Expression of a novel factor, short-type PB-cadherin, in Sertoli cells and spermatogenic stem cells of the neonatal rat testis

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Abstract

In the rodent testis, contact-mediated interactions between gonocytes, or neonatal stem cells, and Sertoli cells are critical for development. Previously, we showed that the neural cell adhesion molecule (NCAM) serves as a Sertoli cell–gonocyte attachment factor in neonates. Its expression decreases dramatically by 1 week of age and eventually disappears *in vivo*, and appears to be down-regulated by thyroid hormone (tri-iodothyronine (T_3)). In this study, we used a cDNA microarray to screen for additional adhesion factors which might be important in testes of developing rats and detected expression of a novel factor, short-type PB-cadherin (STPB-C). Next, RT-PCR was used to generate cDNA for STPB-C from total RNA isolated from co-cultures, cDNA was cloned into pPCR-Script Amp SK(+) cloning vector, and plasmid DNA was isolated and sequenced to confirm the fidelity of the STPB-C cDNA portion of the plasmid. *In situ* hybridization analyses of testicular sections indicated that STPB-C expression in neonates is localized in the cytoplasm of many, but not all, gonocytes and in the cytoplasm of most of the surrounding Sertoli cells. Parallel

hybridizations carried out on co-cultures also demonstrated a strong cytoplasmic signal in some gonocytes and in the great majority of the Sertoli cells of the underlying monolayer. With Northern analyses we found that STPB-C is expressed *in vivo* at high levels between days 1 and 5, with a subsequent large drop by day 10 and thereafter, suggesting that its expression may be associated with Sertoli or germ cell differentiation. Subsequent analyses of co-cultures exposed under a variety of conditions to T_3 suggest that, unlike NCAM, STPB-C is not regulated by this hormone. Next, we studied production of STPB-C protein by using an antiserum recognizing a peptide sequence unique to this factor in Western blotting and in immunolocalization. Signal was detected both intracellularly and at cell surfaces in most Sertoli cells and many gonocytes, although many of the latter cell type were also found to be negative for the protein, suggesting a potential role for STPB-C in survival and further development of some of these germ cells from which all subsequent spermatogenic cells originate.

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Introduction

Fertility in adult male rats is dependent on the production of adequate numbers of normal, mature spermatogenic cells. These critical cells all arise from a parent germ cell type, the gonocyte or neonatal stem cell, which in rats begins its postnatal development just after birth. Maturation of gonocytes in the newborn rat as well as sperm development in adults depends at least in part on proper communication between the germ cells and the somatic cell population, the Sertoli cells. This intercellular communication results in intracellular signaling events (Vleminckx & Kemler 1999) and no doubt involves maintenance of physical contact between the two cell types. For example, our work (Orth *et al.* 1988, Orth & Boehm 1990, Orth & McGuinness 1991, Orth & Jester 1995, Li *et al.* 1998) and that of others (Wright 1993)

indicates that both gonocytes from newborns and maturing germ cells from adults require contact with Sertoli cells to survive for any appreciable length of time *in vitro*. Similar germ cell–Sertoli cell interactions are also important *in vivo*, as evidenced by data from numerous sources (Griswold 1995, Weinbauer & Wessels 1999, Orth *et al.* 2000). Thus, identifying the precise mechanisms involved in these interactions and how they are regulated is a crucial step in exploring critical aspects of testicular development and function.

Recently, new information has become available clarifying the molecular basis for at least some Sertoli cell–germ cell interactions in newborn rats. We demonstrated that a non-cadherin, the neural cell adhesion molecule (NCAM), maintains attachment between neonatal Sertoli and germ cells both *in vivo* and *in vitro* (Orth & Jester 1995, Li *et al.* 1998). We also found that NCAM is substantially

down-regulated after postnatal day 5 *in vivo* and absent from the adult seminiferous epithelium. Thus, it is likely that other adhesion factors are also important in the developing and mature testis since NCAM becomes unavailable as the newborn matures. Interestingly, the adult rat testis also expresses a number of cadherin superfamily members, such as E-, N- and P-cadherin, cadherin-6, -8, -10 and -11, and members of the PCDH α family of cadherins (Johnson *et al.* 2000). The presence of a variety of cadherins suggests that one or more of these factors may also be important for interactions between germ cells and Sertoli cells of developing rats.

