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Epab and *Pabpc1* Are Differentially Expressed During Male Germ Cell Development

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Abstract

Modification of poly(A) tail length constitutes the main posttranscriptional mechanism by which gene expression is regulated during spermatogenesis. Embryonic poly(A)-binding protein (EPAB) and somatic cytoplasmic poly(A)-binding protein (PABPC1) are the 2 key proteins implicated in this pathway. In this study we characterized the temporal and spatial expression of *Epab and Pabpc1* in immature (D6-D32) and mature (D88) mouse testis and in isolated spermatogenic cells. Both *Epab* and *Pabpc1* expression increased during early postnatal life and reached their peak at D32 testis. This was due to an increase in both spermatogonia (SG) and spermatocytes. In the mature testis, the highest levels of *Epab* were detected in SG, followed by round spermatids (RSs), while the most prominent *Pabpc1* expression was detected in spermatocytes and RSs. Our findings suggest that PABPC1 may play a role in translational regulation of gene expression by cytoplasmic polyadenylation, which occurs in spermatocytes, while both EPAB and PABPC1 may help stabilize stored polyadenylated messenger RNAs in RSs.

Keywords

EPAB, PABPC1, spermatogenesis, testis, polyadenylation

Introduction

Spermatogenesis is a complex process of proliferation and differentiation transforming spermatogonia (SG) into mature spermatozoa.[1-4] During this tightly regulated process, male germ cells utilize unique mechanisms of transcription initiation/repression including dedicated transcription factors, tissue-specific promoters, and somatic gene silencing.[5] Transcription factors unique to spermatogenic cells have been identified and include SPRM1, TAK-1, OCT-2, CREB, and CREM.[6] Transcription ceases at mid-spermiogenesis (step 10 spermatids in mouse), as the remodeled condensed chromatin structure of the late-stage spermatids is incompatible with transcription.[7-9] Thereafter, gene expression is regulated posttranscriptionally and is dependent upon dormant paternally stored messenger RNAs (mRNAs) that are polyadenylated within the nucleus and exported to the cytoplasm, where they await alteration of their poly(A) tails for subsequent activation.[10,11]

Interestingly, unlike in the oocyte where the extension of the poly(A) tail results in translational activation, shortening of poly(A) tails in spermatids seems to trigger translation.[12] Indeed, stored mRNAs in spermatids usually have poly(A) tails longer than 150 nucleotides, but these polyadenylated mRNAs are not associated with ribosomes. They appear to undergo deadenylation until approximately 30 nucleotides of poly(A) remain; this is coincident with their association with ribosomes and an increase in translation.[12,13]

In addition, translational activation by cytoplasmic elongation of poly(A) tail, similar to that seen in oocytes, also occurs during spermatogenesis. Targeted disruption of cytoplasmic polyadenylation element-binding protein (CPEB)[14] and testis-specific cytoplasmic poly(A) polymerase[10] result in the arrest of spermatogenesis at the pachytene stage of the first meiotic division and during spermiogenesis (step 7 round spermatids [RSs]), respectively.

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Overall, while posttranscriptional regulation of gene expression by polyadenylation or deadenylation seems to play a key role during spermatogenesis, molecular mediators of these processes in male germ cells remain to be elucidated. It is likely that poly(A)-binding proteins (PABPs)[15-26] that bind and stabilize poly(A) tail in somatic and germ cells play a role in this process.

Embryonic poly(A)-binding protein (EPAB) has initially been identified in *Xenopus* as the predominant cytoplasmic PABP in oocytes and early embryos until zygotic genome activation (ZGA), when it is replaced by the somatic cytoplasmic poly(A)-binding protein, PABPC1.[16] Orthologues of *Xenopus* EPAB have been identified and characterized in mouse and human.[17,27] In the mouse, *Epab* mRNA is exclusively expressed in the gonads, oocytes, and 1- and 2-cell embryos but becomes undetectable in 4-cell, 8-cell, and blastocyst stage mouse embryos.[17] By contrast, *Pabpc1* mRNA is expressed at low levels in oocytes and in early preimplantation embryos, however, its expression is significantly increased at 8-cell and blastocyst stages.[17] A similar expression pattern for *Epab* and *Pabpc1* has been reported during human oocyte and early embryo development.[27] As ZGA occurs at the 2-cell stage in mouse[28] and 4- to 8-cell stage in human embryos,[29] EPAB seems to be the predominant PABP during mammalian oocyte and early embryo development until ZGA, when it is replaced by PABPC1. This tightly controlled expression suggests that EPAB may play a key role in mediating translational regulation of gene expression during early development. Indeed, in *Xenopus*, EPAB is associated with protein complexes that stabilize maternally stored mRNAs and regulate their translational activation.[16,30-32]

Although *Epab* expression during mouse oogenesis and early embryo development has been characterized, its expression in the postnatal mouse testis and in specific spermatogenetic cell types has not yet been studied. In this study, we aimed to characterize *Epab* and *Pabpc1* expression during male germ cell development and maturation by assessing immature and mature postnatal testis (D6-D88) and isolated spermatogenic cells.

Methods

Animals and Collection of Testes

The C57BL/6 mice were bred and maintained according to US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training, and all protocols were approved by Yale University's Institutional Animal Care and Use Committee (IACUC #2008-11207). Mice were housed under a 12-hour light cycle with free access to food and water. Testes from immature (days of 6, 8, 16, 20, 29, and 32) and mature mice (day 88) were collected under sterile conditions following euthanasia. Absence of sperm cells in cauda epididymis of 32-day-old immature mice was confirmed by evaluation under inverted microscope, following mincing and incubation in Dulbecco Modified Eagle Medium (DMEM): F12 media (Gibco, Carlsbad, California) at 37°C for 30 minutes.

Determination of Relative Abundance of Germinal Epithelial Cell Types in Postnatal Male Mouse Testis

To determine the relative abundance of each germinal epithelial cell type in testis from immature and mature male mice, hematoxylin and eosin (HE) staining was performed. For this purpose, whole testes obtained from 6-, 8-, 16-, 29-, 32-, and 88-day-old mice were fixed in Bouin fixative for 12 hours at +4°C and dehydrated in increasing concentrations of ethanol. Then, they were cleared in xylene and embedded in paraffin. Paraffin blocks were cut serially at 5 μm thickness using the rotary microtome (Leica, Nussloch, Germany). For HE staining, slides were incubated at 60°C for 1 hour, treated twice with fresh xylene, and then rehydrated in decreasing ethanol series followed by wash in tap water. Slides were then stained with hematoxylin followed by eosin. After additional washes in distilled water, slides were dehydrated in increasing concentrations of ethanol and cleared in fresh xylene.

The HE-stained male germinal epithelial cells demonstrate well-defined morphological appearances. As previously described, SG were identified by stippled and deeply stained nucleus surrounded by a thin rim of cytoplasm; spermatocytes were identified by strongly stained woolly masses of chromatin around the nucleus and abundant cytoplasm; RSs were identified by a small round nucleus with a heavily stained irregular nucleolus in the center and abundant cytoplasm; elongated spermatids (ESs) were identified by hook-shaped nucleus surrounded by a small amount of cytoplasm at the surface and by the presence of a flagella.[33-35] For each developmental stage studied (D6, D8, D16, D29, D32, and D88), randomly selected 10 seminiferous tubules from 4 testis sections were assessed by 2 independent investigators under bright field light microscope (Optiphot 300, Nikon, Japan). The percentage of each spermatogenic cell was then calculated.

