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Differential effects of testosterone and TGF-β3 on endocytic vesicle-mediated protein trafficking events at the blood-testis barrier[§]

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Abstract

The homeostasis between protein endocytosis, transcytosis, recycling and endosome- or ubiquitin-mediated protein degradation determines the junction integrity in epithelial cells including Sertoli cells at the blood-testis barrier (BTB). Studies have shown that androgens and cytokines (e.g., TGF-β3) that are known to promote and disrupt BTB integrity, respectively, accelerate protein endocytosis at the BTB. We hypothesized that testosterone-induced endocytosed proteins are transcytosed and recycled back to the Sertoli cell surface, whereas cytokine-induced endocytosed proteins are degraded so that androgens and cytokines have opposing effects on BTB integrity. Herein, we report that both testosterone and TGF-β3 induced the steady-state level of clathrin, an endocytic vesicle protein. Testosterone and TGF-β3 also induced the association between internalized occludin (a BTB integral membrane protein) and clathrin, as well as early endosome antigen-1 (EEA-1). Interestingly, testosterone, but not TGF-β3, also induced the levels of proteins that regulate protein transcytosis (e.g., caveolin-1) and recycling (e.g., Rab11), and their association with internalized occludin and N-cadherin from the cell surface. In contrast, TGF-β3, but not testosterone, induced the level of ubiquitin-conjugating enzyme E2 J1 (Ube2j1), a protein crucial to the intracellular protein degradation pathway, and its association with internalized occludin. Based on these findings and recent reports in the field, we hypothesize that the concerted effects of testosterone and TGF-β3 likely facilitate the transit of preleptotene spermatocytes at the BTB while maintaining the immunological barrier in that testosterone induces the assembly of "new" tight junction (TJ)-fibrils below migrating spermatocytes via protein transcytosis and recycling *before* cytokines induce the disassembly of "old" TJ-fibrils above spermatocytes via endocytic vesicle-mediated degradation of internalized proteins.

Keywords

testis; spermatogenesis; blood-testis barrier; testosterone; TGF-β3; protein endocytosis; protein recycling; protein transcytosis; protein degradation; seminiferous epithelial cycle

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Introduction

The concept of the "blood-testis barrier" (BTB) was conceived almost a century ago when dyes administered to animals failed to stain the brain and the seminiferous tubules of the testis [1–2]. Subsequent studies showed that the BTB is one of the tightest blood-tissue barriers in mammals [3]. However, the BTB, unlike other blood-tissue barriers (e.g., blood-brain or blood-retinal barriers) which are constituted by tight junctions (TJ) between endothelial cells, is constituted by specialized junctions between adjacent Sertoli cells near the basement membrane in the adult mammalian testis, such as in rodents and humans [1,4–5]. While the BTB is extensively restructured at stage VIII of the seminiferous epithelial cycle of spermatogenesis to accommodate the transit of preleptotene spermatocytes [6], the immunological barrier conferred by the BTB cannot be compromised, even transiently. This is because the BTB sequesters meiosis and all events relating to post-meiotic germ cell development in a specialized microenvironment behind this immunological barrier known as the apical (or adluminal) compartment of the seminiferous epithelium. In this way, the host's immune system cannot develop antibodies against germ cell specific antigens, some of which appear transiently, during spermatogenesis. However, the molecular mechanism(s) that regulates immunological barrier function while facilitating the transit of primary spermatocytes at stage VIII of the epithelial cycle remains unknown. Recent studies have suggested that "new" TJ-fibrils may be formed below a migrating preleptotene spermatocyte via the action of testosterone which is known to promote BTB integrity [7–11] by mediating *de novo* synthesis of integral membrane proteins (e.g., occludin) [7] at the site. This occurs before "old" TJ-fibrils found above migrating spermatocytes are completely disassembled, which is likely mediated by cytokines (TGF-β2, TGF-β3 and TNFα) that perturb BTB integrity [12–14]. In short, the immunological barrier is likely maintained during the transit of preleptotene spermatocytes the BTB at stage VIII of the epithelial cycle via the opposing effects of testosterone and cytokines at the microenvironment of the BTB.

Two recent reports indeed support such a hypothesis [15–16]. For instance, both testosterone and cytokines were shown to accelerate the endocytosis (i.e., internalization of integral membrane proteins via endocytic vesicle so that they can either be recycled back to the same cell surface, transcytosed to a different cellular compartment or cellular site, or be sent to degradation mediated via the endosome or the ubiquitin pathway [17–19]) of integral membrane proteins at the Sertoli cell BTB using the techniques of biotinylation and a biochemical endocytosis assay [15–16]. However, testosterone, but not TGF-β2, was shown to promote the recycling of endocytosed proteins back to the Sertoli cell surface based on a recycling assay which tracked the reappearance of internalized biotinylated proteins (e.g., occludin) on the cell surface [15]. Interestingly, TGF-β2, but not testosterone, appeared to direct endocytosed proteins for intracellular degradation since TGF-β2-treated Sertoli cells were shown to sequester internalized occludin which associated more extensively with Rab9 [15], a known late endosome marker [20–21]. Thus, we postulated that testosterone-induced protein recycling targets endocytosed proteins to the "new" BTB site behind transiting spermatocytes via transcytosis (namely, relocation of endocytosed proteins from a specific cellular site to another location within an epithelial cell, and in some cases to an adjacent epithelial cell, crossing the cell border [22–23]) in order to assemble "new" TJ-fibrils prior to the disassembly of the "old" BTB site above spermatocytes via endosome-mediated degradation promoted by cytokines (e.g., TGF-β2) [15]. Indeed, this postulate is also supported by a recent study which illustrated that treatment of Sertoli cells with testosterone facilitated redistribution of TJ-proteins (e.g., occludin and claudin-11) to the Sertoli cell-cell interface [24], thereby making the TJ-barrier tighter. In order to further confirm this hypothesis which is based on biochemical endocytosis and recycling assays [15–16], we sought to use dual-labeled immunofluorescence analysis to assess the differential effects of testosterone and TGF-β3 on BTB dynamics using markers for protein endocytosis (e.g.,

clathrin, EEA-1), transcytosis (e.g., caveolin-1), recycling (e.g., Rab11), and endosome- or ubiquitin-mediated protein degradation (e.g., Ube2j1, ubiquitin-conjugating enzyme E2, J1) in Sertoli cells cultured *in vitro* with an established functional TJ-barrier that mimics the BTB *in vivo*. This is the subject of the present investigation.

Materials and Methods

Animals

Sprague-Dawley male rats at 20 days of age were purchased from Charles River Laboratories, Inc. (Kingston, NY). The use of animals for studies in this report was approved by the Rockefeller University Institutional Animal Care and Use Committee (IACUC) with Protocol Numbers 06018 and 09016.

