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The MAPK/ERK-Signaling Pathway Regulates the Expression and Distribution of Tight Junction Proteins in the Mouse Proximal Epididymis[1]

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ABSTRACT

The initial segment (IS) in rodents is functionally and structurally distinct from other epididymal segments and plays an important role in sperm maturation. The MAPK/ERK1/2 pathway is maintained active in the IS by testicular luminal factors and plays crucial roles in the maintenance and differentiation of the IS epithelium. Tight junctions (TJs) are constituents of the blood-epididymis barrier, which mediates the paracellular transport of ions, solutes, and water and controls epithelial cell differentiation, thereby contributing to the establishment of a unique luminal environment. We examine here the role of the MAPK/ERK1/2 pathway in the regulation of TJ proteins in the IS. Inhibition of mitogen activated protein kinase kinase (MAPKK or MEK1/2) with PD325901, followed by reduction of ERK1/2 phosphorylation (pERK), decreased zonula occludens (ZO)-2 expression and increased ZO-3 expression in TJs but had no effect on ZO-1 expression. In control mice, in addition to being located in TJs, claudin (Cldn)-1, Cldn-3, and Cldn-4 were detected in the basolateral membrane of epithelial cells, with enriched expression of Cldn-1 and Cldn-4 in basal cells. PD325901 reduced the expression of Cldn-1 and Cldn-4 at all locations without affecting Cldn-3. Occludin was undetectable in the IS of control mice, but PD325901 triggered its expression in TJs. No effect was observed for any of the proteins examined in the other epididymal regions. Our results indicate the participation of the MAPK/ERK1/2 pathway in the regulation of cell-cell events that control the formation and maintenance of the blood-epididymis barrier.

epididymis, epithelial cell biology, male reproductive tract

INTRODUCTION

After leaving the testis, spermatozoa enter the epididymis, where they acquire their ability to find and fertilize an egg. The epididymis is lined by a pseudostratified epithelium that establishes and maintains an optimum luminal environment for sperm maturation, protection, transport, and storage [1–5]. It is divided into four anatomical regions: the initial segment (IS), caput, corpus, and cauda. In rodents, the IS plays an essential role in sperm maturation and is functionally and structurally distinct from other epididymis segments [6–14]. The IS epithelium contains tall columnar cells, the principal cells and narrow cells, as well as basal cells [15, 16]. Principal and narrow cells feature distinct apical and basolateral membrane domains that express unique sets of transporters [17–19]. Basal cells are not polarized per se, but they exhibit a polarized plasticity by extending a long body projection between adjacent epithelial cells toward the apical region of the epithelium [20–23]. In the mouse IS, these basal cell projections can cross the blood-epididymis barrier (BEB) to reach the luminal compartment [20].

The highly organized architecture of the epididymal epithelium requires cell-cell junctions, including tight junctions (TJs), which participate in the establishment of cell polarity, allowing for the unidirectional transcellular transport of ions, water, and solutes, and control the permeability of the paracellular transport pathway. The first protein identified as a TJ constituent in epithelia was ZO-1, a peripheral membrane protein that connects transmembrane TJ proteins to the actin cytoskeleton and other cytosolic proteins [19, 24–27]. Two more proteins, ZO-2 and ZO-3, were subsequently identified as members of the ZO protein family [28]. Occludin was the first TJ transmembrane protein identified [29]. In adult mice, occludin is present in TJs of the caput, corpus, and cauda epididymidis but it is absent from the IS despite the presence of extensive TJs in this segment [30, 31]. Other important TJ transmembrane proteins are claudins (Cldn), which are part of a large family of tetra-membrane-spanning proteins that modulate the paracellular permeability to ions and water [25, 26]. Several claudins, including Cldn1, Cldn3, and Cldn4, were localized in epididymal TJs and play essential roles in the barrier function of the human and rat epididymis [21, 31–34]. Interactions among these TJ proteins control the highly polarized nature of epididymal epithelial cells and are essential for the physiological, cellular, and molecular regulation of the epididymis.

The maturation, differentiation, and establishment of cell polarity in the IS epithelium require several factors, including luminal testicular factors, which activate signaling pathways, including the MAPK/ERK pathway in this segment [6]. ERK is an essential player in many cellular and physiological functions, including proliferation, survival, apoptosis, motility,

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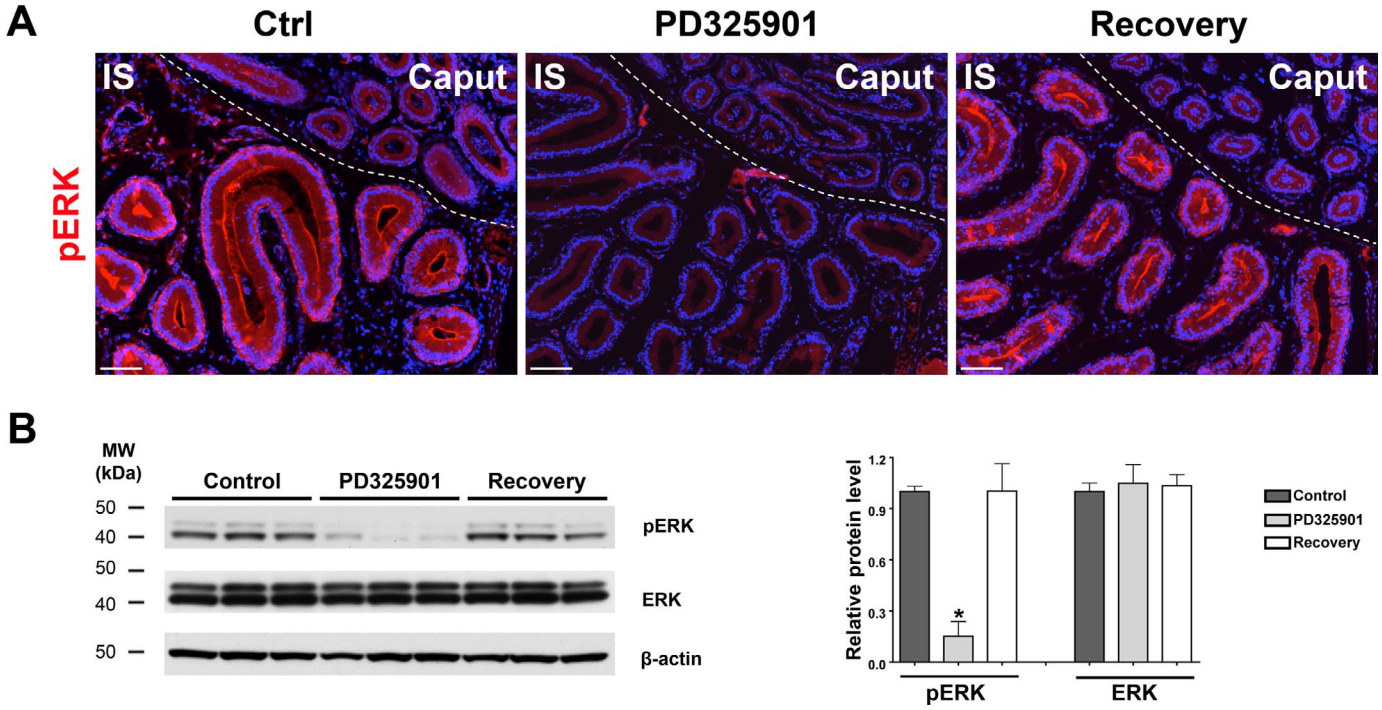


FIG. 1. The MEK1/2 inhibitor PD325901 reduces ERK1/2 phosphorylation in the IS. **A**) The proximal mouse epididymis was labeled using an antibody that recognizes phosphorylated ERK1/2 (p-ERK). In control mice, ERK1/2 is highly phosphorylated in the IS compared to caput regions (left). PD325901 abolished ERK1/2 phosphorylation in the IS of mice that were treated for 3 days (middle). In mice allowed to recover for 72 h, ERK1/2 phosphorylation in the IS returned back to control level (right). Bars = 50 μm. **B**) Western blot and quantification of proteins extracted from the IS and probed for pERK and total ERK. Three different protein extracts are shown for each group. β-actin was used as a loading control. Significant decreases in pERK were detected in the PD325901 group, followed by return back to normal levels in the recovery group. No change in total ERK expression was detected. Band density was calculated and normalized to β-actin using Image J software. Data are expressed as mean ± SEM relative to control values (*$P < 0.01$).

transcription, metabolism, and differentiation [35]. In the epididymis, the MAPK/ERK signaling pathway plays an important role in cell proliferation and differentiation in the developing IS as well as in maintaining cell survival in the adult IS [6, 8]. We examined here the regulation of TJ proteins in the mouse epididymal IS via modulation of the MAPK/ERK signaling pathway.

