RAB13 regulates Sertoli cell permeability barrier dynamics through protein kinase A

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Abstract

In mammalian testes, the blood–testis barrier (BTB), created by specialized junctions between Sertoli cells near the basement membrane of the seminiferous epithelium, provides an indispensable immune-privileged microenvironment for spermatid development. However, the BTB must experience restructuring during the epithelial cycle to facilitate the transit of preleptotene spermatocytes upon the testosterone-induced new TJ fibrils forming behind these cells, which is intimately related to the extensive dynamics of junction protein complexes between Sertoli cells. As key regulators of protein traffic, Rab GTPases participate in delivery of proteins between distinct cellular sites and cross talk with proteins that constitute tight junction and adherens junction. Using primarily cultured Sertoli cells *in vitro* with an established tight junction permeability barrier that mimics the BTB *in vivo*, RAB13 was shown to decrease during the testosterone-induced TJ integrity enhancement, accompanied with an increment in protein kinase A (PKA) activity. Furthermore, knockdown of *Rab13* was found to resemble the effect of testosterone on Sertoli cell TJ permeability by reinforcing filamentous actin and occludin distribution at the cell–cell interface and promoting the direct interaction between ZO-1 and occludin. Interestingly, the effects of testosterone and *Rab13* knockdown on Sertoli cell epithelium were revealed to be antagonized by PKA activity inhibition. In summary, RAB13 serves as a regulatory component in the assembly and restructuring of the TJ fibrils between adjacent Sertoli cells.

Key Words

- ▶ RAB13
- ▶ Sertoli cell
- ▶ blood–testis barrier
- ▶ tight junction
- ▶ protein kinase A

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Introduction

In mammals, the blood–testis barrier (BTB) that divides the seminiferous epithelium into the basal and the adluminal (apical) compartment provides an independent environment for the development of meiosis I/II and postmeiotic spermatid in the apical part, segregating it from the basal part and the systematic circulation (Cheng & Mruk 2010, 2012). This separation serves as an ‘immune barrier’ by avoiding, at least in part, the production of antibodies against the germ cell origin antigens expressed transiently during spermatogenesis. As the spermatogonia and the

preleptotene spermatocytes reside closely to the basement edge of the seminiferous epithelium, the BTB must undergo extensive restructuring during the epithelial cycle, though it is one of the tightest blood–tissue barriers, to facilitate the transit of preleptotene spermatocytes into the apical compartment at stage VIII (Russell 1977, Cheng *et al.* 2011b). This process is mediated by the establishment of new tight junction fibrils below the traversing spermatocytes before the disruption of the old ones above the traversing spermatocytes (Cheng & Mruk 2010, 2012).

Thus, zygotene spermatocytes can differentiate into diplotene spermatocytes and prepare for meiosis without compromising the immunological barrier function of the BTB (Cheng & Mruk 2010). Recent studies have demonstrated that integrity and dynamics of the BTB as a unique ultrastructure in the testis are conferred by coexisting junctions between Sertoli cells, namely tight junction (TJ), basal ectoplasmic specialization (ES; a testis-specific adherens junction type that also exists at the Sertoli–germ cell interface as ‘apical ES’), gap junction (GJ), and desmosome (Cheng *et al.* 2011a,b). Multiple physiological factors and biochemical molecules have been shown to participate in the regulation of BTB dynamics, such as cytokines (i.e. tumor necrosis factor α (TNF α (TNF)), and transforming growth factor β 3 (TGF β 3 (TGFB3)); Lui *et al.* 2001, Li *et al.* 2006, Xia *et al.* 2006), steroids (i.e. testosterone; Yan *et al.* 2008), actin-binding proteins (i.e. epidermal growth factor pathway substrate 8 (EPS8), actin-related protein 3 (ARP3 (ACTR3)), drebrin E, filamin A, etc.) (Lie *et al.* 2009, 2010a, Li *et al.* 2011, Su *et al.* 2012a), polarity proteins (i.e. PAR3/6, CDC42, scribble protein complex, etc.; Wong *et al.* 2008, 2010, Su *et al.* 2012b), and non-receptor protein tyrosine kinases (i.e. c-Yes, focal adhesion kinase (FAK); Xiao *et al.* 2011, Lie *et al.* 2012), whereas the underlying mechanisms by which these molecules regulate BTB still remain elusive.

During epithelial morphogenesis, the actin cytoskeleton substantially contributes to maintain TJ integrity (Madara *et al.* 1986, 1992, Balda *et al.* 1993), but the regulatory pathways that associate actin skeleton to TJ assembly still need to be elucidated. In the seminiferous epithelium, filamentous actin (F-actin) bundles at the Sertoli cell interface were found to lie perpendicular to the Sertoli cell plasma membrane and sandwiched between the cisternae of endoplasmic reticulum and the apposing Sertoli cell membrane (Vogl *et al.* 2008, Cheng & Mruk 2010, 2012). This unique arrangement of F-actin bundles at the BTB confers its remarkable adhesive strength, which was quantified to be even stronger than desmosome at the Sertoli–pre-step 8 spermatid interface, one of the strongest anchoring junctions (Wolski *et al.* 2005, Green & Simpson 2007, Green *et al.* 2010). In the past decades, small GTPases such as Rho/Rac were found to participate in the reorganization of F-actin and paracellular permeability in mammalian epithelia (Takaishi *et al.* 1997, Benais-Pont *et al.* 2003). Rab proteins (i.e. RAB3B, RAB13, and RAB8 (RAB8A)), another small GTPase subfamily that belongs to the Ras superfamily, are localized at the cell–cell interface in epithelial cells (Huber *et al.* 1993, Weber *et al.* 1994, Zahraoui *et al.* 1994) and have been revealed to be