Separation of the Spermatogenic Cells From Immature and Mature Mouse Testes

Spermatogenic cell isolation was performed using previously described protocols[33,36,37] with modifications. Testes from 2 mice for each of the postnatal ages of 6, 8, 16, 29, 32, and 88 days were analyzed. Briefly, testes were decapsulated and digested in DMEM:F12 medium containing 0.5 mg/mL of collagenase (Roche, Indianapolis, Indiana) for 20 minutes at 34°C. Following collagenase treatment, seminiferous tubules were washed twice with fresh media and were gently pipetted with glass Pasteur pipette for 20 minutes at 34°C in medium containing 0.25 mg/mL of trypsin to obtain mixed spermatogenic cells. Following centrifugation at 1600 rpm for 6 minutes, the pellet containing mixed spermatogenic cells was suspended in fresh DMEM:F12 medium containing 0.5% bovine serum albumin ([BSA] American Bioanalytical Inc, Natick, Massachusetts) and 1 μg/mL DNase (Roche). Following a second centrifugation at 1600 rpm for 6 minutes, the pellet was resuspended in media including 0.5% BSA and then filtered through 70 and 40 μm cell strainers (BD biosciences, California).

Spermatogenic cell types were fractionated using a discontinuous 2% to 4% BSA gradient prepared in a 15 mL tube (Falcon, BD biosciences) by first adding 1 mL of 10% BSA and then 1 mL of 4.0%, 3.8%, 3.6%, 3.4%, 3.2%, 3.0%, 2.8%, 2.6%, 2.4%, 2.2%, and 2.0% BSA. Filtered spermatogenic cells were counted using a Makler chamber (Sefi-Medical, Haifa, Israel), and 1 mL of mixed spermatognic cells (3.5×10^5 cells/mL) was placed onto the gradient. Finally, 1 mL of 0.2% BSA was added. The tube was then incubated at + 4°C for 4 hours with gentle rocking at the rate of 15 tilts/min to enhance gravity sedimentation. Following incubation, 1 mL fractions were collected and centrifuged at 1600 rpm for 6 minutes. Cells were then suspended and assessed for their morphology under inverted microscope (Diaphot 300, Nikon). In addition, cells from each fraction were stained with HE and their morphology was assessed as previously described using established criteria.[33-35] Spermatocytes were found at fractions 3 to 6, RSs from spermiogenesis steps 1 to 13 at fractions 7 to 8, ESs from spermiogenesis steps 14 to 16 at fractions 12 to 14. Fractions with ≥77% purity for each specific spermatogenic cell type were pooled together and used for RNA isolation. In addition to morphologic parameters, mRNA expression for specific markers such as protamine 2 (*Prm2*) and synaptonemal complex 3 (*Scp3*) were assessed to confirm effective cell separation.

Mature spermatozoa were isolated from cauda epididymis of mature male mice of day 88 (D88). Dissected cauda epididymides were minced in 2.5 mL fresh DMEM:F12 medium and incubated at 37°C for 30 minutes to allow the release of spermatozoa into the medium. Following centrifugation at 1600 rpm for 6 minutes, the pellet was used for the total RNA isolation.[38]

RNA Isolation, Polymerase Chain Reaction, and Quantitative Real-Time PCR

Total RNA was isolated from immature (D6, D8, D16, D20, D29, and D32) and mature mice testes (D88) using Trizol (Invitrogen, Carlsbad, California) according to the manufacturer's instructions and kept at −80°C until use. RNAqueous-Micro Kit (Ambion, Austin, Texas) was used for total RNA isolation from the isolated spermatogenic cells. RNA was treated for genomic DNA contamination using DNase I (Ambion). The quality and concentration of the isolated total RNA were determined at the absorbance 260 and 280 nm. Reverse transcription reactions were performed using the Omniscript kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Equal amounts of total RNA (2 μg) were reverse transcribed to complementary DNA (cDNA) using random decamer priming at 37°C for 1 hour.

Polymerase chain reactions (PCRs) were performed on cDNAs using *Taq* DNA polymerase (Qiagen). Amplifications were carried out with 27 to 35 cycles of PCR (32 cycles for *Epab* and *Pabpc1*, 27 for *Prm2*, 35 for *Scp3*, and 30 for β-actin [*Actb*]), in which the initial 5 minutes denaturation at 95°C was followed by a "touch-down" program for 10 cycles of 92°C for 30 seconds, 65°C for 30 seconds (−1°C per cycle), and 72°C for 30 seconds; and then 17 to 25 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 10 minutes, in a volume of 25 μL of reaction mixture containing 10 × PCR buffer (Qiagen), 0.125 mmol/L of each deoxyribonucleotide triphosphate (Roche), 0.5 μmol/L of each primer (Keck Facility, Connecticut), and 2 units of SuperTaq polymerase (Qiagen). All PCR products were separated on 1.5% agarose/Tris–acetate–EDTA gels and visualized by ethidium bromide staining.

Quantitative real-time PCRs (qRT-PCRs) were conducted on an iCycler (Bio-Rad, Hercules, California) and assayed in triplicate. Each 25 μL reaction mixture contained 12.5 μL of 2 × SybrGreen supermix (Biorad, Hercules, CA, USA), 0.4 μmol/L of each primer (Keck Facility, Connecticut) and 1 μL of cDNA template. Unique PCR products were confirmed by melting curve analysis. Relative amounts of the *Epab*, *Pabpc1*, and *Actb* genes were determined based on the standard curve generated using serially diluted mouse cDNA. The PCR efficiency was >92% for all qRT-PCR reactions. Relative fold change for the *Epab* and *Pabpc1* genes were determined after normalization to *Actb* levels.

The primers used for PCR and RT-PCR reactions were *Epab*: forward: 5′-ATG AAC GGG CGC ATC GTG GGC-3′, reverse: 5′-ATG TGG CTG GGC TGT CCA CCT-3′; *Pabpc1*: forward: 5′-CCC AGC GCC CCC AGC TAC-3′, reverse: 5′-CAC GTT CCG CGT CC GCC-3′; *Actb*: forward: 5′-TGC GTG ACA TCA AAG AGA AG-3′, reverse: 5′-CGG ATG TCA ACG TCA CAC TT-3′, *Prm2*: forward: 5′-CGC TAC CGA ATG AGG AGC CCC AGT G-3′, reverse: 5′-TTA GTG ATG GTG CCT CCT ACA TTT CC-3′; *Scp3*: forward: 5′-TCA AAG CCA GTA ACC AGA-3′, reverse: 5′-TCG AAC ATT TGC CAT CTC-3′

RNA In Situ Hybridization

Testes obtained from immature and mature male mice were fixed in Bouin fixative for 4 hours at room temperature and dehydrated in increasing concentrations of ethanol. They were then cleared in xylene and embedded in paraffin. Paraffin blocks were stored at 4°C. Testis sections (5 μm) were cut using the rotary microtome (Leica).

Epab and *Pabpc1* cDNA probes for in situ hybridization (ISH) were produced by PCR, using cDNA from mouse ovary and testis. Amplifications were carried out in 35 cycles of PCR as above, with the exception of 1 minute extension steps in a volume of 50 μL reaction mixture as above including 0.02 mmol/L Dig-UTP (Roche). Both *Epab* and *Pabpc1* primers were located on exons 1 and 2 of respective genes. The PCR products were fractionated on a 1.5% agarose gel and purified using QIAEX II gel extraction kit (Qiagen). The efficiency of labeling was confirmed by comparison with a labeled control DNA in a dot blot according to the manufacturer's instructions (Roche). The labeled probe was stored at −20°C until use.