MATERIALS AND METHODS

Antibodies

All antibodies used in the present study are listed in Supplemental Table S1 (primary antibodies; Supplemental Data are available online at www.biolreprod.org) and Supplemental Table S2 (secondary antibodies).

Animals

Adult C57BL//CBAF1 male mice were purchased from Jackson Laboratories. All procedures described here have been reviewed and approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experiments were performed on at least five animals for each group.

PD325901 Administration

Adult male mice were divided into a control group (n = 10) and a PD325901-treated group (n = 10). For the PD325901-treated group, mice received a dose of 10 mg/kg per day every 12 h for 36 h by i.p., as described previously [36]. In recovery experiments, one side of the epididymis was removed from the body 36 h after PD325901 treatment and used as the treated group, and the other side of the epididymis was removed 72 h after recovery and used as the recovery group.

Efferent Duct Ligation

Unilateral efferent duct ligation (EDL) was performed to prevent lumicrine factors from entering the epididymis, as described previously [20]. Mice were anesthetized with isoflurane (3%–4% in oxygen, inhaled), and the testis and efferent ducts were exposed through a low midline incision under sterile conditions. A needle leading a 4-0 nylon suture was passed around the ducts. The suture was tied tightly around the efferent ducts while taking care to avoid damage to nearby blood vessels. The testes and epididymides were placed back into the abdomen, and the surgical site was closed using nylon sutures.

Tissue Fixation and Immunofluorescence

Mice were killed by inhalation of CO_2, and the epididymides were harvested and fixed by immersion in periodate-lysine-paraformaldehyde containing 4% paraformaldehyde for ~5 h at room temperature as described previously [20, 37] and rinsed three times in PBS for 5 min each. Tissues were cryoprotected by incubating them in a solution of 30% sucrose in PBS for at least 24 h. Tissues were embedded in OCT compound (Tissue-Tek; Sakura Finetek), mounted on a cutting block, and frozen. The tissue was then cut at 10- to 16-μm thickness using a Leica 3050 cryostat (Leica Microsystems). Sections were picked up onto Fisher Superfrost /Plus microscope slides (Fisher Scientific) and refrigerated until use. Sections were hydrated in PBS and heated by microwaving in an alkaline buffer (Vector Laboratory) three times for 2 min each time, with 5-min intervals for antigen retrieval. To block nonspecific-binding, 1% bovine serum albumin in PBS was applied for 30 min at room temperature. The sections were then incubated with primary antibodies in a moist chamber for 90 min at room temperature or overnight at 4°C. The samples were washed in PBS and incubated with secondary antibodies for 60 min at room temperature. All antibodies were diluted in DAKO antibody diluent (DAKO).

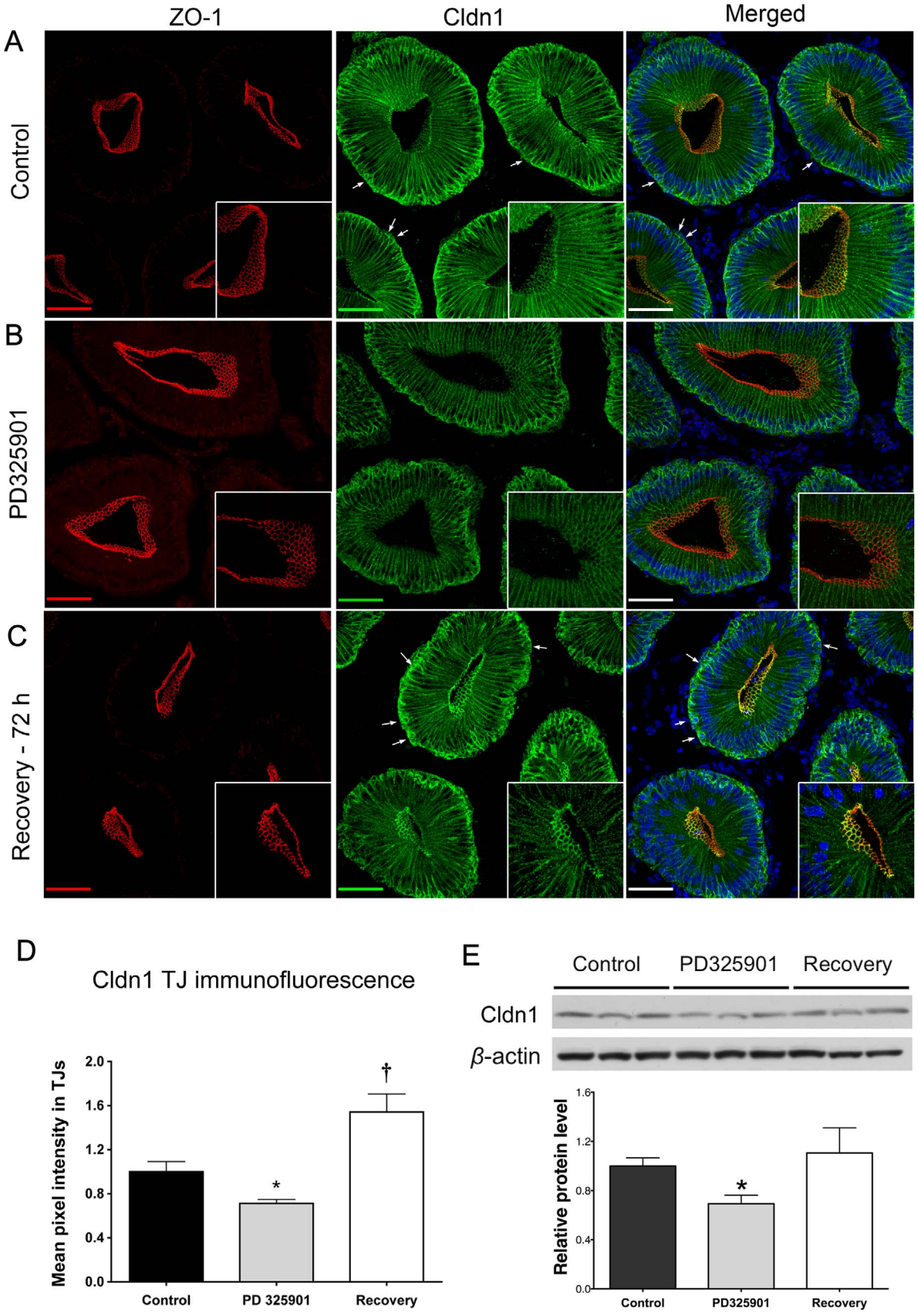


FIG. 2. PD325901 decreases Cldn1 expression in the IS. The epididymis IS was immunolabeled for ZO-1 (red) and Cldn1 (green). **A**) In control mice, Cldn1 is detected in TJs, positive for ZO-1, and in the basolateral membrane of epithelial cells. Higher expression is detected in basal cell plasma membrane (arrows). **B**) PD325901 treatment induced a decrease in Cldn1-associated immunofluorescence in TJs and basolateral membrane. **C**) Cldn1 labeling was brighter in the IS of mice that were allowed 72 h of recovery without the drug. No change was detected in ZO-1 labeling, which was restricted to TJs. Higher expression is detected in the plasma membrane of basal cells (arrows) compared to adjacent epithelial cells. Bars = 50 μm. **D**)

Protein Extraction and Immunoblotting

Tissues were lysed for 30 min on ice in RIPA buffer (Boston Bioproducts) containing complete protease inhibitors (Roche) and phosphatase inhibitors (Roche). Centrifugation was performed at 16 000 × *g* for 30 min at 4°C, and the supernatant was collected. Protein concentration was determined using the bicinchoninic acid assay (Thermo Scientific). For electrophoresis, samples were prepared in LDS Sample Buffer (Invitrogen) with 4% β-mercaptoethanol and incubated for 30 min at room temperature; 30–100 μg of protein were loaded into each well of 4%–12% NuPAGE gels (Invitrogen). After SDS-PAGE separation, proteins were transferred onto Immun-Blot polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dry milk and then incubated overnight at 4°C with the primary antibody diluted in TBS containing 2.5% milk. After three washes in TBS containing 0.1% Tween 20, membranes were incubated with secondary antibody for 1 h at room temperature. After three or four additional washes, antibody binding was detected with the Western Lightning Chemiluminescence reagent (Perkin Elmer Life Sciences) and Kodak imaging films.

RNA Extraction and Real-Time PCR

RNA was isolated from pooled epididymides from control, PD325901-treated, and recovery groups using the RNeasy Macro Kit (Qiagen); cDNA was synthesized using iScript Advanced cDNA Synthesis Kit (Bio-Rad). All quantitative PCR (qPCR) assays were carried out using the SYBR Green Mixes (Applied Biosystems) as previously described [38]. The primer sequences are listed in Supplemental Table S3.

