and Cy5 mono-Reactive Dye (Amersham Biosciences, Piscataway, NJ) and purified away from free dye using PD-10 Desalting Columns (Amersham Biosciences, Piscataway, NJ). The microarray slides were scanned with a GenePix 4000 B instrument (Axon Instruments, Union City, CA) and subsequent data analysis was carried out according to the manufacturer's directions, as follows. GenePix Pro 4.0 microarray analysis software (Axon Instruments) was used to obtain Cy5/Cy3 fluorescent signal ratios for all coordinates and the Ab Microarray Analysis Workbook (BD Biosciences Clontech) was used to convert these data into Internally Normalized Ratios (INRs). For each coordinate, an INR (transgenic/control) greater than or equal to 2.0 was considered to represent increased expression, whereas a ratio less than or equal to 0.5 was considered to represent decreased expression.

Immunoprecipitation and Western blotting

Protein from testes or cells was extracted as described above and Western blotting or immunoprecipitation was performed as described previously (Orth et al., 1996), with some modifications. For Western blotting, membranes were exposed to anti-p-JAK2 (phosphorylated Janus activated kinase 2, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-JAK2 (1:200, Santa Cruz Biotechnology, Inc.), anti-p-STAT3 (phosphorylated Signal Transducer and Activator of Transcription 3, 1:100, Santa Cruz Biotechnology, Inc.), anti-STAT3 (1:1000, BD Bioscience Clontech), anti-STAT5 (1:200, BD Bioscience Clontech), anti-SH2-B (1:100, BD Bioscience Clontech), or anti-STPB-C (1:200, Resgen). For immunoprecipitation, protein samples were first pre-cleared in 1% normal rabbit serum for 1 h at 4°C followed by addition of protein A-agarose for an additional hour and centrifugation. Supernatant was then incubated with anti-STPB-C (Resgen) for 2 h at 4°C, followed by addition of protein A-agarose and overnight incubation. The immunoprecipitate was collected by centrifugation, washed with extraction buffer (BD Bioscience Clontech), resuspended in sample buffer, and separated by electrophoresis as described previously (Orth et al., 1996) for Western analysis. Membranes were immunodetected with appropriate antibodies to anti-STPB-C (1:200, Resgen, positive control), anti-SH2-B (src homology 2-B, 1:100, BD Bioscience Clontech), anti-JAK2 (1:200, Santa Cruz Biotechnology, Inc.), or pre-immune serum (negative control).

Morphological analysis and TUNEL reaction

For comparison of Tg ($n = 6$) and wild-type ($n = 9$) pups, body and testis weights were determined on post-natal day 4 by direct measurement. Testes from both groups ($n = 3$/group) were then fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and then cleared by routine methods. For morphological analysis, sections were stained in 1% Toluidine Blue in 1% Na Borate and seminiferous cord diameters and number of gonocytes/cord profile were determined as described previously (McGuinness and Orth, 1992). For the TUNEL reaction, additional sections were cut and subjected to proteinase K digestion (20 μg/ml, 15 min, RT), followed by incubation at 37°C for 60 min in a TdT (terminal deoxynucleotidyl transferase) cocktail containing 1 mM dATP (Sigma, St. Louis, MI), 0.25 units TdT/μl (Promega, Madison, WI), and 6 μM biotin-11-dUTP (Perkin-Elmer, Branchburg, NJ) in One-Phor-All Buffer (Amersham/Pharmacia, Piscataway, NJ). After washing,

endogenous peroxidase activity was suppressed by incubation in a suppression solution (Pierce Chemical, Rockford, IL) for 15 min at RT and positive nuclei were visualized with a Histostain-SP Kit using a red AEC chromogen, per the manufacturer's directions (Zymed Labs, San Francisco, CA). For each animal, 20 sections were examined, each section containing a minimum of 5 cross-sectioned cords (3–5 gonocytes/cord profile). Thus, 300–500 gonocytes were assessed in each rat for the presence or absence of reaction. Representative sections were photographed with a Leitz Orthoplan microscope equipped with a Magna-Fire digital camera (Optronics, Inc., Muskogee, OK).

Immunolocalization

Gonocyte–Sertoli cell co-cultures prepared on day 2 after birth were treated with either anti-STPB-C (5 ng/μl) or pre-immune serum in the presence of the Chariot reagent as already described and then fixed with 4% paraformaldehyde (15 min, RT) at 4, 10, or 21 h after treatment. Routine methods were used to immunolocalize Caspase-3 in chambers from both groups, as follows. After rinsing and then permeabilizing in ice-cold methanol, cultures were incubated in blocking solution (10% NGS in PBS, 60 min RT), followed by rinsing and overnight incubation in primary antibody at 4°C (Rabbit polyclonal Anti-Caspase-3, dil 1:100; Chemicon, Temecula, CA). After extensive rinsing, co-cultures were incubated in darkness with rhodamine-conjugated secondary antibody (Goat anti-rabbit IgG, dil 1:500, 1 h 4°C; Jackson Immunoresearch, West Grove, PA), then rinsed, mounted in DAPI-containing medium, and viewed and photographed with a Nikon inverted microscope equipped with a Magna-Fire digital camera (Optronics Inc., Muskogee, OK).

L cell culture and transfection

L cells were cultured at 37°C in a complete medium (Eagle's minimum essential medium with 2 mM L-glutamine and Earle's BSS, 0.1 mm non-essential amino acids, and 1.0 mm sodium pyruvate, 10% horse serum, 100u/ml penicillin, 100 μg/ml streptomycin) and transfected with pCMV-STPB-C or empty pCMV vector, using a calcium phosphate transfection kit (Invitrogen, Carlsbad, CA). Stably transfected cell lines were selected for resistance to neomycin (G418, 400 μg/ml, Invitrogen) for more than 20 days in vitro. Individual clones were screened by semi-quantitative RT-PCR and clones exhibiting high expression of STPB-C mRNA were used to assay for cell aggregation and for expression of JAK2, STAT3, p-JAK2, and p-STAT3.

Semiquantitative RT-PCR

Total RNA was isolated from cells transfected with either pCMV-STPB-C or empty vector (mock) using the Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen). cDNA was synthesized by reverse transcription of 1.5 μg total RNA using an oligo$(dT)_{12-18}$ primer (Invitrogen) with superscript II RNase H^- reverse transcriptase (Invitrogen). PCR was carried out in a volume of 50 μl containing 10 μl reverse transcriptase reaction mixture, 1× PCR buffer, 1.5 mM magnesium chloride, 200 μM of dNTP mix, 200 nM of each primer (50 nM of each primer for GAPDH), and 2 U Taq polymerase (Invitrogen). Primers were designed according to published sequences of the rat STPB-C mRNA (accession number D83349) or the rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA (accession number M17701). Primer sequences for STPB-C were: sense, 5′-TTACTGTGTTGGCCATGGAA-3′; antisense, 5′-GTTGCTGGGTTCCTCTGGTA-3′, yielding a product of 236 bp; PCR was performed using the following temperature cycle profile: 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 58°C, 42 s at 72°C, and 10 min at 72°C. PCR products were subjected to horizontal agarose gel electrophoresis. Amplification products were eluted, subcloned into pCR Blunt-TOPO (Invitrogen), and sequenced for verification. As a control, PCR was performed simultaneously for both GAPDH and STPB-C. Primers for GAPDH were: sense, 5′-CCCATCA-CCATCTTCCAGGAGC-3′, antisense, 5′-AGAGCAATG-CCAGCCCCAGC-3′, yielding a product of 692 bp.