The ISH method was modified from Seli et al.[17] Briefly, paraffin sections (5 μm) from mature male mice testis were

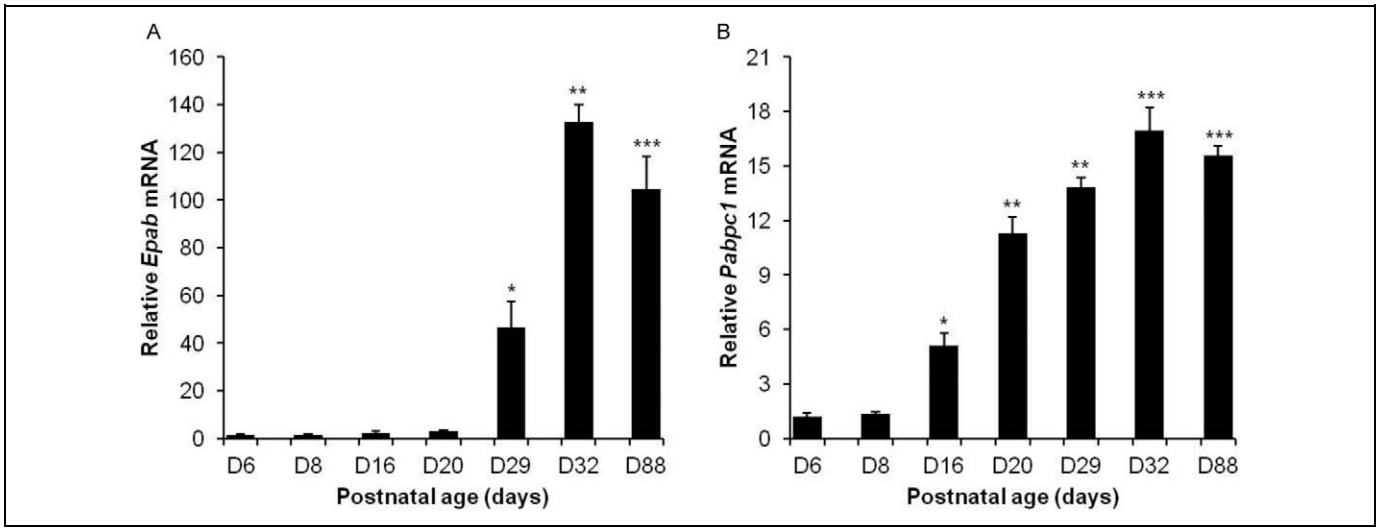

Figure 1. Expression analysis of *Epab* and *Pabpc1* in postnatal mouse testis. Quantification of *Epab* or *Pabpc1* mRNA expression in D6 to D88 postnatal mouse testes using real-time polymerase chain reaction (PCR). *Epab* or *Pabpc1* expression was normalized to β-actin expression. A, *Epab* messenger RNA (mRNA) level was significantly increased at D29 and reached its highest level at D32. **P* < .05 for D29 versus D20, D16, D8, and D6; ***P* < .05 for D32 versus D29; ****P* < .05 for D88 versus D32. B, *Pabpc1* mRNA level was significantly increased at D16 and reached its highest level at D32. **P* < .05 for D16 versus D8, and D6; ***P* < .05 for D20 and D29 versus D16; ****P* < .05 for D32 and D88 versus D29 and D20.

incubated at 60°C for 1 hour prior to ISH. Then, sections were treated twice with fresh xylene and rehydrated in decreasing concentrations of ethanol followed by rinsing in water treated with 0.1% diethylpyrocarbonate (Sigma-Aldrich, St. Louis, MO, USA). Permeabilization with 5 μg/mL proteinase K (Roche) in 1 × phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBST) at 37°C for 30 minutes was followed by postfixation in 4% paraformaldehyde in PBS for 5 minutes at room temperature. After washing with PBST, sections were incubated in prehybridization buffer (50% formamide, 5 mmol/L EDTA, 4 × SSC, 0.1% Tween-20, and 1% blocking reagent (Roche), 100 μg/mL of sonicated salmon sperm DNA (Stratagene, Jolla, CA, USA), 0.5% CHAPS (Sigma-Aldrich)] at 37°C for 2 hours. Hybridization with 300 ng/mL of digoxigenin-labeled *Epab* or *Pabpc1* probes was performed at 42°C overnight. After hybridization, sections were washed twice in 2 × SSC, once in 0.1 × SSC for 20 minutes at 65°C, and twice in MABT (0.1 mol/L maleic acid [Sigma-Aldrich], 0.15 mol/L NaCl, pH 7.5, and 0.1% Tween-20) for 10 minutes at room temperature. Blocking was performed using 1% (wt/vol) blocking reagent (Roche) in MAB (0.1 mol/L maleic acid [Sigma-Aldrich]/0.15 mol/L NaCl) for 30 minutes at room temperature. Sections were then incubated in antidigoxigenin antibody conjugated to alkaline phosphatase (1:800, Roche) for 1 hour at room temperature. After six 10-minute washes with MABT, sections were equilibrated in NaCl-Tris-MgCl2-Tween-buffer (NTMT) buffer (0.1 mol/L NaCl, 0.1 mol/L Tris-HCl, 50 mmol/L $MgCl_2$ [Sigma-Aldrich], 0.1% Tween-20) and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in NTMT (Roche) for ∼16 hours. Color development was stopped with several washes in PBST, and slides were mounted with Immu-Mount (Thermo Shandon, Pittsburgh). Sections were analyzed under bright-field microscope (Carl Zeiss Inc, Thornwood, New York).

Statistical Analysis

Because the data were normally distributed (as determined by the Kolmogorov-Smirnov test), comparisons of samples were analyzed with one-way analysis of variance followed by a post hoc Student-Newman-Keuls test where appropriate. Statistical calculations were performed using SigmaStat for Windows, version 3.0 (Jandel Scientific Corp, San Rafael, California). Statistical significance was defined as $P < .05$.

Results

Expression Analysis of Epab and Pabpc1 in Postnatal Mouse Testis

During postnatal development, testis undergoes maturation and the relative abundance of spermatogenic cells changes in a predictable manner. We first assessed *Epab* and *Pabpc1* mRNA expression in postnatal mouse testis from D6, D8, D16, D20, D29, D32, and D88. Quantitative RT-PCR showed that the level of *Epab* mRNA was very low on D6, D8, D16, and D20, but increased on D29. A further 3-fold increase was detected on D32 (Figure 1A). The amount of *Pabpc1* mRNA on D6 and D8 were low and increased significantly on D16. Further significant increases in *Pabpc1* mRNA were observed on D20, D29, and D32 (Figure 1B). Both *Epab* and *Pabpc1* mRNA expression reached their highest level at D32 and showed a slight decrease (statistically significant only for Epab [Epab written in a italic form]) on D88 (Figure 1).