Quantification of Immunofluorescence of Proteins in TJs

Epididymis sections were double-immunolabeled for ZO-1 and Cldn-1, Cldn-3, Cldn-4, or occludin. Images were acquired with a 40× objective using a Nikon A1R laser scanning confocal microscope. For comparisons between groups, at least four animals were used in each group. To quantify the level of each protein expression in TJ, Image J software (National Institutes of Health) was used to generate a mask corresponding to the ZO-1 positive regions of the section. This mask was then applied to the channel corresponding to the protein being quantified (Cldn-1, Cldn-3, Cldn-4, or occludin). The mean pixel intensity of the immunofluorescence labeling for each protein was quantified in the regions overlapping with ZO-1 labeling.

Statistical Analysis

The numeric data were analyzed using GraphPad Prism (version 4; GraphPad Software Inc.) using one-way analysis of variance followed by a two-tailed unpaired *t*-test. Values are presented as mean ± SEM.

RESULTS

The MAPK/ERK1/2 Pathway Regulates the Expression and Localization of Cldn-1 and Cldn-4 but Not Cldn-3 in the Mouse IS

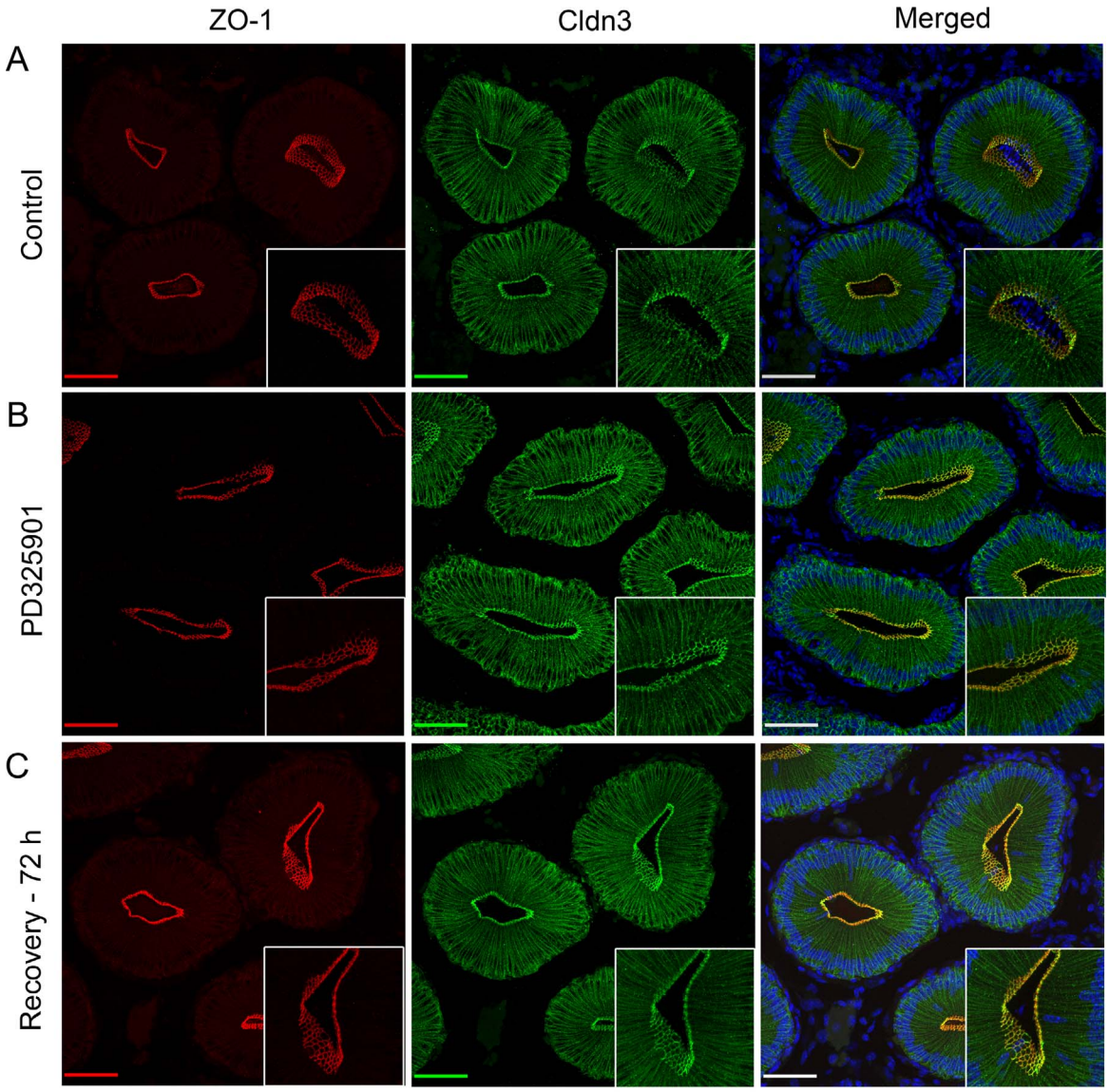
To test for the role of the MAPK pathway in the regulation of TJ proteins, we treated adult male mice with the MEK1/2 inhibitor PD325901 (10 mg/kg/day; i.p.) for 1.5 days. This treatment induced a significant reduction in ERK1/2 phosphorylation in the epididymal IS, compared to control (Fig. 1A, middle and left panels). In recovery experiments, the contralateral epididymides from the same mice were examined after removal of PD325901 treatment. ERK1/2 phosphorylation returned back to normal levels after 72 h of recovery (Fig. 1A, right panel). Western blotting confirmed the reduction of ERK1/2 phosphorylation by PD325901 and restoration back to normal levels after 72 h of recovery (Fig. 1B). Total ERK expression remained stable during and after PD325901 treatment. These results show the efficacy of PD325901 to inhibit the MAPK pathway.

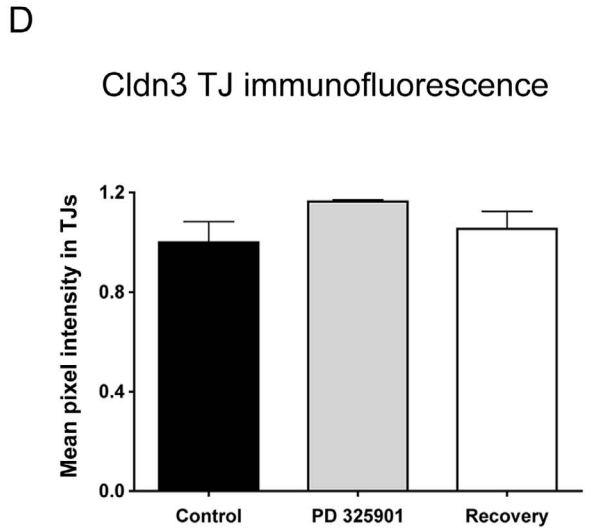
We then determined whether the reduction of pERK by PD325901 altered the expression patterns of TJ proteins. Figure 2A shows that in control mice, Cldn1 is located not only in TJs (positive for ZO-1; insets) but also in the basolateral membrane of epithelial cells, with a higher expression observed in basal cells (arrows), as was previously described in the rat epididymis [21, 34]. PD325901 induced a significant reduction of Cldn1 immunofluorescence labeling, while ZO-1 expression in TJs remained unaffected (Fig. 2B) compared to control (Fig. 2A). Cldn1 labeling was brighter at the end of the recovery period (Fig. 2C) compared to control. Quantification of the mean pixel intensity of Cldn1-associated immunofluorescence in TJs showed a significant reduction by PD325901 followed by an increased signal after 72 h of recovery (Fig. 2D) compared to control. Western blot analysis confirmed the reduction of Cldn1 protein expression by PD325901 but did not show an increase after recovery. As shown in Figure 3, Cldn3 was also detected in TJs (insets) as well as in the basolateral membrane of epithelial cells, but no enrichment was observed in basal cells compared to principal cells (Fig. 3A). PD325901 did not affect Cldn3 immunofluorescence labeling intensity (Fig. 3, A–D). Western blot confirmed the absence of effect of PD325901 on Cldn3 protein expression (Fig. 3E). Similarly to Cldn1, Cldn4 was enriched in the basolateral membrane of basal cells (Fig. 4, arrows) in addition to being present in adjacent epithelial cells and in TJs (insets). PD325901 induced a reduction of Cldn4 basolateral membrane and TJ immunofluorescence labeling (Fig. 4A, middle panels, and Fig. 4B) compared to control (Fig. 4A, left panels, and Fig. 4B). A partial rescue of Cldn4 expression in TJs was observed at the end of the recovery period (Fig. 4A, right panels, and Fig. 4B). Western blot showed reduction of total Cldn4 protein expression by PD325901, followed by a partial recovery after 72 h of recovery (Fig. 4C). Cldn4 expression was not affected by PD325901 in other epididymal regions (Supplemental Figure S1).