In spite of the obvious importance of physical interactions between Sertoli and germ cells, the identity and regulation of factors that support their adhesion remains largely unknown. In our previous work (Laslett *et al.* 2000), we found the tri-iodothyronine (T_3)-stimulated down-regulation of NCAM is accompanied by detachment of approximately 80% of gonocytes from co-cultures. However, the remaining 20% of these cells retain attachment and survive, presumably due to the presence of an alternative adhesion factor. Thus, we undertook the current study to identify new adhesion factors in the neonatal testis which might be responsible for continued attachment and survival of some gonocytes during this period of development.

Initially, we screened well-characterized Sertoli cell-gonocyte co-cultures with a cDNA microarray to identify candidate molecules and found a novel and potentially important factor, short-type PB-cadherin (STPB-C), which was hitherto undetected in the testis. We studied its expression both *in vivo* and *in vitro* with *in situ* hybridization and subsequently found with Northern analysis that it is expressed at highest levels in neonates and at substantially diminished levels thereafter. In addition, because of the recognized role of T_3 in testicular development (Palmero *et al.* 1989, 1995, Francavilla *et al.* 1991, Jannini *et al.* 1993, Van Haaster *et al.* 1993, Cook *et al.* 1994, Simorangkir *et al.* 1995, 1997), we also asked whether STPB-C expression might be regulated by this hormone. We utilized Northern analysis of co-cultured cells under various conditions of T_3 treatment and found no clear-cut relationship between the hormone and STPB-C, at least under the conditions tested. Finally, we used Western analysis and immunolocalization to examine STPB-C protein. We found that STPB-C is produced *in vivo* in neonatal testes and that its cellular localization suggests that it acts to modify adhesion between Sertoli cells and gonocytes, the stem cells from which all subsequent generations of germ cells arise.

Materials and Methods

Animals and collection of testis tissue

Male pups were obtained by mating Sprague-Dawley rats (Charles River Breeding Labs, Kingston, RI, USA). All

animals were maintained in an environmentally controlled facility with water and lab chow freely available, in accordance with the National Institute of Health guidelines for the care and use of laboratory animals. Testes obtained from adult rats and rat pups (1–5, 10 and 23 days old) were either decapsulated, snap-frozen in liquid nitrogen, and stored at -70°C until extraction of RNA or fixed in freshly prepared 4% formaldehyde in PBS overnight, followed by rinsing in PBS and routine processing and embedding in paraffin.

Gonocyte-Sertoli cell co-cultures

Gonocytes and Sertoli cells were isolated and cultured as previously described (Orth & Boehm 1990, Orth & Jester 1995). In brief, Sertoli cells and gonocytes were isolated from testes of 2-day-old pups by sequential digestion with collagenase/hyaluronidase and collagenase. A single cell suspension was obtained by incubating cell aggregates in cell dissociation buffer (Life Technologies, Inc., Gaithersburg, MD, USA), and cells were plated on Matrigel (Collaborative Research, Waltham, MA, USA) in either eight-chamber culture slides (Lab-Tek-Nunc, Naperville, IL, USA) or 35 mm plastic Pertri dishes. Gonocytes and Sertoli cells were cultured in hormone- and serum-free Eagle's D-valine MEM (Sigma, St Louis, MO, USA) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 3 mM sodium lactate, 5 $\mu\text{g}/\text{ml}$ transferrin, and 50 ng/ml retinol in a 5% CO_2 atmosphere at 37°C for up to 60 h. Some co-cultures were treated 24 h after plating with 0, 0.5, 1, 5 or 10 nM T_3 (Calbiochem, San Diego, CA, USA), or with 1 nM T_3 or vehicle for 6, 12, 24 or 36 h in hormone- and serum-free Eagle's D-valine MEM. Each of these incubation regimens was carried out on each of three separate sets of cultures. Results from each set of these incubations were subjected to densitometry and the results analyzed and compared statistically, as described below. For Western or immunofluorescent analysis of STPB-C protein, additional cultures were prepared as above. For immunoblotting, cells were scraped from the dishes, rinsed and frozen, followed by isolation of protein as described below. For immunolocalization, cultures were fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min, rinsed and processed as described below.