Northern blotting

Total RNA was extracted as described above for semiquantitative RT-PCR. Samples (10 μg RNA/lane) were run on a 1% agarose-formaldehyde gel and transferred by capillary action to a nylon membrane (Amersham Pharmacia Biotech) in 20× SSC overnight. Prehybridization and hybridization were done as described above for Southern blotting, with reagents supplied in the kit used at recommended concentrations. The probe was a Kpn1–Sac1 fragment (1327 bp) containing STPB-C cDNA (1225 bp), produced as described previously (Wu et al., 2003).

Cell aggregation assay

The cell aggregation assay was performed five times as described by others (Takeichi, 1977) with modifications. A suspension of 5×10^5 L cells was incubated for 25 min at 37°C, with or without 1 mM $CaCl_2$ on a gyro-shaker at 80 rpm. After incubation, the number of aggregated particles in the cell suspension was measured, and the ratio of particles to initial cell number was calculated.

Statistical analysis

All experiments were repeated 3–5 times, findings quantified as appropriate to the experiment, and means determined for each sample group. For each experiment, the

Student's *t* test was used to identify significant differences between mean values obtained for control and treatment groups.

Results

Transgenic rats overexpress STPB-C

To study the role of STPB-C in male germ cell development, we produced transgenic rats overexpressing STPB-C (Tgs) by using a novel approach to generate mosaic rat founders carrying StealthGene-STPB-C in some germ cells. Tg offspring were identified as follows. First, the StealthGene vector was amplified with real-time PCR and two transgenic lines were identified, one with higher copy number than the other (Fig. 1A,b, pink and yellow arrows, respectively). Next, Southern analysis was carried out and the transgene identified in samples from each of the two Tg lines with a probe recognizing exon 6 of the STPB-C genomic sequence (Fig. 1B). Quantitative densitometric analysis indicated that these two transgenic lines have 3 (lane 1) and 5 (lane 2) copies of the transgene. Finally, Western blot analysis was performed

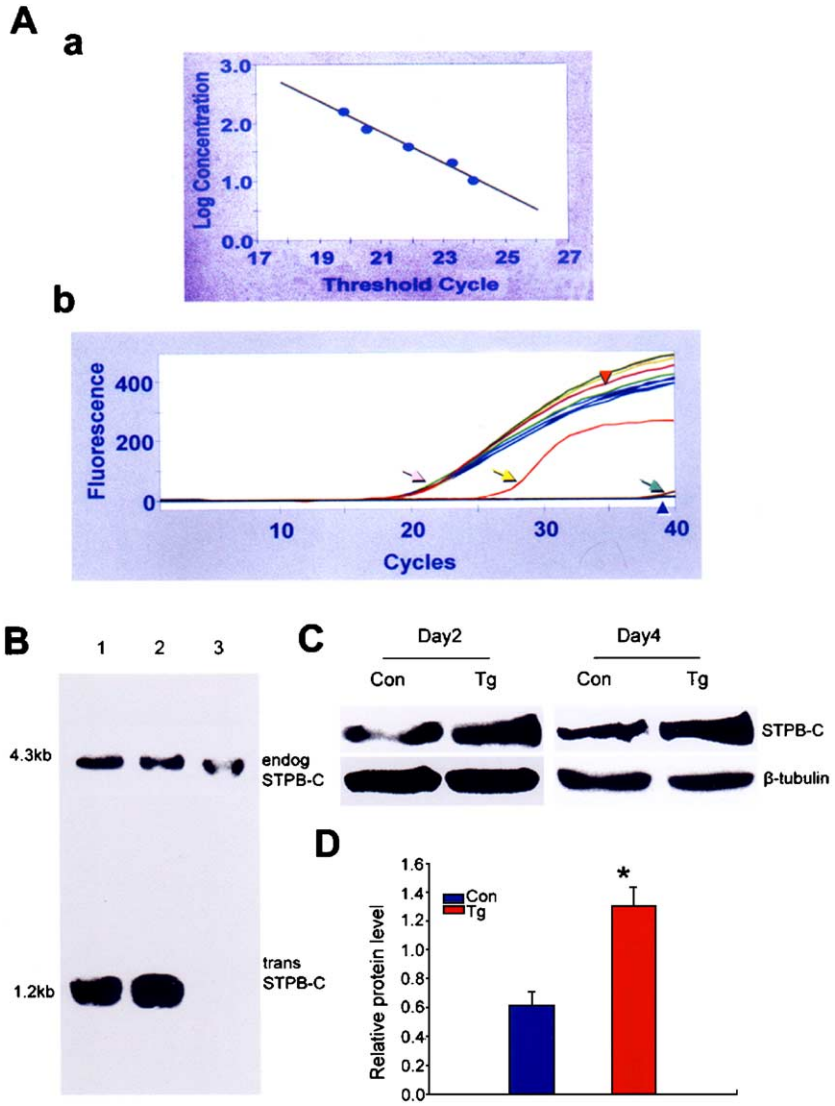

Fig. 1. Establishment of a transgenic rat model overexpressing STPB-C. (A) Real-time PCR data as used to identify STPB-C transgenic rats. (a) A typical standard curve generated by amplification, containing the equivalent of 0.0625–1 mg DNA input/tube. (b) Amplification profiles of genomic DNA from rat tails. Red arrowhead, positive control; blue arrowhead, negative control. Two lines of transgenic rats are indicated, line 1 by the pink arrow with apparently higher copy number, line 2 with apparently lower copy number by the yellow arrow (green arrow, negative rat). (B) Southern blotting of tail DNA with a probe recognizing exon 6 of the STPB-C genomic sequence. DNA was digested with *Bcl*I and *Hin*cII. Lanes 1 and 2: transgenic rats, lines 2 and 1, respectively; lane 3, negative rat. Densitometric analysis indicates that line 1 has 5 copies of the transgene [not shown]. (C) Representative Western analysis of testicular protein obtained from line 1 neonatal transgenic (Tg) or control rats (Con), to confirm overexpression of STPB-C in 2-day-old and 4-day-old transgenic pups. (D) Western blots from 5 separate experiments were quantified by densitometry and the data were expressed as a ratio of STPB-C:β-tubulin (means ± SEM). Line 1 Tg rats express approximately twice the normal amount of STPB-C. $*P < 0.05$.