Primary Sertoli cell cultures

Sertoli cells were isolated from 20-day-old rat testes as previously described [25–26]. Cells were plated either on Matrigel [(BD Biosciences, Franklin Lakes, NJ), diluted 1:7 in serum-free F12/DMEM (Sigma-Aldrich, St. Louis, MO)]-coated 12-well dishes (Corning Inc., Corning, NY) at 0.5×10^6 cells/cm^2 or on 25-mm glass coverslips (Thomas Scientific, Swedesboro, NJ) at 0.05×10^6 cells/cm^2 and cultured in F12/DMEM supplemented with 1.2 g/L sodium bicarbonate, 5 μg/ml human transferrin, 10 μg/ml bovine insulin, 2.5 ng/ml epidermal growth factor (EGF) and 10 μg/ml bacitracin [25,27]. Cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 (v/v) at 35 °C. About 48-hr thereafter, cells were treated with a hypotonic buffer (20 mM Tris, pH 7.4 at 22 °C) for 2.5 min to lyse residual germ cells [28]. As such, these Sertoli cells had a purity greater than 98% with negligible contamination of germ cells, Leydig cells and peritubular myoid cells. Media supplemented with growth factors were replaced every 24-hr. On day 4 *in vitro*, cells were treated with or without testosterone (2×10^{-7} M, dissolved in ethanol; Sigma-Aldrich) or TGF-β3 [3 ng/ml, dissolved in F12/DMEM (Sigma-Aldrich) containing 0.1% BSA] for specific time points at 0-, 5-, 15-, 30-, and 60-min, and cultures were terminated with IP lysis buffer [50 mM Tris, pH 7.4 at 22 °C containing 0.15 M NaCl, 10% glycerol (v/v), 1% NP-40 (v/v), and protease (Sigma-Aldrich, Cat. # D2714) and phosphatase inhibitor cocktails (Sigma-Aldrich, Cat. #s P2850 and P5726)] to obtain cell lysates or fixed with paraformaldehyde for immunofluorescence microscopy. Vehicle controls consisted of incubating Sertoli cells with ethanol or growth factor supplemented F12/DMEM containing 0.1% BSA. We have earlier shown that a functional Sertoli cell TJ-permeability barrier was established by ~day 2–3 [7,29] with ultrastructures of TJ, basal ES, and desmosome that mimicked the BTB *in vivo* as detected by electron microscopy ~48-hr after cell plating [30]. Thus, these cultures had formed an intact Sertoli cell epithelium similar to the BTB *in vivo* when they were used on day 3 or day 4 for our experiments. The rationale of utilizing a Sertoli cell density at 0.5×10^6 cells/cm^2 and 0.05×10^6 cells/cm^2 for cultures to be subsequently used for lysate preparation and for fluorescent microscopy dual-labeled immunofluorescence analysis is as follows. Earlier studies have shown that at $0.5–0.75 \times 10^6$ cells/cm^2, the Matrigel-coated culture dishes or bicameral units were covered with a densely and tightly packed monolayer of Sertoli cells in columnar shape, suitable for assessing the TJ-permeability barrier in a physiological assay (e.g., quantifying the transepithelial electrical resistance, TER, across the Sertoli cell epithelium) [31–32]. Also, sufficient total protein could be obtained either in 6- or 12-well dishes for immunoblot analysis against multiple Sertoli cell BTB markers when lysates were prepared from these cultures. For dual-labeled immunofluorescence analysis, if a cell density at 0.5×10^6 cells/cm^2 was used, cells were too densely populated with the distance between cell nuclei too close, so that TJ- (e.g., occludin, claudin-11) or basal ES-proteins (e.g., N-cadherin) localized to the cell-cell interface would be difficult to be visualized and the changes in their

distribution following different treatments would be even more difficult to be detected. Thus, a 10-fold less cell density, 0.05×10^6 cells/cm^2, was selected based on initial pilot experiments; however, at this cell density, 150-mm dishes would have to be used to obtain sufficient protein lysates for immunoblot analysis, but the cost of Matrigel would become prohibitively expensive to perform such experiments.

Dual-labeled immunofluorescence analysis

Sertoli cells were plated on Matrigel-coated 25-mm coverslips at 0.05×10^6 cells/cm^2 and hypotonically treated 48 hr after plating to lyse residual germ cells, washed in F12/DMEM and incubated at 35°C in a humidified environment with 95% air/5% CO2 (v/v) for another 24-hr. On day 4, cells were incubated with F12/DMEM supplemented with or without testosterone (2×10^{-7} M) or TGF-β3 (3 ng/ml) at 35 °C for 60 min. Dual-labeled immunofluorescence analysis was performed as described [15–16]. In short, cells were washed twice with PBS/CM buffer (10 mM NaH_2PO_4, pH 7.4 at 22 °C containing 0.15 M NaCl, 0.9 mM $CaCl_2$ and 0.33 mM $MgCl_2$) to remove residual F12/DMEM, fixed in 4% paraformaldehyde (w/v) for 10 min, and permeabilized with 0.1% Triton X-100 (v/v) for 10 min. Non-specific binding sites were then blocked with 10% normal goat serum (NGS, v/v) in PBS (10 mM NaH_2PO_4, pH 7.4 at 22 °C containing 0.15 M NaCl) at room temperature for 1 hr. Sertoli cells were incubated with primary antibodies (Table 1, note: each antibody used in this study was pre-screened by immunoblotting using Sertoli cell lysates to ensure that it cross-reacted with a protein band of expected molecular weight corresponding to the target protein before being used for immunofluorescence microscopy) at 1:50 dilution in PBS containing 1% NGS (normal goat serum) at room temperature overnight. Thereafter, cells were briefly washed with PBS and incubated with secondary antibodies [goat anti-rabbit or goat anti-mouse IgG conjugated to either Alexa Fluor 488 or 555 (Invitrogen, Carlsbad, CA)] (Table 1) at 1:100 dilution in PBS containing 10% NGS (v/v) at room temperature for 30 min, followed by mounting with ProLong Gold antifade reagent containing DAPI (Invitrogen). Negative controls included the use of: (i) corresponding normal rabbit or mouse IgG, depending on the source of the primary antibodies, at appropriate dilution (see Table 1) to substitute the primary antibody, and (ii) PBS to substitute the secondary antibody. Both of these negative controls yielded undetectable fluorescence signals (data not shown). Images were acquired in an HP xw8200 Workstation using MicroSuite FIVE software (Version 1.224, Olympus Soft Imaging Solutions Corp., Lakewood, CO) and an Olympus DP71 12.5 MPa digital camera attached to an Olympus BX61 fluorescence microscope (Olympus America, Inc., Center Valley, PA). Images were uniformly adjusted for brightness, contrast and overlay (merge) using Adobe Photoshop (Version 10.0, Adobe Systems, San Jose, CA).

Co-immunoprecipitation

Sertoli cell lysates were prepared by scraping cells with a rubber policeman in a minimal volume of IP lysis buffer, sonicating cells on ice and centrifuging samples to collect clear supernatants. Approximately 400 μg lysate protein from Sertoli cells treated with or without testosterone or TGF-β3 for 60-min was subjected to immunoprecipitation as described [33]. In brief, lysates were first pre-cleared with 2 μg non-immune rabbit or mouse IgG for 1 hr, and interacting proteins were precipitated with 10 μl Protein A/G PLUS agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, supernatants were collected and incubated with either 2 μg primary antibody (IgG), or non-immune rabbit or mouse IgG at room temperature overnight. Thereafter, 15 μl Protein A/G PLUS agarose was used to pull-down interacting proteins. Agarose beads were washed with IP lysis buffer, centrifuged to collect beads and immunocomplexes were extracted with SDS-sample buffer. This was followed by SDS-PAGE and immunoblotting as described [27].