cDNA microarray preparation and hybridization

After 24 h of exposure to 10 nM T_3 or to vehicle, total RNA was extracted from co-cultures with the Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The total RNA was then treated with DNase and subsequent lack of contamination of each sample was verified using glyceraldehyde phosphate dehydrogenase (G3PDH) PCR without prior reverse transcription. Only DNA-free total RNA was

Figure 1 A rat cDNA microarray using RNA isolated from co-cultures with (lower panel) or without (upper panel) prior treatment with 10 nM T_3 . The white arrows indicate signal corresponding to STPB-C in each panel.

radiolabeled by reverse transcription to generate cDNA probes, which were hybridized to Atlas Rat 1.2 cDNA Expression Arrays (Clontech, Heidelberg, Germany) following the manufacturer's instructions. The membranes were then exposed to PhosphorImage screens overnight and scanned on a Fujifilm FLA-3000 PhosphorImager system (Tilburg, The Netherlands).

Probes

Total RNA was extracted from co-cultures as described above for cDNA microarray preparation. cDNA was synthesized by reverse transcription of 2 µg total RNA using oligo(dT)₁₂₋₁₈ primer (Life Technologies) with Superscript II RNase H⁻ reverse transcriptase according to the manufacturer's instructions (Life Technologies). PCR was carried out in a volume of 50 µl containing 10 µl reverse transcriptase reaction mixture, 1 × PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM potassium chloride), 1.5 mM magnesium chloride, 200 µM of dNTP mix, 200 nM each of primers (forward primer, 5'-TCAG TGTCCAGCTAGTCTCTC-3'; reverse primer, 5'-AC CCCCCACTTTCTCTTTCC-3'), and 2 U *Taq* polymerase (Life Technologies). Thirty-two cycles of amplification were performed: denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, followed by extension at 72 °C for 1.5 min. Amplified DNA (1225 bp) for STPB-C

Figure 2 Expression of STPB-C mRNA in co-cultures after 48 h *in vitro*. *In situ* hybridization was carried out with DIG-labeled cRNA antisense (A) or sense (B) probes and viewed with DIC optics. The focal plane of the image is at the level of the gonocytes, recognizable by their large round nuclei and their position, attached to the upper surfaces of the underlying Sertoli cells. In (A) cytoplasmic reaction product indicating the presence of the STPB-C message was prominent in many gonocytes (arrows) in antisense incubations, but other gonocytes lacking product were also common (asterisk in inset). Virtually all of the closely packed cells in the underlying monolayer are Sertoli cells and most of these cells contain a modest amount of signal in their scant cytoplasm. No reaction product was detected in any co-cultures exposed to sense cRNA probes (B; arrows indicate gonocytes). Bar = 10 µm.

was separated by agarose gel electrophoresis and sub-cloned into the pPCR-Script Amp SK (+) plasmid vector (Stratagene, La Jolla, CA, USA). Plasmid DNA was cut with the restriction enzyme Kpn1 and Sac1, and analyzed by agarose gel electrophoresis. To confirm the fidelity of the STPB-C cDNA portion of the plasmid, plasmid DNA containing the STPB-C insert was sequenced (98% identity).

In situ hybridization

Digoxigenin (DIG)-labeled cRNAs were produced with a DIG RNA labeling kit (Roche Molecular Biochemicals,

Figure 3 Representative views of *in situ* hybridizations for STPB-C carried out on tissue sections from 1-day-old (A), 5-day-old (B and C) and adult rats (D). In (A, C and D) sections were incubated with antisense cRNA probes, while in (B) sense probes were used. At all ages prior to day 23, substantial cytoplasmic product was detected in both gonocytes (asterisks in A and C insets) and the surrounding Sertoli cells. However, some gonocytes lacking product (arrow in C inset) were also observed. In older rats (D), product was restricted to the nuclei of apparent primary spermatocytes in most tubules (PS in inset: L, lumen).