to confirm overproduction of STPB-C protein in testes of neonatal Tgs on days 2 and 4 after birth (Figs. 1C,D). For all subsequent analyses, we used the Tg line with 5 copies of the transgene. Based on our earlier finding in normal rats that STPB-C expression is high in neonates and low in adults (Wu et al., 2003), we focused our initial analyses of Tgs on post-natal day 4 (Fig. 2). Testis weights in neonatal Tgs tended to be higher than in controls, but this difference was not significant, nor were seminiferous cord diameters altered. However, the number of gonocytes per cross-sectioned seminiferous cord in Tgs was more than two times that seen in controls ($P < 0.001$), suggesting that overexpression of STPB-C may increase production and/or survival of gonocytes. In a preliminary analysis of *adult* transgenic rats, Tg rats were mated and the outcome compared to that from matings between wild-type rats. Both litter frequency and litter sizes were similar in the two groups. In addition, routine examination indicated that testes of adult Tgs were generally normal in morphology, with all stages of the spermatogenic cycle represented and abundant mature spermatids present in the lumens of many tubules. Finally, when serum FSH, LH and triiodothyronine were measured and compared between the groups, no significant differences were identified (data not shown).

STPB-C promotes survival of gonocytes in vivo and in vitro

To determine if our finding of higher numbers of gonocytes in Tg pups reflects enhanced survival of these cells, the TUNEL reaction was used to compare gonocyte apoptosis in vivo between 4-day-old control and Tg rats (Fig 3A). In normal animals, occasional TUNEL-positive gonocytes were routinely encountered within the seminiferous cords in virtually all of the sections examined. In Tgs, however, no TUNEL-positive cells were seen in any of the 20 sections examined for each of three pups, suggesting that overexpression of STPB-C may suppress gonocyte apoptosis. To examine this possibility directly, we tested the effect of STPB-C antiserum on normal gonocytes co-cultured under standard conditions in vitro (Fig. 3B). Gonocyte–Sertoli cell co-cultures were treated with either anti-STPB-C or pre-immune serum in the presence of the Chariot reagent to facilitate entry of the antiserum into the cells. After 24 h, it was obvious upon visual inspection of cultures exposed to STPB-C anti-

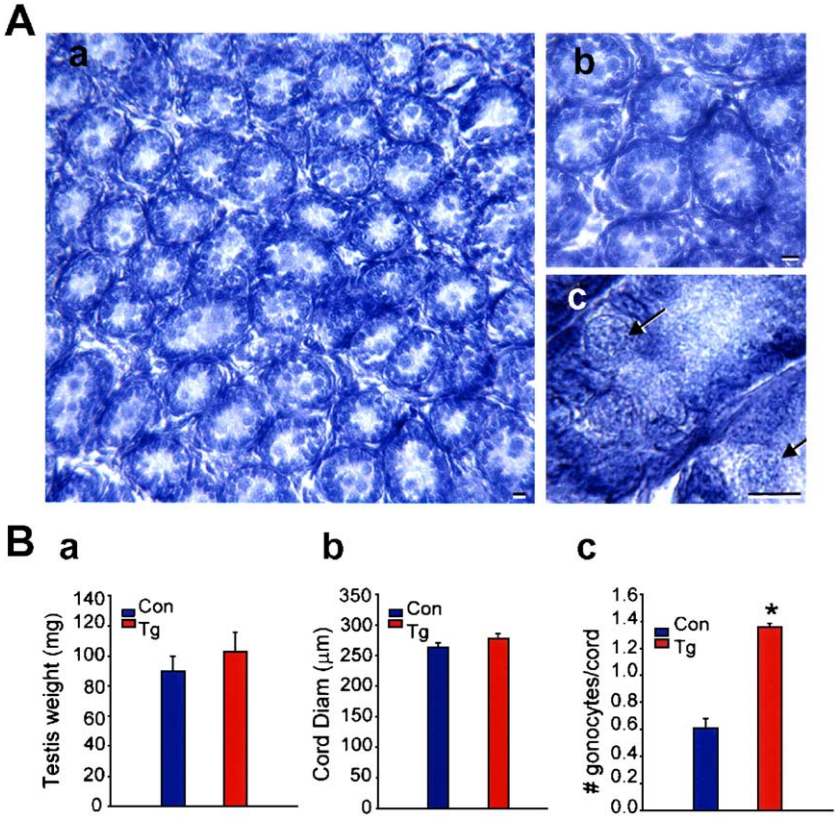

Fig. 2. (A) Representative morphology of testes from 4-day-old transgenic rats, demonstrating that the overall morphology of testicular cords is normal with many gonocytes present (a and b). As would be expected on day 4, gonocytes moving towards or already in contact with the basement membrane are common; two examples are indicated by arrows at higher magnification in panel c. Scale bar = 20 μm. (B) Morphometric analysis of transgenic and control testes. Testis weights (a) and cord diameters (b) were similar in transgenics and controls. However, significantly higher numbers of gonocytes/cord (c) were quantified in transgenics (Tg) compared to controls (Con). *$P < 0.001$.

