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BIOLOGY OF REPRODUCTION (2015) **92**(3):66, 1–11
Published online before print 7 January 2015.
DOI 10.1095/biolreprod.114.120824

Tricellulin and Its Role in the Epididymal Epithelium of the Rat[1]

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ABSTRACT

Tricellulin is a tight-junction protein present at tricellular tight junctions. It has been suggested that basal cells are implicated in the blood-epididymis barrier. Basal cells express claudins, a component of tight junctions; however, there is no information regarding the potential architecture or regulation of basal cell-principal cell interactions. The present objectives were to determine the expression and localization of tricellulin in rat epididymis in relation to occludin, basal cell-principal cell interactions, and other junctional proteins. Tricellulin levels were similar in all segments of the adult epididymis, and the protein was localized to the apical region of the epithelium. Postnatal development showed that tricellulin levels increased with age and localization changed from cytoplasmic to membrane-bound as a function of age. Colocalization with occludin indicated that both proteins are in the region of the tight junction. In the initial segment, the proteins did not colocalize compared to the epididymis where they were both colocalized. Tricellulin did not colocalize with cytokeratin 5, a marker of basal cells, in any region of the epididymis, including the corpus and cauda epididymidis, where apical projections of basal cells were apparent. Tricellulin knockdown studies using small interfering RNA in rat caput epididymal principal cells resulted in decreased transepithelial resistance and was correlated with decreased levels of Cldn3, Cldn1, and occludin. Tight-junction protein1, also known as ZO-1, and cadherin1 levels were unchanged. This is the first report of tricellulin in the epididymis and on the interaction between tricellulin and other tight-junction proteins.

barrier, basal cells, claudins, epididymis, male reproductive tract, sperm maturation, tight junctions, tricellulin

INTRODUCTION

In the epididymis, the tight junctions between adjacent epithelial cells that line the lumen of the epididymis form a tissue barrier that is commonly referred to as the blood-epididymis barrier (BEB) [1]. The tight junctions in the epididymis are created during embryonic development and become less permeable during postnatal development [2]. In mink (*Mustela vison*), the barrier appears to be impermeable to lanthanum by the time of birth [3]. The BEB is associated with the protection of sperm from the immune system and provides a unique environment in the epididymal lumen that is critical for sperm maturation [4]. In addition to its barrier function, other components the BEB are involved in ion movement across the epithelium, secretion of small molecules, transportation and excretion of molecules from epithelial cells, and immunoprotection [5–8].

Tight junctions of the epididymis are composed of occludin and a large number of different claudins (CLDNs) [9, 10]. Human studies have reported that both obstructive and nonobstructive azoospermia is associated with a loss of CLDNs [11]. Furthermore, while the human epididymis contains a large number of CLDNs, the loss of a single CLDN is sufficient to cause a loss in the integrity of the tight junction and thus compromise the BEB [11]. The localization and levels of various CLDNs have been shown to vary as a function of development; these levels may be regulated by the endocrine system and by cytokines [5].

The epithelium of the epididymis is composed of multiple cell types. These include epithelial principal cells, basal cells, clear cells, narrow cells, and apical cells. Principal cells are responsible mainly for fluid transport and secretion/reabsorption [12, 13]. Apical, narrow, and clear cells play a role in acidification of the intraluminal fluid [14–16]. Basal cells are present at the base of the epithelium and have been implicated in regulation of principal cell functions [17, 18] and immune defense [19–22]. Recent studies have reported that dendritic cells are also present in the epithelium and that the immune functions attributed to basal cells may in fact be attributable to dendritic cells [23]. Whether or not this is the case remains to be demonstrated. It has been reported that basal cells have both basal and apical projections [24, 25], and the latter have been shown to reach the lumen of the epididymis [25]. It has been suggested that these apical projections can cross the BEB. In addition to bicellular junctions, which are formed by occludin and CLDNs, studies have shown that cells can form tripartite tight junctions and that these are mediated by a protein termed tricellulin, or MarvelD2 [26]. Tricellulin lies at the three corner contact sites of epithelial cells [26]. This protein contains a tetra-spanning MARVEL domain (MAL and related proteins for vesicle trafficking and membrane link) and has 32% homology with occludin [26]. Tricellulin has been identified in epithelial cells of the intestine, stomach, and kidney [27] as well as in the stratified epithelium of epidermis [28], pancreatic duct epithelial cells [29], nasal epithelial cells [30], and immune cells [31]. Tricellulin is also necessary for hearing as mutations in tricellulin can lead to sensorial deafness [32]. There are no studies to date regarding the presence of tricellulin in the epididymis or its role in mediating tight junctions between basal and principal cells. The objective of this study was to determine the presence and localization of tricellulin in the rat epididymis, to determine whether or not it is regulated as a function of postnatal development, and to assess its role in the maintenance of epididymal tight junctions, using an in vitro approach.

[1]Supported by a CIHR operating grant 84576 to D.G.C. M.M. was the recipient of a studentship from the Fondation Armand-Frappier. This work was presented in part at the Annual Meeting of the Society for the Study of Reproduction (SSR), July 2013, Montreal, QC, Canada.
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Received: 28 April 2014.
First decision: 7 June 2014.
Accepted: 17 December 2014.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

MATERIALS AND METHODS

Experimental Protocols

Male Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. Different ages (14, 21, 42, 56, and 91 days postnatally; n = 3–4 animals per age) were selected, based on the morphological and physiological development of the epididymis. By Day 14, the BEB is not complete and the epithelial cells are largely undifferentiated [33], whereas by Day 21, epithelial cells have differentiated into columnar principal cells and the tight junctions are impermeable to lanthanum throughout the epididymis [34]. Rat epididymal epithelial cells are fully differentiated and testosterone levels have reached adult levels by Day 42, but there are no spermatozoa in the epididymis [33]. By Day 56, spermatozoa are present throughout the epididymis [35]; Day 91 is considered adult.

Rats were maintained under a constant 12L:12D photoperiod and received food and water ad libitum. At the time of sampling, rats were anesthetized with CO_2 and killed by cervical dislocation. Depending on the experiment, epididymides were dissected and subdivided into either four (initial segment, caput, corpus, and cauda) or two separate regions (initial segment-caput-corpus and cauda). Tissues were immediately frozen in liquid nitrogen and stored at −86°C, or frozen in optimal cutting temperature compound for cryosections (Fisher Scientific) on dry ice and stored at −86°C until sectioning. All the animal protocols used in this study were approved by the university animal care committee.

Cell Culture

Rat caput epididymal (RCE) principal cells [36] were grown on 35-mm Petri dishes coated with 5% mouse collagen IV (BD Biosciences) in Dulbecco-modified Eagle medium (DMEM)/Ham F12 culture medium containing antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin), L-glutamine (2 mM), and nutrients (10 μg/ml insulin; 10 μg/ml transferrin; 80 ng/ml hydrocortisone; 10 ng/ml epidermal growth factor; 10 ng/ml cAMP; and 5 nM testosterone) at 32°C in a humidified chamber with 5% CO_2 [36].

Immunofluorescence

Cryopreserved rat epididymides were sectioned (10 μm) with a cryostat and fixed in ice-cold methanol for 20 min at −20°C. After rehydration in PBS-Tween (0.05%), the sections were permeabilized in a solution of 0.3% Triton X-100 in PBS at room temperature for 20 min. Sections were blocked with PBS-bovine serum albumin (5%) for 30 min. Sections were then incubated with a polyclonal anti-tricellulin antibody (1.25 μg/ml; Life Technologies Inc.) diluted in blocking solution at 4°C overnight, washed three times with PBS-Tween, and subsequently incubated with an anti-rabbit Alexa 488 (green)-conjugated secondary antibody (20 μg/ml, Life Technologies) at 37°C for 1 h. Finally, sections were washed twice with PBS-Tween and once with PBS and mounted with Vectastain mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Sections were examined under a Leica DMRE microscope (Leica Microsystems, Inc.).