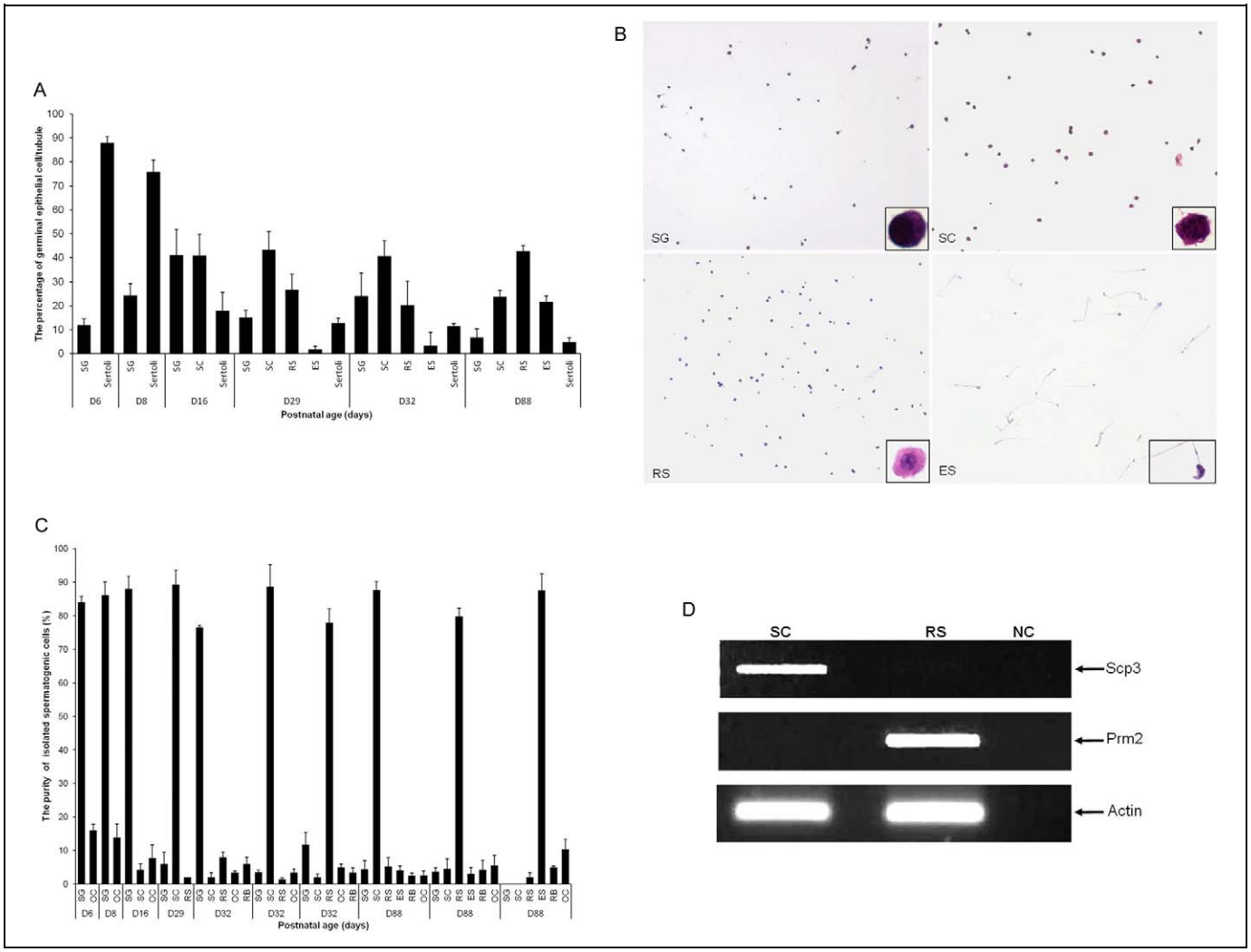

Figure 2. A, Percentage of spermatogenic cell types in testis from D6 to D88 mouse testis. The age of the mice and counted spermatogenic cell types are specified for each fraction. B, Isolation of spermatogenic cells by fractionation of mixed germ cells from D6 to D88 mouse testis in a 2% to 4% bovine serum albumin (BSA) gradient. Photomicrographs of air-dried and hematoxylin–eosin-stained smears of spermatogenic cells isolated from immature or mature testis. The images were taken at an original magnification of ×100; inserts were at ×1000 magnification. C, Percentage of spermatogenic cell types in fractions obtained from D6 to D88 mouse testis by separation of mixed germ cells using a 2% to 4% BSA gradient. One hundred hematoxylin and eosin (HE)-stained cells were assessed for each fraction. The age of the mice and isolated spermatogenic cell types are specified for each fraction. D, Expression of *Scp3* (synaptonemal complex 3) and *Prm2* (protamine 2) in isolated SC and RS fractions. *Scp3*, a gene expressed specifically in spermatocytes, was detected in the SC fraction, but not in the RS fraction. *Prm2*, a gene expressed specifically in round spermatids, was detected in the RS fraction, but not in the SC fraction, suggesting efficient isolation. β-Actin was used as an endogenous control. SG indicates spermatogonia; SC, spermatocytes; RS, round spermatids; ES, elongated spermatids; RB, residual bodies; OC, other cells.

Isolation of Spermatogenic Cells by Fractionation of Mixed Germ Cells From D6 to D88 Mouse Testis

First, we determined the relative abundance of each germinal epithelial cell type in testis from immature and mature male mice. The HE staining was performed, assessing 10 tubules from 4 testis sections for each developmental stage (D6, D8, D16, D29, D32, and D88). We found that the relative abundance of male germinal epithelial cells changes during testicular maturation, with decreasing abundance of SG and sertoli cells and increasing abundance of RS and ES as previously described (Figure 2A).[36]

In order to study cell type-specific expression of *Epab* and *Pabpc1*, spermatogenic cells were isolated from immature (D6-D32) and mature mouse testes (D88) by fractionation using 2% to 4% BSA gradient. Isolated spermatogenic cells in fractions enriched for SG, spermatocytes, RSs, or ESs were stained with HE (Figure 2B), and percentage of spermatogenic cell types were determined using previously described morphologic characteristics (Figure 2C).

Enrichment of more than 77% was achieved for each spermatogenic cell type (Figure 2B), sufficient to assess cell type-specific *Epab* and *Pabpc1* expression. In addition to