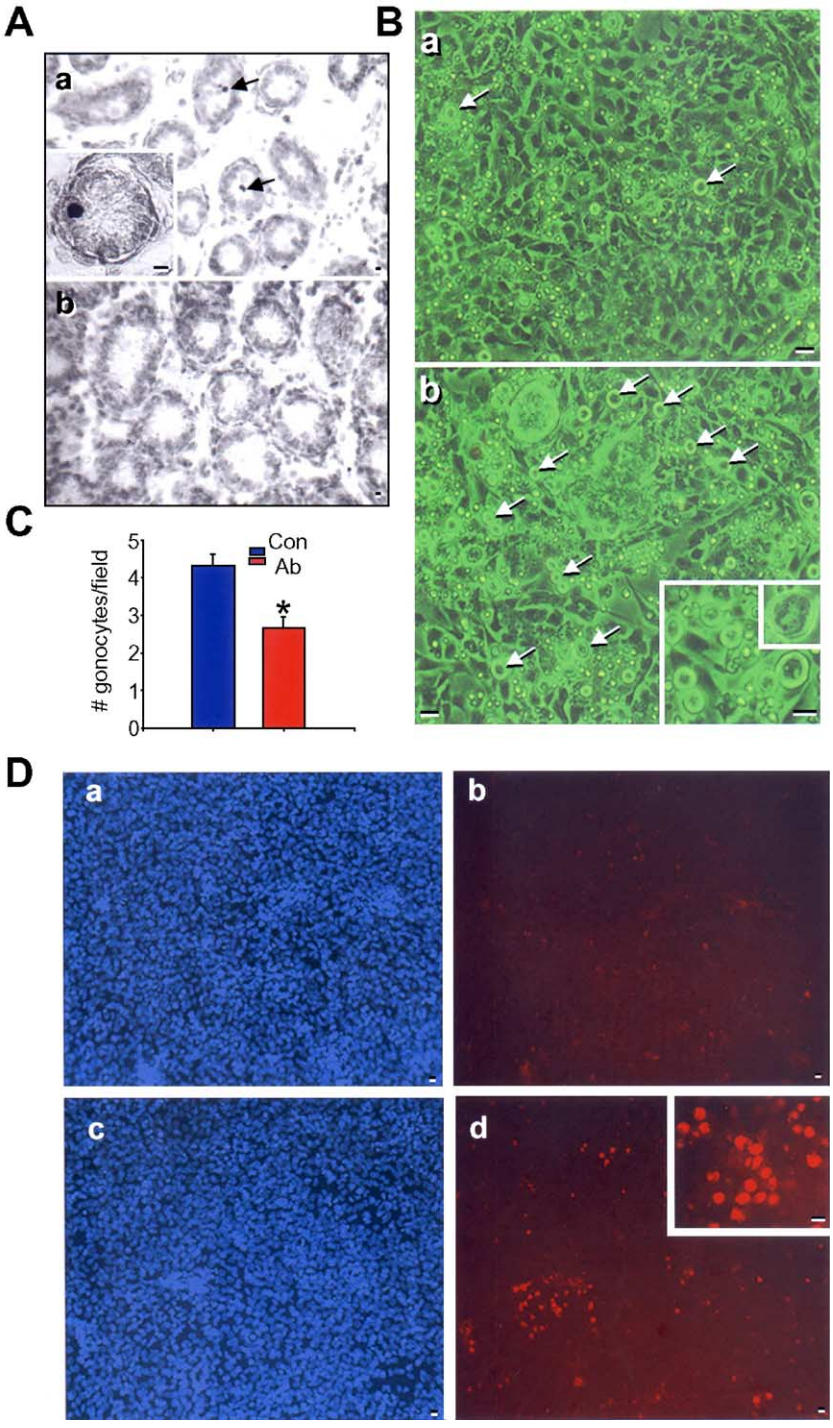

Fig. 3. The role of STPB-C in gonocyte apoptosis in vivo and in vitro. (A) Representative views 4-day-old testis sections from Tg pups and control pups subjected to the TUNEL reaction. Occasional positive gonocytes were seen in controls (a), while no apoptotic cells were detected within the seminiferous cords of any of the sections examined from Tg pups (b). (B) The effect of anti-STPB-C treatment on gonocyte survival in vitro. Gonocyte–Sertoli cell co-cultures from normal 4-day-old pups were exposed for 24 h to either pre-immune serum (a) or STPB-C antiserum (b), both in the presence of the Chariot reagent to facilitate entry of antiserum into the living cells. Examples of gonocytes either rounding up or floating are indicated by arrows. Note the obviously higher number of detaching cells in the antiserum-treated culture. Small inset: Gonocyte showing typical nuclear morphology with several nucleoli. (C) The number of gonocytes remaining adherent was quantified and compared between anti-STPB-C-treated and control co-cultures 24 h after start of the experiment. Approximately twice as many gonocytes remained in control vs. treated cultures (mean ± SEM, $n = 3$ experiments; $^*P < 0.02$). (D) The effect of anti-STPB-C treatment on expression of caspase-3 in co-cultured gonocytes. Cultures were exposed to either pre-immune serum (a,b) or to anti-STPB-C (c,d) on the day after plating and then fixed 10 h later. (a,c) DAPI immunofluorescence. (b,d) Caspase-3 immunofluorescence. Note the obvious increase in caspase-3 positive cells in anti-STPB-C treated cultures. Judged by their size and position, most if not all of the immunopositive apoptotic cells in these cultures are gonocytes. Scale bar = 20 μm.

serum that many gonocytes had detached from the monolayer and were floating (Fig. 3B,a) while only a few floating gonocytes were seen in controls (Fig. 3B,b). After rinsing all chambers, we next quantified and compared the number of adherent gonocytes in antiserum-treated and control cultures (Fig. 3C). We found that significantly fewer gonocytes were present in the anti-STPB-C treated cultures compared to controls ($P < 0.02$). Finally, apoptosis of gonocytes exposed to anti-STPB-C or to pre-immune serum was evaluated directly by immunolocalizing caspase-3 at 4, 10 or 21 h after start of treatment (Fig. 3D). In the co-cultures treated with anti-STPB-C at each of the tested times, we detected many caspase-3-positive cells either singly or in clusters (panels a,b), while few positive cells occurred in any of the controls (panels c,d). Based on previously documented attributes of gonocytes (Orth and Boehm, 1990) such as nuclear size and adhesion to the underlying monolayer, most if not all of these apparent apoptotic cells were judged to be gonocytes. Importantly, anti-STPC-C treatment appeared to leave the underlying Sertoli cell monolayer unaffected, as judged by the persistence of confluency and normal cellular morphology in treated cultures compared to controls (panel a vs. panel c).

Protein microarray analysis of Tg and control rats

To provide prelimary information on proteins whose expression is potentially linked to STPB-C function, we used an antibody microarray to compare profiles of proteins between testes of day 4 old control and Tg rats. Quantitative analysis of protein expression in the microarrays and comparison of levels between controls and Tgs, as described in Materials and methods, indicated a total of 40 proteins that show significantly increased expression in testes of Tgs. These proteins are listed in Table 1. Among the overexpressed proteins in the Tgs, SH2-B and STAT-3 were of particular interest. Their increased level of expression was subsequently confirmed by Western analysis and densitometric quantification (see Figs. 4B,C). Since SH2-B$^{\beta}$ binds to and stimulates the kinase activity of JAK2 and increases tyrosine phosphorylation of STAT3 and STAT5B (Rui et al., 1997; Rui and Carter-Su, 1999), our results raised the novel possibility that STPB-C may activate the JAK-STAT signaling pathway in neonatal testes. Subsequent experiments were therefore aimed at testing this possibility.

STPB-C binds to and activates JAK2 and/or SH2-B, up-regulating STAT3

To determine whether STPB-C activates the JAK-STAT pathway, we first used immunoprecipitation to clarify whether SH2-B or JAK2 is an STPB-C binding partner. Proteins isolated from Tg rat testes were immunoprecipitated with STPB-C and immunoblotted with anti-SH2-B or anti-JAK2. Each blot was then stripped and re-probed with anti-STPB-C to confirm the presence of STPB-C. As shown in Fig. 4A, we found that both SH2-B and JAK2 co-precipitate with STPB-C, indicating that at least one of these JAK-STAT pathway components is a binding partner for STPB-C. Next, to determine whether other JAK-STAT components are also overexpressed in Tgs, we compared expression of STAT5 and JAK2 by immunoblotting protein from control and Tg rats with anti-STAT5 and anti-JAK2 and using densitometry to quantify differences between the samples. Fig. 4B shows the Western blots and Fig. 4C the quantitative data from this analysis. With the latter, we detected a significant increase in JAK2 expression in Tgs compared to controls ($P < 0.01$), while STAT5 was equal in the two groups. Thus, our findings from both immunoprecipitation analysis and immunoblotting suggest that STPB-C binds to JAK2 and/or SH2-B, enhanc-