Reciprocal Regulation of ZO-2 and ZO-3 by the MAPK Pathway

We next examined whether the two other ZO proteins, ZO-2 and ZO-3, were affected by the reduction of pERK1/2 induced by PD325901. In control mice, ZO-2 is highly expressed together with ZO-1 in TJs of the epididymal IS (Fig. 5A, left panels). PD325901 treatment induced a significant reduction of ZO-2 labeling without altering ZO-1 expression (Fig. 5A, middle panels). ZO-3 also colocalized with ZO-1 in TJs in control mice (Fig. 5B, left panels), but its expression was significantly increased after PD325901

Quantification of Cldn1 labeling in regions associated with TJs (labeled for ZO-1) confirmed the reduction of Cldn1 expression in TJs by PD325901 and higher expression after 72 h of recovery compared to controls. Data are expressed as means ± SEM relative to control values (*$P < 0.05$ compared to control and †$P < 0.05$ compared to PD325901). **E**) Western blot and quantification of Cldn1 expression from the IS of control mice, PD325901-treated mice, and mice allowed to recover without drug for 72 h (recovery). Three different protein extracts are shown for each group. β-actin was used as a loading control. Significant decreases in total Cldn1 expression were detected in the PD325901 group, followed by return back to normal levels in the recovery group. Band density was calculated and normalized to β-actin using Image J software (*$P < 0.05$ compared to control).





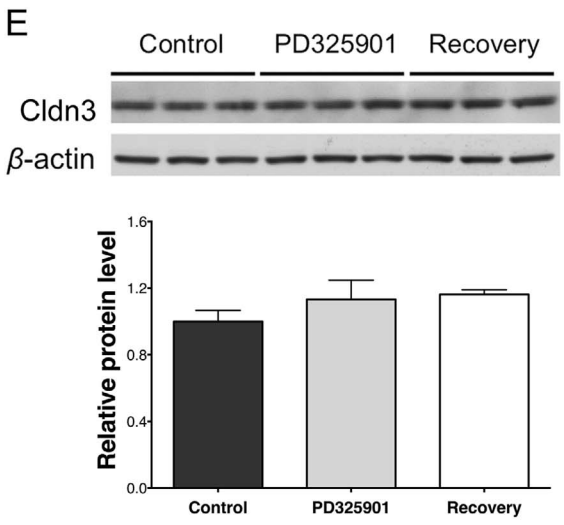


FIG. 3. PD325901 does not affect Cldn3 expression in the IS. The IS was immunolabeled for ZO-1 (red) and Cldn3 (green). **A**) In control mice, Cldn3 is detected in TJs, positive for ZO-1, and in the basolateral membrane of epithelial cells. Uniform expression was expressed among all epithelial cell types. **B**) PD325901 treatment had no effect on Cldn3-associated immunofluorescence labeling. **C**) Cldn3 remained unaffected during the recovery period. No change was detected in ZO-1 labeling, which was restricted to TJs. Bars = 50 μm. **D**) Quantification of Cldn3 labeling in regions associated with TJs (labeled for ZO-1) confirmed the absence of effect of PD325901 on Cldn3 expression. Data are expressed as means ± SEM relative to control values.

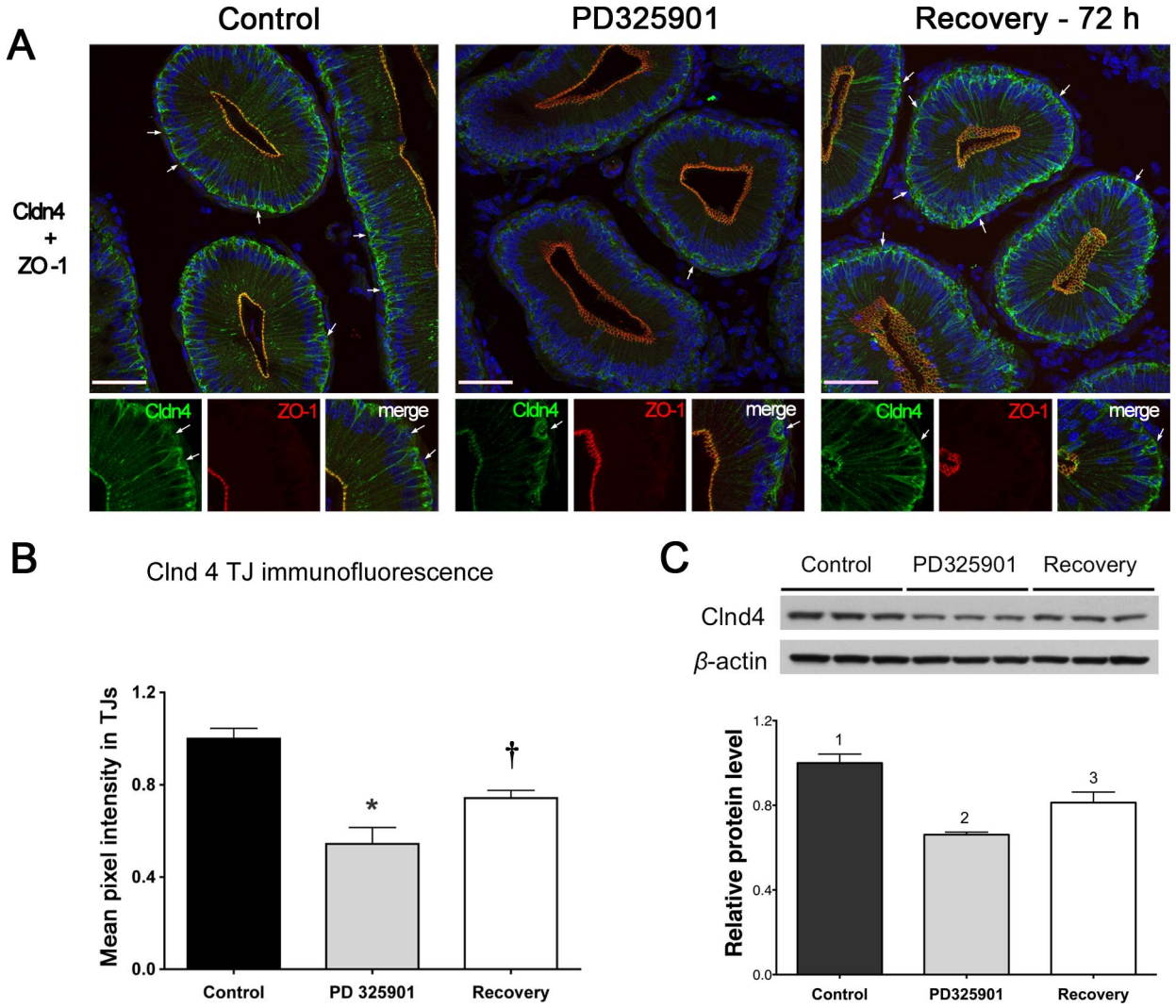


FIG. 4. PD325901 decreases Cldn4 expression in the IS. **A**) The epididymis IS was immunolabeled for ZO-1 (red) and Cldn4 (green). Left) In control mice, Cldn4 is detected in TJs, positive for ZO-1, and in the basolateral membrane of epithelial cells. Higher expression is detected in basal cell plasma membrane (arrows). Middle) PD325901 treatment induced a decrease in Cldn4-associated immunofluorescence in TJs and basolateral membrane. Right) Partial recovery of Cldn4 labeling is detected in the IS of mice that were allowed 72 h of recovery without the drug. No change was detected in ZO-1 labeling, which was restricted to TJs. Bars = 50 μm. **B**) Quantification of Cldn4 labeling in regions associated with TJs (labeled for ZO-1) confirmed the reduction of Cldn4 expression in TJs by PD325901 and partial recovery of TJ expression after 72 h without drug compared to controls. Data are expressed as means ± SEM relative to control values (*$P < 0.05$ compared to control and †$P < 0.05$ compared to PD325901). **C**) Western blot and quantification of Cldn1 expression from the IS of control mice, PD325901-treated mice, and mice allowed to recover without drug for 72 h (recovery). Three different protein extracts are shown for each group. β-actin was used as a loading control. Significant decreases in total Cldn1 expression were detected in the PD325901 group, followed by return back to normal levels in the recovery group. Band density was calculated and normalized to β-actin using Image J software [$^{2}P < 0.05$ compared to control (1) and $^{3}P < 0.005$ compared to PD325901 (2)].

treatment (Fig. 5B, middle panels). Both ZO-2 and ZO-3 immunofluorescence labeling returned back to control levels after 72 h of recovery (Fig. 5, A and B, right panels). Western blot confirmed the stability of ZO-1, reduction of ZO-2, and increase of ZO-3 expression during PD325901 treatment and the recovery of ZO-2 and ZO-3 back to control levels at the end of the recovery period (Fig. 6A). In addition, qPCR analysis showed that PD325901 did not affect the levels of ZO-1 and ZO-2 mRNA but increased ZO-3 mRNA (Fig. 6B). These results indicate that pERK controls the stability of ZO-2 protein without altering its transcription. However, both ZO-3 transcription (mRNA) and translation (protein) were activated after inhibition of ERK phosphorylation by PD325901.