Colocalization experiments were done using cryosections prepared in the same fashion as described above. Sections were incubated with anti-tricellulin antisera (1.25 μg/ml) overnight at 4°C, rinsed in PBS, and subsequently incubated for 1 h at 37°C with Alexa 488-conjugated anti-rabbit antisera (20 μg/ml; Life Technologies). Sections were then rinsed three times in PBS-Tween and incubated with one of the second primary antibodies: anti-occludin monoclonal antibody (1 μg/ml; Santa Cruz Biotechnology) or anti-cytokeratin V monoclonal antibody (1 μg/ml; Santa Cruz Biotechnology). Incubations were carried out for 2 h at room temperature. Sections were subsequently washed three times in PBS-Tween and incubated for 1 h at 37°C with an Alexa 594-conjugated anti-mouse secondary antibody (20 μg/ml; Life Technologies). Finally, the sections were washed three times in PBS and mounted with Vectastain containing DAPI. Sections were examined with a Leica DMRE fluorescent microscope.

Three-dimensional confocal microscopy was done using cryopreserved epididymal sections (20 μm). Tissue sections were prepared in the same fashion as for fluorescent microscopy with the exception that lower antibody concentrations of anti-tricellulin and secondary were used (0.25 μg/L anti-tricellulin; 0.25 μg/L Alexa 488-conjugated anti-rabbit antisera). Nuclei were stained with Hoechst dye (1 μg/ml) (Biotium) and mounted with Fluoromount-G (Southern Biotech). Sections were examined under a Zeiss LSM780 confocal microscope (Carl Zeiss Canada Ltd.), and the data was analyzed using the Zen software (Zeiss). Movies were created with 150 total frames. Z-stack images were then exported in TIFF or AVI format.

Western Blot Analysis

Cells and tissues were lysed in cold RIPA lysis buffer (PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 100 μg/ml phenylmethylsulfonyl fluoride (Sigma-Aldrich), 100 μM sodium orthovanadate (Sigma-Aldrich), a protease inhibitor cocktail (Sigma-Aldrich), and a phosphatase inhibitor cocktail (PhosStop 1×; Roche). Samples were homogenized, briefly sonicated, and centrifuged at 10 000 × *g* at 4°C for 10 min to remove cellular debris. The supernatants were collected and protein concentrations were measured using a Pierce BCA Protein assay kit (Thermo Scientific). Proteins (50ug) were diluted in Laemmli loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue), heated at 95°C for 5 min, and loaded onto a 6%, 10%, or 15% polyacrylamide gel for separation by electrophoresis (140 V) for 1 h. Proteins were then transferred onto a polyvinylidene fluoride membrane at 25V and 2.5A for 10 min (Turbo-Blotter; Bio-Rad). The resulting blots were stained with 0.6% Ponceau red S to evaluate transfer efficiency. Membranes were blocked for 1 h at room temperature with 5% nonfat dry milk diluted in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) and subsequently incubated overnight at 4°C for 2 h at room temperature with either a polyclonal anti-tricellulin rabbit (1.25 μg/ml; Life Technologies), a monoclonal anti-occludin mouse antibody (1 μg/ml; Santa Cruz Biotechnology), a rabbit polyclonal anti-claudin-3 (1.25 μg/ml; Santa Cruz Biotechnology), a rabbit polyclonal anti-claudin-1 (1 μg/ml; Life technologies), a rabbit polyclonal anti-cadherin1 (1 μg/ml; Santa Cruz Biotechnology), or a rabbit polyclonal anti-zonula occludens-1 (0.5 μg/ml; Life Technologies). Membranes were then washed three times in Tris-buffered saline with 0.05% Tween-20 (Fisher Scientific) for 10 min and probed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antisera (130 ng/ml; Santa Cruz Biotechnology). The membranes were once again washed three times for 10 min in Tris-buffered saline with 0.05% Tween-20. Detection was done using the Clarity Western ECL Substrate (Bio-Rad) according to the manufacturer's instructions and visualized using a Chemidoc scanner (Bio-Rad). Protein levels in each lane were normalized using a rabbit polyclonal anti-tubulin antibody (60 ng/ml; Life Technologies).

Small Interfering RNA

Small interfering RNAs (siRNA) (1.0 μM) against rat tricellulin (NM_001108936, XM_001062343, XM_345145; Qiagen) and a nonsense siRNA (Qiagen) were transfected into RCE cells cultured in DMEM on collagen IV-coated glass chamber slides (BD Biosciences) using Hi-Perfect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Cells were cultured for 48 h under normal culture conditions (32°C and 5% CO_2) and then lysed for Western blot analysis.

Transepithelial Resistance

RCE cells were seeded at a density of 5×10^4 cells/ml on Costar Transwell 12-mm cell culture inserts (polyester membrane, pore size 0.4 μm; Corning) coated with mouse collagen IV (BD Biosciences) and cultured in DMEM/Ham F12 culture medium containing antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin), 2 mM L-glutamine, and 5% fetal bovine serum. Cells were seeded onto inserts at the time of transfection and, once confluent, were exposed to 1.8 mM $CaCl_2$ for 48 h. Transepithelial resistance (TER) was measured in quadruplicate wells at regular intervals for 48 h using an EVOM2 epithelial volt-ohmmeter (World Precision Instruments). TER was normalized to the area of the filter, after removal of background resistance of a blank filter that contained only medium, and calculated as Ω/cm^2.

Methylthiazolyldiphenyl-Tetrazolium Bromide Assay

Aliquots of 2500 cells per well were plated in 24-well culture plates coated with collagen IV. The number of cells was determined using trypan blue staining and a hemocytometer (Invitrogen Inc.). The next day, once the cells had adhered, the medium was changed, and this first time point was designated as time zero. Each time point was done in triplicate. Medium was changed every 24 h. At different time points, the culture medium was removed from three of the wells and replaced with 20 μl methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution (0.5 mg/ml in culture medium) (Sigma-Aldrich) to measure cellular proliferation [36]. After 2.5 h, the MTT solution was removed and the formazan crystals were solubilized in 200 μl dimethylsulfoxide. Absorbance (570 nm) was measured using a microtiter plate reader (Power Wave X; Bio-Tek Instruments Inc.).

Statistical Analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using a Student *t*-test or a one- or two-way ANOVA when appropriate. Significance was established as $P \leq 0.05$. For comparisons of protein expression by Western blot analysis, a Student-Newman-Keuhls test for multiple comparisons or a Dunnett test was done. For comparisons of TER, a Bonferroni post hoc test was used. All the analyses were performed using GraphPad Prism software.

RESULTS

Expression and Localization of Tricellulin in Adult Rat Epididymis

To determine whether or not tricellulin was expressed in the epididymis and to determine if the levels were similar throughout the epididymis, Western blot analyses were performed on the different segments (initial segment, caput, corpus, and cauda) of adult rat epididymis (91 days, n = 4). The results revealed that tricellulin was expressed in all the segments of the epididymis and that protein levels were similar between the different segments (Fig. 1A). Tricellulin was localized to the apical margins of adjacent epididymal epithelial cells throughout the epididymis (Fig. 1B and Supplemental Movie S1; supplemental movies are available online at www.biolreprod.org). The pattern of punctate immunostaining and localization of tricellulin remained consistent throughout the epididymis (Fig. 1B).