Mannheim, Germany) from linearized pPCR-Script Amp SK (+) plasmid containing an STPB-C insert, using T3 and T7 RNA polymerase for sense and antisense probes respectively. Sections of paraffin-embedded tissue were cut (5 µm thick), placed on Super-Frost slides (Fisher Scientific, Irvine, CA, USA), dehydrated and cleared in xylene. Co-cultures and paraffin sections were subjected to *in situ* hybridization with a minor modification of the method described previously (Orth *et al.* 1996). Hybridization was at 60 °C overnight, and washes were in gradually decreasing concentrations of formamide and saline–sodium citrate (SSC), with final stringent washes in 0.2 × SSC, all at 60 °C. After completion of post-hybridization rinses, sections were preblocked in blocking solution (1% blocking reagent in maleic acid buffer) and then exposed to anti-DIG antibodies conjugated to alkaline phosphatase (Roche; 1:500) at room temperature for 2 h. A nucleic acid detection kit (Roche) was used to visualize hybridized probe in the co-cultures, with reagents supplied in the kit used at recommended concentrations. The color reaction was allowed to proceed for the same length of time for all slides in a single analysis (25–35 min). After rinsing, culture slides were mounted in

PBS:glycerol (1:1). Slides in each group were viewed and photographed with a Leitz Orthoplan 2 microscope (Leitz, Rockleigh, NJ, USA) equipped with differential interference contrast (DIC) optics (Leica, Malvern, PA, USA) and images captured with a digital Optronics Magnafire camera (Optical Apparatus, Ardmore, PA, USA).

Northern blotting

Total RNA was isolated from testis tissue or co-cultures using the protocol as described above. 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, Arlington Heights, IL, USA) in 20 × SSC overnight. After prehybridizing for 30 min in Rapid-Hyb (Amersham Pharmacia Biotech), blots were hybridized with a ³²P-labeled cDNA probe produced with the Rediprime kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The probe was a Kpn1-Sac1 fragment (1327 bp) containing STPB-C cDNA (1225 bp), obtained from a subclone in a pPCR-Script Amp SK (+) plasmid vector, as described above. After hybridization, blots were

Figure 4 (A) Representative Northern blotting analyses of total RNA from samples obtained from testes on postnatal days 1–5, 10 and 23, or from adult testes (Ad). The expression of G3PDH was used as an internal standard to control for evenness of loading. (B) In each of three replicate analyses, Northern blots were quantified, and the results were expressed as the ratio of STPB-C:G3PDH. The bars represent the means $\pm$ S.E.M. of the data for each age and bars marked with different letters are significantly different from each other ($P < 0.01$).

washed in decreasing concentrations of SSC, 0.1% SDS. Autoradiography was performed using Kodak BioMax film (Eastman Kodak, Rochester, NY, USA). Each blot was then stripped using the StripEz DNA kit (Ambion, Austin, TX, USA) according to the manufacturer's directions, and re-probed for G3PDH with an antisense DNA probe produced from a commercially available rat template (Ambion).

Western blotting and immunolocalization

Antiserum recognizing a peptide sequence unique to STPB-C (GHRGTSNKEDHQCPAS) was raised in rabbits by a commercial source (Resgen, Huntsville, AL,

USA) and utilized for all Western blotting and immunolocalization in this study. Immunoblotting and immunolocalization methods were essentially as previously described (Laslett *et al.* 2000). In brief, for immunoblotting cells were lysed in buffer containing 50 mM Tris (pH 7.4), 1% Triton X-100, 5 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin, followed by incubation at 4 °C for 60 min. Lysates were then centrifuged at 14 000 *g* at 4 °C for 30 min and supernatants were collected into fresh tubes. Protein concentrations of the supernatants were determined using a bicinchoninic protein assay kit (Pierce, Rockford, IL, USA). Proteins were equally loaded on each gel (50 μ g total protein/lane) and separated by electrophoresis on a

Figure 5 (A) Representative Northern analysis of STPB-C in total RNA samples isolated from co-cultures after incubation with 0, 0.5, 1, 5 or 10 nM T_3 for 24 h. The expression of G3PDH was used as an internal standard to control for evenness of loading. (B) Northern blots from each of three replicate sets of cultures were quantified by densitometry and the densitometric data were expressed as a ratio of STPB-C:G3PDH for each sample. Means $\pm$ S.E.M. were obtained for each treatment from the three sets of cultures, then plotted and analyzed statistically. No statistically significant differences were seen among the groups.