General methods and statistical analysis

Protein estimation was performed using the BioRad D*c* Protein Assay kit with BSA as a standard and a BioRad Model 680 Microplate Reader (Hercules, CA). All experiments reported herein were repeated 4–5 times using different batches of cells from independent cultures. Changes in protein levels were quantified by scanning immunoblots with Scion Image software package (Version 1.1, NIH, Bethesda, MD). In studies by immunoblot analysis to estimate the effects of testosterone and TGF-β3 on the production of proteins pertinent to endocytic vesicle-mediated protein trafficking (e.g., clathrin, EEA-1, caveolin 1, Rab11 and Ube2j1), we selected the time point of 5-, 15-, 30- and 60-min *versus* time 0 so that all the samples including treatment *versus* control groups could be processed simultaneously in an experimental session by a single investigator to avoid inter-experimental variations. Also, recent analysis has estimated that the average time to add an amino acid to the nascent polypeptide chain is 40 ms, or 25 amino acids per sec at 20 °C for peptide/protein synthesis [34], thus a rapid synthesis rate could be obtained at 35 °C such as in Sertoli cells. It is noted that the findings regarding the rate of *de novo* peptide synthesis are consistent with an earlier report [35]. As such, for a protein of 20 kDa (e.g., caveolin-1 with ~180 amino acid) to 180 kDa (e.g., clathrin with ~1600 amino acids), a synthesis time of 7.2- to 64-sec was anticipated, which is well within the selected time point range for analysis. Statistical analysis was performed using the GB-STAT statistical analysis software package (Version 7.0, Dynamic Microsystems, Inc., Silver Spring, MD). Multiple comparisons were performed using ANOVA followed by Dunnett's significant difference test.

Results

Testosterone and TGF-β3 up-regulate the steady-state level of clathrin at the Sertoli cell barrier

It is known that Sertoli cells cultured *in vitro* under the conditions reported herein establish a functional TJ-permeability barrier [10,36–37] with TJs, basal ES and desmosome-gap junctions by day 2–3 [30] that mimic the BTB *in vivo*. We have used this *in vitro* system in earlier studies to examine the effects of testosterone, TGF-β2 and TGF-β3 on protein endocytosis and recycling [15–16]. To further understand the role of testosterone and cytokines on the events of protein trafficking at the Sertoli cell BTB, we examined the effects of testosterone and TGF-β3 on the steady-state levels of two endocytic vesicle-associated proteins, namely clathrin and early endosome antigen-1 (EEA-1). While testosterone and TGF-β3 had no effects on occludin or EEA-1 in Sertoli cells, they both induced the steady-state level of clathrin (Fig. 1A*a-d*). Fig. 1B*a, b* illustrates the specificities of anti-clathrin and anti-EEA-1 antibodies used in this study since this initial experiment was to be followed by immunofluorescence microscopy (Table 1). By co-immunoprecipitation, we also demonstrate an increase in the association between occludin and clathrin following treatment of Sertoli cells with testosterone and TGF-β3 when compared to the control (Fig. 1C*a, b*). Because this increase in association may be due to the increase in the steady-state level of clathrin as shown in Fig. 1A*a, c*, we used dual-labeled immunofluorescence analysis to further assess this change in association.

Testosterone and TGF-β3 increase occludin-clathrin, occludin–EEA-1, claudin-11-clathrin and claudin-11-EEA-1 association in the Sertoli cell epithelium

The findings shown in Fig. 1 were confirmed and expanded in this experiment which used dual-labeled immunofluorescence analysis to investigate the localization of occludin, clathrin and EEA-1 as well as their co-localization in Sertoli cells following treatment with testosterone or TGF-β3 for 60 min (Fig. 2A*a-l*, B*a-l*). Treatment of Sertoli cells with either testosterone or TGF-β3 increased clathrin (Fig. 2A*f, j vs. b*), as well as induced the

association between occludin (green) and clathrin (red) (Fig. 2A) and between occludin and EEA-1 (red) (Fig. 2B) even though the steady-state level of EEA-1, unlike clathrin, did not change following treatment (Fig. 2B*f, j vs. b*; also see Fig. 1A*a, d*). An increase in association between occludin and clathrin (Fig. 2A*g,h,k,l*), as well as between occludin and EEA-1 (Fig. 2B*g,h,k,l*) (see white arrowheads which point to merged areas that appear yellow-orange in color) following testosterone or TGF-β3 treatment was detected due to the internalization of proteins induced by testosterone and TGF-β3. DAPI was used to visualize nuclei (Fig. 2A, B). These observations were reproduced using claudin-11, another putative TJ-marker at the BTB, which demonstrated an increase in association between claudin-11 and clathrin or EEA-1 (Fig. 3A, B). Thus, these findings shown in Figs. 2 and 3 support and further expand our previously published results based on biochemical assays of endocytosis and protein recycling that both testosterone and TGF-β3 induce endocytic vesicle-mediated protein internalization [15].

Testosterone, but not TGF-β3, up-regulates the steady-state levels of caveolin-1 and Rab11 at the Sertoli cell barrier

Next, we examined if testosterone and TGF-β3 can differentially affect the steady-state levels of markers known to mediate protein transcytosis (e.g., caveolin-1) [38–39] and protein recycling (e.g., Rab11) [40–41] (Fig. 4). Fig. 4 illustrates that testosterone and TGF-β3 had no apparent effects on the Sertoli cell steady-state level of N-cadherin from 0 to 60 min following treatment (Fig. 4A). However, testosterone, but not TGF-β3, was shown to induce the steady-state levels of caveolin-1 and Rab11 (Fig. 4A*a, c, d*). The specificities of the anti-N-cadherin, anti-caveolin-1 and anti-Rab11 antibodies are shown in Fig. 4B *a-c*, and these results along with the negative controls in which the blot was probed with either normal mouse or rabbit IgG, illustrating that each antibody recognized its corresponding target protein when Sertoli cell lysates were used in conjunction with different antibodies for immunoblotting (Table 1). Thus, these antibodies are also suitable to be used for dual-labeled immunofluorescence analysis as shown in Fig. 5A, B.

Testosterone, but not TGF-β3, increases the association of caveolin-1 or Rab11 with endocytosed integral membrane proteins in cell cytosol internalized from cell surface at the Sertoli cell BTB

The effects of testosterone and TGF-β3 on the association between markers of protein transcytosis (e.g., caveolin-1) and protein recycling (e.g., Rab11) with occludin (a TJ-integral membrane protein) and N-cadherin (a basal ES-integral membrane protein) are shown in Fig. 5A*a-l* and 4B*a-l*. Occludin (green) and N-cadherin (green) are constituent proteins at the Sertoli cell BTB and were found at the cell-cell interface (Fig. 5A*a* and 5B*b*). However, treatment of the Sertoli cell epithelium with testosterone for 1-hr was shown to induce endocytosis of occludin (Fig. 5A*e-h vs.* 5A*a-d*) and N-cadherin (Fig. 5B*e-h vs.* 5B*a-d*), similar to the effects of TGF-β3 (Fig. 5A*i-l vs. a-d*; Fig. 5B*i-l vs. a-d*) Treatment of Sertoli cells with testosterone, but not with TGF-β3, was shown to induce caveolin-1 (red) (Fig. 5A*f vs. b* and *j*) and Rab11 (red) (Fig. 5B*f vs. b* and *j*), and these findings are consistent with immunoblotting data shown in Fig. 4 that testosterone stimulated the steady-state protein levels of caveolin-1 and Rab11. Furthermore, testosterone, but not TGF-β3, also increased the association of caveolin-1 and Rab11 with endocytosed occludin (green) (Fig. 5A*g, h vs. c, d* and *k, l*) and N-cadherin (green) that were internalized from the cell surface (Fig. 5B*g, h vs. c, d* and *k, l*). Thus, these findings illustrate that treatment of Sertoli cells with a functional BTB with testosterone increased protein transcytosis and recycling, as manifested by an increase in association of integral membrane proteins with markers of protein transcytosis and recycling. However, TGF-β3 treated cells in the same experiment exhibited no apparent effects to induce association of internalized occludin and/or N-

cadherin with markers of transcytosis (e.g., caveolin-1) or recycling (e.g., Rab11) (Fig. 5A, B).