involved in multiple cellular events like endocytic and exocytic processes (Schimmoller *et al.* 1998, Chavrier & Goud 1999), vesicle movement (Echard *et al.* 1998, Nielsen *et al.* 1999, White *et al.* 1999), and actin organization during the regulation of epithelial barrier function (Peranen *et al.* 1996). Among these Rab proteins, RAB13 has been shown to play a crucial role in structures and functions of TJs in polarized epithelial cells by mediating protein transportation to distinct membrane domains (Schimmoller *et al.* 1998, Takai *et al.* 2001, Pfeffer 2007). In Madin-Darby canine kidney (MDCK) cells, the active form of RAB13 was revealed to inhibit protein kinase A (PKA)-dependent phosphorylation and perturb TJ recruitment of the actin remodeling protein, namely vasodilator-stimulated phosphoprotein (VASP; Kohler *et al.* 2004). In the testis, RAB13 was found to interact directly with two putative actin-binding proteins, vinculin and espin, and function in ES dynamics at the Sertoli–germ cell interface (Mruk & Lau 2009). Herein, we investigated the involvement of RAB13 in Sertoli cell BTB function and the possible pathway by which it regulates the permeability of Sertoli cell TJ barrier.

Materials and methods

Animals and antibodies

Twenty-day-old male Sprague Dawley rats were obtained from the Department of Laboratory Animals, China Medical University (CMU). All experiments were performed at CMU in accordance to the NIH Guidelines for the Care and Use of Laboratory Animals. The protocol for animal handling and the treatment procedures were approved by the CMU Animal Care and Use Committee. Antibodies used for various experiments were commercially obtained from different vendors as described in Table 1. Reagents, unless otherwise specified, were from Sigma–Aldrich.

Primary Sertoli cell cultures

A widely used *in vitro* system was used in this study for isolation and culturing of Sertoli cells as described (Byers *et al.* 1986, Janecki *et al.* 1991a,b, 1992, Okanlawon & Dym 1996, Grima *et al.* 1998, Lui *et al.* 2001, Mruk & Cheng 2011). Sertoli cells were isolated from 20-day-old male Sprague Dawley rats (CMU) and cultured in serum-free DMEM/F12 (Gibco) at 35 °C in a humidified atmosphere of 5% CO₂ with epidermal growth factor, insulin, transferrin, and bacitracin as described (Mruk &

Table 1 Antibodies used for different experiments in this report. Antibodies used herein were found to cross-react with the corresponding proteins in the rat as indicated by the manufacturers

Antibody	Catalog no.	Host	Vender	Conjugation	Working dilution		
					IB	IF	IP
RAB13	ab136414	Rabbit	Abcam	–	1:1000		1:50
PKA α cat	sc-903	Rabbit	Santa Cruz Biotechnology	–	1:500		
Occludin	71-1500	Rabbit	Zymed/Invitrogen	–	1:250	1:50	
JAM-A	sc-25629	Rabbit	Santa Cruz Biotechnology	–	1:500		
ZO-1	61-7300	Rabbit	Zymed/Invitrogen	–	1:250		1:40
Actin	sc-1616	Goat	Santa Cruz Biotechnology	–	1:500		
Rabbit IgG	sc-2370	Bovine	Santa Cruz Biotechnology	HRP	1:3000		
Goat IgG	sc-2768	Rabbit	Santa Cruz Biotechnology	HRP	1:3000		
Rabbit IgG	A-31572	Donkey	Invitrogen	Alexa Fluor 555		1:100	

IB, immunoblotting; IF, immunofluorescence microscopy; IP, immunoprecipitation.

Cheng 2011). It was noted that Sertoli cells from this age of rats were differentiated and stopped to divide and were morphologically and functionally similar to those isolated from adult rats (Orth 1982, Li *et al.* 2001). Specifically, isolated Sertoli cells were plated on Matrigel (BD Biosciences)-coated bicameral units, culture dishes, or microscopic coverslips at different cell densities depending on different use in the following experiments: i) Millicell-HA inserts (Millipore) at $1.2 \times 10^6/\text{cm}^2$ for transepithelial electrical resistance (TER) measurement to assess the TJ–permeability barrier function of the Sertoli cells; ii) 12-well culture dishes at $0.5 \times 10^6/\text{cm}^2$ for lysate preparation; and iii) coverslips at $0.05 \times 10^6/\text{cm}^2$ for immunofluorescence analysis or F-actin staining. These cell densities for different studies were determined based on pilot experiments so that Sertoli cell TJ barrier function could be quantified, or sufficient proteins could be obtained for lysate preparation before immunoblot, or fluorescence microscopy could be performed to visualize protein distribution at the evenly arranged cell–cell interface. It has been confirmed by electron microscopy that a functional TJ barrier with ultrastructures of TJ, basal ES, GJ, and desmosome be established by Sertoli cells cultured on these Matrigel-coated utensils, mimicking the BTB *in vivo* (Lee & Cheng 2003, Siu *et al.* 2005, Lie *et al.* 2010b). On day 2 after isolation, a hypotonic treatment with 10 mM This (pH 7.4) at room temperature for 2 min was performed to lyse residual germ cells, so that these Sertoli cell cultures were with negligible contaminations of germ, Leydig, and myoid cells (Lee *et al.* 2004). For each experiment, triplicate culture units were used per time point and each experiment was repeated at least three times besides pilot experiments to determine the optimal experimental conditions.