The MAPK Pathway Represses Occludin Expression

Our immunofluorescence results confirmed that occludin is absent from the epididymal IS in control mice (Fig. 7, A

E) Western blot and quantification of Cldn3 expression from the IS of control mice, PD325901-treated mice, and mice allowed to recover without drug for 72 h (recovery). Three different protein extracts are shown for each group. β-actin was used as a loading control. Band density was calculated and normalized to β-actin using Image J software. No change in total Cldn3 expression was detected.

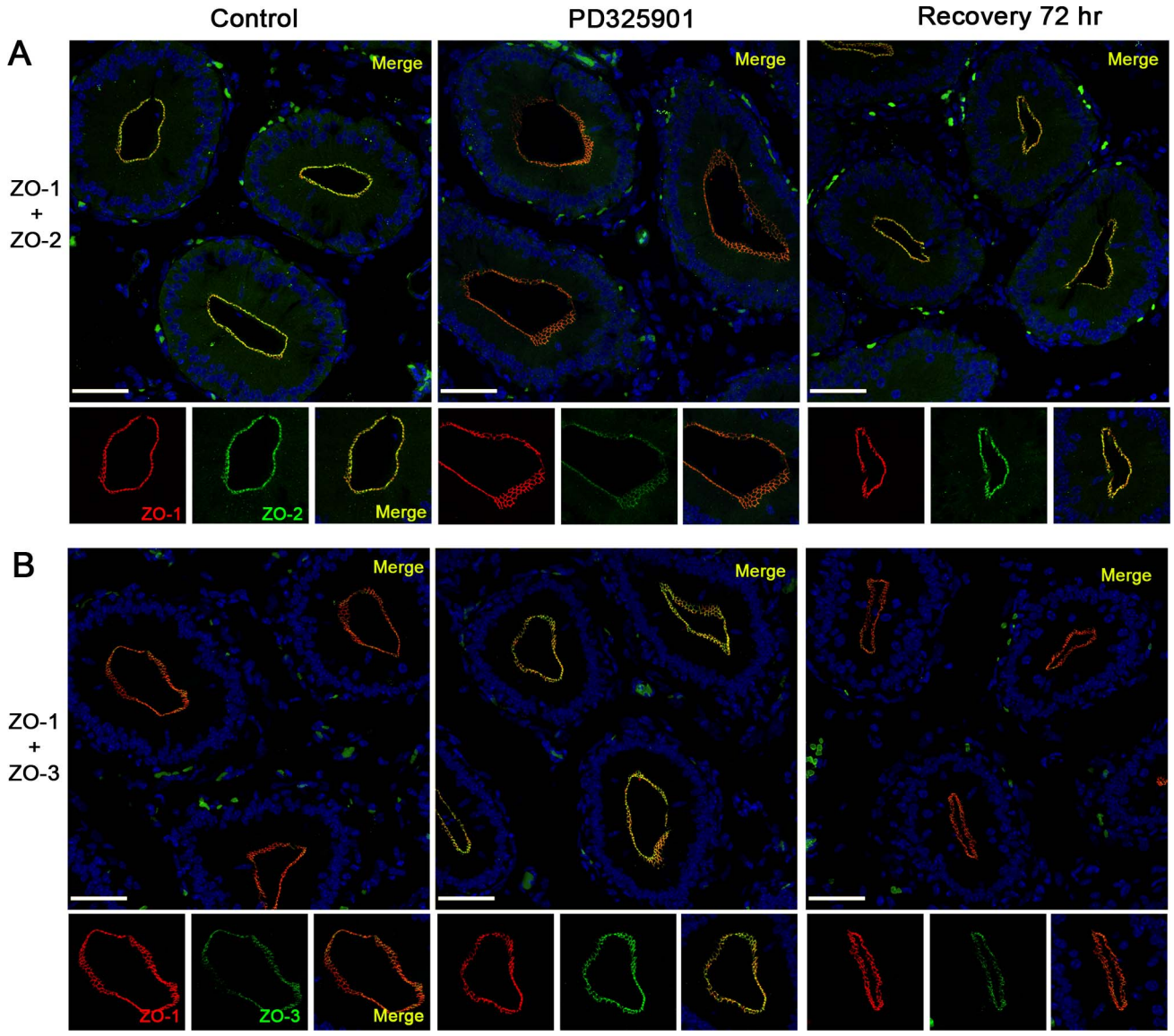


FIG. 5. PD325901 decreases ZO-2 and increases ZO-3 expression in TJs. **A**) Epididymal IS was double labeled for ZO-1 (red) and ZO-2 (green). Left) Both ZO-1 and ZO-2 are restricted to TJs in control mice. Middle) A decrease in ZO-2 labeling is seen in the TJ of mice treated with PD325901 for 3 days, while ZO-1 labeling remained unaffected. Right) Recovery of ZO-2 labeling is detected after 72 h of recovery without the drug. **B**) Epididymal IS was double labeled for ZO-1 (red) and ZO-3 (green). Left) Both ZO-1 and ZO-3 are restricted to TJs in control mice. Middle) An increase in ZO-3 labeling is seen in the TJ of mice treated with PD325901 for 3 days, while ZO-1 labeling remained unaffected. Right) A return back to control level was detected for ZO-3 labeling after 72 h of recovery without the drug. Bars = 50 μm.

and D) but is located in TJs in other epididymal regions (Supplemental Figure S2), as was previously reported [30]. PD325901 induces the expression of occludin in the IS (Fig. 7, B and D), without affecting its expression in other regions (Supplemental Figure S2). Occludin expression returned back to nondetectable levels after 72 h of recovery (Fig. 7, C and D). A band at around 50 kDa was detected by Western blot, in addition to the 63-kDa occludin predicted band (Fig. 7E). This band was nonspecific, as it was also detected in the epididymis of occludin knockout (KO) mice (Fig. 7E, bottom panel, KO). In wild-type (WT) mice, occludin was detected only after PD325901 treatment and was undetectable in control mice and after 72 h of recovery (Fig. 7E). In contrast, qRT-PCR analysis showed constant occludin mRNA levels before, during PD325901 treatment, and after removal of the drug (Fig. 7F). These results show that ERK phosphorylation represses occludin translation without affecting transcription.

Effect of PD325901 on Epithelial Cell Apoptosis

We next tested whether the effects of PD325901 on TJ gene and protein expression could be secondary to an indirect effect on epithelial cell viability. To do so, we compared the effect of PD325901 with the effect elicited by EDL, a procedure known to induce a wave of apoptosis in epithelial cells [6, 20, 39]. As expected, after EDL, several epithelial cells in the IS were positive for the apoptotic marker caspase 3 (Fig. 8). In contrast, we found that PD325901 induced apoptotic cell death only in a few scattered cells. In control mice, rare apoptotic cells were detected. We conclude that the changes in gene and protein