Levels and Localization of Tricellulin During Postnatal Development

To correlate the expression and the localization of tricellulin with the development of the male reproductive tract, Western blot analyses and immunofluorescent microscopy were done. Western blots analyses showed that tricellulin is already present at Day 14 and that the levels increased as a function of age and peaked in the adult rat. The pattern of tricellulin levels as a function of age was similar in both the proximal and distal (cauda) regions of the epididymis (Fig. 2, A and B).

Immunofluorescent microscopy showed differences in the localization of tricellulin during development. At Day 14, the immunostaining was present both along the apical region of the epithelium as well as in the cytoplasm throughout the epididymis. At Day 21, tricellulin was localized to the lateral margins of the plasma membranes of adjacent cells, although some cytoplasmic staining remained, especially in the initial segment. At Day 42, the immunostaining was apical along the plasma membrane and became somewhat more intense by Day 56, when the immunostaining was similar to that observed in the adult rat (Fig. 3) in all regions of the epididymis.

Colocalization of Tricellulin and Occludin

Merged images of occludin and tricellulin indicated that in the initial segment of the adult rat epididymis, tricellulin and occludin did not colocalize even though both proteins were present in the area of the BEB (Fig. 4, photomicrograph A). In other regions of the epididymis, both occludin and tricellulin colocalized in the region of the tight junction of the BEB, although some occludin immunostaining occurred independent of tricellulin (Fig. 4, photomicrographs B–D). Vertical and horizontal sectional images were extensively analyzed using Z-stacked images generated by laser scanning confocal microscopy. Three-dimensional reconstruction of epididymis was done using stacked images

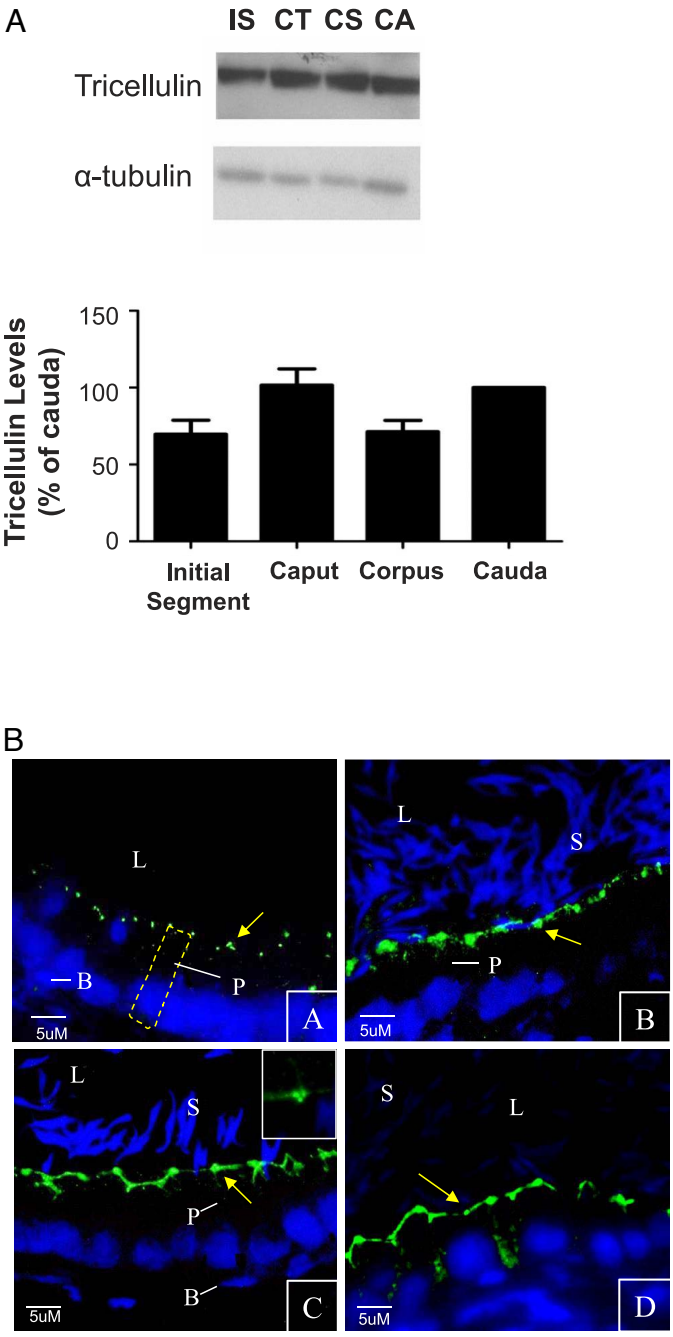

FIG. 1. Expression of tricellulin in the adult rat epididymis. **A**) Western blot analysis of tricellulin in the different segments (initial segment [IS], caput [CT], corpus [CS], and cauda [CA]) of adult rat epididymis. Tricellulin levels were similar in all the regions of the epididymis. Tricellulin level were normalized to α-tubulin, which was used as a loading control. Data is expressed as the mean ± SEM (n = 4). **B**) Photomicrographs of tricellulin immunofluorescent localization in different segments of the adult rat epididymis. Tricellulin (green; yellow arrow) was localized to the apical region of the epithelium in every region of the epididymis (**A**, initial segment; **B**, caput; **C**, corpus; **D**, cauda). A tripunctate tricellulin staining pattern was observed (inset, **C**) in certain areas of the caput, corpus, and cauda epididymidis. Nuclei were stained with DAPI (blue). A dotted yellow line in the panel of the initial segment displays the area of a typical principal cell. P, principal cell; B, basal cell; L, lumen; S, sperm. See also Supplemental Movie S1.

and the Ortho function in the Zen software (Zeiss). These data confirmed the colocalization of tricellulin with occludin in caput, corpus, and cauda epididymidis, and showed that