TGF-β3, but not testosterone, up-regulates the steady-state level of Ube2j1, a marker for endosome- or ubiquitin-mediated intracellular protein degradation

Interestingly, TGF-β3, but not testosterone, was shown to significantly induce the steady-state level of Ube2j1, a protein involved in the degradation of intracellular proteins [42] within 15-min after treatment of Sertoli cells which persisted until the end of this experiment by 60-min (Fig. 6A*a, b*). The specificity of the two anti-Ube2jl antibodies (see Table 1) were demonstrated by immunoblot analysis using Sertoli cell lysates which yielded a prominent protein band, but not when normal rabbit or mouse IgG was used, with an electrophoretic mobility at 35 kDa (Fig. 6B*a*, *b*).

TGF-β3, but not testosterone, increases the association of Ube2j1 with endocytosed occludin in Sertoli cell cytosol

Next, we used dual-labeled immunofluorescence analysis (Fig. 6C*a-i*, and Fig. 6D*a-i*) to assess the increase in Ube2j1 which was seen following TGF-β3, but not testosterone, treatment (Fig. 6C*h vs. b, e* and Fig. 6D*h vs. b, e*), and any changes in its co-localization with occludin (Fig. 6C*c,f,i versus a,d,g* & *b,e,h*) or claudin-11 (Fig. 6D*c,f,i versus a,d,g* & *b,e,h*). The induction in the Ube2j1 steady-state level also resulted in an increase in the co-localization of Ube2j1 with occludin and claudin-11 after TGF-β3, but not with testosterone, treatment (see white arrowheads which point to merged areas in Fig. 6C*i vs. c* and *f*; Fig. 6D*i vs. c*, and *f*). These findings thus support that notion that TGF-β3 promotes intracellular degradation of endocytosed proteins, such as occludin and claudin-11, internalized from the cell surface at the Sertoli cell BTB.

Discussion

Is the *in vitro* Sertoli cell model a reliable system for studying BTB dynamics and applicable to BTB *in vivo*?

The use of primary Sertoli cells isolated from rat testes and cultured *in vitro* in serum-free chemically defined media is known to establish a cell epithelium that mimics the physiology (e.g., establishing a TJ-permeability barrier, restricting the flow of substances from the apical to the basal compartment and *vice versa* across the Sertoli cell epithelium on a Matrigel-coated bicameral unit, and polarized secretion of Sertoli cell proteins such as transferrin, androgen binding protein) and morphology (e.g., columnar shape) of Sertoli cells *in vivo* was first established in the late 1980s in the laboratories of Dym and Steinberger [43–44]. Subsequent studies from these two laboratories have shown that this *in vitro* culture system can reliably be used to study the Sertoli cell BTB function *in vivo* [10,45–48]. We have since adopted such a system for our initial characterization of Sertoli cell BTB dynamics since the early 1990s [36,49]. For instance, using this *in vitro* Sertoli cell culture system, we have shown that the Sertoli cell BTB dynamics were regulated by cytokines (e.g., TGF-β3) via the p38 MAPK signaling pathway [50]. These findings were validated by subsequent studies *in vivo* using a cadmium model, demonstrating unequivocally that the TGF-β3-p38 MAPK is the critical signaling pathway that regulates BTB dynamics [12,51], including a later study using an *in vivo* BTB integrity assay to assess the reversible disruption of TGF-β3 on the BTB integrity in adult rats [16]. Collectively, these findings thus demonstrate clearly that Sertoli cells cultured *in vitro*, forming an intact cell epithelium with a functional TJ-permeability barrier plus the ultrastructures of TJ, basal ES, and desmosome when examined by electron microscopy [30], mimics the BTB *in vivo* is a reliable system to assess the BTB function *in vivo*. However, there are studies that can only be performed using this *in vitro* system because of the limitations in available technology.

For instance, the present study that examined the differential effects of testosterone and TGF-β3 on the endocytic vesicle-mediated protein trafficking at the BTB can only be performed using Sertoli cells cultured *in vitro* because careful analysis of changes in protein distribution and/or redistribution at the Sertoli-Sertoli cell interface following treatment with testosterone or TGF-β3 is difficult to perform in seminiferous tubules in life animals. Yet, results obtained from studies *in vitro* can be interpreted in the context of the Sertoli cell BTB *in vivo* based on the more than two decades of studies using primary Sertoli cells as discussed above. Nonetheless, some of the findings reported herein will be validated and expanded in future studies when better technology is available in the field.

The differential role of cytokines and testosterone on the BTB integrity provides a unique mechanism to facilitate the transit of preleptotene spermatocytes at the BTB while maintaining the immunological barrier during spermatogenesis

It is increasingly clear that the endocytosis of proteins and the balance between recycling, transcytosis, and endosome- or ubiquitin-mediated degradation of internalized proteins play a crucial role in the regulation of junction restructuring, as well as in the adhesive status of cell junctions in multiple epithelia [17,22,52–55] which includes the seminiferous epithelium particularly at the BTB [56–58]. The BTB, unlike other blood-tissue barriers, such as the blood-brain and blood-retinal (or blood-ocular) barriers, is a highly dynamic ultrastructure since it must undergo restructuring at stage VIII of the seminiferous epithelial cycle to accommodate the transit of preleptotene spermatocytes, many of which are connected by intercellular bridges and in clones [59–60]. Thus, a unique mechanism must exist in the testis to avoid disrupting, even transiently, the immunological barrier conferred by the BTB. This is because meiosis and post-meiotic germ cell development take place in an immunologically priviledged microenvironment behind the BTB known as the apical compartment to avoid the production of antibodies against germ cell-specific antigens which would lead to infertility. Recent studies have shown that testosterone which is known to promote BTB integrity [8–9] and cytokines (e.g., TGF-β2, TGF-β3 and TNFα) which are known to reversibly disrupt BTB integrity [12,14,16] can both accelerate the endocytosis of BTB integral membrane proteins, such as occludin, N-cadherin, and JAM-A, based on a biochemical endocytosis assay [15–16]. However, testosterone further promoted recycling of endocytosed biotinylated proteins (e.g., occludin) based on a biochemical recycling assay to estimate the recovery of biotinylated/internalized proteins at the Sertoli cell surface, whereas TGF-β2 promoted their endosome-mediated degradation by associating internalized occludin with Rab9 [15], which is a late endosome marker [20–21]. However, the possibility of protein transcytosis was not examined in these two earlier studies [15–16]. Nonetheless, these findings led us to hypothesize that testosterone may facilitate the assembly of “new” TJ-fibrils (note: occludin is a putative component of TJ-fibrils in epithelial tight junctions [61]) below migrating preleptotene spermatocytes by inducing *de novo* synthesis of occludin [7], as well as by mediating its recycling and/or transcytosis from above preleptotene spermatocytes [62]. Indeed, studies in epithelial cells have shown that a pool of TJ-proteins (e.g., occludin) exists that is less phosphorylated and ‘stored’ at the basolateral region of cells [63–64], which can be rapidly phosphorylated at Thr/Ser and/or Tyr resides and be delivered, perhaps via transcytosis, to sites that require “new” [64–65] TJ-fibril assembly. Once the “new” BTB that is constituted by “new” TJ-fibrils is formed, the “old” BTB situated above migrating preleptotene spermatocytes is disrupted, and this is likely mediated by cytokines (e.g., TGF-β2, TGF-β3 and TNFα) via an acceleration in protein endocytosis [15–16] and endosome-mediated intracellular protein degradation [15]. Herein, we provide additional proof to support this hypothesis besides the two earlier studies that were based on biochemical assays of protein endocytosis [15–16] and recycling [15]. In short, testosterone and TGF-β3 were shown to induce endocytic vesicle-mediated protein endocytosis via their effects to induce the steady-state levels of clathrin, as well as the association between