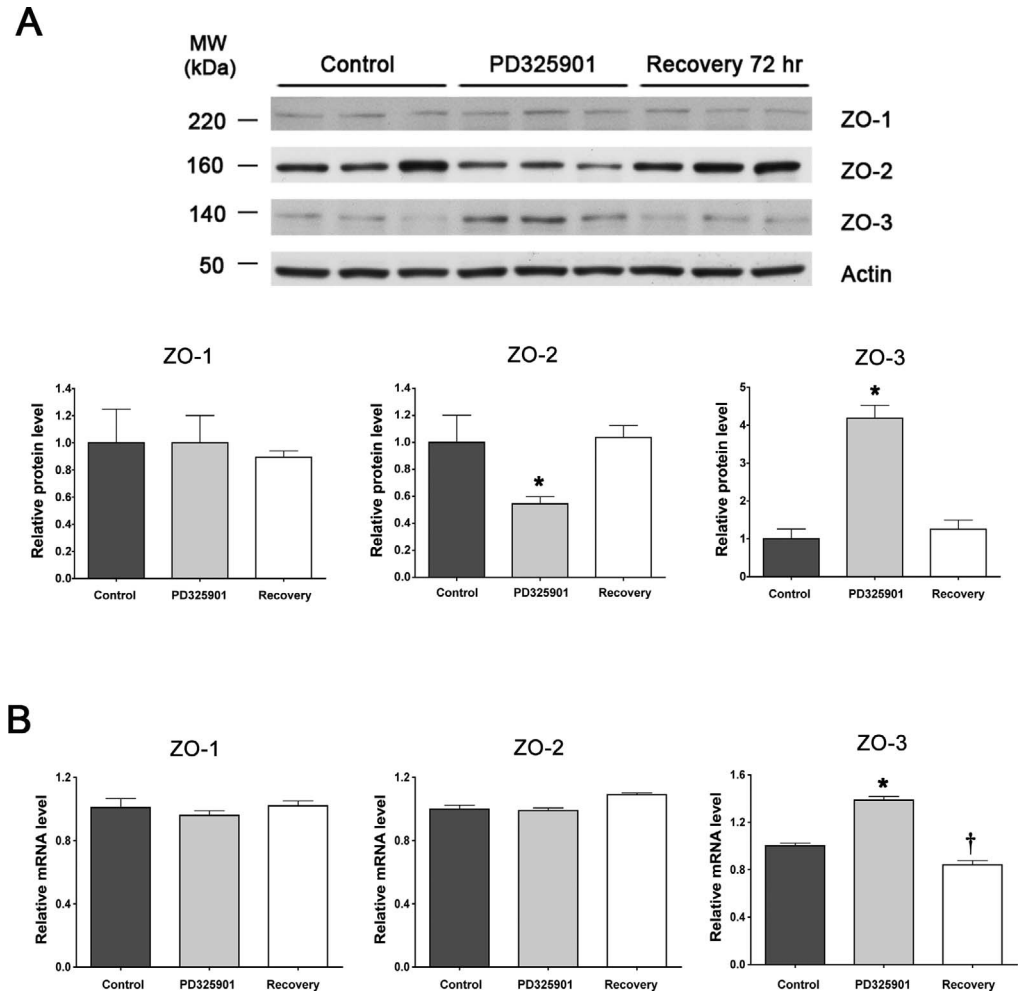


FIG. 6. Effect of PD325901 on total ZO-1, ZO-2, and ZO-3 protein and mRNA expression. **A**) Western blots for ZO-1, ZO-2, and ZO-3 in triplicate protein extracts from the IS of control mice, PD325901-treated mice, and mice allowed to recover without drug for 72 h (recovery). β-actin was used as a loading control. No effect on ZO-1 expression is shown, while a decrease in ZO-2 and an increase in ZO-3 expression are shown. Quantification of band intensity normalized to β-actin confirmed these results. Results represent means ± SEM (*$P < 0.05$ compared to control). **B**) Quantitative PCR showed no effect on ZO-1 and ZO-2 mRNA expression but showed an increase in ZO-3 mRNA expression by PD325901. ZO-3 mRNA level returned back to control value at the end of the recovery period. Results are expressed as means ± SEM relative to control values (*$P < 0.001$ compared to control and †$P < 0.001$ compared to PD325901).

expression induced by PD325901 occurred in the absence of epithelial cell apoptosis.

DISCUSSION

TJs are important cell structural and functional components that play critical roles in epithelial barrier function. In the epididymis, TJs participate in the formation of the BEB, which mediates the unidirectional transport of ions, solutes, and water, therefore contributing to the establishment and maintenance of a specific luminal microenvironment for sperm maturation. Modulation of TJ proteins by the MAPK/ERK pathway has been previously reported with variable effects ranging from down-regulation to up-regulation of expression, depending on the cell type and protein examined [40–45]. We report here that inhibition of MEK1/2, followed by reduction of ERK1/2 phosphorylation, reduces the expression of Cldn1, Cldn4, and ZO-2 but increases the expression of ZO-3 and occludin without affecting ZO-1 and Cldn3 expression in the mouse IS. PD325901 induced apoptotic cell death only in a few epithelial cells, indicating that the effects observed on TJ protein expression were for the most part not secondary to cells undergoing apoptosis. In addition, no effect of PD325901 was observed in the other epididymal segments, indicating the role of the MAPK/ERK pathway in the IS specifically.

While occludin is an integral membrane protein located in TJs, its role in TJ physiology remains controversial. Early observations first indicated that occludin is necessary for the structure and function of TJs [46–49]. However, a later study showed that occludin KO mice are viable and have normal TJ morphology, although they develop a complex phenotype, including male infertility, testicular atrophy, and chronic inflammation and hyperplasia of the gastric epithelium [50]. Our results confirm the previous observation that occludin is not expressed in the TJs of the epididymal IS but is present in all other regions [30]. This result confirms that occludin is not necessary for the formation of TJs, which are well developed in the IS [51]. In addition, we found that inhibition of the MAPK/ERK pathway with PD325901 triggered the expression of occludin protein in the IS but had no effect on occludin

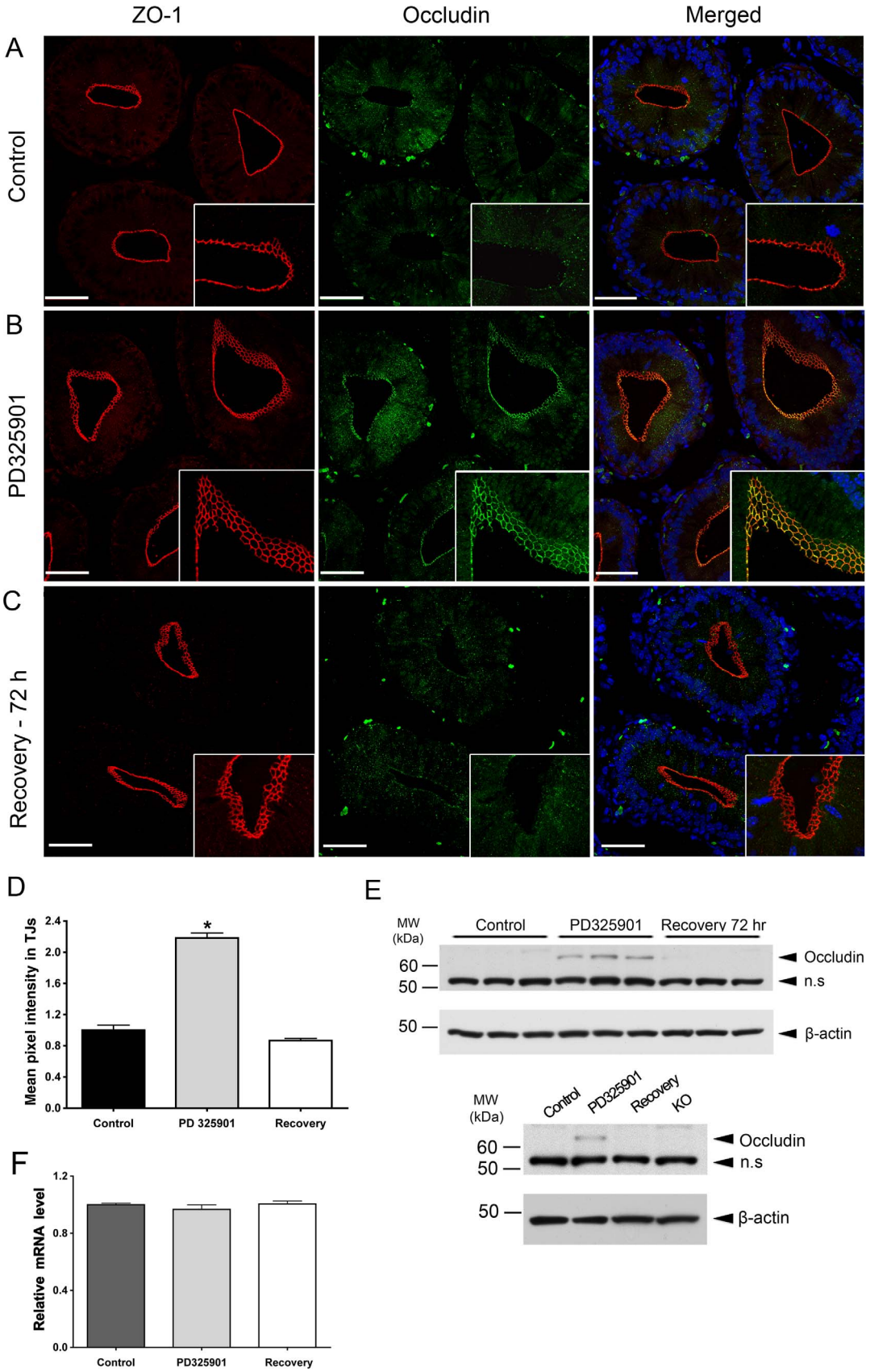


FIG. 7. PD325901 increases occludin expression in the IS. The epididymis IS was immunolabeled for ZO-1 (red) and occludin (green). **A**) In control mice, occludin was undetectable. **B**) PD325901 treatment triggered the expression of occludin in TJs (positive for ZO-1). **C**) After 72 h of recovery, occludin expression returned back to the undetectable levels seen in control mice. No change was detected in ZO-1 labeling, which was restricted to TJs. Bars = 50 μm. **D**) Quantification of occludin labeling in regions associated with TJs (labeled for ZO-1) confirmed the increase in occludin expression by PD325901 and the return back to low levels after 72 h of recovery compared to controls. Data are expressed as means