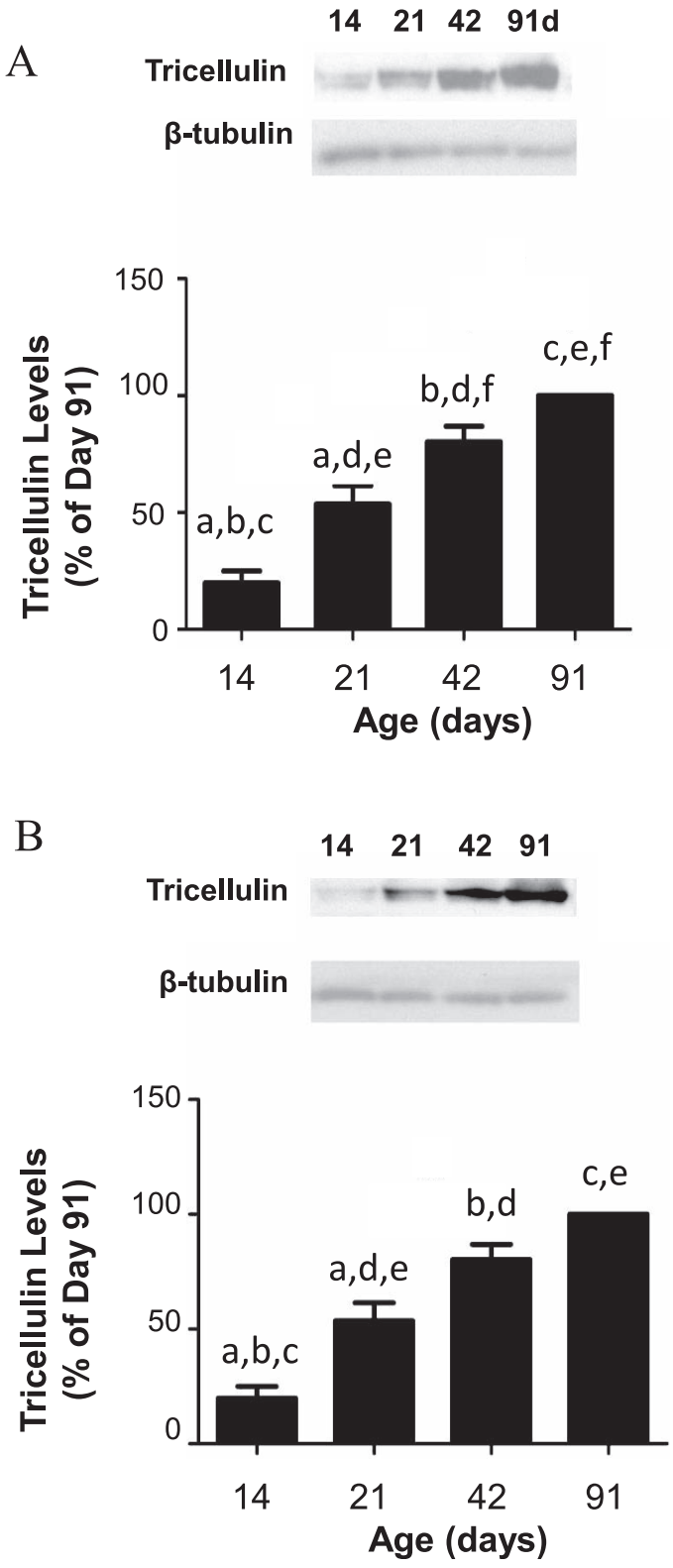

FIG. 2. Expression and localization of tricellulin at different ages. Western blot analyses of tricellulin in epididymidis from rats at 14, 21, 42, and 91 days of age. Epididymides were divided into two regions: proximal (initial segment, caput, and corpus; upper panel, **A**) and distal (cauda; lower panel, **B**) region. Tricellulin levels were normalized to α-tubulin, which was used as a loading control. Data are expressed as the mean ± SEM (n = 4). Groups with the same letter indicate a statistical difference ($P \leq 0.05$; ANOVA).

in the initial segment, there was greater separation of signal between tricellulin and occludin (Fig. 5).

Colocalization of Tricellulin and Cytokeratin 5

Previous studies have reported that apical projections of basal cells could cross the tight junctions of the BEB to reach the lumen of the epididymis in the corpus and cauda epididymidis [25]. To determine whether or not tricellulin was localized at contact points between basal and principal cells, colocalization experiments were done with tricellulin and cytokeratin 5 (KRT5), a marker of basal cells [37, 38].

In the initial segment and caput epididymidis, no apical projections of basal cells were detected and, as such, tricellulin and KRT5 were not colocalized (Fig. 6). In the corpus and cauda epididymidis, however, basal cell projections were observed, as previously reported [21]. Tricellulin and KRT5 were not colocalized at the sites where basal cells reached the apical region of the epithelium, suggesting that basal cells do not form tripartite cellular junctions with principal cells in the area of the tight junction (Fig. 6). Three-dimensional reconstructions confirmed the absence of colocalization between tricellulin and KRT5 in the corpus and cauda where there were cytoplasmic projections of basal cells (Fig. 6 and Supplemental Movie S2).

Transepithelial Resistance

To assess the role of tricellulin in the barrier function of epididymal tight junctions, we determined the TER in RCE cells in which the formation of tight junctions was stimulated using changes in calcium concentration (i.e., calcium switch) [39]. MTT assays were done to assess the viability of the cells treated with either nonsense or tricellulin siRNA. In all cases, there were no significant differences in cell viability (data not shown) and tricellulin levels were decreased by approximately 65% (Fig. 7A). TER in cells treated with nonsense siRNA increased until 48 h following the calcium switch (Fig. 7B; approximately 180 Ω/cm^2). In cells treated with tricellulin siRNA, the TER decreased significantly at 6 h and remained significantly less than in cells treated with nonsense siRNA at all subsequent time points (Fig. 7B).

Effects of Tricellulin Knockdown on Other Junctional Proteins

To determine the role of tricellulin in the expression of other junctional proteins, Western blot analyses were done on cells treated with either nonsense or tricellulin siRNA for 48 h (Fig. 8A). Tricellulin siRNA treatment significantly decreased tricellulin levels by approximately 80%. Likewise, levels of occludin, Cldn3, and Cldn1 were also decreased (Fig. 8B). There were no significant differences in levels of either TJP1 or Cdh1 protein levels (Fig. 8B).

DISCUSSION

Tricellular, or tripartite, tight junctions are structurally unique forms of tight junctions. Freeze-fracture electron microscopy micrographs have shown that the tight-junction belt around the cell is not continuous in the area of tricellular contacts. In this region, the long strands of apical tight junctions tend to bend in a basal direction [40]. These strands are connected with shorter strands that form a network at the contact points of the tricellular junction. Hence, the architecture and protein composition of tricellular tight junctions differs