occludin and clathrin/EEA-1. More important, testosterone, however, but not TGF-β3, was shown to promote protein transcytosis *and* recycling, whereas TGF-β3 promoted intracellular protein degradation. Thus, these findings further support the hypothesis [62] regarding the differential role of testosterone and cytokines in the events of intracellular protein trafficking that facilitates the transit of preleptotene spermatocytes at the BTB while maintaining the immunological barrier function as summarized above.

In this context, it is of interest to note that there are some concerns regarding the suitability of using occludin, an integral membrane protein, as a putative marker for the Sertoli cell BTB since occludin knock-out mice (occludin$^{-/-}$) by 6-wk of age (at the time the first wave of spermiation occurs) were fertile and the BTB integrity, based on immunofluorescence microscopy, appeared to be intact because other TJ-proteins (e.g., claudin-3) were present at the BTB to confer the barrier function [66]. However, it is noted that as occludin$^{-/-}$ mice age, a somewhat different picture regarding their fertility emerges. For instance, seminiferous tubules of adult occludin knockout mice by age 60-wk were devoid of all spermatids and spermatocytes and were infertile [66]. These observations [66] were recently confirmed in occludin knockout mice which were generated using techniques of gene targeting in multipotent germline stem cells [67]. In short, a knock-out of occludin does not affect fertility in young adults by 6-wk [66] to 10-wk [67] of age; however, the loss of occludin indeed impedes male fertility in these occludin$^{-/-}$ mice by 36- to 60-wk of age and the tubules were devoid of all spermatocytes and spermatids, and perhaps the BTB was also disrupted since the BTB integrity was not examined in these older occludin$^{-/-}$ male rats [66–67]. Collectively, these findings do not negate the significance of occludin being served as a crucial TJ structural protein at the Sertoli cell BTB. They only illustrate that the barrier function conferred by occludin can be *temporarily* substituted by other BTB-proteins, such as claudin-11, claudin-3, JAM-A, CAR, and N-cadherin, however, as animals “age”, the “lost” function of occludin can no longer be replaced. Nonetheless, we have also included other BTB markers in the present study including N-cadherin and claudin-11. It is of interest to note that claudin-11 knockout mice were reported to be infertile with disrupted BTB function [68]. Since the phenotypic changes in claudin-11 and N-cadherin were similar to occludin following treatment of Sertoli cell epithelium with TGF-β3 or testosterone, it is obvious that occludin can serve as a valuable marker for BTB function, similar to its use in other epithelial TJ-barriers [69–70].

Cytokines and testosterone have different effects on the endocytic vesicle-based intracellular trafficking at the Sertoli cell BTB

Earlier studies have shown that clathrin is involved in testosterone- and cytokine-mediated protein endocytosis at the Sertoli cell BTB in studies using either inhibitors (e.g., phenylarsine oxide, a clathrin-mediated pathway inhibitor [71]) [15–16] or RNA interference (RNAi) with clathrin specific siRNA duplexes [16]. Indeed, both testosterone and TGF-β3 were shown to induce the steady-state level of clathrin, a crucial component of endocytic vesicles even though they had no apparent effects on EEA-1 (an early endosome marker). Additionally, both testosterone and TGF-β3 induced the association of clathrin and EEA-1 with endocytosed integral membrane proteins (e.g., occludin) in the cell cytosol internalized from cell surface. However, testosterone, but not TGF-β3, induced protein transcytosis and recycling as manifested by an increase in the steady-state levels of the transcytosis marker caveolin-1 [38,72] and the protein recycling marker Rab11 [40–41], as well as their association with either endocytosed occludin or N-cadherin. Furthermore, TGF-β3, but not testosterone, induced intracellular protein degradation by up-regulating the steady-state level of Ube2j1, a marker for endosome- or ubiquitin-mediated degradation [42,73]. It also increased the association of endocytosed occludin with Ube2j1 by dual-labeled immunofluorescence analysis. These findings thus support a current hypothetical

concept [18] that the differential effects of testosterone and cytokines on the "fate" of endocytosed proteins can regulate the assembly of "new" TJ-fibrils or the dissolution of "old" TJ-fibrils within the microenvironment of the BTB via the restricted and spatial expression of the androgen receptors or cytokine receptors below or above 'clones' of preleptotene spermatocytes in transit.

Much work is needed to understand the intracellular sorting mechanism(s) in early endosomes in which testosterone and TGF-β3 determine the 'fate' of endocytosed proteins in the microenvironment of the BTB during spermatogenesis since the barrier remains relatively quiescence except at stage VIII of the epithelial cycle when it must restructure to facilitate the transit of preleptotene spermatocytes at the BTB. For instance, there must be a 'switch' mechanism in place at the BTB to determine the 'proportion' of the endocytosed integral membrane proteins that should be 'diverted' (i.e., transcytosed) to the new BTB site to assemble 'new' TJ-fibrils, or to be sent to late endosomes for degradation. It is likely that polarity proteins (e.g., Par6, Cdc42 and 14-3-3) and actin regulators (e.g., Arp2/3) may play a crucial role in this event [74–75], and this possibility should be investigated in future studies.