5% SDS–polyacrylamide gel, then electrophoretically transferred to a nitrocellulose membrane. After incubation in 5% dried non-fat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) at 4 °C overnight, membranes were probed for 60 min at room temperature with STPB-C antiserum (dilution 1:5000), or with pre-immune serum (control). After four washes of at least 10 min each in TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution 1:10 000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antibodies specifically bound to the membrane were visualized by chemiluminescence with a SuperSignal kit (Pierce) applied according to the manufacturer's directions. For immunolocalization of STPB-C in cells, freshly fixed cultures were blocked in 10% normal goat serum at room temperature for 60 min and then incubated with primary antiserum diluted 1:100 overnight at 4 °C. Control chambers were incubated either in pre-immune serum or without primary antibody. Following extensive washing in PBS, chambers were incubated in rhodamine-conjugated goat anti-rabbit secondary antibody (dilution 1:1000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 2 h at room temperature. After rinsing, slides

were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and then viewed with epifluorescence optics on a Leitz Orthoplan microscope and images captured as described above.

Data analysis

For each Northern analysis, densitometry was used to obtain a numerical value for STPB-C signal and for G3PDH signal, and the final data for each sample were expressed as a ratio of these values. Each analysis was performed in triplicate, and the means obtained from these three values for each treatment group were then analyzed statistically with one-way ANOVA and a Student–Newman–Keuls test.

Results

cDNA microarray analysis

To identify potential adhesion factors relevant to germ cell–Sertoli cell interactions, RNA was isolated from Sertoli cell–gonocyte co-cultures. Some of these cultures were treated with T_3 (10 nM, 24 h) while others received vehicle alone. A cDNA microarray was used to screen for expression of 1176 genes (Atlas Rat 1.2 Array (#7854–1); Clontech) and signal for several members of the cadherin family of adhesion factors, including STPB-C (grid #A02 h in Fig. 1), was seen in both sample groups. Based on this observation, we initiated more detailed analyses of STPB-C expression in neonatal testicular cells.

In situ hybridization of STPB-C mRNA in co-cultures and in sections

To identify and localize STPB-C transcripts in testicular cells *in vitro* and *in vivo*, we used DIG-labeled cRNA probes and subsequent immunolocalization with an alkaline phosphatase detection system, as described in Materials and Methods. Co-cultures were fixed after 2 days of culture and then hybridized to either antisense or sense probes. Antisense probes recognizing STPB-C mRNA were predominantly localized to the cytoplasm of many, but not all gonocytes, with a lower amount of product also seen in many Sertoli cells (Fig. 2A). In cultures hybridized to sense cRNA probes, essentially no signal was detected (Fig. 2B). When the same techniques were applied to testicular sections (Fig. 3A and C), a strong signal was seen in the cytoplasm of most but not all gonocytes and also in the cytoplasm of Sertoli cells. In testes from 23-day-old and adult animals, a weaker signal for STPB-C message was observed in some cross-sectioned tubules (Fig. 3D). In these areas, product was localized to the nuclei of germ cells whose size and

Figure 6 (A) Representative Northern analysis of STPB-C in total RNA samples isolated from co-cultures after incubation with T₃ (1.0 nM) or vehicle for 6, 12, 24 or 36 h. The expression of G3PDH was used as an internal standard to control for evenness of loading. (B) Northern blots from each of three replicate sets of cultures were quantified by densitometry and the densitometric data were expressed as a ratio of STPB-C:G3PDH. Means $\pm$ S.E.M. were obtained for each time point from the three replicate sets of cultures, then plotted and analyzed statistically. No statistically significant differences were seen among the groups.

position suggest that they are primary spermatocytes. No signal was seen in the cytoplasm of these germ cells. Finally, at all ages essentially no signal was detected in tissue hybridized to sense probes (see Fig. 3B for an example).

STPB-C expression during development in vivo

To assess the developmental pattern of STPB-C expression in rat testes, total mRNA was obtained from testes on days 1, 2, 3, 5, 10 or 23, or from adults. Northern blotting analysis was applied to these samples (Fig. 4) and the resulting signals from three sets of samples were quantified with densitometry and subjected to statistical analysis. Relative expression of STPB-C (STPB-C mRNA/

G3PDH mRNA) was strong and approximately equivalent on postnatal days 1–5. However, by day 10 a significantly decreased level of STPB-C expression was detected compared with that on days 1–5 ($P < 0.01$). Thereafter, on postnatal day 23 and in adults, expression of STPB-C remained relatively low, at levels significantly below those seen on days 1–5 ($P < 0.01$) and not different from that seen on day 10.