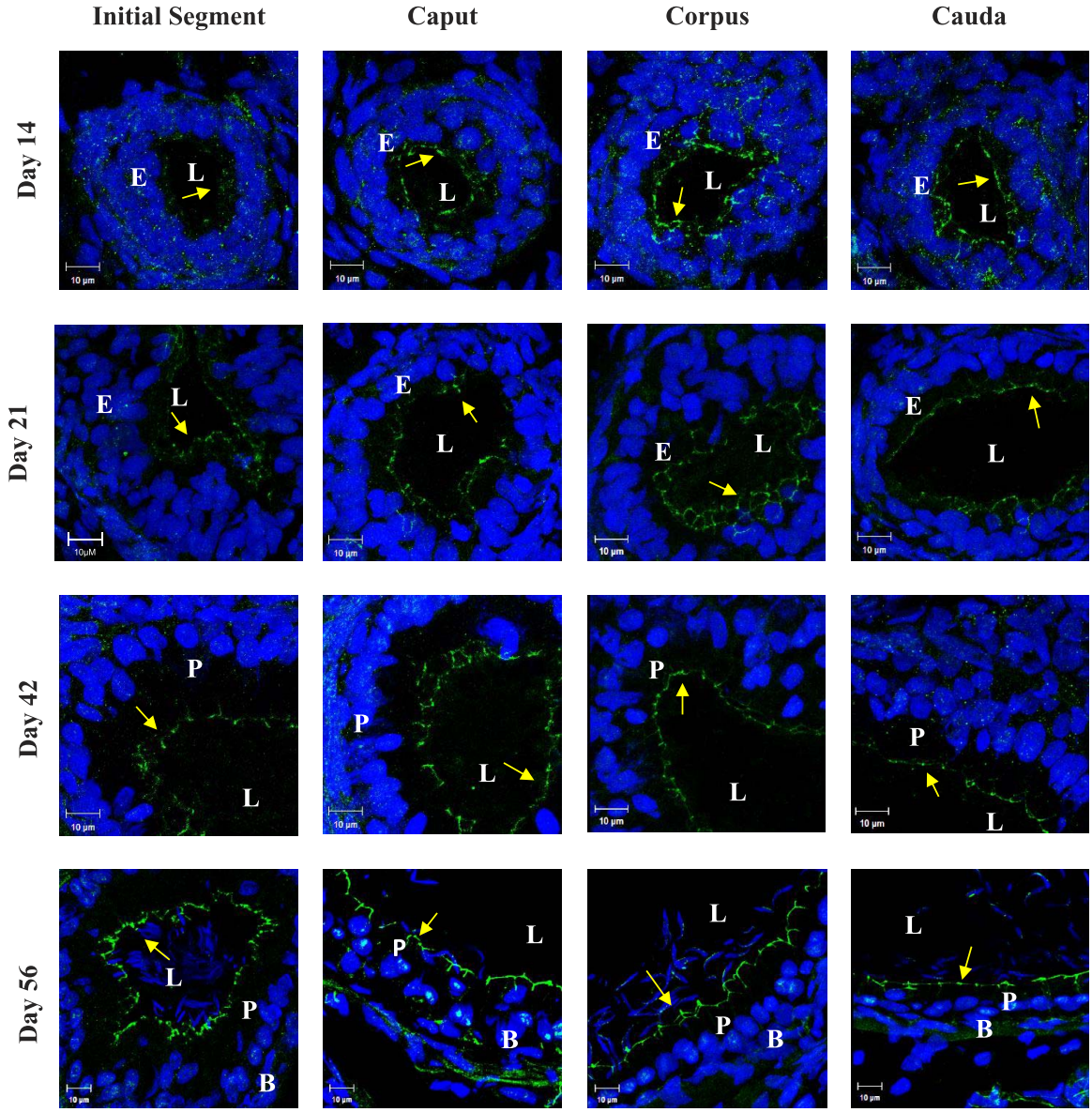

FIG. 3. Immunolocalization of tricellulin during epididymal development. Photomicrographs of immunofluorescent localization of tricellulin (green) in the initial segment, caput, corpus, and cauda epididymidis. At Day 14, the BEB tricellulin was present along the apical region of the epithelium as well in the cytoplasm of the cells lining the lumen (yellow arrow). At Day 21, tricellulin was localized to the lateral margins of the plasma membrane, although there is still some staining in the cytoplasm, in particular in the initial segment (yellow arrow). By Day 42, tricellulin was localized primarily to the apical lateral plasma membrane of the epithelium (yellow arrow). At Day 56, the immunostaining appears more intense and localized to the apical lateral plasma membrane (yellow arrow). Nuclei are stained with Hoechst dye (blue). L, lumen; E, epithelium; P, principal cells; B, basal cells.

from that of bicellular tight junctions [26, 41]. While occludin and CLDNs may occur in the area of the tricellular junction, tricellulin was the first protein identified that specifically concentrates at the level of the tricellular tight junction [26]. More recently the lipolysis-stimulated lipoprotein receptor (LSR) members of the angulin family of proteins (ILDR-1, -2) have also been shown to be important for tricellular tight junctions and, in particular, in the recruitment of tricellulin to these junctions [42, 43].

Our present data indicates that tricellulin is expressed along the entire length of the rat epididymis, in the apical region of the epithelium, and that the levels are similar between the different regions of the epididymis. This pattern differs from that of many of the CLDNs and of occludin, which are present in bicellular tight junctions, and whose levels can vary dramatically between different regions of the epididymis [9, 11, 44]. It is, therefore, likely that the regulation of tricellulin is distinct from the regulation of other tight-junction proteins, such Cldn10, Cldn8, or occludin, for example, which display segment-specific expression levels in the epididymis [45]. The immunolocalization of tricellulin revealed a tripunctate staining that is more evident in the caput, corpus, and cauda regions than in the initial segment. This tripunctate staining has been observed in the liver and MTD-1A cells and is believed to be related to the localization of tricellulin at both edges of bicellular tight junctions [27, 46].

Postnatal developmental expression and localization of tricellulin varies significantly with age based on our Western blot and immunofluorescent data. In young rats (14-days old), tricellulin levels were low and displayed cytoplasmic localization throughout the epididymis. Tricellulin levels increased as a function of age, and by Day 42, the immunostaining became

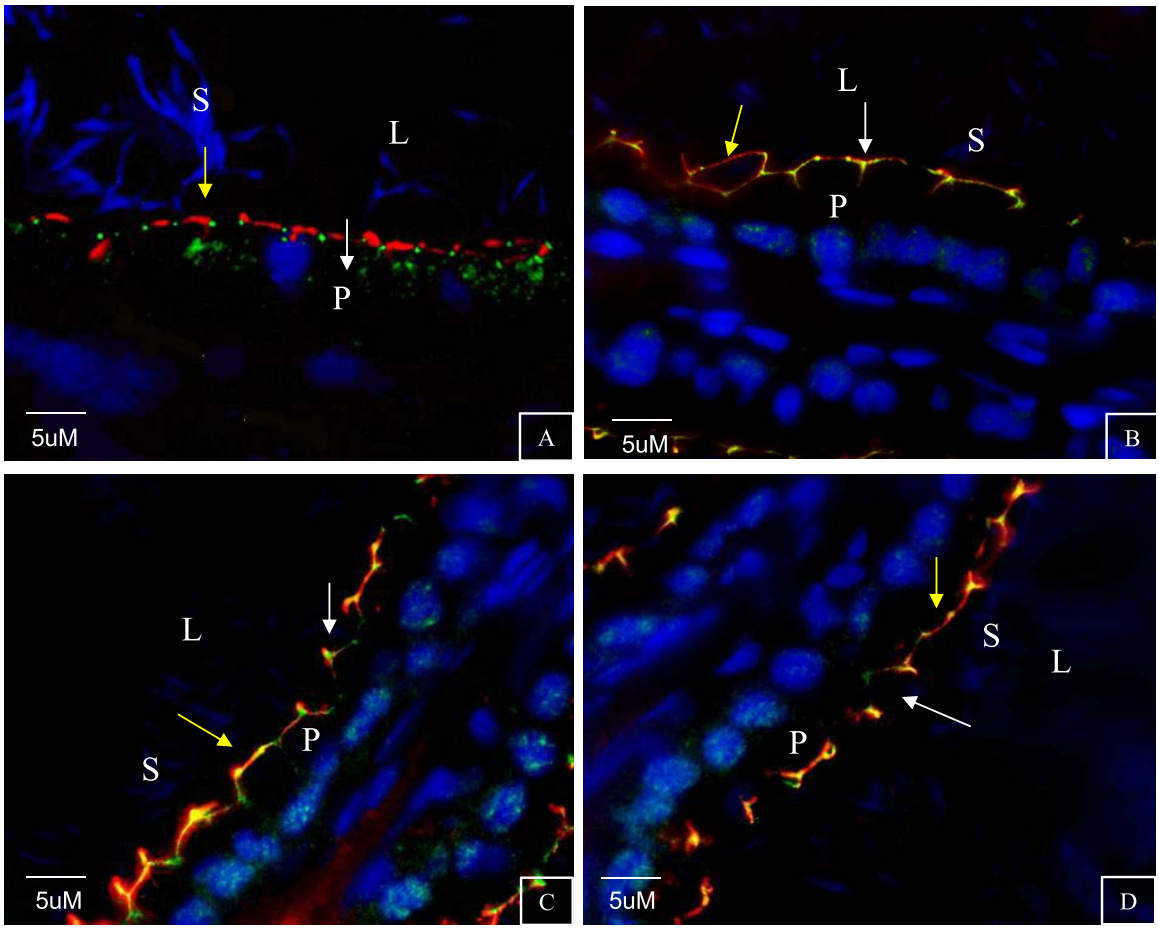

FIG. 4. Colocalization of tricellulin and occludin in adult rat epididymis. Photomicrographs of tricellulin (green) and occludin (red) immunostaining in the adult rat epididymis. Merged images show that in the initial segment (**A**) occludin and tricellulin do not overlap and appear to be present in different areas of the BEB (white arrows). In the caput (**B**), corpus (**C**), and cauda (**D**) epididymidis, merged images suggest colocalization of tricellulin and occluding (yellow arrows in the apical area of the epithelium where tight junctions are localized, although some areas show occludin immunostaining without tricellulin (white arrows). Nuclei are stained with DAPI (blue). L, lumen; P, principal cell; S, spermatozoa.