In summary, we have demonstrated herein the differential effects of testosterone and cytokines (e.g., TGF-β3) on the events of protein transcytosis, recycling and intracellular degradation that are critical to maintain the homeostasis of the BTB, in particular at stage VIII of the epithelial cycle during the transit of preleptotene spermatocytes across the immunological barrier. It is obvious that future studies should include a carefully investigation on the restricted distribution of androgen receptor (and/or testosterone) as well as cytokines (and their corresponding receptors) in the microenvironment of the BTB near the basal compartment surrounding the clones of preleptotene spermatocytes during their transit at the BTB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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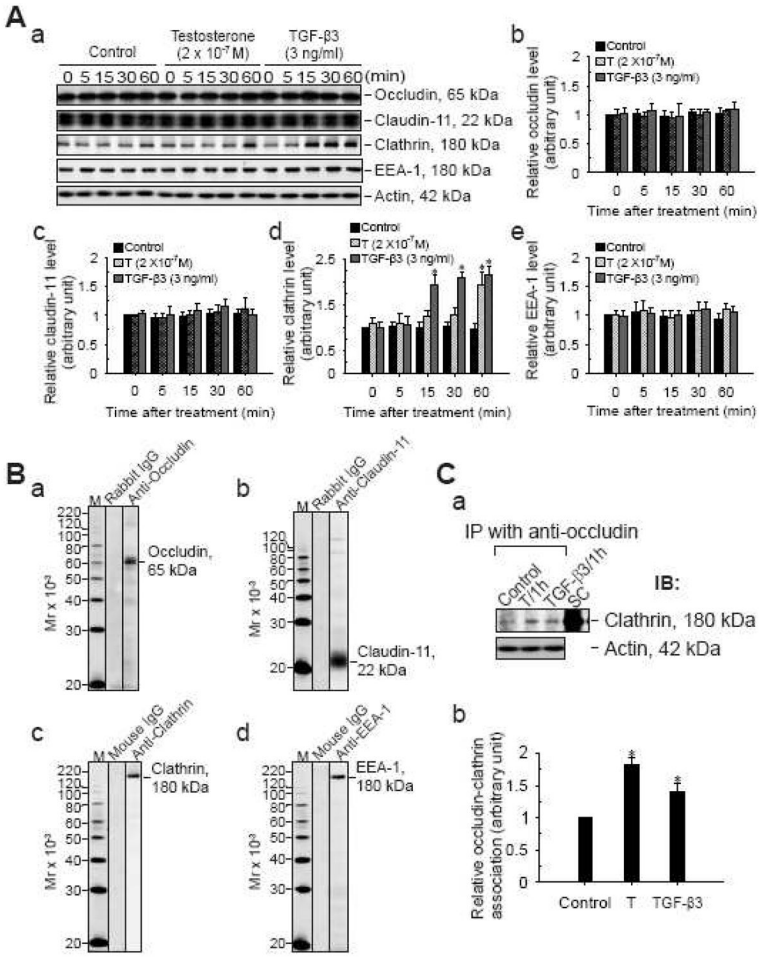

Fig. 1. Effects of testosterone and TGF-β3 on the steady-state levels of markers of endocytic vesicles (e.g., clathrin) and early sorting endosomes (e.g., early endosome marker-1, EEA-1) in Sertoli cell cultures having an established TJ-permeability barrier, as well as on the association between clathrin and occludin

(A) As described in *Materials and Methods*, Sertoli cells were cultured at 0.5×10^6 cells/cm^2 for 4 days to allow the assembly of a functional TJ-permeability barrier, and with the ultrastructures of TJs, basal ES and desmosome-like junctions when examined by electron microscopy as described [30]. Thereafter, cells were incubated with testosterone (2×10^{-7} M) or TGF-β3 (3 ng/ml) for 0-, 5-, 15-, 30- and 60-min *vs.* controls. Equal amounts of protein (30 μg from each sample) were used for immunoblot analysis using anti-occludin, anti-clathrin or anti-EEA-1 antibodies (*a*). Actin was used as an equal protein loading control. Histograms in *b, c* & *d* represent composite results from several immunoblots such as the ones shown in *a*. Each data point was normalized against actin, and time 0 in each experimental group was arbitrarily set at 1 against which statistical analysis was performed. Each bar is the mean±SD from 4–5 independent experiments (with each experiment having at least duplicate 12-wells) using different batches of isolated Sertoli cells. *, $P<0.05$. **(B)** Immunoblot analysis (7.5% *T* gel) using lysates from Sertoli cells (SC) (30 μg protein) and probed with the corresponding antibody and either the rabbit or mouse IgG illustrating the specificities of the anti-occludin (*a*), anti-claudin-11 (*b*) anti-clathrin (*c*) and anti-EEA-1 (*d*) antibodies (see Table 1). M, MagicMark XP protein markers (Invitrogen). **(C)** Co-immunoprecipitation (Co-IP) was performed using ~400 μg protein lysate from control *vs.* treated Sertoli cells [i.e., testosterone (2×10^{-7} M) or TGF-β3 (3 ng/ml)] harvested at 60-

min (*a*). Lysates were immunoprecipitated with anti-occludin, subjected to SDS-PAGE and then probed with anti-clathrin. Sertoli cell lysates (30 μg protein) without IP served as a positive control. (*b*) Histogram summarizing Co-IP results. Each data point was normalized against actin. Each bar is the mean±SD from 3 independent experiments using different batches of isolated Sertoli cells treated with or without testosterone and TGF-β3.

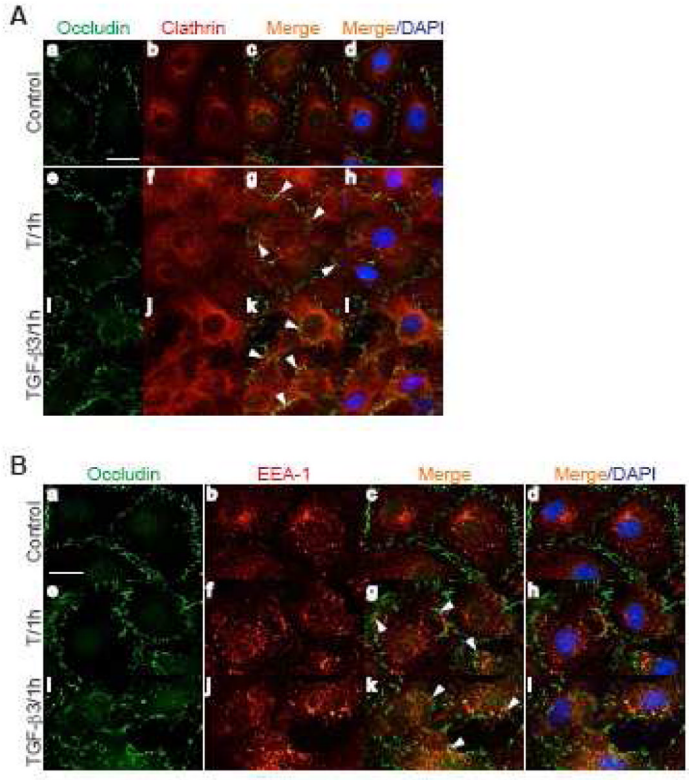

Fig. 2. Dual-labeled immunofluorescence analysis to assess the co-localization of occludin with clathrin or EEA-1 in Sertoli cells and the redistribution of these proteins following testosterone or TGF-β3 treatment

Sertoli cells were cultured at 0.05×10^6 cells/cm^2 on Matrigel-coated glass coverslips for 4 days, and incubated with testosterone (2×10^{-7} M) or TGF-β3 (3 ng/ml) *vs.* controls at 35 °C for 60 min. This was followed by dual-labeled immunofluorescence analysis as shown in (**A***a-l*) and (**B***a-l*) to assess changes in the distribution of occludin (green), clathrin (red) and EEA-1 (red). DAPI (blue) was used to visualize nuclei. An increase in clathrin (**A***f, j vs. b*), but not EEA-1 (**B***f, j vs. b*), was noted when Sertoli cells were treated with testosterone or TGF-β3 *vs.* controls, consistent with immunoblotting data shown in Fig. 1. White arrowheads in micrographs in **A***g,k* and **B***g,k* indicate an increase in the co-localization of occludin with clathrin or EEA-1 after testosterone or TGF-β3 treatment. Scale bar = 10 μm in **A***a* and **B***a*, which applies to **A***b-l* and **B***b-l*.