Functional assessment of the Sertoli cell TJ permeability barrier *in vitro*

Using a digital resistance meter (DT-9208, Shanghai Jiajia, Shanghai, China), the TER across the Sertoli cell epithelial was measured and recorded daily (days 1–7) after isolation to assess the ability of an intact cell epithelium with functional TJ to resist current passage between the two electrodes placed respectively at the apical and the basal compartment of the bicameral units as described previously (Grima *et al.* 1998, Lui *et al.* 2001, Mruk & Cheng 2011). For each time point, triplicate bicameral units were set and each experiment was repeated at least three times using different cell cultures.

Treatment of testosterone and H89

To assess the effects of testosterone on Sertoli cell TJ barrier assembly, the steady-state level of *Rab13* expression, F-actin organization, and the role of PKA activity in these processes, Sertoli cells in some experiments were incubated with vehicle or testosterone (0.2 μM) with or without H89 (30 μM , a specific PKA inhibitor) in DMEM/F12 supplemented with growth factors from day 4 after isolation for TER assay or F-actin staining and terminated at different time points for immunoblot or PKA activity assay. The concentrations of testosterone and H89 used herein were selected based on earlier studies (Yan *et al.* 2008, Lie *et al.* 2010a, Su *et al.* 2012a). Sertoli cell cultures in triplicates were used for each time point and each experiment was repeated at least three times using different batches of Sertoli cells.

Transfection of siRNA duplexes in cultured Sertoli cells

Three siRNA duplexes specifically against rat *Rab13* were designed and synthesized by GenePharma and one was

selected after a preliminary experiment according to the knockdown efficiency. The selected sequence was subjected to BLAST to ensure no significant homology with other genes. The scrambled sequence was chemically synthesized by GenePharma as a non-targeting control. The siRNA sequences were as follows (sense and antisense respectively): *Rab13*, 5'-GAUGUAAGUGCUGUUGAAGtt-3' and 5'-CUUCAACAGCACUUACATCtt-3'; scrambled control, 5'-AGGAGAUGUGAUUUGGCUtt-3' and 5'-AGCCAAUAUCACAUCUCCUtt-3'.

Sertoli cells obtained from 20-day-old rats were cultured alone for 3 or 4 days before an intact cell epithelium was established. Thereafter, Sertoli cells cultured on different Matrigel-coated culture units were transfected with siRNA duplexes that specifically target *Rab13* or with scrambled siRNA control using Lipofectamine 2000 reagent (Invitrogen). The siRNA and Lipofectamine 2000 reagents were mixed in Opti-MEM (Invitrogen) before transfection. The final transfection volume was 0.5 ml DMEM/F12 (containing 3 µl Lipofectamine 2000) in the apical chamber of the bicameral unit, which seated in a well of a 24-well plate containing another 0.5 ml medium in the basal chamber, 1 ml DMEM/F12 (containing 4 µl Lipofectamine 2000) in a 12-well plate, or 2.5 ml DMEM/F12 (containing 8 µl Lipofectamine 2000) in a six-well plate with cells on coverslips. Based on pilot experiments for different cell densities with optimal phenotypes and without detectable cytotoxicity, the siRNA concentration used was selected at 150 nM for TER measurement, 100 nM for lysate preparation, and 80 nM for immunofluorescence analysis or F-actin staining. After transfection for 24 h, cells were rinsed and replenished with fresh DMEM/F12 and incubated for another 48 h before termination for lysate preparation or for another 24 h before fixation for immunofluorescence or F-actin staining. In groups to determine the role of PKA in regulation of Sertoli cell TJ barrier function after knockdown of *Rab13*, PKA inhibitor H89 (30 µM) was added into the culture medium daily after transfection for TER assay, immunofluorescence, F-actin staining, and co-immunoprecipitation (Co-IP) experiments.

Lysate preparation and immunoblot

Nonidet P-40 IP lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EGTA, 10% glycerol (v/v), 1% Nonidet P-40 (v/v), 2 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, 2 mM *N*-ethylmaleimide, 1 µg/ml leupeptin, and 1 µg/ml aprotinin, pH 7.4) was used to lyse testes and Sertoli cells. Protein concentrations were detected

using the protein assay kit (Bio-Rad Laboratories). Approximately 80 µg protein from testis or 30 µg protein from Sertoli cells were applied per lane and resolved by SDS–PAGE under reducing conditions for immunoblot with specific antibodies with actin serving as a loading control. Enhanced chemiluminescence was performed using a kit from Pierce Chemical Co. according to the manufacturer's protocols.

Co-immunoprecipitation

Co-IP was used to detect changes in protein–protein interactions in this study. A precleaning step was applied to avoid nonspecific IgG interaction. In brief, 2 µg normal rabbit IgG were added to 500 µg protein lysate from Sertoli cells and pre-incubated for 1 h. After addition of 10 µl protein A/G agarose (GenDEPOT) and incubation for another 1 h, the lysate was concentrated at 4 °C and the supernatant was collected for subsequent steps. The supernatant was then incubated with 2 µg normal rabbit IgG, which served as negative control, or 2 µg anti-RAB13 or anti-zonula occluden-1 (ZO-1) antibody overnight at room temperature. Thereafter, 20 µl protein A/G agarose beads were added to precipitate immunocomplexes, followed by gentle washing with IP lysis buffer. The immunocomplexes were at last extracted with an SDS–PAGE sample buffer at 100 °C for 5 min before being subjected to SDS–PAGE resolving and immunoblot analysis.