increasingly localized to the apical region of the lateral plasma membrane. Previous studies have reported a similar change in immunolocalization of other tight-junction proteins, including Cldn3, Cldn4, and Cldn10 [9, 44]. These data support the notion that the assembly of tight junctions is completed postnatally in the rat. While Guan et al. [44] reported that the rat epididymis was impermeable to lanthanum by Postnatal Day 7, there is clearly a maturation of the tight junctions that occurs later during postnatal development. Suzuki and Nagano [2] reported that the strands of the tight junctions observed in freeze-fracture electron microscopy change as a function of age until Day 42. The changes in localization of tricellulin in this study and previously reported changes in the cellular localization of CLDNs may reflect the changes in the architecture of epididymal tight junctions as a function of age and development and suggest a type of reinforcement of the tight-junction complex.

Tricellulin and occludin share approximately 32% sequence homology, and have been shown to colocalize in the tight junctions of the human nasal mucosa [30]. In the initial segment, occludin and tricellulin did not colocalize even though both proteins were present in the area of the tight junctions. Previous studies have reported differences in the expression and localization of occludin in the initial segment of the mouse, where levels are low or completely absent [10] as compared to other regions of the epididymis. Other studies have also reported differences in the regulation of junctional proteins in the initial segment relative to other segments [47, 48]. Colocalization studies of occludin and tricellulin in other segments indicate that there are overlapping and nonoverlapping staining of the proteins within the area of the BEB. While tricellulin appears to be present at the same region as occludin, there is occludin immunostaining that occurs in the absence of tricellulin. Because these proteins are localized to different areas of the tight junctions, these results are not necessarily surprising and are similar to those observed in a human pancreatic cell line in vitro [49].

Previous studies have reported that basal cell projections can migrate apically and reach the lumen of the epididymis [24, 25]. Shum et al. [25] suggested that these projections cross the tight junctions of the BEB. To determine if tricellulin was expressed at the apical contact points between basal and principal cells, a colocalization study of tricellulin and KRT5, a specific marker of basal cells [38, 50], was done. We did not observe any apical projections of basal cells in the initial segment and caput epididymidis; in the corpus and cauda epididymidis, where basal cell apical projections were noted, there was no colocalization of KRT5 and tricellulin. These data suggest that basal cells do not appear to form tricellular tight junctions in the epididymis.

To assess the role of tricellulin in the function of epididymal tight junctions, TER was assessed using RCE cells treated with

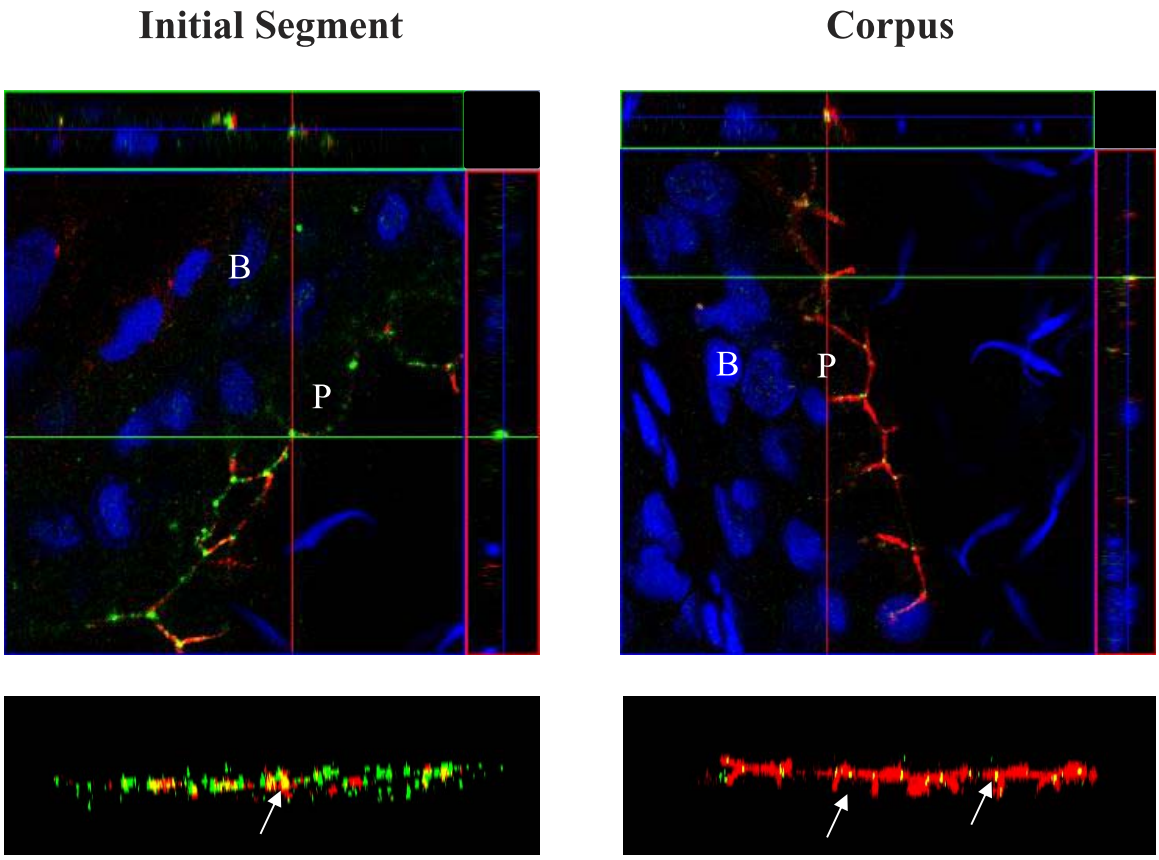

FIG. 5. Photomicrographs using confocal microscopy of tricellulin (green) and occludin (red) immunostaining in adult rat epididymis. Stacked images of initial segment and corpus are analyzed with the Ortho feature of the Zen software (upper panels). Corpus is used as representative of caput, corpus, and cauda. Three-dimensional reconstructions (lower panels) show that in the initial segment, tricellulin and occludin have distinct signals, while in the corpus epididymidis, tricellulin colocalizes with occludin. Nuclei are stained with Hoechst dye (blue). P, principal cell; B, basal cells. Original magnification ×378.

siRNA against tricellulin. Knockdown of tricellulin caused a decrease in TER, indicating the importance of tricellulin in tight-junction function. Similar results were observed in breast epithelial cells (Eph4 cells) [26], and more recently in human pancreatic cell line cells [49]. Ikenouchi et al. [26] showed that tricellulin knockdown resulted in an increase in paracellular permeability of Eph4 cells. Furthermore, overexpression of human tricellulin-a (Tric-a), an isoform of tricellulin, increased TER by 3-fold in MDCK II cells (Mardin-Darby canine kidney cell line), which normally exhibit low levels of resistance [51]. To explain differences in tricellulin knockdown studies, it has been proposed that the paracellular pathway can be divided into two separate pathways: a porous pathway (limited pore size of 0.4 nm) and a leaky pathway, which contributes to the passage of macromolecules between adjacent cells [52, 53]. Tricellular tight junctions appear to be responsible for regulating the leaky pathway [43]. Analysis of the physiological functions of tricellulin has revealed that it may also alter bicellular tight junctions by increasing paracellular electrical resistance and decreasing permeability of ions and larger solutes [51]. At tricellular contacts, tricellulin specifically seals epithelial cell sheets, thereby preventing the passage of macromolecules without affecting ion permeation [51]. Whether or not this is the case in the epididymis is currently not known; however, regulation of paracellular movement of ions and macromolecules across the tight junctions offers an intriguing mechanism by which tight junctions can regulate the luminal environment of the epididymis.