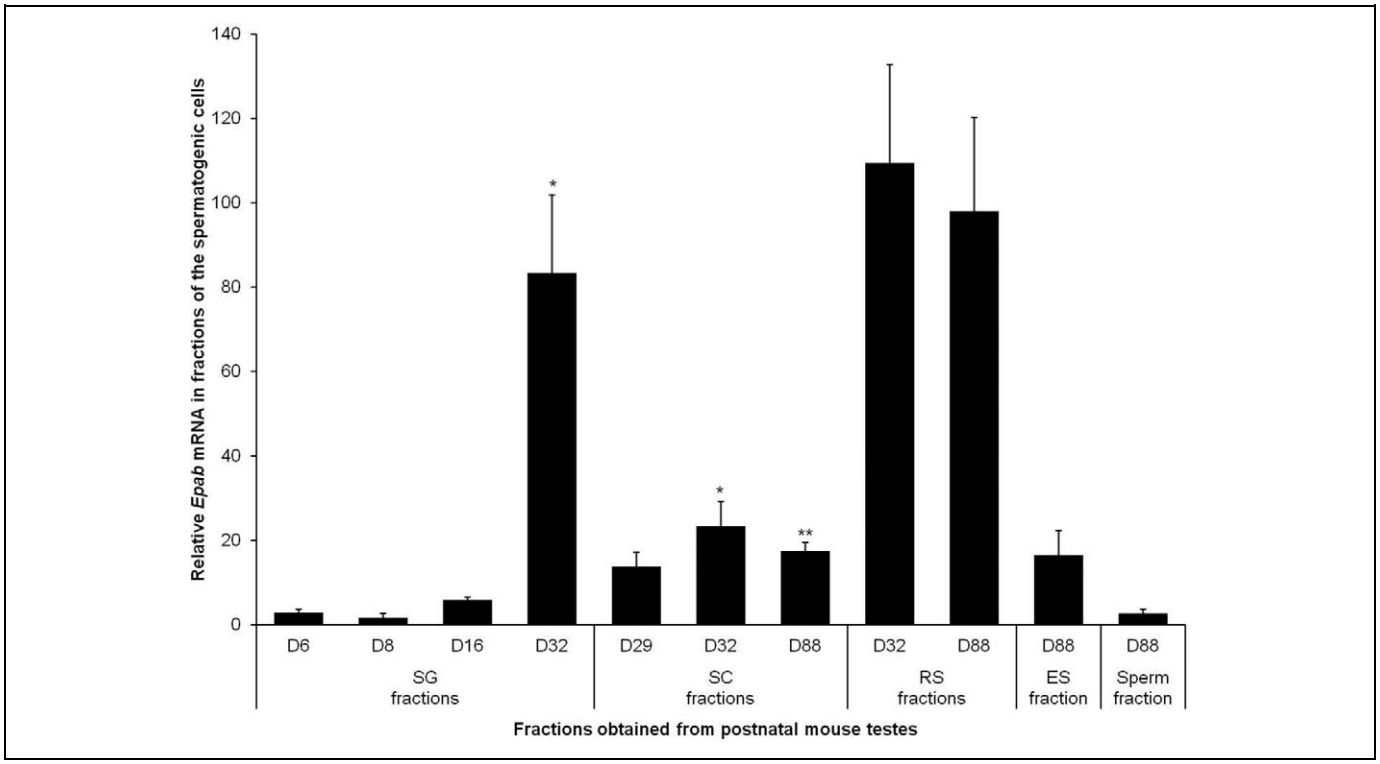

Figure 3. Quantification of *Epab* messenger RNA (mRNA) expression in isolated spermatogenic cells from immature and mature mouse testes. Qualitative real-time polymerase chain reaction (qRT-PCR) in all samples was normalized to β-actin expression used as an internal control. In spermatogonia, *Epab* mRNA expression was low on D6, D8, and D16 (immature mice), and reached the highest value at D32. In spermatocytes *Epab* expression reached its highest level at D32. *Epab* mRNA expression in round spermatids isolated from 32- (immature) or 88-day-old (mature) mice was high, but there was no remarkable change between the 2 time points. SG indicates spermatogonia; SC, spermatocyte; RS, round spermatid; ES, elongated spermatid. SG: $^{*}P < .05$ for D32 versus D16, D8, or D6; SC: $^{*}P < .05$ for D32 versus D29; $^{**}P < .05$ D88 and D32.

morphologic characteristics, expression of cell-specific genes was tested by RT-PCR to confirm efficient cell isolation. *Scp3*, a marker of spermatocytes was expressed in the spermatocyte fraction and not in the RS fraction. Similarly, *Prm2* expression was detected in the RS fraction and not in the spermatocyte fraction (Figure 2D). Morphologic assessment of HE stained slides and expression of the 2 cell-specific markers suggested efficient fractionation and isolation of spermatogenic cell types.

Quantification of Epab and Pabpc1 mRNA Expression in Isolated Spermatogenic Cells From Immature and Mature Mouse Testes

Epab and *Pabpc1* expression in isolated spermatogenic cells obtained from immature and mature mouse testes was determined using qRT-PCR. *Epab* levels were low in SG isolated from D6, D8, and D16 testes but significantly increased in D32 (Figure 3). Similar to SG, *Epab* expression in the spermatocytes from D32 testis was remarkably higher than spermatocytes from D29. We found that *Epab* expression in spermatocytes decreased by D88 (Figure 3). *Epab* expression in RSs was high but did not differ significantly between D32 and D88 (Figure 3).

Pabpc1 mRNA expression in SG was low on D6, D8, and D16 testes and significantly increased by D32 (Figure 4). Similar to *Epab*, *Pabpc1* expression in spermatocytes was found to be increased on D32 compared to D29, and decreased by D88 (Figure 4), and there was no significant difference in *Pabpc1* expression of RSs between D32 and D88 (Figure 4).

Localization of Epab and Pabpc1 mRNA in Mature Male Mouse Testis

To assess cell-specific expression of *Epab* and *Pabpc1* in mature male mice, we applied qRT-PCR and ISH. We first carried out qRT-PCR using spermatocytes, RSs, ESs isolated from testis, and spermatozoa isolated from cauda epididymis of mature male mice. We found that RSs have significantly higher *Epab* expression compared with spermatocytes, ESs, or spermatozoa (Figure 5A). Spermatogonia could not be isolated from adult mice because of very low abundance. Qualitative RT-PCR also revealed *Pabpc1* expression in RSs and spermatocytes to be higher than ESs or spermatozoa (Figure 5B).

To confirm our findings and to assess expression of *Epab* and *Pabpc1* in SG, we performed RNA ISH for *Epab* and *Pabpc1* mRNAs in mature mouse testis. High expression of *Epab* mRNA was detected in SG settled at the basal layer of the