Immunofluorescence analysis and F-actin staining

Sertoli cells cultured on Matrigel-coated coverslips at 0.05×10^6 cells/cm² were fixed with paraformaldehyde (4%, w/v in PBS) at room temperature for 10 min before permeabilization with 0.1% Triton X-100 (v/v in PBS) for 4 min. Then the Sertoli cells were treated with 5% BSA (w/v in PBS) for 30 min for blocking before anti-occludin antibody (diluted in 1% BSA at 1:50) was added and incubated overnight at room temperature. Secondary antibody conjugated with CY3-555 (red) was diluted in 1% BSA (1:150) and incubated at room temperature for 1 h. For F-actin staining, Sertoli cells were incubated with Acti-stain 488 phalloidin (cytoskeleton) at the same time with the secondary antibody. Thereafter, the coverslips were mounted with Prolong Gold Antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were visualized with an Olympus BX60 fluorescence microscope (Olympus Optical Co.) and captured using a SpotRT digital camera (Diagnostic Instruments, Inc.) in TIFF format. Photoshop in Adobe Creative Suite Design

Premium (version 3.0) was used to analyze and adjust for contrast/brightness and image overlay. We note that all micrographs listed herein are representative images of at least three independent experiments using different batches of cells.

PKA activity assay

The activity of PKA was determined by the phosphorylation state of kemptide (Enzo Biochem), which is a specific synthetic substrate peptide (LRRASLG) of PKA, according to Grieco *et al.* (1994), with minor modification. In brief, fresh Sertoli cell lysate was diluted to 0.1 µg/µl with IP lysis buffer described earlier before 5 µl were transferred to an Eppendorf tube. Then a total of 25 µl reaction buffer (54 mM β-glycerophosphate, 24 mM 3-(N-morpholino)-propanesulfonic acid, 14.5 mM *p*-nitrophenylphosphate, 14.5 mM MgCl₂, 1 mM dithiothreitol, 14.5 mM EGTA, 0.12 mM EDTA, 75 mM genistein (a tyrosine kinase inhibitor), 10 µM ML-9 (a myosin light-chain kinase inhibitor), 1 mg/ml kemptide, 1 µg/ml each of leupeptin, aprotinin, pepstatin, chymostatin, and trypsin–chymotrypsin inhibitor) was added to the tube and the PKA reaction was started by adding 25 µl 20 µCi/ml [γ-³²P]ATP (Beijing FuRui Biotechnology, Beijing, China) and then incubated for 7 min at 30 °C. Thereafter, 25 µl reaction buffer were extracted and spotted on Whatman p81 paper and the reaction was stopped by 5% phosphorous acid. After thorough washing, the radioactivity on the paper was counted using a BECKMAN scintillation counter.

To confirm the phosphorylation state of kemptide, a parallel experiment was performed by incubating 1.5 µg protein lysate with 50 µl reaction buffer described earlier containing 50 µCi/ml [γ-³²P]ATP at 37 °C for 30 min. Then equal amount of 2×SDS sample buffer was added to stop the reaction before resolving the sample by SDS–PAGE and the phosphorylation state of kemptide was detected by autoradiography.

Statistical analysis

GB-STAT statistical analysis software (version 7.0; Dynamic Microsystems) was used to analyze data from the TER experiment, immunoblot, and Co-IP analysis. All data presented here are results from at least three independent experiments. Statistical significance was determined with one-way ANOVA coupled with two-tailed Dunnett's test. Values represent mean ± s.d.

Results

The steady-state level of Rab13 in Sertoli cell BTB *in vitro* and its interaction with catalytic subunit of PKA

Using the *in vitro* system of Sertoli cell culturing (Mruk & Cheng 2011), we found that Rab13 was expressed both in the rat testis and in the Sertoli cells cultured *in vitro* using an antibody specific to RAB13 (Fig. 1A). However, the

Figure 1

The expression of Rab13 in the rat testis and in Sertoli cell BTB *in vitro* and its interaction with the catalytic subunit of PKA. (A) Immunoblot analysis of RAB13 in lysate of rat testis (80 µg protein) or Sertoli cells (SCs) (30 µg protein) cultured *in vitro* with a functional TJ barrier established by approximately days 2–3 after isolation. Actin served as a loading control. (B) Histogram corresponds to the result of (A) describing the relative RAB13 level in SCs from days 1 to 7 after each data point was normalized against actin. RAB13 level at day 1 was arbitrarily set at 1 against which statistical comparison was performed. Each bar is a mean ± s.d. of *n* = 3 batches of Sertoli cells. **P* < 0.05 and ***P* < 0.01. (C) Structural interaction between RAB13 and the catalytic subunit of PKA was examined by Co-IP analysis using 500 µg protein lysate of SCs cultured for 4 days with an established TJ permeability barrier. SCs lysate (30 µg protein) alone without Co-IP and with Co-IP using normal rabbit IgG to substitute for the precipitating antibody respectively served as the corresponding positive and negative controls. The blot of IgG_H and IgG_L chains in the bottom panel illustrated equal IgG/IP-antibody loading in this Co-IP experiment. These findings are representative data from three independent experiments.

protein level of *Rab13* was detected to decrease during the assembly of the TJ permeability barrier from days 1 to 7 after isolation (Fig. 1A and B). Furthermore, RAB13 was also found to structurally interact with the catalytic subunit of PKA by Co-IP (Fig. 1C), which implied the possible participation of RAB13 in Sertoli cell TJ permeability barrier assembly by regulating the activity of PKA.