To further understand the mechanism by which tricellulin knockdown decreased TER in RCE cells, we examined levels of other tight-junction proteins. These experiments indicated that tricellulin knockdown resulted in decreased levels of occludin, Cldn3, and Cldn1. There were no effects on the levels of either TJP1 or Cdh1. The loss of occludin and of CLDNs indicates that the composition of the epididymal tight junctions was altered by the loss of tricellulin. Ikenouchi et al. [27] reported that tricellulin may be incorporated into claudin-based tight junctions and may play a role in the secondary organization, or structure, of tight-junctional strands and that it can interact with Cldn3, which we have shown to be present in the epididymis [9, 11]. Furthermore, cotransfection of cells with tricellulin and Cldn1 can alter the network of tight-junction strands [54]. Studies in HEK293 cells, which do not form tight junctions, have shown that tricellulin alone was not sufficient to induce the formation of tight junctions, but that cotransfection of Cldn1 and tricellulin resulted in an enrichment of tricellulin at the points of cellular contact, indicating that tricellulin interacts with Cldn-based junctions [54]. Interestingly, while tricellulin knockdown in RCE cells resulted in a decrease in both Cldn-3 and -1, studies in Caco-2 cells have reported that Cldn1 knockdown had no effect on levels of tricellulin [54].

Tricellulin knockdown did not cause any changes in either TJP1 or Cdh1 levels. It has been reported that tricellulin, unlike occludin or CLDNs, does not require TJP1

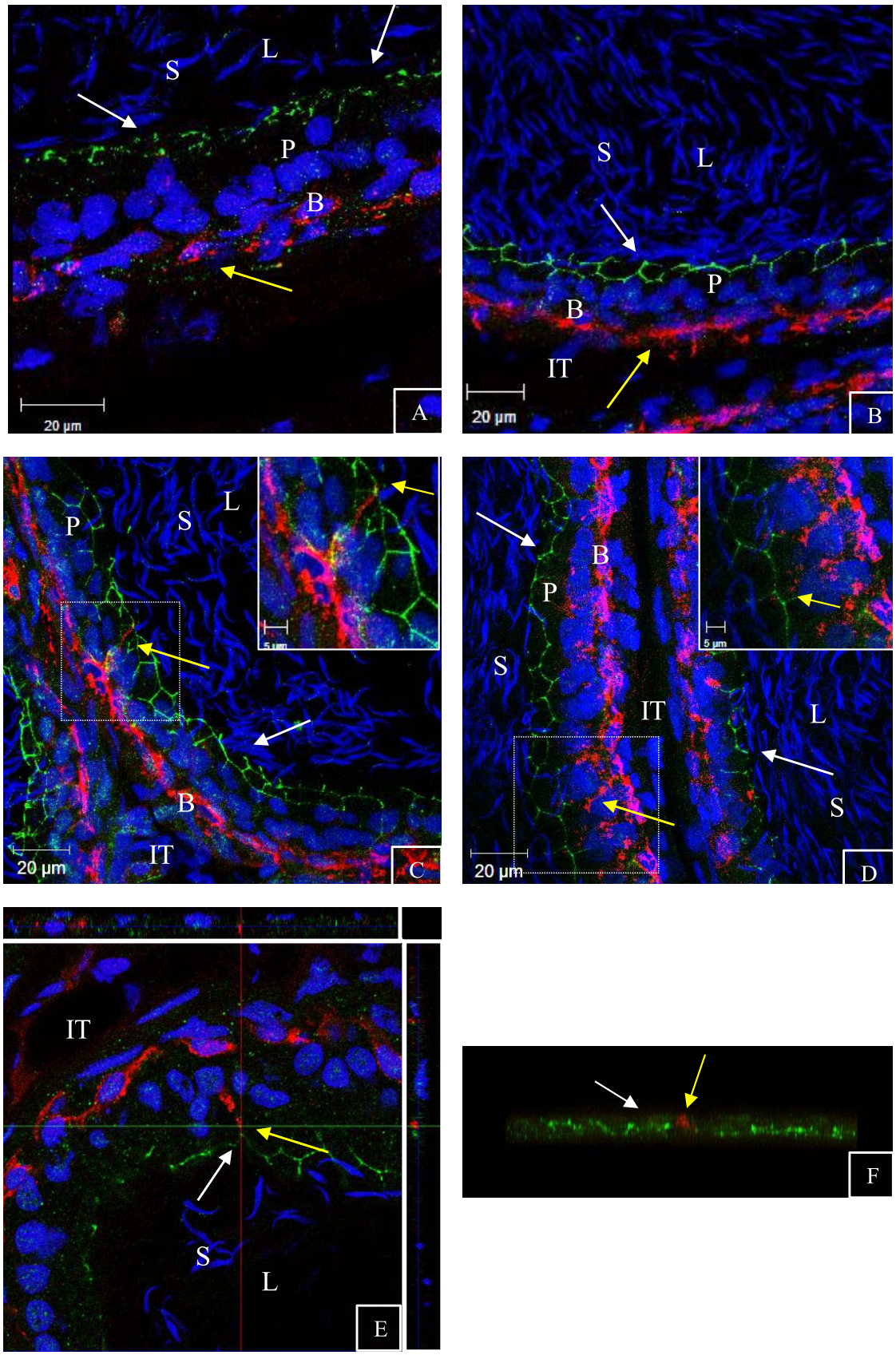

FIG. 6. Confocal microscopy images of the colocalization of tricellulin and KRT5 in adult rat epididymis. Photomicrographs of tricellulin (green; white arrow) and KRT5 (red; yellow arrow) immunostaining in the adult rat epididymis (**A**, initial segment; **B**, caput; **C**, corpus; **D**, cauda). Merged images show that in the initial segment and caput epididymidis KRT5 and tricellulin do not overlap because KRT5 is restricted to the basal compartment of the epithelium while tricellulin is localized to apical region. In the corpus and cauda epididymidis, some KRT5 immunostaining was present in the apical region of the epithelium (yellow arrow). Tricellulin and KRT5 did not colocalize at areas where basal cell projection reach the lumen of the epididymis (yellow arrow; insets). Stacked images (**E**) analyzed with the Ortho feature of the Zen software show that in the corpus epididymidis, KRT5 staining is not