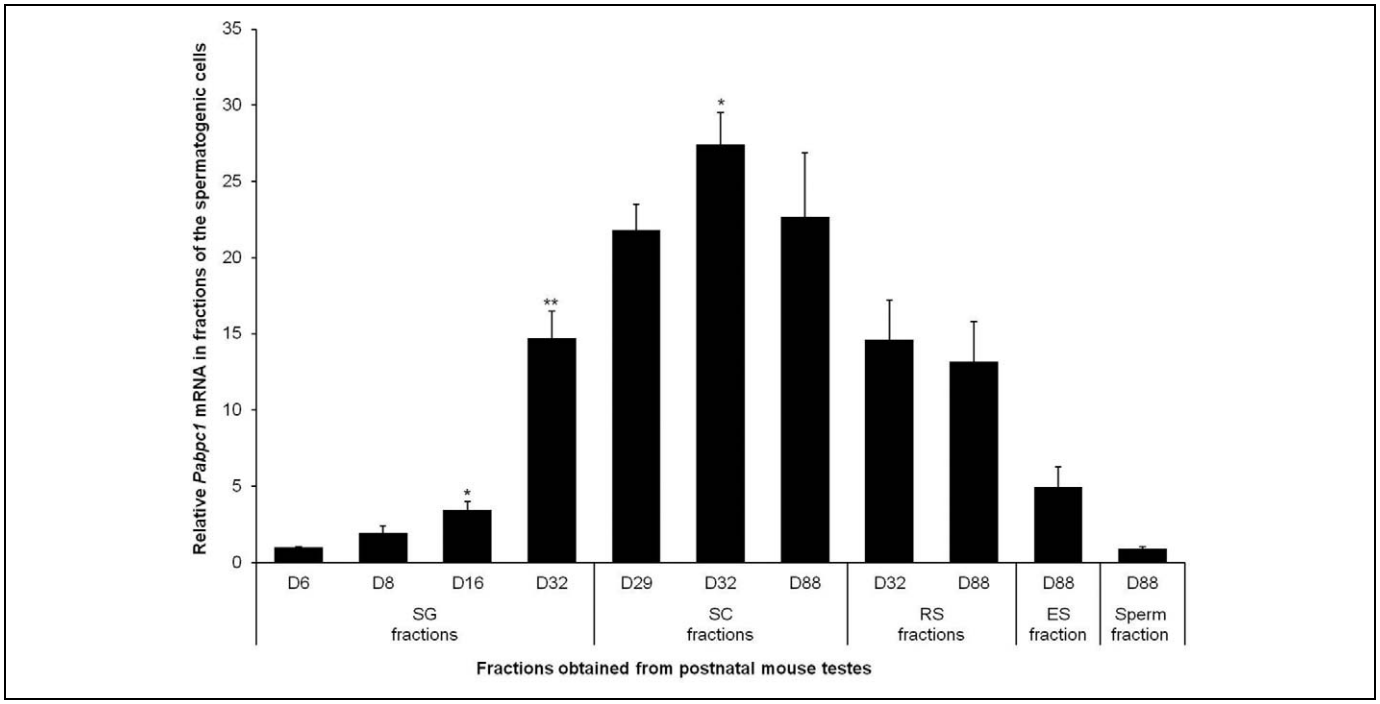

Figure 4. Quantification of *Pabpc1* messenger RNA (mRNA) expression in isolated spermatogenic cells from immature and mature mouse testes. Qualitative real-time polymerase chain reaction (qRT-PCR) was normalized to β-actin expression used as an internal control. *Pabpc1* expression began to rise at D8 and reached the highest value at D32. *Pabpc1* mRNA expression reached its highest level at D32. In round spermatid fractions isolated from 32- (immature) and 88-day-old (mature) mice there was no remarkable change in *Pabpc1* expression. SG indicates spermatogonia; SC, spermatocyte; RS, round spermatid; ES, elongated spermatid. SG: **P* < .05 for D16 versus D8; ***P* < .05 for D32 versus D16; SC: **P* < .05 for D32 versus D88 and D29.

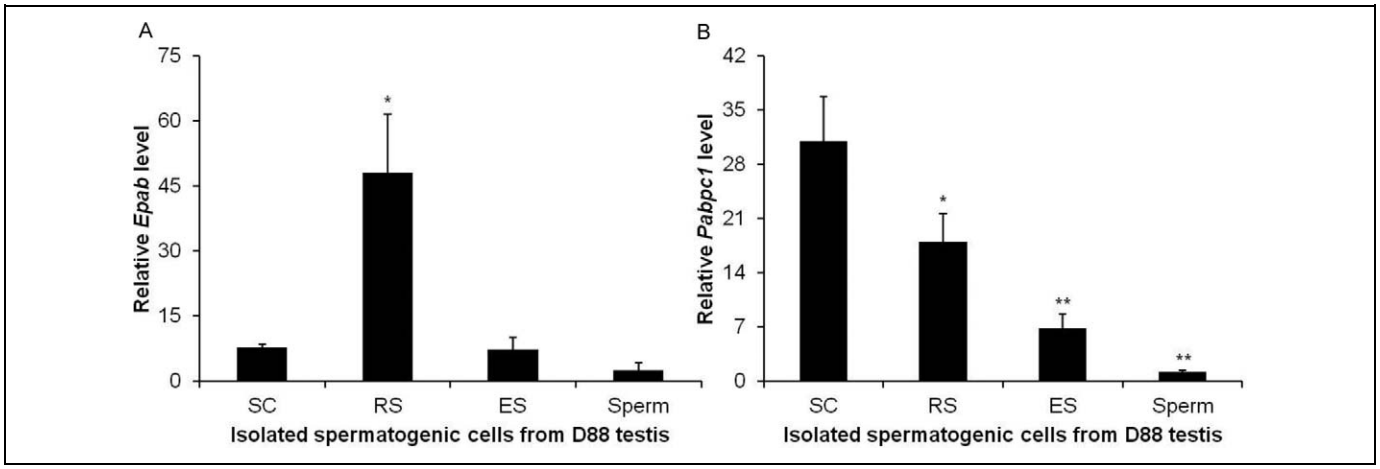

Figure 5. Quantification of *Epab* and *Pabpc1* messenger RNA (mRNA) expression in isolated spermatogenic cells from mature male mouse using Qualitative real-time polymerase chain reaction (qRT-PCR). Spermatocytes, round spermatids, and elongated spermatids were isolated from mature male testis, and spermatozoa were isolated from cauda epididymis. The qRT-PCRs for *Epab and Pabpc1* were normalized to β-actin expression used as an internal control. A, Comparable with RNA in situ hybridization (ISH), round spermatids showed significantly higher *Epab* expression than spermatocytes, elongated spermatids, and spermatozoa. **P* < .05 RS versus SC, ES, and spermatozoa. B, Comparable with RNA ISH, *Pabpc1* expression in spermatocytes, and round spermatids was higher than elongated spermatids and spermatozoa. Moreover, qRT-PCR revealed that SC fractions had significantly more *Pabpc1* mRNA than RS. SC indicates spermatocyte; RS, round spermatid; ES, elongated spermatid. **P* < .05 RS versus SC; ***P* < .05 ES and spermatozoa versus RS.

seminiferous tubules. *Epab* was also expressed in spermatocytes, RSs, and ESs, at lower levels. It is noteworthy that, consistent with qRT-PCR findings, *Epab* mRNA expression seemed to be higher in RSs compared with spermatocytes or ESs (Figure 6A). In contrast to *Epab* expression, *Pabpc1* was barely present in SG, but, consistent with qRT-PCR findings,