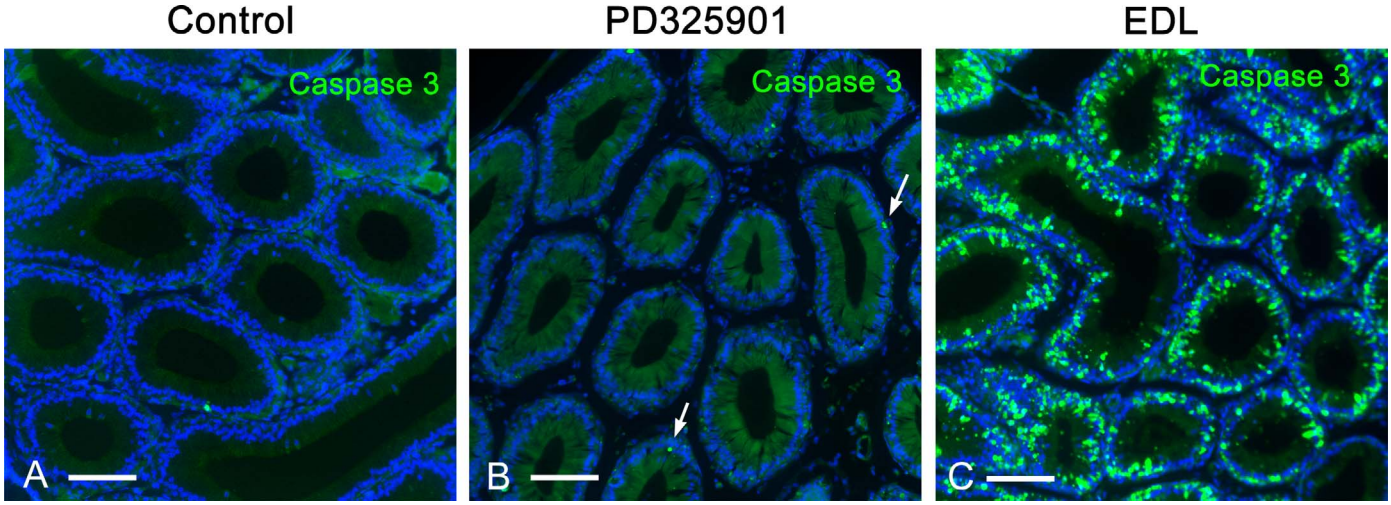


FIG. 8. Effect of PD325901 and EDL on apoptotic cell death in the IS. The epididymal IS was immunolabeled for caspase 3, a marker of apoptosis (green). **A**) No apoptotic epithelial cells were detected in control mice. **B**) A few rare apoptotic epithelial cells were detected after 36 h of treatment with PD325901 (arrows). **C**) Several apoptotic cells were detected 24 h after EDL. Nuclei are labeled in blue with DAPI. Bars = 50 μm.

expression in the other epididymis regions. Interestingly, qPCR showed the presence of occludin mRNA in the IS of control mice, while mRNA levels remained unaffected during and after PD325901 treatment. These observations indicate that maintenance of the MAPK/ERK pathway in an activated state in the IS might prevent the translation of occludin in this segment.

Claudins are transmembrane proteins that are essential organizers of TJs [24–26]. Several claudins have been described in the epididymis [21, 33, 34], but in contrast to other epithelia where claudins are restricted to TJs, in the epididymis Cldn1, Cldn3, and Cldn4 are also located in the basolateral membrane of epithelial cells. The significance of this location outside of TJs remains unknown, but we previously proposed that Cldn1, which is enriched in basal cells, could provide a ''molecular ladder'' that would support the elongation of basal cell projections between adjacent epithelial cells [21]. The present study now includes Cldn4, which is enriched in basal cells, as an additional potential mediator of basal cell elongation. The expression of several claudins in a given epithelium raised the possibility that some claudins functionally interact with each other, forming clusters that would determine tissue-specific paracellular permeability [26]. Cldn1, Cldn3, and Cldn4 are members of the group of claudins that provide ''sealing'' or ''barrier-enhancing'' functions to epithelia [24–26], in agreement with the establishment of a tight BEB that contributes to maintaining a unique luminal environment in the epididymis. Epididymal Cldn1 was shown to be partially regulated by androgens in the rat IS [34]. In addition, TGFβ1 and TGFβ3 reduced the expression of Cldn1 in an immortalized mouse epididymal cell line [52]. Our study now shows the contribution of the MAPK/ERK pathway to the maintenance of Cldn1 and Cldn4 expression, while it does not regulate Cldn3 expression.

Expression of all three ZO proteins in epididymal epithelial cells suggests their potential redundant roles in this organ. A previous study demonstrated that ZO-1 binds to both ZO-2 and ZO-3, whereas ZO-2 and ZO-3 do not bind to each other [53], indicating a more fundamental role for ZO-1 in TJ dynamics. We show here that PD325901 did not affect ZO-1 expression but induced a decrease in ZO-2 expression and an increase in ZO-3 expression. ZO proteins contain a PDZ binding domain, and they interact directly with claudins to mediate their recruitment to TJs [25]. The stability of Cldn3 after PD325901 treatment is compatible with a predominant role for ZO-1 in its recruitment to TJs. Alternatively, Cldn1 and Cldn4 might require the participation of ZO-2 for their recruitment to TJs because all three proteins had a reduced TJ expression during PD325901 administration. In addition, the localization of Cldn1, Cldn3, and Cldn4 outside of TJs, in the basolateral membrane of epididymal epithelial cells where ZO proteins are absent, indicates that their recruitment to this membrane domain does not require the participation of ZO-1, ZO-2, or ZO-3. Finally, while we observed an almost complete disappearance of ERK phosphorylation, we still observed Cldn1, Cldn4, and ZO-2 levels after PD325901 treatment, indicating that other pathway(s) may be responsible for the regulation of these proteins.

In conclusion, the present study indicates a major role for the MAPK/ERK signaling pathway in the regulation of several TJ proteins in the epididymis IS. Testicular luminal factors maintain the MAPK/ERK pathway in a constitutively active state in this segment. Modulation of TJs following ERK phosphorylation might be part of complex cell-cell

± SEM relative to control values (*$P < 0.001$ compared to control). **E**, top) Western blots of occludin expression from the IS of WT control mice, PD325901-treated WT mice, and WT mice allowed to recover without drug for 72 h (recovery). Three different protein extracts are shown for each group. β-actin was used as a loading control. A single 50-kD band was detected in all groups, and a 67-kD band corresponding to the predicted molecular weight of occludin was detected in the IS of PD325901-treated mice. **E**, bottom) The lower 50-kD band was also detected in occludin KO mice, showing that it is nonspecific (n.s.). Here again, the 67-kD band was detected only in WT mice treated with PD325901. **F**) qPCR showing that occludin RNA levels in the IS remained constant before, during, and after PD325901 treatment.

signaling events that control 1) epithelial cell death and survival, 2) the establishment of cell polarity, and 3) the paracellular permeability of the epididymis epithelium. All of these processes contribute to the production and maintenance of the optimal luminal environment that is necessary for sperm maturation and storage in the epididymis.