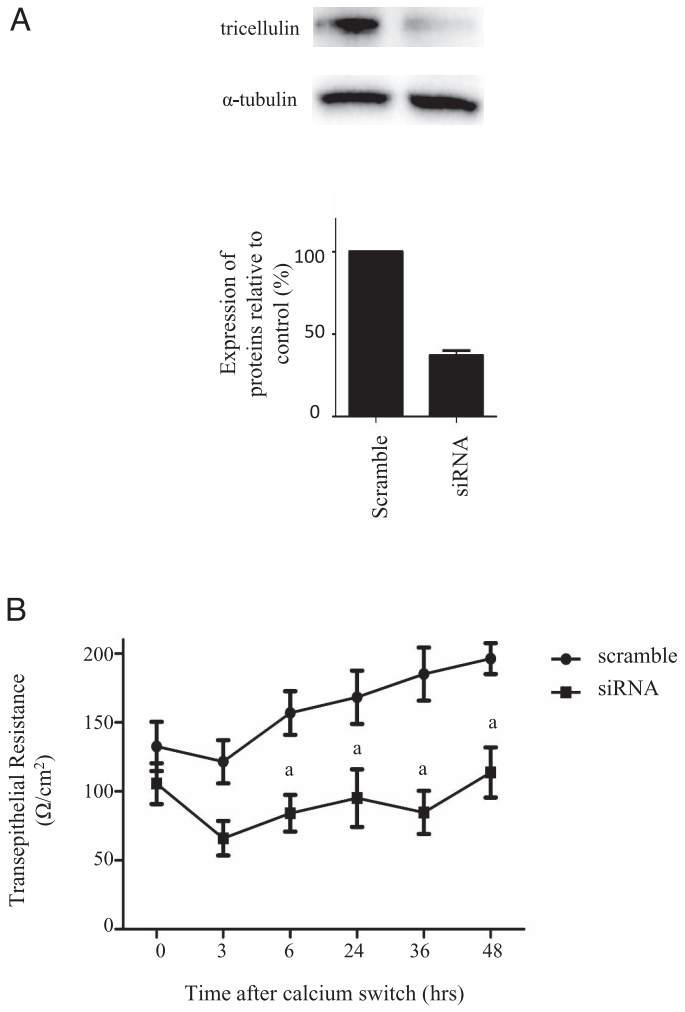

FIG. 7. Role of tricellulin in maintaining the integrity of epididymal tight junctions. **A**) Western blot analyses were done on RCE cells treated with scramble (control) and tricellulin siRNA (n = 3). Tricellulin levels were decreased about 65% in cells treated with the tricellulin siRNA. **B**) Transepithelial resistance (TER) was measured in RCE cells at different time points after switching from low to normal calcium-containing medium with a final concentration of 1.8 mM (n = 4). A peak in TER was seen 48 h after calcium switch. In cells treated with a tricellulin siRNA, TER was significantly ($P \leq 0.05$; ANOVA) lower from 6 h onward. Data are expressed as the mean ± SEM; the letter (a) indicates a significant difference from scramble siRNA controls.

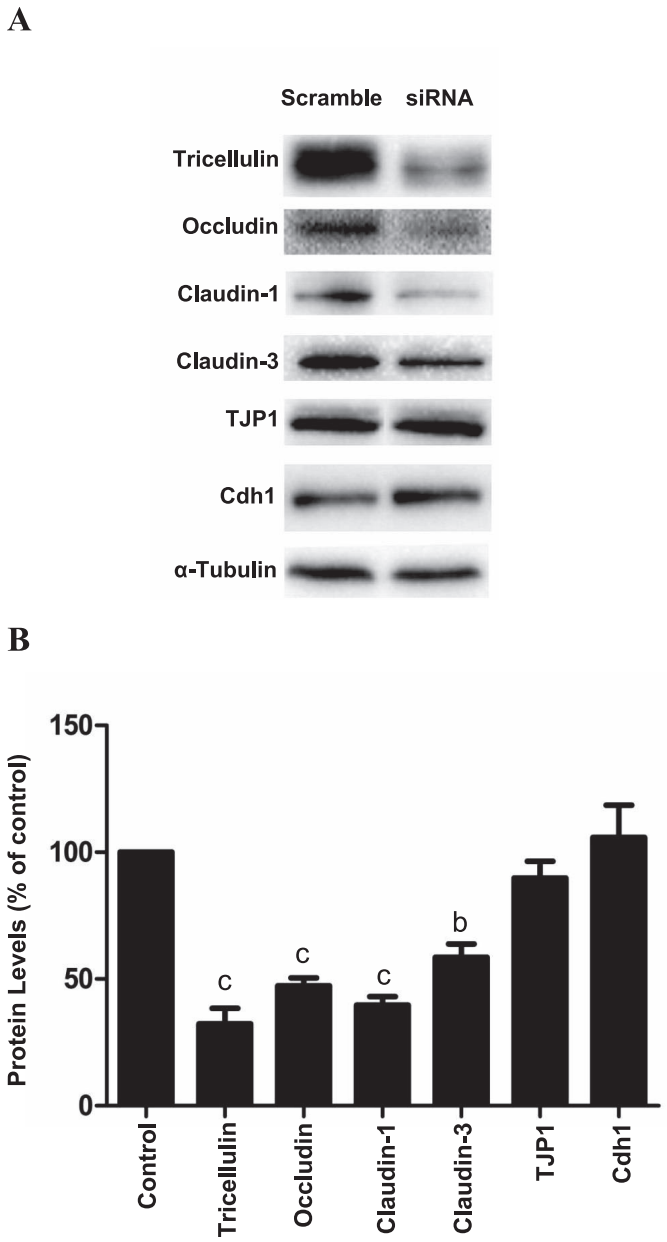

FIG. 8. Effects of tricellulin knockdown on other junctional proteins in RCE cells. **A**) Western blot analyses were done on cells treated with scramble (control) and tricellulin siRNA (n = 4; for Cldn1 n = 3). **B**) Tricellulin levels were significantly decreased by approximately 70% in cells treated with the tricellulin siRNA. There was a significant decrease in occludin, Cldn1, and Cldn3 levels in cells treated with the tricellulin siRNA. There were no significant differences in either TJP1 or Cdh1 levels. Statistical analyses were performed using a one-way ANOVA and Dunnett post test. Letters designate a significant difference from scramble siRNA controls. (b = $P \leq 0.01$; c = $P \leq 0.001$).

at cell-cell contacts to form a link with the cytoskeleton of the cell [27]. Hence, the loss of tricellulin is unlikely to result in changes in TJP1. Cdh1, a cell adhesion protein, was also not affected by tricellulin knockdown. We have previously shown that during the formation of tight junctions in the epididymis, the adherens junctions play a role in the localization of TJP1 [55]. It is therefore conceivable that the roles of Cdh1 and TJP1 are upstream from tricellulin with respect to the formation of epididymal tight junctions.

In conclusion, tricellulin is implicated in the formation and maintenance of tight junctions of the BEB. Its expression and localization are correlated with the development of the epididymis, and it interacts with both occludin and Cldn-based tight junctions of the epididymis. Based on changes in TER following tricellulin knockdown by siRNA, tricellulin appears to be important for maintaining the integrity of epididymal tight junctions through these interactions.

present in the same region as tricellulin (**E** and **F**). See also the Supplemental Movie S2. Nuclei are stained with Hoechst dye (blue). L, lumen; P, principal cell; B, basal cells; S, spermatozoa; IT, interstitium. Original magnification ×378.

ACKNOWLEDGMENT

Julie Dufresne, Mary Gregory, Jessy Tremblay, and Dr. Isabelle Plante (INRS) are thanked for their suggestions and assistance.

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