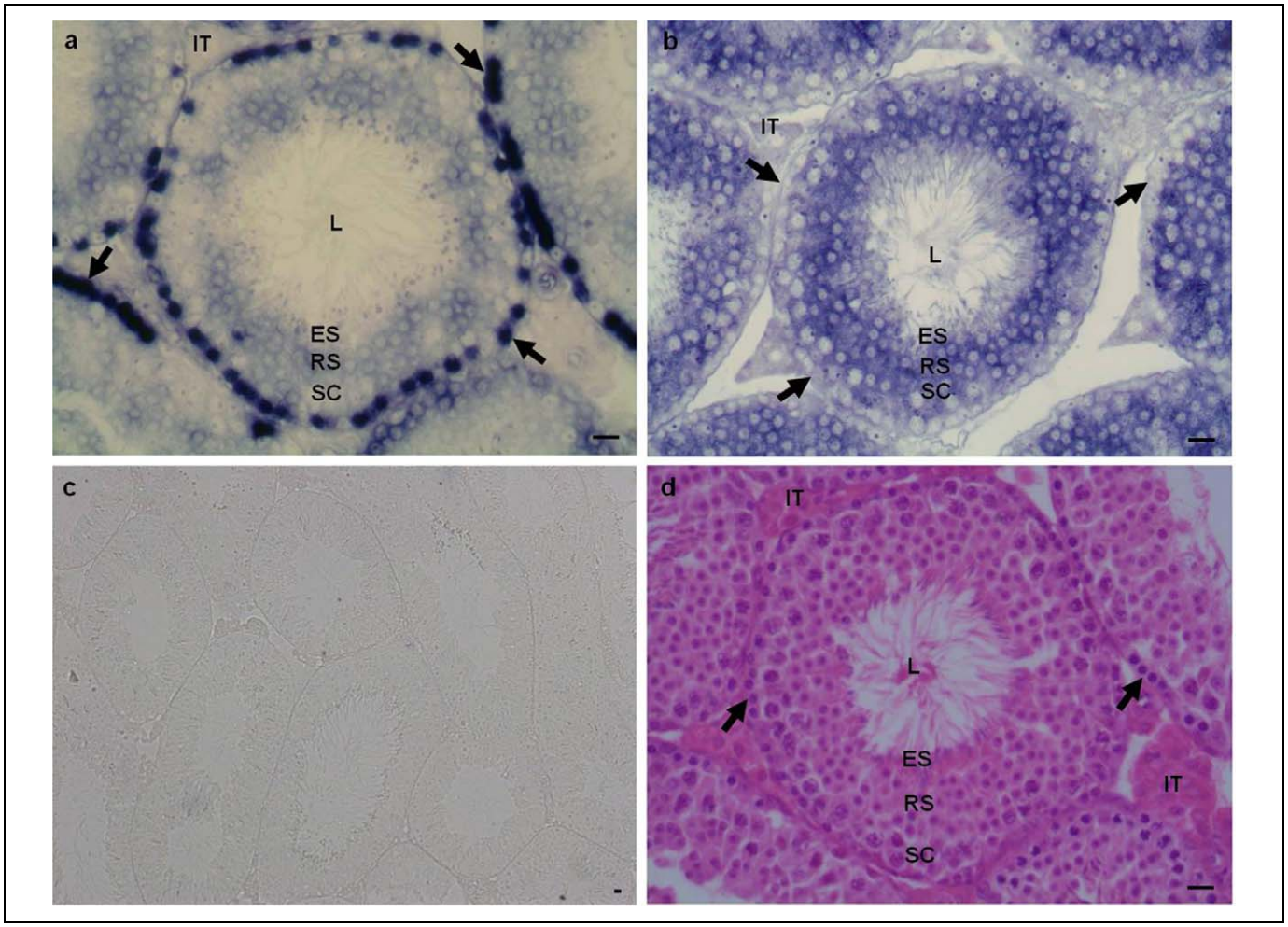

Figure 6. Localization of *Epab* and *Pabpc1* messenger RNA (mRNA) by in situ hybridization in mature male mouse testis. A, *Epab* expression in mature male mouse testis. *Epab* expression was assessed by in situ hybridization using a *Epab* exon 2 probe. *Epab* expression was highest in spermatogonia (arrows) localized at the periphery of the tubules. In addition, RSs had higher expression compared with spermatocytes or elongated spermatids. B, *Pabpc1* expression in mature male mouse testis. *Pabpc1* expression was assessed by in situ hybridization using a *Pabpc1* exon 1-2 probe. *Pabpc1* expression was cytoplasmic and was localized to the seminiferous tubules as well as the intertubular areas. In seminiferous tubules, spermatocytes and RSs had higher *Pabpc1* expression than spermatogonia or elongated spermatids. C, Negative control for in situ hybridization. D, Hematoxylin and eosin stained mature male mouse testis. L indicates lumen of the seminiferous tubule; ES, elongated spermatid; RS, round spermatid; SC, spermatocyte; IT, intertubular area.

its expression was remarkably increased in spermatocytes and RSs. Similar to *Epab*, *Pabpc1* expression in ESs and spermatozoa was found to be very weak (Figure 5B). Therefore, using RNA ISH and cell separation combined with qRT-PCR, we established germ cell-specific expression of the 2 predominant poly(A)-binding proteins in mature mouse testis.

Localization of Epab mRNA in Immature Male Mouse Testis

We detected a high expression of Epab in SG in mature male mouse testis using ISH. However, qRT-PCR revealed that *Epab* was present at very low levels in postnatal D6 or D8 (Figure 1A), when SG is the predominant germ cell in the seminiferous tubule (Figure 2A). To determine whether there is an increase in spermatogonial *Epab* expression in spermatogonial cells during postnatal development, as suggested by the qRT-PCR findings (Figure 1A), we performed ISH to assess *Epab* expression in testis from 6-, 8-, 16-, 29-, and 32-day-old mice (Figure 7). While *Epab* expression in SG was very weak on D6, D8, and D16, it increased significantly by D29 and D32. This finding was consistent with qRT-PCR findings presented in Figure 1.

Discussion

In this study, we characterized temporal and spatial expression of *Epab* in male germ cells and gonads in mouse and compared and contrasted *Epab* expression to that of *Pabpc1*. Immature testes from 6-, 8-, 16-, 29-, and 32-day-old mice were analyzed as these time points are characterized by a predictable spermatogenic cell type content of increasing maturity (Figure 2A).[36]