The protein level of *Rab13* in the Sertoli cells decreases with increasing PKA activity under the treatment of testosterone

Testosterone was known to promote Sertoli cell BTB integrity by mediating recycling of integral membrane proteins and strengthening the cortical actin filament (F-actin) at the cell–cell interface, resulting in a surge in TJ barrier function (Meng *et al.* 2005, Yan *et al.* 2008, Lie *et al.* 2010a, Su *et al.* 2012a). Therefore, we herein used the testosterone-treated Sertoli cells as a model of enhanced TJ barrier assembly to explore whether an increased Sertoli cell TJ assembly is accompanied with changes in protein level of *Rab13*. Sertoli cells cultured *in vitro* with an established permeability barrier were treated with either testosterone or vehicle solution from day 4 after isolation and terminated at specified time points. While the protein level of *Rab13* decreased steadily during the 48-h experimental period in the vehicle control group, its level in the testosterone-treated group displayed a sharper descending trend, significantly lower vs the corresponding vehicle control from 12 h after treatment (Fig. 2A and B).

In order to understand whether testosterone can affect PKA activity as the catalytic subunit of PKA was found to directly interact with RAB13, an *in vitro* phosphorylation system was used and the PKA activity was reflected by the [³²P] incorporation level of kemptide, a known substrate of PKA, and measured by a radioactivity scintillation counter. Opposite from *Rab13* expression, PKA activity in Sertoli cell lysate exhibited an increasing trend after testosterone treatment with significant difference from the control group at time points after 12 h (Fig. 2C). This result was further supported by a parallel autoradiography experiment with corresponding samples described earlier (Fig. 2D). These results together suggest that RAB13 may participate in the Sertoli cell BTB assembly via affecting PKA activity.

Inhibition of PKA activity antagonizes the enhancing effect of testosterone on Sertoli cell TJ barrier function

To confirm the role of PKA in testosterone-induced Sertoli cell BTB enhancing, Sertoli cells cultured *in vitro* were

Figure 2

Effects of testosterone on the protein level of *Rab13* and PKA activity in Sertoli cells (SCs) cultured *in vitro*. (A) Immunoblot analysis of *Rab13* protein level using lysates of SC (30 µg protein) treated with testosterone (0.2 µM) from day 4 after isolation for the specified time, with actin serving as a loading control. The relative RAB13 level decreased after 12-h treatment of testosterone compared with the vehicle control. (B) Histogram summarizing the results shown in (A) after normalizing each data point against actin. RAB13 level at time 0 was arbitrarily set at 1. Protein level changes were compared between treatment and the corresponding vehicle control at the same time point. Each bar is a mean $\pm$ s.d. of $n=3$ independent experiments. ** $P<0.01$. (C and D) PKA activity analysis using SC lysates after testosterone treatment. For each time point, SCs were lysed and PKA activity was examined by scintillation counting (C) using 0.5 µg protein per time point and autoradiography (D) using 1.5 µg protein per time point, with kemptide serving as the PKA substrate. PKA activity changes were compared between treatment and the corresponding vehicle control at the same time point in (C). Each bar is a mean $\pm$ s.d. of $n=3$ independent experiments. * $P<0.05$ and ** $P<0.01$. The autoradiograph shown in (D) was representative data from three independent experiments.

treated with H89, a known inhibitor of PKA (Lochner & Moolman 2006, Murray 2008), in combination with testosterone from day 4 after isolation. Indeed, testosterone alone was shown to tighten the TJ barrier by causing a significant increase in TJ integrity compared with the vehicle control as presented by the TER assay (Fig. 3A). Interestingly, the addition of the PKA inhibitor H89 then exhibited a statistically significant inhibition on the effect of testosterone by decreasing TJ integrity, though not to the level as low as the vehicle control (Fig. 3A).

Figure 3

Inhibition of PKA activity by H89 perturbs the effect of testosterone on Sertoli cell TJ integrity via changes in F-actin conformation. (A) Sertoli cells were cultured at a density of $1.0 \times 10^6/\text{cm}^2$, and the establishment of a TJ permeability barrier was assessed by TER measurement across the cell epithelium. The TJ barrier was established by day 3 with a stable TER. Cells were then treated with vehicle, testosterone (0.2 μM), or a mixture of testosterone and H89 (testosterone (0.2 μM) + H89 (30 μM)) from day 4 to 6 (see black arrows). Each data point is the mean $\pm$ s.d. of $n=3$ replicates. The significant difference was analyzed between testosterone-treated and vehicle control groups (see black stars), or between testosterone- and testosterone + H89-treated groups (see blue stars). $*P < 0.05$ and $**P < 0.01$. (B) F-actin (green) was stained by acti-stain 488 phalloidin (cytoskeleton) in testosterone- or testosterone + H89-treated Sertoli cells ($0.05 \times 10^6/\text{cm}^2$) vs vehicle control. Cell nuclei were visualized with DAPI (blue). Testosterone treatment led to a more intense localization of F-actin at the cell cortex (see arrowheads), whereas testosterone + H89 treatment kept the F-actin organization similar as in the vehicle control. Scale bar in (B, a), 25 μm for all micrographs in (B).