Table 1
Expression of proteins in testicular samples of 4-day-old transgenic rats compared to controls

Name	Swiss prot accession #	Fold increase over controls
PI-3 kinase (p110a)	P42336	2.17
Nestin	P48681	2.01
Neuroglycan C	O95196	2.20
HAX-1	O00165	2.07
c-Myc	P01106	2.74
CD3z	P20963	2.28
PKC lambda	n/a	2.01
Endothelin receptor	P25101	2.15
Synaptophysin	P08247	2.19
Stat 3	P40763	3.07
Ikka/1	O15111	2.79
Calretinim	P22676	2.07
CDC27	P30260	2.17
CrmA	P07385	2.05
Bog	O75884	3.46
TLS	P35637	2.16
NFAT-1	Q13469	2.01
PU.1 (Spi-1)	P17947	2.08
PKArIIb	P31323	2.06
Gap1m	Q15283	2.35
Amphiphysin	P49418	2.07
FBF	Q12828	2.34
Fnk	Q9H4B4	2.79
MUPPI	O75970	2.46
Serotonin receptor (5-HT2AR)	P28223	2.00
Cytochrome *c*/Apaf-2	P00001	2.34
SH2-B	Q9NRF1	2.08
DDX1	Q92499	2.84
TFII-I/BAP-135	Q15359	2.05
P140mDia	O60610	4.16
Glucocorticoid receptor	P04150	2.08
Nup88	Q99567	3.15
E2F2	Q14209	2.46
53BP2	Q13625	3.55
XPA	P23035	2.67
M33	P30658	2.89
PCNA	P12004	2.26
SCP3	P70281	2.39
Apo E	P02649	3.04
CLIP-115	Q9UDT6	2.02

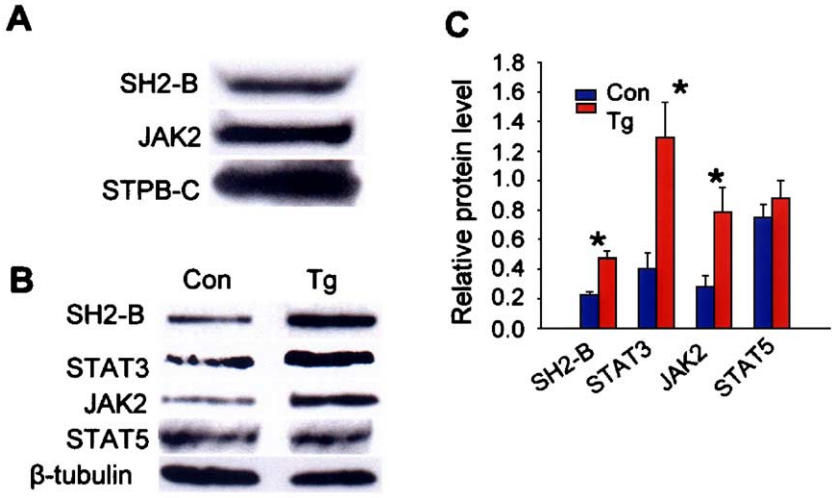

Fig. 4. STPB-C activates the JAK-STAT signaling pathway. (A) Protein obtained from testes of TG rats was immunoprecipitated with STPB-C and then probed with SH2-B (top panel) or JAK2 (middle panel). Subsequently, blots were stripped and re-probed with anti-STPB-C to confirm the presence of STPB-C in the immunoprecipitate (e.g., lower panel). (B) Western analysis of SH2-B, STAT3, JAK2, or STAT5 in protein isolated from testes of day 4 transgenic rats (Tg) or controls (Con). (C) Western blots in panel B were quantified by densitometry and the data were expressed as a ratio of each protein:β-tubulin. This analysis confirmed overexpression of SH2-B in Tgs compared to controls. In addition, STAT3 and JAK2, but not STAT5, were significantly elevated in Tg rats compared to controls (*$P < 0.01$).

ing their activity and subsequently activating STAT3, but not STAT5.

STPB-C activates the JAK-STAT pathway via phosphorylation of JAK2 and STAT3

We next used gonocyte–Sertoli cell co-cultures to examine the time course of changes in expression of JAK-STAT components following anti-STPB-C treatment for increasing times (15 min–32 h). As part of this analysis, we also determined whether STPB-C antiserum caused changes in levels of *phosphorylated* JAK-2 and/or STAT3 (Fig. 5). Densitometric data were obtained and results expressed as a ratio of signal for treated cells vs. controls (Fig. 5B). By 30 min post-treatment, p-JAK2 was significantly suppressed and remained decreased through 1 h, returning to normal by

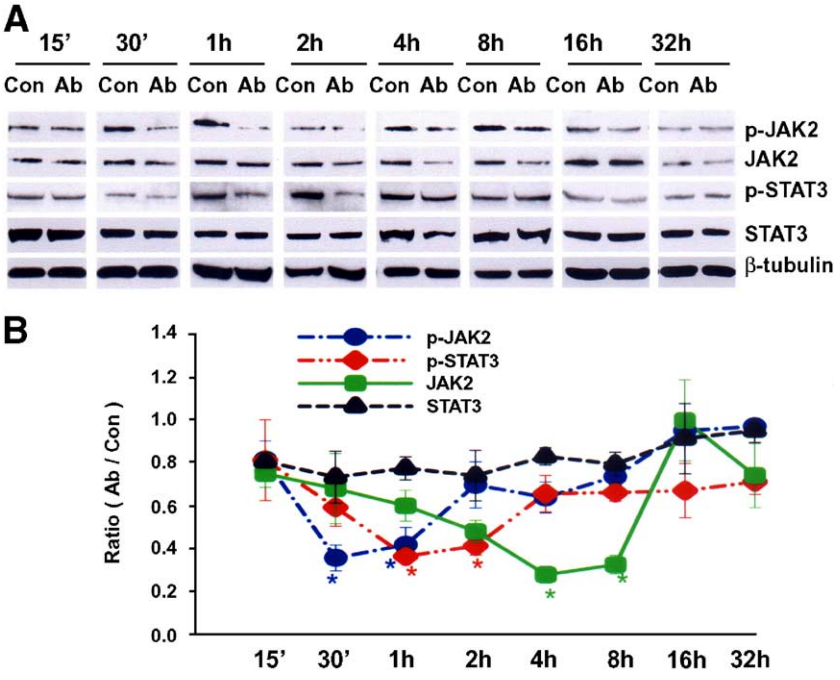

Fig. 5. The time course of changes in JAK2 and STAT3 and in their phosphorylation following anti-STPB-C treatment of co-cultures. (A) Representative Western analysis of p-JAK2, JAK2, p-STAT3, or STAT3 in protein isolated from co-cultures 15 min–32 h after exposure to anti-STPB-C (Ab) or pre-immune serum (Con). (B) Western blots from each of five replicate sets of cultures were quantified, and the results were expressed as a ratio of anti-STPB-C (Ab):pre-immune serum (Con). Asterisk: $P < 0.05$ (mean ± SEM of five experiments).