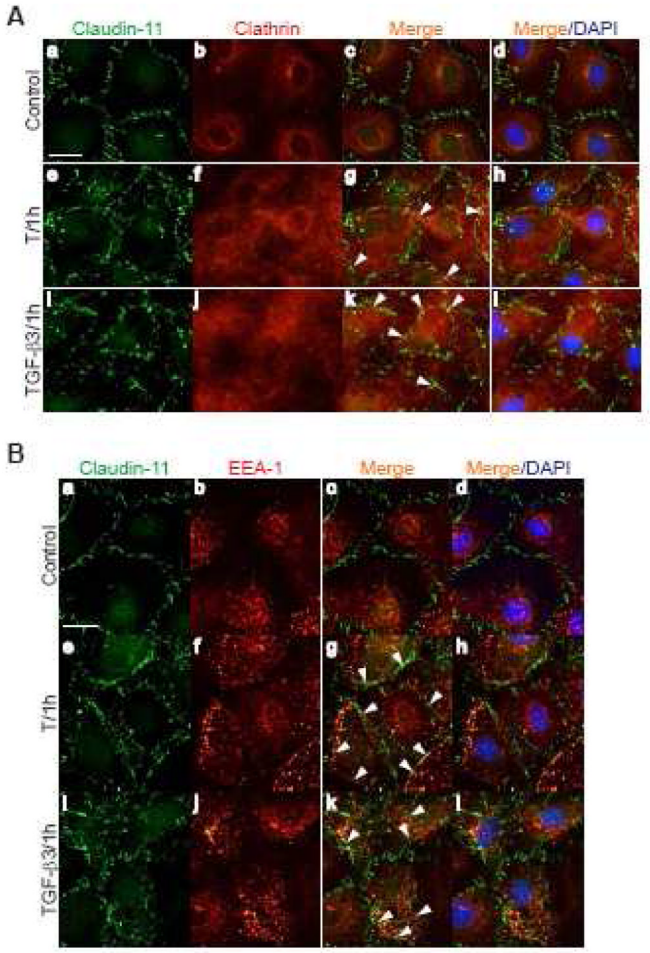

Fig. 3. Dual-labeled immunofluorescence analysis to assess the co-localization of claudin-11 with clathrin or EEA-1 in Sertoli cells and the redistribution of these proteins following testosterone or TGF-β3 treatment

Sertoli cells were cultured at 0.05×10^6 cells/cm^2 on Matrigel-coated glass coverslips for 4 days, and incubated with testosterone (2×10^{-7} M) or TGF-β3 (3 ng/ml) *vs.* controls at 35 °C for 60 min. This was followed by dual-labeled immunofluorescence analysis as shown in (**A***a-l*) and (**B***a-l*) to assess changes in the distribution of claudin-11 (green), clathrin (red) and EEA-1 (red). DAPI (blue) was used to visualize nuclei. An increase in clathrin (**A***f, j vs. b*), but not EEA-1 (**B***f, j vs. b*), was noted when Sertoli cells were treated with testosterone or TGF-β3 *vs.* controls, consistent with immunoblotting data shown in Fig. 1. White arrowheads in micrographs in **A***g,k* and **B***g,k* indicate an increase in the co-localization of claudin-11 with clathrin or EEA-1 after testosterone or TGF-β3 treatment. Scale bar = 10 μm in **A***a* and **B***a*, which applies to **A***b-l* and **B***b-l*.

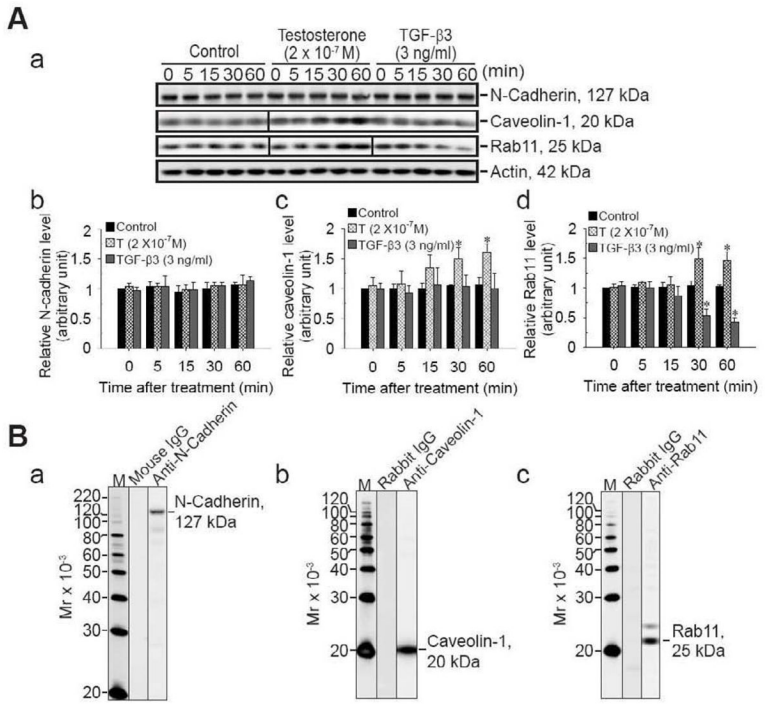

Fig. 4. Effects of testosterone and TGF-β3 on the steady-state levels of caveolin-1 (a transcytosis marker) and Rab11 (a recycling marker)

(A) Sertoli cells were cultured and treated as described in *Materials and Methods* and the legend to Fig. 1. Equal amounts of protein (30 μg from each sample) were used for immunoblot analysis using anti-N-cadherin, anti-caveolin-1 or anti-Rab11 antibodies (*a*). Actin was used as an equal protein loading control. Histograms shown in *b-d* represent composite results from several immunoblots such as the ones shown in *a*. Each data point was normalized against actin, and time 0 in each experimental group was arbitrarily set at 1 against which statistical analysis was performed. Each bar is the mean±SD from 4 independent experiments (for each experiment, each time point has duplicate or triplicate culture wells including treatment and control groups) using different batches of Sertoli cells from different group of rats. *, $P<0.05$. **(B)** Immunoblot analysis (12.5% *T* gel) using lysates from Sertoli cells (30 μg protein) and the blots were probed with the corresponding antibody as well as either normal mouse IgG or rabbit IgG, illustrating the specificities of the anti-N-cadherin (a), anti-caveolin-1 (*b*) and anti-Rab11 (*c*) antibodies used for immunoblot analysis and the subsequent dual-labeled immunofluorescence analysis (see Fig. 5). M, MagicMark XP protein markers (Invitrogen).