Relationship between T₃ and expression of STPB-C in co-cultures

The observation of a substantial decrease in STPB-C mRNA after postnatal day 10 raises the possibility that a

differentiation factor such as T_3 may play a role in regulating this adhesion factor (see Discussion). For this reason, we determined whether STPB-C expression is affected by either the level or extent of exposure of co-cultures to T_3 . First, Northern blotting was applied to

RNA isolated from co-cultures exposed to vehicle or to 0.5–10 nM T_3 for 24 h. When densitometry was used as described in Materials and Methods to quantify and compare signals on Northern blots, we noted a trend suggesting a slight increase in STPB-C signal between

0.5 and 5.0 nM of T_3 compared with corresponding controls (Fig. 5). However, statistical analysis revealed no significant differences among these samples. An examination of STPB-C expression in cultures exposed to T_3 (1 nM) or vehicle for shorter (6–12 h) or longer (36 h) periods of incubation (Fig. 6) also revealed no significant effect of the hormone on STPB-C expression.

Western analysis and immunolocalization of STPB-C protein

An antiserum generated against a peptide sequence unique to STPB-C was used in Western analysis of protein samples obtained from testes of rats aged 1 or 5 days. The antiserum recognized a single prominent band of approximately 85 kDa in immunoblots (Fig. 7A). Subsequently, the antiserum was used in immunofluorescent localizations of STPB-C in Sertoli cell–gonocyte co-cultures prepared on postnatal day 2 and analyzed after 24 h in culture. Figure 7B shows representative cultures stained either with DAPI (subpanels A, C and E) to visualize the cells present or with the STPB-C antiserum (subpanels B, D and F). Signal for STPB-C was strong in the great majority of Sertoli cells with only an occasional negative cell (Fig. 7B). In addition, when various planes of focus were examined in these cells, it was clear that fluorescent signal was both intracellular within the cell cytoplasm as well as localized to the surfaces of Sertoli cells (subpanel D). When gonocytes in these cultures were examined, many of these cells also displayed strong signal at cellular surfaces (subpanel F) as well as within the cells (not shown). However, in contrast to Sertoli cells many gonocytes were easily located which were totally lacking in signal. Two examples of negative gonocytes are identified in subpanel F. In additional cultures treated identically except for either omission of primary antiserum or substitution of pre-immune serum for the latter, no immunofluorescent signal was detected (not shown).

Discussion

Our finding of STPB-C expression in the developing rat testis *in vivo* and *in vitro* provides new evidence for a potential role of this adhesion factor in the testis.

Moreover, because Sertoli cell–gonocyte interactions play a crucial role in germ cell development, appropriate expression of STPB-C may be critical for normal onset of spermatogenesis and ultimate attainment of fertility in males. The final size of the gonocyte population is determined during the neonatal period of testicular maturation *in vivo* (Orth *et al.* 2000), when many of these germ cells undergo apoptosis (Knudson *et al.* 1995) while others survive. The postnatal wave of apoptosis and subsequent development of the surviving germ cells has been shown to be essential for normal quality and quantity of sperm production in adults (Knudson *et al.* 1995, Lee *et al.* 1997). Thus, surviving gonocytes which avoid apoptosis act as stem cells for all future generations of spermatogenic cells. In the light of the above information, it is clear that any factor which ensures continued attachment of maturing gonocytes to Sertoli cells will be important for further development of the spermatogenic population.

In previous studies from our laboratory, we documented that a non-cadherin, NCAM, is at least partly responsible for Sertoli cell–gonocyte adhesion in neonates (Laslett *et al.* 2000) and that its expression decreases substantially by day 15 and disappears in adults (Li *et al.* 1998). We also found that T_3 treatment of neonatal co-cultures results in down-regulation of NCAM and, at higher concentrations or times of exposure, in detachment of approximately 80% of gonocytes from the underlying Sertoli cells (Laslett *et al.* 2000). Our current findings extend our understanding of the relationship between neonatal Sertoli and germ cells by identifying STPB-C as a potentially important factor expressed by both cell types at this time. Moreover, its pattern of expression *in vivo* differs from that of NCAM, with sustained levels of STPB-C expression between days 1 and 5 after birth, followed by a notable decline by day 10 to low levels sustained thereafter (Fig. 4). In our observations in *in situ* hybridizations and in immunolocalizations, most Sertoli cells in neonates expressed STPB-C while few if any were positive for STPB-C in adults. We also observed strong signal in some gonocytes while many others were negative. In post-pubertal testes, expression of STPB-C mRNA appeared to be restricted to the nuclei of primary spermatocytes. This pattern of expression suggests that STPB-C expression may be related, directly or indirectly, to differentiation of Sertoli cells which cease