2 h. Anti-STPB-C treatment also suppressed p-STAT3, albeit somewhat later at 1 h post-treatment, followed by a return to normal by 4 h through 32 h post-treatment. Thus, the decrease in phosphorylation of JAK-2 induced by anti-STPB-C preceded that of STAT3 by about 30 min and also recovered somewhat more rapidly. In addition, the *total* amount of JAK2 was significantly decreased by antiserum treatment at 4 h post-treatment, while no significant change was detected in the total pool of STAT3 in the treated cultures. Thus, blockade of STPB-C in co-cultures suppresses overall JAK2 expression, consistent with our findings from Tg rats where increased STPB-C results in enhanced JAK2 expression. In addition, direct blockade of STPB-C in vitro results in reduced phosphorylation of both JAK2 and STAT3 in a temporal pattern consistent with the known sequence of these pathway components (Aaronson and Horvath, 2002).

Conversely, to establish that expression of STPB-C is sufficient to *up-regulate* the JAK-STAT pathway under conditions which alter cell function, we transfected L cells which lack endogenous cadherins (Sugimoto et al., 1996) with a construct containing STPB-C cDNA (pCMV-STPB-C). Stable transformants were isolated by screening with G418 and expression of STPB-C was examined by semi-quantitative RT-PCR (Fig. 6). Clones exhibiting high expression of STPB-C mRNA were used for further experiments. Individual transfected cells appeared morphologically similar to parental cells and Northern and Western blot analyses showed a band of predicted size in the cells transfected with the STPB-C expression vector (Fig. 7), indicating expression of apparently normal STPB-C by the transfected cultures. Next, to determine whether transfection with STPB-C produced functional, Ca^{2+}-dependent cell–cell adhesion in the transfected cultures, a cell aggregation assay was performed. STPB-C-transfected, mock-transfected (plasmid only), or parental monolayers were trypsinized to provide single cells and the resulting cells were incubated ±1 mM $CaCl_2$ to allow the potential formation of cellular aggregates. We found that only the STPB-C-transfected cells formed aggregates, and only if Ca^{2+} was present in the incubation media (Fig. 8). Aggregates were of various size, containing from 2 or 3 cells to large numbers of cells. No aggregates were seen in any other incubations, including that with transfected cells in the absence of Ca^{2+}. Thus, transfection of L cells with STPB-C induced functional cadherin-mediated adhesion.

Finally, we determined whether STPB-C transfection of L cells produced up-regulation of JAK-STAT components, parallel to the down-regulation we had observed in anti-STPB-C treated testicular co-cultures. For this, we compared both total expression and phosphorylation levels of JAK2 and STAT3 in STPB-C-transfected and mock-transfected L cells (Figs. 7C,D). We found that phosphorylation of both JAK2 and STAT3 were substantially increased ($P < 0.001$) in transfected cells compared

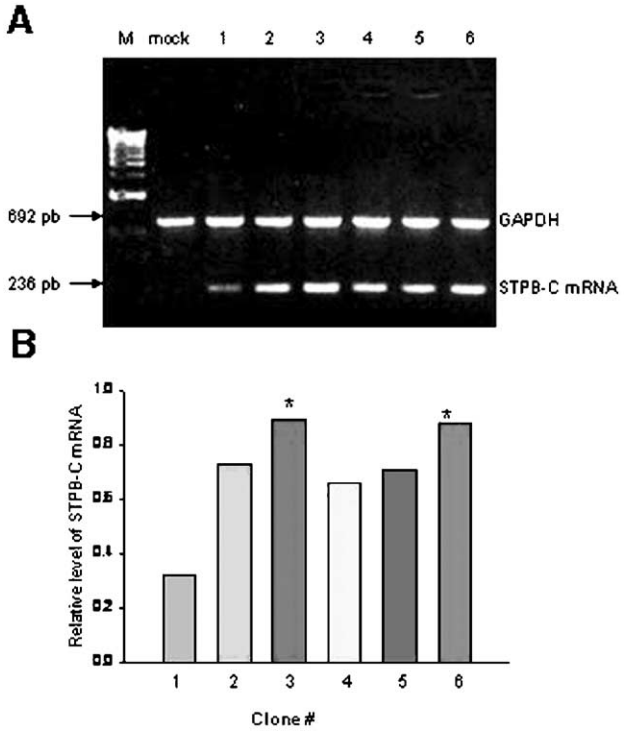

Fig. 6. An example of semi-quantitative RT-PCR analysis of transfected L cells. (A) RT-PCR of mRNA from cells transfected with either pCMV-STPB-C or empty vector. The expression of GAPDH was used as an internal standard. M, 1 kb DNA ladder; Mock, empty vector; lanes 1–6, samples from individual clones transfected with STPB-C. (B) RT-PCR was semi-quantified by densitometry and the densitometric data were expressed as a ratio of STPB-C:GAPDH for each sample. Clones exhibiting high expression of STPB-C mRNA (e.g., *) were used for further experiments.

to controls. In addition, there was also an increase in total JAK2 ($P < 0.005$) in the transfected cultures, results paralleling exactly our findings in anti-STPB-C treated co-cultures. Taken together, our observations on transfected L cells, along with our findings from transgenic rats and from anti-STPB-C treated co-cultures, indicate that STPB-C activates the JAK-STAT signaling pathway by inducing phosphorylation of JAK2 and STAT3.

Discussion

In adult rodents, the number of differentiating spermatogonia available to enter spermatogenesis is controlled by regulation of both proliferation and cell death of their ancestors, the self-renewing Type A spermatogonia (Sharpe, 1994). Continuous availability of these spermatogonia is responsible for lifelong fertility of males. In this study, we examined a rare cadherin, STPB-C, previously found by us to be highly expressed in neonatal testes (Wu et al., 2003), asking whether it has a role in promoting survival of gonocytes which are the progenitors in neonates of the first generations of spermatogonia that develop at puberty. First, we found that overexpression of this factor in transgenic rats