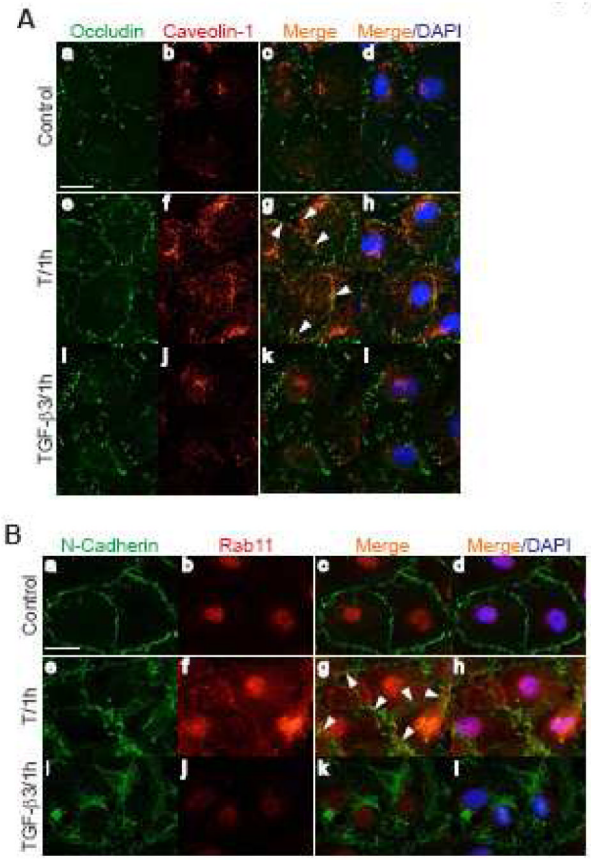

Fig. 5. Dual-labeled immunofluorescence analysis to assess changes in the localization of occludin *vs.* caveolin-1 (a protein transcytosis marker) or Rab11 (a protein recycling marker) in Sertoli cells following testosterone or TGF-β3 treatment

Sertoli cells were cultured at 0.05×10^6 cells/cm^2 on Matrigel-coated glass coverslips for 4 days, followed by incubation with testosterone (2×10^{-7} M) or TGF-β3 (3 ng/ml) *vs.* controls at 35 °C for 60 min. Thereafter, Sertoli cells were fixed, permeabilized and subjected to dual-labeled immunofluorescence analysis for occludin (green) and caveolin-1 (red) in **A***a-l* and for N-cadherin (green) and Rab11 (red) in **B***a-l*. An increase in caveolin-1 (**A***f vs. b, j*) and Rab11 (**B***f vs. b, j*) was detected after testosterone, but not TGF-β3 treatment, consistent with immunoblotting data shown in Fig. 3. Occludin also increased its association with both caveolin-1 and Rab11 after testosterone, but not TGF-β3, treatment (**A***g vs. c, k*; **B***g vs. c, k*). DAPI (*blue*) was used to visualize nuclei. White arrowheads in **A***g* and **B***g* indicate an increase in co-localization of occludin with caveolin-1 or Rab11 after testosterone treatment (**A***g* and **B***g versus* **A***c,k* and **B***c,k*). Scale bar = 10 μm in **A***a* and **B***a*, which applies to **A***b-l* and **B***b-l*.

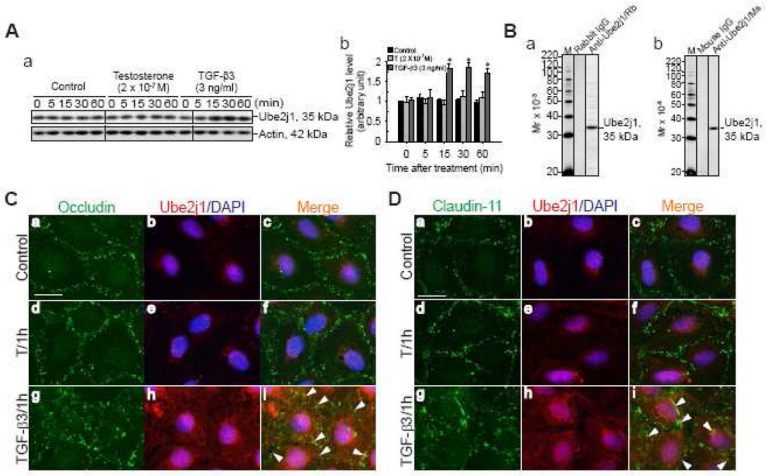

Fig. 6. Effects of testosterone and TGF-β3 on the steady-state level of Ube2j1 (a marker of intracellular protein degradation) and its co-localization with occludin

(A) Sertoli cells were cultured and treated as described in *Materials and Methods* and the legend to Fig. 1. Blots were probed with an anti-Ube2j1 antibody (see Table 1) using actin which served as a protein loading control. TGF-β3, but not testosterone, was shown to induce the steady-state level of Ube2j1 in the Sertoli cell epithelium (*a*). Histogram shown in *b* represents composite results from several immunoblots (n = 3–4), such as the one shown in *a*. Each data point was normalized against actin, and time 0 in each experimental group was arbitrarily set at 1 against which statistical analysis was performed. *, P<0.05. **(B)** Immunoblot analysis (7.5% *T* gel) using lysate from Sertoli cells (SC) (30 μg protein) and the corresponding rabbit (Rb) (**B***a*) or mouse (Ms) (**B***b*) anti-Ube2j1 antibody (see Table 1) as well as the normal rabbit or mouse IgG, illustrating the specificity of the anti-Ube2j1 antibody. M, marker. **(C)** Dual-labeled immunofluorescence analysis in *a*-*i* illustrating an increase in co-localization between occludin (green) and Ube2j1 (red) after TGF-β3, but not testosterone, treatment (**C***i versus c*,*f*). DAPI (blue) was used to visualize nuclei. White arrowheads in **C***i* indicate an increase in the co-localization of occludin with Ube2j1 after TGF-β3 treatment (**C***i versus* **C***c*,*f*). Scale bar = 10 μm in **C***a*, which also applies to **C***b*-*i*. **(D)** The study shown in **(C)** was performed using claudin-11 (green), another putative TJ-protein at the BTB, with similar results. Scale bar = 10 μm in **D***a*, which also applies to **D***b*-*i*.

Table 1

Primary antibodies used for different experiments in this report.

Primary antibody	Catalog no.	Lot no.	Host	Vendor	Working dilution		
					IB	IF	IP
Occludin	71–1500	672381A	rabbit	Invitrogen/Zymed	1:250	1:50	1:40
	33–1500	138461	mouse	Invitrogen/Zymed	1:250	1:50	
Claudin-11	36–4500	387613A	rabbit	Invitrogen/Zymed	1:250	1:50	
N-Cadherin	sc-7939	H0907	rabbit	Santa Cruz Biotechnology	1:200		
	33–3900	423674A	mouse	Invitrogen/Zymed	1:250	1:50	
Clathrin	610499	13357	mouse	BD Biosciences	1:1000	1:100	
EEA-1	610457	46554	mouse	BD Biosciences	1:2000	1:100	
Caveolin-1	ab18199	128833	rabbit	Abcam	1:250	1:100	
Rab11	sc-9020	K239	rabbit	Santa Cruz Biotechnology	1:200	1:100	
Ube2j1	600-401-991	19066	rabbit	ROCKLAND	1:1600	1:150	
	WH0051465M1	09258-6A12	mouse	Sigma-Aldrich	1:300	1:100	
Actin	sc-1616	H0808	goat	Santa Cruz Biotechnology	1:200		
Rabbit IgG	I5006	-	rabbit	Sigma-Aldrich	*	*	
Mouse IgG	02-6502	1394578	mouse	Invitrogen/Zymed	*	*	

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

Abcam (Cambridge, MA); BD Biosciences (San Jose, CA); ROCKLAND (Gilbertsville, PA); Santa Cruz Biotechnology (Santa Cruz, CA); Invitrogen/Zymed (South San Francisco, CA).

*Use the same concentration of IgG as its corresponding primary antibody.