This finding was further supported by an experiment to visualize actin filaments in the Sertoli cells. It was observed that treatment of testosterone alone led to a more extensive alignment of F-actin at the cell junction sites while the addition of H89 attenuated this effect and weakened F-actin staining at these positions (Fig. 3B). These results implicated that PKA activity inhibition can reverse the enhancing effect of testosterone on Sertoli cell TJ barrier assembly, at least in part, by maintaining the normal distribution and organization of F-actin, especially at the cell-cell interface.

Knockdown of *Rab13* by RNAi in Sertoli cells leads to an increase in PKA activity

To discriminate whether the change of PKA activity in testosterone-treated Sertoli cells is a result of a decrease in *Rab13* expression, *Rab13* was knocked down by transfecting Sertoli cells with specific *Rab13* siRNA duplexes vs scrambled siRNA control as stated in the Materials and methods section. The immunoblot analysis showed that when *Rab13* was silenced by $\sim 70\%$ compared with the scrambled control, no off-target effect was detected when TJ-associated proteins, namely occludin, JAM-A, and ZO-1, and PKA catalytic subunit α were monitored (Fig. 4A and B). However, the knockdown of *Rab13* by 70% was revealed to lead to an increase in PKA activity vs the control group in the Sertoli cell lysates by [^{32}P] incorporation tests (Fig. 4C and D). This finding further prompted the notion that RAB13 may participate in Sertoli cell TJ barrier assembly by regulating the activity of PKA.

RAB13 participates in Sertoli cell TJ dynamics by regulating F-actin organization, junction protein recruitment, and protein-protein interaction through PKA

As the steady-state level of RAB13 decreased during the increment of Sertoli cell TJ barrier caused by testosterone, RAB13 was also knocked down in a TJ integrity assay to reveal its relationship with Sertoli cell BTB assembly *in vitro*. After transfection with the siRNA duplexes targeting *Rab13* in Sertoli cells cultured on Matrigel-coated bicameral unit for 24 h from day 3 after isolation, the Sertoli cell TJ permeability barrier was shown to increase significantly compared with the non-targeting control (Fig. 5A). Notably, the addition of the PKA inhibitor H89 was found to antagonize the effect of *Rab13* silencing by reducing the TJ barrier function vs the group only treated with *Rab13* siRNA.

To explore the mechanism by which PKA activity affects the phenotype of Sertoli cells caused by *Rab13*

silencing, F-actin, the cytoskeleton that participates in the formation of Sertoli cell BTB by lying perpendicular to the cell plasma and sandwiched between the cisternae of endoplasmic reticulum and the apposing Sertoli cell plasma membranes (Vogl *et al.* 2000, Cheng & Mruk 2010, 2012), and occludin, the TJ constitution protein (Nelson & Yeaman 2001, Mruk *et al.* 2008), were visualized under fluorescence microscopy. The results showed that F-actin staining after *Rab13* silencing resembled that after testosterone treatment, i.e. with a remarkably concentrated signal at the cell cortex near the plasma membrane (Fig. 5B b and e vs a and d), while the inhibition of PKA by

H89 was found to resist the effect of *Rab13* knockdown on F-actin reorganization and present an even F-actin distribution (Fig. 5B c and f vs b and e). A very similar variation in occludin localization was visualized by immunofluorescence: an intensified signal at the cell-cell interface after *Rab13* silencing (Fig. 5C b and e vs a and d) but this remained unchanged when H89 was applied in addition to *Rab13* siRNA duplexes (Fig. 5C c and f vs b and e), which may lead to the changes in TER together with F-actin reorganization.

Because *Rab13* knockdown by RNAi was found to increase the Sertoli cell TJ barrier *in vitro* without affecting

Figure 4

Knockdown of *Rab13* by RNAi in Sertoli cells affects the activity of PKA. (A) Sertoli cells ($0.5 \times 10^6/\text{cm}^2$) were cultured alone for 4 days before being transfected with specific *Rab13* siRNA vs scrambled control siRNA duplexes for 24 h. Cultures were terminated 72 h later to obtain lysates for immunoblot analysis, which detected no off-target effect on several BTB proteins (i.e. occludin, ZO-1, and JAM-A) and the catalytic subunit α of PKA (PKA α cat) when *Rab13* was knocked down significantly. (B) Histograms based on immunoblot results shown in (A) after normalizing the data

against actin. The upper panel illustrates that the efficacy of *Rab13* RNAi is $\sim 70\%$. Protein levels in the control were arbitrarily set at 1. Each bar is mean $\pm$ s.d. of $n=3$ independent experiments. $**P<0.01$. (C and D) PKA activity analysis using Sertoli cell lysates after *Rab13* knockdown by scintillation counting (C) and autoradiography (D). The incorporation of [P^{32}] in kemptide was increased by about 30% in the *Rab13* silencing group vs the scrambled control group, representing an increment in PKA activity. Bar is mean $\pm$ s.d. of $n=3$ independent experiments. $**P<0.01$.