REFERENCES

1. Robaire B, Hinton BT, Orgebin-Crist MC. The epididymis. In: Neill JD (ed.), Physiology of Reproduction. New York: Elsevier; 2006:1071–1148.
2. Shum WW, Ruan YC, Da Silva N, Breton S. Establishment of cell-cell cross talk in the epididymis: control of luminal acidification. J Androl 2011; 32:576–586.
3. Belleannee C, Thimon V, Sullivan R. Region-specific gene expression in the epididymis. Cell Tissue Res 2012; 349:717–731.
4. Hinton B, Cooper T. The epididymis as a target for male contraceptive development. In: Habenicht UF, Aitken RJ (eds.), Fertility Control. New York: Springer; 2010:117–137.
5. Cornwall GA. New insights into epididymal biology and function. Hum Reprod Update 2009; 15:213–227.
6. Xu B, Abdel-Fattah R, Yang L, Crenshaw SA, Black MB, Hinton BT. Testicular lumicrine factors regulate ERK, STAT, and NFKB pathways in the initial segment of the rat epididymis to prevent apoptosis. Biol Reprod 2011; 84:1282–1291.
7. Xu B, Yang L, Hinton BT. The role of fibroblast growth factor receptor substrate 2 (FRS2) in the regulation of two activity levels of the components of the extracellular signal-regulated kinase (ERK) pathway in the mouse epididymis. Biol Reprod 2013; 89:48.
8. Xu B, Yang L, Lye RJ, Hinton BT. p-MAPK1/3 and DUSP6 regulate epididymal cell proliferation and survival in a region-specific manner in mice. Biol Reprod 2010; 83:807–817.
9. Yeung C-H, Breton S, Stetiawan I, Xu Y, Lang F, Cooper TG. Increased luminal pH in the epididymis of infertile c-ros knockout mice and the expression of sodium-hydrogen exchangers and vacuolar proton pump H+-ATPase. Mol Reprod Dev 2004; 68:159–168.
10. Murashima A, Xu B, Hinton BT. Understanding normal and abnormal development of the Wolffian/epididymal duct by using transgenic mice. Asian J Androl 2015; 17:749–755.
11. Krutskikh A, De Gendt K, Sharp V, Verhoeven G, Poutanen M, Huhtaniemi I. Targeted inactivation of the androgen receptor gene in murine proximal epididymis causes epithelial hypotrophy and obstructive azoospermia. Endocrinology 2011; 152:689–696.
12. Bjorkgren I, Saastamoinen L, Krutskikh A, Huhtaniemi I, Poutanen M, Sipila P. Dicer1 ablation in the mouse epididymis causes dedifferentiation of the epithelium and imbalance in sex steroid signaling. PLoS One 2012; 7:e38457.
13. Sipila P, Cooper TG, Yeung CH, Mustonen M, Penttinen J, Drevet J, Huhtaniemi I, Poutanen M. Epididymal dysfunction initiated by the expression of simian virus 40 T-antigen leads to angulated sperm flagella and infertility in transgenic mice. Mol Endocrinol 2002; 16:2603–2617.
14. Sonnenberg-Riethmacher E, Walter B, Riethmacher D, Godecke S, Birchmeier C. The c-ros tyrosine kinase receptor controls regionalization and differentiation of epithelial cells in the epididymis. Genes Dev 1996; 10:1184–1193.
15. Robaire B, Viger RS. Regulation of epididymal epithelial cell functions. Biol Reprod 1995; 52:226–236.
16. Hermo L, Robaire B. Epididymal cell types and their functions. In: Robaire B, Hinton BT (eds.), The Epididymis: From Molecular to Clinical Practice. A Comprehensive Survey of the Efferent Ducts, the Epididymis and the Vas Deferens. New York: Kluwer Academic/Plenum; 2002:81–102.
17. Pietrement C, Sun-Wada GH, Da Silva N, McKee M, Marshansky V, Brown D, Futai M, Breton S. Distinct expression patterns of different subunit isoforms of the V-ATPase in the rat epididymis. Biol Reprod 2006; 74:185–194.
18. Da Silva N, Pietrement C, Brown D, Breton S. Segmental and cellular expression of aquaporins in the male excurrent duct. Biochim Biophys Acta 2006; 1758:1025–1033.
19. Ruan YC, Wang Y, Da Silva N, Kim B, Diao RY, Hill E, Brown D, Chan HC, Breton S. CFTR interacts with ZO-1 to regulate tight junction assembly and epithelial differentiation via the ZONAB pathway. J Cell Sci 2014; 127:4396–4408.
20. Kim B, Roy J, Shum WW, Da Silva N, Breton S. Role of testicular luminal factors on basal cell elongation and proliferation in the mouse epididymis. Biol Reprod 2015; 92:9.
21. Shum WWC, Da Silva N, McKee M, Smith PJ, Brown D, Breton S. Transepithelial projections from basal cells are luminal sensors in pseudostratified epithelia. Cell 2008; 135:1108–1117.
22. Veri JP, Hermo L, Robaire B. Immunocytochemical localization of the Yf subunit of glutathione S-transferase P shows regional variation in the staining of epithelial cells of the testis, efferent ducts, and epididymis of the male rat. J Androl 1993; 14:23–44.
23. Shum WW, Smith TB, Cortez-Retamozo V, Grigoryeva LS, Roy JW, Hill E, Pittet MJ, Breton S, Da Silva N. Epithelial basal cells are distinct from dendritic cells and macrophages in the mouse epididymis. Biol Reprod 2014; 90:90.
24. Hou J. The kidney tight junction (review). Int J Mol Med 2014; 34: 1451–1457.
25. Lingaraju A, Long TM, Wang Y, Austin JR II, Turner JR. Conceptual barriers to understanding physical barriers. Semin Cell Dev Biol 2015; 42: 13–21.
26. Markov AG, Aschenbach JR, Amasheh S. Claudin clusters as determinants of epithelial barrier function. IUBMB Life 2015; 67:29–35.
27. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 1986; 103:755–766.
28. Haskins J, Gu L, Wittchen ES, Hibbard J, Stevenson BR. ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. J Cell Biol 1998; 141:199–208.
29. Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 1993; 123:1777–1788.
30. Cyr DG, Hermo L, Egenberger N, Mertineit C, Trasler JM, Laird DW. Cellular immunolocalization of occludin during embryonic and postnatal development of the mouse testis and epididymis. Endocrinology 1999; 140:3815–3825.
31. Cyr DG, Gregory M, Dube E, Dufresne J, Chan PT, Hermo L. Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans. Asian J Androl 2007; 9:463–475.
32. Dube E, Dufresne J, Chan PT, Hermo L, Cyr DG. Assessing the role of claudins in maintaining the integrity of epididymal tight junctions using novel human epididymal cell lines. Biol Reprod 2010; 82:1119–1128.
33. Gregory M, Cyr DG. Identification of multiple claudins in the rat epididymis. Mol Reprod Dev 2006; 73:580–588.
34. Gregory M, Dufresne J, Hermo L, Cyr D. Claudin-1 is not restricted to tight junctions in the rat epididymis. Endocrinology 2001; 142:854–863.
35. Ramos JW. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. Int J Biochem Cell Biol 2008; 40:2707–2719.
36. Mulholland DJ, Kobayashi N, Ruscetti M, Zhi A, Tran LM, Huang J, Gleave M, Wu H. Pten loss and RAS/MAPK activation cooperate to promote EMT and metastasis initiated from prostate cancer stem/progenitor cells. Cancer Res 2012; 72:1878–1889.
37. Ruan YC, Shum WW, Belleannée C, Da Silva N, Breton S. ATP secretion in the male reproductive tract: essential role of CFTR. J Physiol 2012; 590: 4209–4222.
38. Kim B, Kim Y, Cooke PS, Rüther U, Jorgensen JS. The Fused Toes locus is essential for somatic-germ cell interactions that foster germ cell maturation in developing gonads in mice. Biol Reprod 2011; 84: 1024–1032.
39. Robaire B, Fan X. Regulation of apoptotic cell death in the rat epididymis. J Reprod Fertil Suppl 1998; 53:211–214.
40. Mitic L, Anderson J. Molecular architecture of tight junctions. Annu Rev Physiol 1998; 60:121–142.
41. Li D, Mrsny RJ. Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. J Cell Biol 2000; 148:791–800.
42. Wang Y, Zhang J, Yi X-J, Yu F-SX. Activation of ERK1/2 MAP kinase pathway induces tight junction disruption in human corneal epithelial cells. Exp Eye Res 2004; 78:125–136.
43. Lipschutz JH, Li S, Arisco A, Balkovetz DF. Extracellular signal-regulated kinases 1/2 control claudin-2 expression in Madin-Darby canine kidney strain I and II cells. J Biol Chem 2005; 280:3780–3788.
44. Yang R, Harada T, Li J, Uchiyama T, Han Y, Englert JA, Fink MP. Bile modulates intestinal epithelial barrier function via an extracellular signal related kinase 1/2 dependent mechanism. Intensive Care Med 2005; 31: 709–717.
45. Basuroy S, Seth A, Elias B, Naren A, Rao R. MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. Biochem J 2006; 393:69–77.

46. Wong V, Gumbiner BM. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J Cell Biol 1997; 136:399–409.
47. McCarthy KM, Skare IB, Stankewich MC, Furuse M, Tsukita S, Rogers RA, Lynch RD, Schneeberger EE. Occludin is a functional component of the tight junction. J Cell Sci 1996; 109:2287–2298.
48. Balda MS, Whitney JA, Flores C, González S, Cereijido M, Matter K. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. J Cell Biol 1996; 134:1031–1049.
49. Chen Y-H, Merzdorf C, Paul DL, Goodenough DA. COOH terminus of occludin is required for tight junction barrier function in early Xenopus embryos. J Cell Biol 1997; 138:891–899.
50. Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, Tsukita S. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell 2000; 11:4131–4142.
51. Suzuki F, Nagano T. Development of tight junctions in the caput epididymal epithelium of the mouse. Dev Biol 1978; 63:321–334.
52. Stammler A, Muller D, Tabuchi Y, Konrad L, Middendorff R. TGFbetas modulate permeability of the blood-epididymis barrier in an in vitro model. PLoS One 2013; 8:e80611.
53. Wittchen ES, Haskins J, Stevenson BR. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. J Biol Chem 1999; 274:35179–35185.