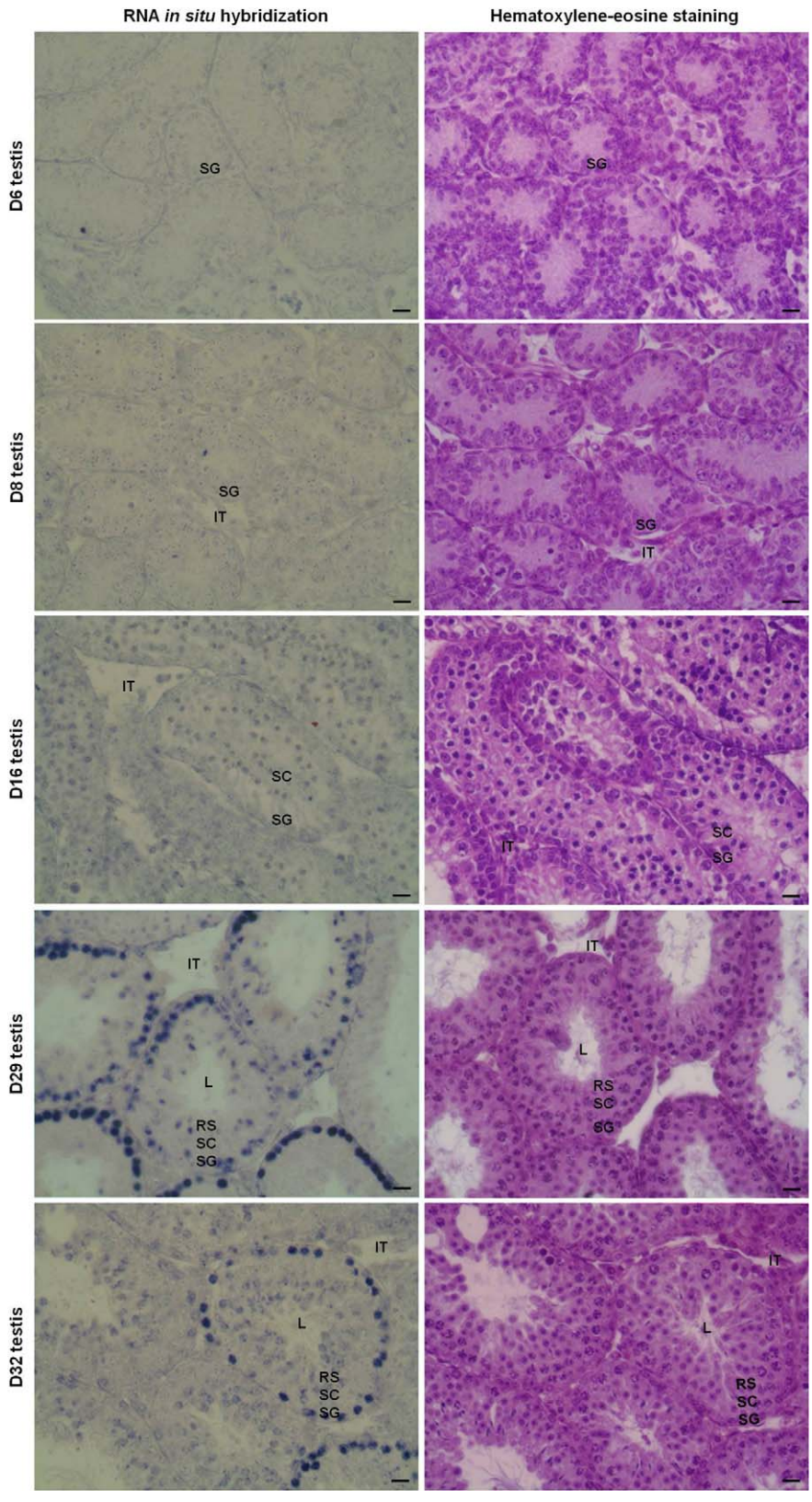

Figure 7. Localization of *Epab* messenger RNA (mRNA) by in situ hybridization in postnatal male mouse testis from D6 to D32. Hematoxylin–eosin staining was also done for each stage. *Epab* expression was assessed by in situ hybridization using a *Epab* exon 2 probe. While *Epab* expression in spermatogonia was very weak on D6, D8, and D16, it increased significantly by D29 and D32. Spermatocytes had slightly higher *Epab* expression in D16 than D29 or D32 testes. In RSs, *Epab* expression in D32 testis was found to be more than D29 testis. L indicates lumen of the seminiferous tubule; ES, elongated spermatid; RSs, round spermatid; SC, spermatocyte; IT, intertubular area.

We found that, *Epab* mRNA expression was low at D6, D8, D16, and D20 testes, but increased significantly at D29, reaching the highest level at D32 and declining at D88 (Figure 1A). *Pabpc1* mRNA levels were also low at D6 and D8 and began to increase at D16. Similar to *Epab, Pabpc1* expression reached its highest level at D32 testis and decreased at D88 (Figure 1B). Our *Pabpc1* findings are consistent with a study by Kimura et al, which examined *Pabpc1* expression during mouse postnatal testicular development at 5 to 60 days using Northern blot and found *Pabpc1* mRNA to be remarkably increased from D18 onward.[39] The peak expression observed for both *Epab* and *Pabpc1* at D32 strongly suggests that these PABPs may play important roles in the process of mature spermatozoa production that starts at around D35 postpartum.[40]

Pabpc1 and *Epab* are expressed in different types of cells and at different time points during development: *Pabpc1* is present in all somatic cells and in the embryonic cells following ZGA, while *Epab* is expressed in gametes and early embryos.[16,17] Therefore, we wanted to determine whether these 2 PABPs differ in the type of germ cells in which they are expressed. Expression analysis of *Epab* and *Pabpc1* in isolated spermatogenic cells was performed between D6 and D88, on days selected based on the elegant study carried out by Bellve[36] as well as our own findings (Figure 2A). We have isolated spermatogenic cell types at high purity from immature and mature testes at specific ages with the aim of obtaining adequate number of a specific cell type for analysis. For example, RSs first appear at D18 mouse testis, when all spermatogenic cell types become detectable.[36] However, the numbers of RSs are very low at D18; therefore, RSs isolation was performed at D32. Similarly, as SG constitute the great majority of spermatogenic cells in early immature mouse testis with decreasing abundance at D32, isolation of SG was performed at D6, D8, D16, and D32. However, due to the low number of SG in mature testis, adequate purity for molecular analysis of SG could not be achieved at D88 testis samples. Other methods for spermatogonial cell isolation include culture on laminin matrix[41], flow cytometry,[42,43] or Magnetic-activated cell sorting (MACS) methods.[44] In this study, we used RNA ISH to assess *Epab* and *Pabpc1* expression in SG from mature testes and to compare with other germ cells.

We found both *Epab* and *Pabpc1* expression to increase in SG and spermatocytes during postnatal life reaching their peak at D32 (Figures 3 and 4). This suggests that rather than an increased expression in a specific spermatogenic cell type, an overall increase in PABP expression in spermatogenic cells together with an increase in the number of germ cells is responsible for the observed increase in *Epab* and *Pabpc1* expression in maturing testis (Figure 1A and B), possibly in preparation for spermatogenesis. Interestingly, in testis, *Epab* and *Pabpc1* expression showed a moderate but statistically significant decrease at D88 compared with D32.

In mature male mouse testis, the highest *Epab* expression was in SG (Figure 6A). Conversely, *Pabpc1* mRNA levels in SG were low compared to other spermatogenic cells in mature testis (Figure 6B). This observation suggests that during the adult life, EPAB may play an important role in SG, possibly helping expression of genes responsible for initiating spermatogenesis. Additional studies are required to determine specific functions of EPAB and its interactions with other proteins and mRNAs in the SG. In addition, as *Pabpc1* is also expressed in SG (especially in immature testes) and as SG have not been reported to require posttranscriptional regulation, it would be important to determine whether spermatogonial function is dependent on the presence of EPAB, or whether EPAB's functions can be compensated by other RNA-binding proteins.

In addition, *Epab* mRNA levels were significantly higher in isolated RSs compared with spermatocytes, ESs, or sperm cells (Figures 5 and 6). As is known, a set of translationally repressed mRNAs that have long poly(A) tails are stored in the RSs.[12] Therefore, increased *Epab* expression in the RSs could play a role in the maintenance of the poly(A) tail length of stored mRNAs. Transcription becomes suppressed in ESs, where gene expression is regulated primarily posttranscriptionally. In these cells, unlike that observed in oocytes or spermatocytes, translational activation of mRNAs requires shortening of poly(A) tail, coinciding with a decrease in *Epab* expression. It is noteworthy that, while at very low levels, spermatozoa contain *Epab* mRNA.

Unlike *Epab*, the highest *Pabpc1* mRNA expression was detected in spermatocytes, followed by RSs; both cell types exhibited significantly higher *Pabpc1* expression compared with SG, ESs, or spermatozoa. Our findings are consistent with a study by Kleene et al,[20] which demonstrated that enriched pachytene spermatocyte and RS fractions have higher *Pabpc1* expression than ESs in mouse, using Northern blot analysis. Expression of *Pabpc1* in pachytene spermatocytes and RSs has also been demonstrated in human, where SG and ESs also express *Pabpc1*.[19] Increased *Pabpc1* expression in spermatocytes suggests that *Pabpc1* may play an important role in regulating poly(A) tail length in spermatocytes, where translational activation is associated with cytoplasmic lengthening of poly(A) tail and depends on CPEB.[14] In addition, similar to *Epab*, elevated levels of *Pabpc1* in RSs may play a role in maintaining polyadenylated mRNAs stored in RSs for later activation, and the decrease in the ES stage may facilitate translational activation of paternally stored mRNAs by ploy(A) tail shortening.

Overall, our findings reveal that both *Epab* and *Pabpc1* have a distinct temporal and cell-specific expression in mouse testes. The expression of these 2 key cytoplasmic PABPs seems to be tightly regulated during postnatal testicular development as well as mature spermatogenesis. As posttranscriptional mechanisms seem to play an important role in regulating gene expression at multiple stages of male germ cell development, further characterization of the role of these proteins, identification of the mRNAs that they stabilize and activate, and isolation of the proteins that they bind would be beneficial in understanding the intricate mechanisms governing male reproduction.

Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

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