Figure 5

RAB13 regulates Sertoli cell BTB integrity *in vitro* by regulating F-actin organization, junction protein recruitment, and protein-protein interaction through PKA. (A) Sertoli cells cultured on Matrigel-coated bicameral units were assessed by TER measurement across the cell epithelium daily after isolation. On day 3, cells were treated with specific *Rab13* siRNA vs scrambled control siRNA duplexes for 24 h. In some *Rab13* siRNA treated groups, H89 (30 μ M) was added to the culture medium daily after transfection (see black arrows). Each data point is the mean $\pm$ s.d. of $n=3$ replicates. The significant difference was analyzed between *Rab13* siRNA and control groups (see black stars) or between *Rab13* siRNA and *Rab13* siRNA+H89 groups (see blue stars). The graph shown here was representative data from three independent experiments. * $P<0.05$ and ** $P<0.01$. (B and C) F-actin (green, B) and occludin (red, C) were stained in *Rab13* siRNA- or *Rab13* siRNA+H89-treated Sertoli cells ($0.05 \times 10^6/\text{cm}^2$) vs scrambled siRNA control. Cell nuclei were visualized with DAPI (blue). *Rab13* knockdown led to a more concentrated distribution of F-actin the

near cell membrane (see white arrowheads in (B)) and a more intense signal of occludin at the cell-cell interface (see yellow arrowheads in (C)), while the addition of H89 treatment kept the distribution of F-actin or occludin similar with that of the control. Scale bar in (B, a), 25 μ m for all micrographs in (B) and (C). (D) Co-IP was performed to assess changes in protein-protein interactions between ZO-1 and occludin using 500 μ g protein lysate from Sertoli cells ($0.5 \times 10^6/\text{cm}^2$) following RNA interference. Co-IP using normal rabbit IgG to substitute for the precipitating antibody served as the negative control. The blot of IgG_H and IgG_L chains in the bottom panel illustrated equal IgG/IP-antibody loading in this Co-IP experiment. This finding is representative data from three independent experiments. (E) Histogram summarizes the result shown in (D) in which the relative association of occludin and ZO-1 in control group was arbitrarily set at 1. Each bar represents a mean $\pm$ s.d. of $n=3$ independent experiments. ** $P<0.01$.

the steady-state levels of junction proteins but upregulating the recruitment of occludin to cell–cell interface, we next performed Co-IP to examine whether there was any alteration of protein–protein interactions in adhesion complexes at the Sertoli cell BTB. As anticipated, there was an increased interaction between TJ protein occludin and ZO-1 after *Rab13* silencing by ~40% compared with the scrambled siRNA control group, which was significantly weakened in the group treated with both *Rab13* siRNA duplexes and H89 (Fig. 5D and E).

Discussion

To date, more than 60 Rab GTPases have been discovered and defined as small GTP-binding proteins of 20–30 kDa that belong to the Ras GTPase superfamily, the key regulators of protein traffic in compartmentalized eukaryotic cells (Olkonen & Stenmark 1997, Schimmoller *et al.* 1998, Deneka *et al.* 2003, Hutagalung & Novick 2011). Rab GTPases exert their roles in multiple cellular events such as transport, recycling, endocytosis, exocytosis, and cytoskeletal organization, primarily by converting between the active GTP-bound and the inactive GDP-bound forms, which is regulated by GDP/GTP exchange factor, GTPase activating protein, and GDP dissociation inhibitor (Hall 1994, Takai *et al.* 1995, 1996, Mruk *et al.* 2005). In epithelial cells, RAB3B, RAB8, and RAB13 have been found to exist at the cell–cell contacts and have cross talk with tight or adherens junctions (Huber *et al.* 1993, Weber *et al.* 1994, Zahraoui *et al.* 1994). For instance, many reports have revealed the mediation of E-cadherin endocytosis by Rab proteins during cell junction disassembly (Maxfield & McGraw 2004, Lock & Stow 2005, Palacios *et al.* 2005).

In the testis, several Rab proteins have been demonstrated to exist and exert their roles during spermatogenesis within the seminiferous epithelium. For example, RAB8B was shown to associate with the cytoskeletal networks and participate in Sertoli–germ cell junction dynamics in the testis (Lau & Mruk 2003). The association between RAB4A and adherens junction signaling protein α - and β -catenin was also reported to increase during the junction restructuring accompanied with the loss of spermatocytes and spermatids from the seminiferous epithelium under the treatment of adjuvant, a male contraceptive known to induce germ cell loss from the epithelium (Cheng *et al.* 2005, Mruk *et al.* 2007). Although RAB13, another member of Rab GTPase family, has been found to participate in the apical ES dynamics between Sertoli and elongating/elongated spermatids

(Mruk & Lau 2009), it seems no investigation was performed to clarify its role in TJ assembly/restructuring between Sertoli cells, which is considered as a crucial ‘switch’ of the BTB for the transit of preleptotene spermatocytes at stage VIII of the epithelial cycle.