Figure 7 (A) Western analysis of testicular protein samples obtained from 1- or 5-day-old pups (50 µg/lane), probed with anti-STPB-C. The position of molecular mass markers is indicated to the right of the blot. (B) Immunofluorescent localization of STPB-C in Sertoli cell–gonocyte co-cultures prepared on postnatal day 2 and analyzed 24 h after plating. Images on the left show nuclear fluorescence for DAPI while those on the right show rhodamine fluorescence, indicating the presence of anti-STPB-C. Subpanels A and B: a view of Sertoli cells, with subpanel B showing an intracellular plane of focus at the level of the nuclei of most of the cells. Most Sertoli cells display strong intracellular signal for STPB-C, although an occasional cell lacking signal is present (two negative cells are indicated by asterisks). Subpanels C and D: a similar view of Sertoli cells as seen in subpanels A and B, with the plane of focus in subpanel D at the level of the cell surface. Note strong punctate fluorescence on the surfaces of several Sertoli cells. Subpanels E and F: a portion of a culture containing four gonocytes, easily recognizable by their large round nuclei and prominent nucleoli in subpanel E. Two of these cells display abundant punctate fluorescence at their surfaces in subpanel F, while two are totally negative. The location of these negative gonocytes has been indicated by white circles in subpanel F. Bar=10 µm.

mitotic activity at about 10 days of age (Orth 1982) and presumably all differentiate at that time or shortly thereafter. Currently available information identifies T₃ as an important influence in Sertoli cell differentiation. For example, experimental hyperthyroidism leads to a shortened period of Sertoli cell proliferation and accelerated Sertoli cell differentiation, while hypothyroidism causes a prolonged period of Sertoli cell proliferation and a delay in differentiation of these cells (Van Haaster *et al.* 1993, Simorangkir *et al.* 1995, 1997). However, data presented here do not support a direct role for T₃ in regulation of STPB-C expression. Further studies in this area should clarify the mechanism whereby the level of STPB-C is regulated as the testis matures and Sertoli cells differentiate.

Although evidence from the literature on the developing testis indicates that several other members of the cadherin family of adhesion factors are produced during testicular maturation, the patterns of their expression and their mechanism of action suggest that STPB-C may play a role distinct from that of these other cadherins. Johnson *et al.* (2000) explored expression of multiple cadherin superfamily members and reported that cadherins with the exception of N-cadherin and cadherin-6 are expressed most abundantly around postnatal day 7. In addition, for all members of the PCDH α family of cadherins, expression levels are generally high at day 7 and then remain at a steady-state from postnatal day 21 through adulthood (Johnson *et al.* 2000), a pattern different from that found for STPB-C. Moreover, a consideration of the molecular structure and subcellular interactions of STPB-C also supports the concept of a distinct role for this factor. Previously, both STPB-C and long-type PB-cadherin (LTPB-C) were found in pituitary and brain of rats (Sugimoto *et al.* 1996). However, unlike LTPB-C and the classic cadherins, STPB-C lacks a catenin-binding domain yet functions via an unknown, alternative mechanism in Ca²⁺-dependent cell-cell interactions (Sugimoto *et al.* 1996). Thus, STPB-C induces functional changes in cells via Ca²⁺-regulated actions that are distinct from those associated with the other cadherins.

In summary, we have detected expression of a novel factor, STPB-cadherin, by neonatal gonocytes and Sertoli cells and we have verified production of STPB-C protein in both cell types. Moreover, our observations suggest that this protein functions at cell surfaces to support contact-mediated interactions which are important in survival of those gonocytes that are the foundation of all subsequent germ cell generations.

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