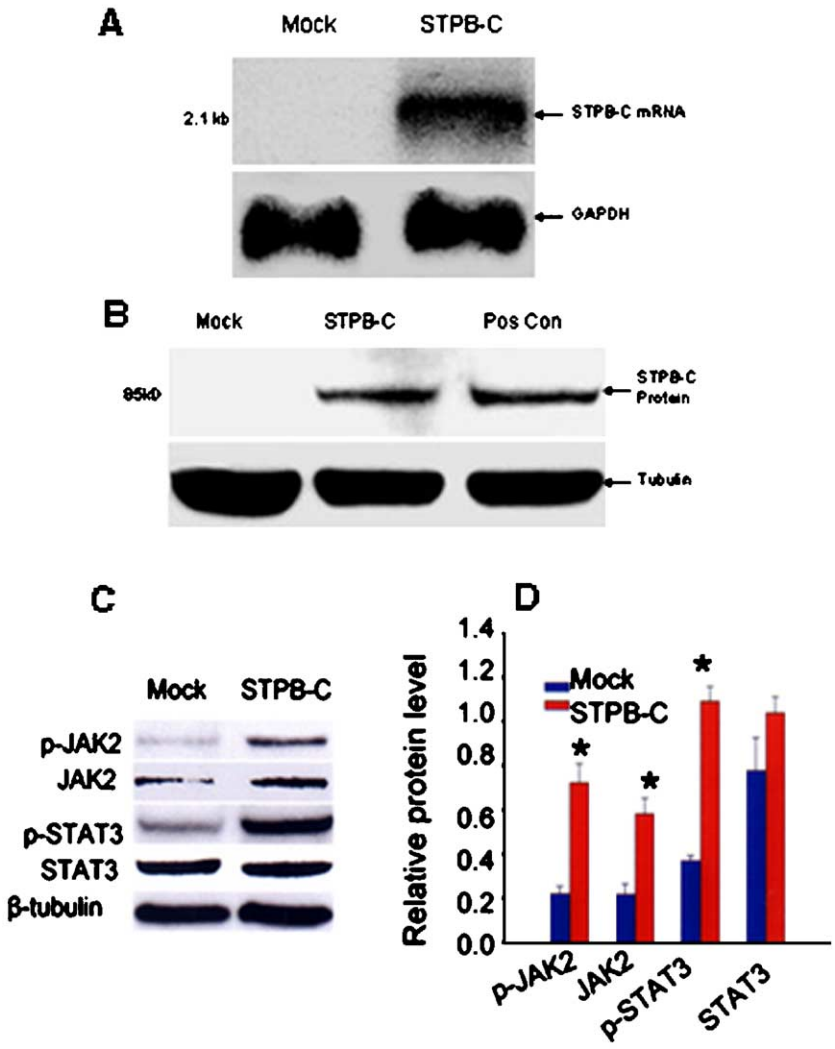

Fig. 7. (A) Northern analysis of STPB-C mRNA and (B) Western analysis of STPB-C protein in stably transfected L cells, confirming their expression of STPB-C compared to mock-transfected cells. (C) Western analysis of p-JAK2, JAK2, p-STAT3, or STAT3 in samples obtained from mock-transfected or STPB-C transfected L cells. (D) Western blots in panel C were quantified by densitometry and the data were expressed as a ratio of protein:β-tubulin for each. Expression of p-JAK2 (*$P > 0.001$), total JAK2 (*$P > 0.005$), and p-STAT3 (*$P > 0.001$) was significantly elevated in transfected L cells compared to mock-transfected controls.

resulted in abnormally high numbers of gonocytes in neonates. Although we cannot as yet rule out a prenatal effect of high STPB-C in these transgenics, perhaps influencing germ cell proliferation in fetuses, we did find that STPB-C overexpression was accompanied by an apparent absence of post-natal apoptosis in this population. Thus, it is likely that reduced apoptosis is at least partly responsible for the elevated numbers of gonocytes in transgenic pups. This likelihood is also supported by our observation that blockade of endogenous STPB-C in testicular co-cultures prepared from normal neonates resulted in a substantial increase in caspase-3 positive gonocytes compared to controls. With longer incubation in STPB-C antiserum, we also observed detachment of large numbers of gonocytes from the underlying Sertoli cells. Thus, expression of STPB-C in neonates appears to support gonocyte survival by suppressing apoptosis.

Formation of a germ cell population of appropriate size during testicular development is critical in establishing spermatogenesis and insuring fertility at adulthood, and this seems to be partly determined by an early and large wave of germ cell apoptosis (Rodriguez et al., 1997). For example, mis-expression of the death repressor gene Bcl-2 in 2-week-old mice leads to abnormally high numbers of spermatogonia and results in disrupted spermatogenesis (Furuchi et al., 1996), while knocking out the Bax gene produces a similar disruption of spermatogenesis and infertility in adult mice (Knudson et al., 1995). Both lines of evidence highlight the need for apoptosis to reduce the numbers of early spermatogenic cells to appropriate levels. However, to date, essentially nothing is known about the mechanisms whereby some gonocytes avoid apoptosis and survive to become founders of the spermatogonial population. Our current data suggest that at least one requirement for gonocytes to