In this report, we demonstrated that RAB13 is the likely regulator of TJ dynamics at the BTB using primarily cultured Sertoli cell epithelium *in vitro*, which has been confirmed to mimic the BTB *in vivo* and is widely used by investigators to study the biology and regulation of BTB dynamics (Byers *et al.* 1986, Janecki *et al.* 1991b, Grima *et al.* 1992, Kato *et al.* 2006, Chen *et al.* 2012, Robillard *et al.* 2012). First, using an antibody specific to Rab13, Rab13 was shown to exist in both testis and Sertoli cell lysates. Interestingly, its protein level decreased during the assembly of the Sertoli cell TJ–permeability barrier *in vitro* after isolation, implying that its expression is relevant to BTB dynamics. This postulate is further supported by findings using an *in vitro* model in which the Sertoli cell BTB is promoted under the treatment of testosterone, which has been verified to increase endocytosis of integral membrane proteins, such as occludin and N-cadherin, at the BTB and induce recycling of endocytosed proteins back to the Sertoli cell surface (Cheng & Mruk 2010, 2012). After the treatment of testosterone for 12 h, the steady-state level of RAB13 was found to decrease significantly compared with the control group, prompting a negative correlation between RAB13 and Sertoli cell TJ assembly. Indeed, a knockdown of *Rab13* by RNAi was shown to associate with an increment of the Sertoli cell TJ–permeability barrier, probably due to the strengthened localization of F-actin and occludin at the cell–cell interface visualized under fluorescence microscopy. More importantly, an increased protein–protein interaction was also detected between occludin and ZO-1 after *Rab13* silencing, though the total level of these junction proteins remained unchanged. These data seem to support the notion that RAB13 participates in Sertoli cell BTB dynamics by regulating junction protein endocytosis and recycling, coinciding with a recent study in epithelial MTD-1A cells, which revealed that a dominant active mutant of *Rab13* inhibited continuous endocytic recycling of occludin to the cell surface (Morimoto *et al.* 2005).

Studies in other epithelia including blood–tissue barriers have indicated that the phosphorylation status of integral membrane proteins (e.g. occludin, claudins, and N-cadherin) and peripheral adaptors (e.g. β -catenin) at cell–cell junctions play a key role in determining adhesive function at the cell–cell interface (Gumbiner 2000, Cheng & Mruk 2002, Dorfel *et al.* 2009, Findley &

Koval 2009, Raleigh *et al.* 2011). Actually, many cellular signaling proteins such as PKA and various PKC isotypes are involved in epithelial cell TJ dynamics (Balda *et al.* 1993, Denisenko *et al.* 1994). For example, it was found in T84 intestinal epithelial cells that an elevation of intracellular cAMP level enhanced the barrier function while an inhibition of PKA diminished barrier recovery (Lawrence *et al.* 2002). In epithelial MDCK cells, PKA signaling was also demonstrated to play a significant role in TJ assembly by recruiting claudin 1 and ZO-1 to the TJ and regulating the phosphorylation and distribution of the actin-binding protein VASP (Kohler *et al.* 2004). In this

study, we detected a structural interaction between RAB13 and the catalytic subunit α of PKA. Furthermore, the decline of RAB13 in Sertoli cell epithelium induced by testosterone was found to be accompanied by an increased PKA activity. In order to discriminate whether this change in PKA activity was caused by RAB13 declination, we performed RNA interference to knockdown *Rab13* in cultured Sertoli cells and found an increment of PKA activity following $\sim 70\%$ knockdown of *Rab13* expression. Importantly, the enhancing effects of both testosterone and *Rab13* silencing on the Sertoli cell TJ barrier can be antagonized by the PKA activity inhibitor H89, which

Figure 6

A schematic drawing illustrating the possible role of RAB13 in BTB restructuring and apical ES disassembly. (A) Seminiferous epithelium with intact cell junctions at the apical ES and the BTB, according to most of the seminiferous epithelial cycle of adult rat testis except stages VIII–IX. Actin filaments at these two sites are tightly bundled to provide powerful support for the junction complexes. The status of the junction integrity at the apical ES and the BTB is maintained, at least in part, by the relative contribution of testosterone, which is known not only to facilitate junction protein recycling for junction assembly but also responsible for F-actin organization underneath the Sertoli cell cortex. (B) Restructuring

seminiferous epithelium with simultaneous happening of apical ES disassembly and BTB restructuring, according to stages VII–IX of the seminiferous epithelial cycle. The lower level of testosterone at these stages increases the *Rab13* expression, which might interact directly with espin or vinculin (Mruk & Lau 2009), two actin-bundling proteins, inducing disassembly of F-actin bundles and then disassembly of the apical ES. On the other hand, the enhancing level of RAB13 at the BTB could lead to an inactivation of PKA in Sertoli cells, which in turn results in a trafficking of TJ protein (i.e. occludin) to cell cytosol and the breakdown of the BTB, facilitating the process of spermatogenesis.

emphasized the possibility that PKA might work as a downstream effector of RAB13 in regulating BTB dynamics. This postulation was inconsistent with a previous study investigating the role of RAB13 in TJ dynamics in MDCK cells (Kohler *et al.* 2004).

In summary, we here suggest that RAB13 is a regulator of Sertoli cell BTB dynamics via its effects on PKA activity. Thus, besides participating in apical ES dynamics at the Sertoli–germ cell interface (Mruk & Lau 2009), RAB13 may also exert its role in integral membrane protein trafficking to facilitate BTB dynamics between adjacent Sertoli cells during the seminiferous epithelial cycle (Fig. 6). This process must involve reorganizations of actin filament distribution at the Sertoli cell cortex. In this context, it will be of interest in future studies to determine the actin-regulating molecules downstream of the RAB13–PKA pathway in rearranging F-actin underneath the Sertoli cell membrane, which is considered as a particular feature that characterizes BTB dynamics (Cheng & Mruk 2002, 2010).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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