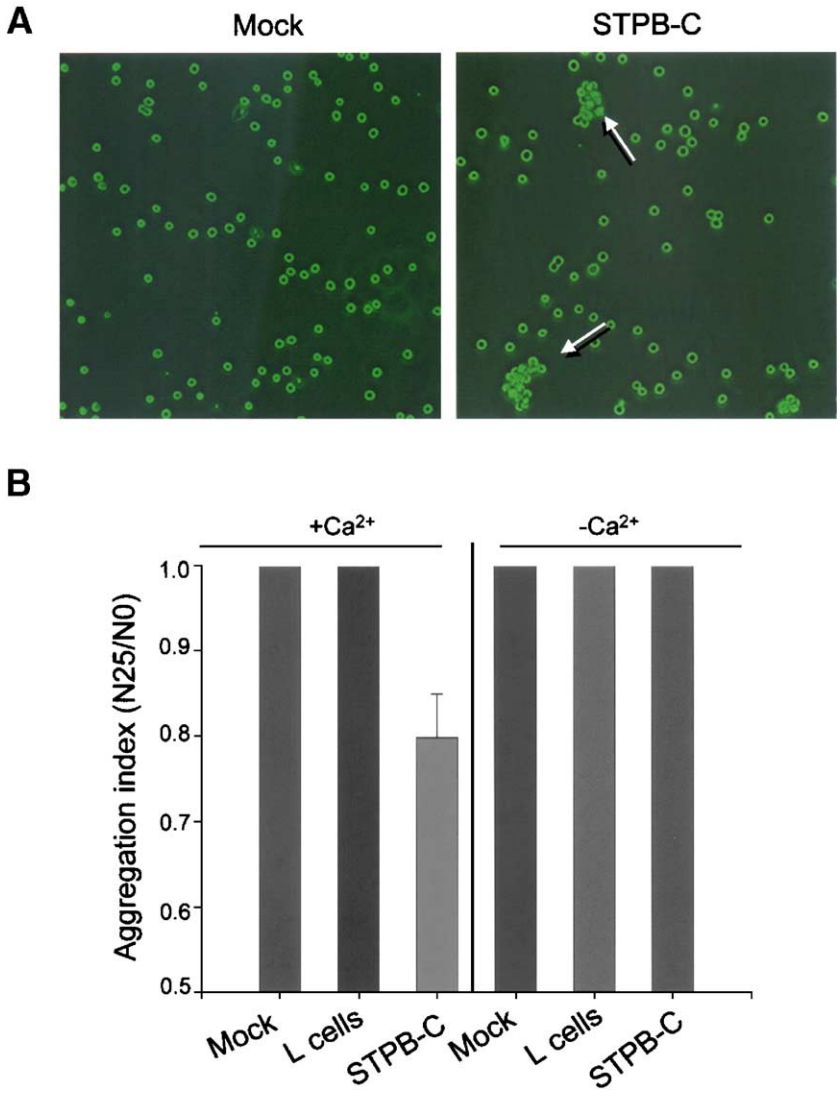

Fig. 8. Cell aggregation assay of STPB-C transfected L cells. (A) Representative view of a cell aggregation assay performed on mock-transfected (left) and STPB-C transfected (right) cells. Both small and large cellular aggregates formed only in the STPB-C transfected cells in the presence of Ca^{2+}. Arrows indicate examples of large cell aggregates. (B) Quantification and Ca^{2+}-dependence of cell aggregation in STPB-C transfected L cells vs. mock-transfected or parental L cells. Equal numbers of cells were added to each incubation at the start of the experiment. Aggregation index (N25/N0) is the ratio of the total number of particles in the cell suspension after 25 min incubation (N25) to the number of particles at the start of the incubation (N0), with or without 1 mM $CaCl_2$.

survive is their ability to express STPB-C, which allows some of these cells to avoid apoptosis while others expressing little or no STPB-C are destined to die.

Although we have not yet fully evaluated the adult testis in transgenic rats overexpressing STPB-C, our preliminary data indicate that these males are fertile and that they have testes of overall normal morphology (data not shown). This is an interesting observation considering our finding that abnormally high numbers of gonocytes were present in neonatal transgenics. Since a complete analysis of the transgenic rats throughout further development and puberty was beyond the scope of the present work, at this time, we can only speculate on the basis for normal fertility in these animals as adults. Little is known about mechanisms that determine survival of germ cells as the testis develops beyond the neonatal period. However, it is clear from other models that long term suppression of germ cell apoptosis is detrimental to fertility. For example, in the Bax knockout mouse, suppression of germ cell apoptosis leads to lifelong interference with the normal occurrence of germ cell apoptosis, producing enormous numbers of spermatogonia in young animals, followed by structural abnormalities in the seminiferous tubules and subsequent infertility in adults (Knudson et al., 1995). However, although STPB-C overexpression also seems to suppress apoptosis, the mechanism whereby this happens is doubtless quite different than that following direct suppression of a “death” gene such as Bax since STPB-C is a cadherin most likely involved in cellular interactions. In addition, our earlier work (Wu et al., 2003) demonstrated that STPB-C expression is highest during perinatal development, dropping significantly to very low levels in adults. Thus, overexpression of STPB-C

in transgenics is likely to have a significant effect primarily in neonates, with subsequent overexpression of potentially less consequence later in life when the protein has less significance. In addition, other as yet unknown mechanisms may operate during prepubertal development to adjust germ cell numbers in these animals. Additional studies are planned with the transgenic model, including a detailed morphometric analysis of cell numbers in seminiferous tubules of adults, and the outcome of these and other studies will clarify in more detail the impact of STPB-C overexpression both later development and in adults.

Our observations on STPB-C also provide additional new evidence highlighting the critical relationship between Sertoli cells and germ cells in neonatal development. STPB-C is expressed in both cell types (Wu et al., 2003) and blockade of its expression in co-cultured cells results in up-regulation of caspase-3 in gonocytes, followed by eventual detachment of gonocytes from Sertoli cells with no apparent changes in the somatic cells. Thus, expression of STPB-C is required for normal adhesion and functional interaction between these cell types in neonates. While alteration in STPB-C expression appears to affect gonocytes without obvious changes in Sertoli cells, our data on signaling events triggered by STPB-C, discussed below, were obtained from testicular homogenates. This leaves open the possibility that STPB-C expression may also be critical for neonatal Sertoli cell function beyond maintaining their adhesion with germ cells, a possibility we will explore in future studies.

Our findings demonstrate, for the first time, a functional link between cadherin-mediated cell behavior and the JAK-STAT pathway. This was first suggested by the outcome of protein microarrays performed on normal and transgenic rats. Since our previous data indicated that Thyroid Hormone is not involved in STPB-C function (Wu et al., 2003) and that gonadotropin levels are normal in transgenics (data not shown), we sought clues to regulatory pathways for STPB-C by using a microarray. The resulting suggestion that JAK-STAT components are involved in STPB-C action was later confirmed by several approaches, revealing up-regulation of JAK2, STAT3, and SH2-B in transgenics and down-regulation of these components in cultured cells exposed to anti-STPB-C. In addition, our studies on L cells transfected with STPB-C confirmed that ectopic expression of this protein is associated with increased JAK-STAT activity and is also accompanied by cell–cell adhesion that is consistent with cadherin-based function. Taken together, these observations provide strong evidence supporting the concept that STPB-C activates this important pathway.

In mammals, the JAK-STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors and STAT3 has been shown to have a central role in suppressing apoptosis. For example, normal T cells that no longer express STAT3 are insensitive to the anti-apoptotic effects of IL-6 (Takeda et al., 1998) and the constitutively-expressed form of STAT3 (STAT3-C) increases resistance to apoptotic challenges (Shen et al., 2001). Moreover, in many cancers including lymphomas, leukemias, multiple myeloma, breast, and others, STAT3 has been found to be persistently activated (Bowman et al., 2000). Thus, our observations of up-regulation of STAT3 in transgenics and in transfected L cells and its down-regulation in antibody treated cells, with no changes in STAT5, are consistent with a similar role for STAT3 in regulation of apoptosis in testicular gonocytes. Similarly, our finding of increased SH2-B in transgenic rats is also consistent with current understanding of this signaling molecule. SH2-B mediates the effects of a number of factors that regulate cell survival and proliferation, including growth hormone, insulin, insulin-like growth factor, nerve growth factor, and platelet-derived growth factor (PDGF). Interestingly, PDGF has been identified as a regulator of gonocyte proliferation in neonatal rats (Li et al., 1997), raising the possibility that it may also activate the JAK-STAT pathway in exerting this effect, a possibility that remains to be explored.

In summary, we have identified STPB-C as an important factor determining the fate of gonocytes in neonatal rats. Its expression in neonates may allow these cells to avoid apoptosis and may also have a role in maintaining proper adhesion with Sertoli cells. We have also demonstrated that STPB-C activates the JAK-STAT pathway by inducing phosphorylation of JAK2 and subsequently of STAT3, implying that STAT3 may prevent apoptosis in these cells as it does in many other systems (Shen et al., 2001; Catlett-Falcone et al., 1999). Thus, our observations identify a new and potentially exciting link among cadherin-mediated cell–cell adhesion, the JAK-STAT pathway and gonocyte apoptosis, indicating that this pathway is operational in the neonatal testis where regulation of germ cell survival is especially critical (Kiger et al., 2001; Tulina and Matunis, 2001).

Acknowledgment

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