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Tight junction protein claudin 1 is down-regulated by TGF-β1 via MEK signaling in benign prostatic epithelial cells

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Abstract

Background: Benign prostatic hyperplasia (BPH) is arguably the most common disease in aging men. Although the etiology is not well understood, chronic prostatic inflammation is thought to play an important role in BPH initiation and progression. Our recent studies suggest that the prostatic epithelial barrier is compromised in glandular BPH tissues. The pro-inflammatory cytokine TGF-β1 impacts tight junction formation, enhance epithelial barrier permeability, and suppresses claudin 1 mRNA expression in prostatic epithelial cells. However, the role of claudin 1 in the prostatic epithelial barrier and its regulation by TGF-β1 in prostatic epithelial cells are not clear.

Methods: The expression of claudin 1 was analyzed in 22 clinical BPH specimens by immunohistochemistry. Human benign prostate epithelial cell lines BPH-1 and BHPrE1 were treated with TGF-β1 and transfected with siRNAs specific to claudin 1. Epithelial monolayer

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Ke Wang and Dr. Feng Li, who performed this study in Pittsburgh, were Visiting Scholars at the University of Pittsburgh from the First Affiliated Hospital of Xi'an Jiaotong University in China, where Prof. Dalin He (hedl@xjtu.edu.cn) is their supervisor. Therefore, Dr. He was included as a co-author in this article. Dr. He has no conflict of interest for this study and was involved in the final approval process of the manuscript.

Disclosure of conflicts of interest

The authors have nothing to disclose.

permeability changes in the treated cells were measured using trans-epithelium electrical resistance (TEER). The expression of claudin 1, E-cadherin, N-cadherin, snail, slug, and activation of mitogen activated proteins kinases (MAPKs) and AKT was assessed following TGF-β1 treatment using western blot analysis.

Results: Claudin 1 expression was decreased in glandular BPH tissue compared to adjacent normal prostatic tissue in patient specimens. TGF-β1 treatment or claudin 1 knockdown in prostatic epithelial cell lines increased monolayer permeability. TGF-β1 decreased levels of claudin 1 and increased levels of snail and slug as well as increased phosphorylation of the MAPK extracellular signal regulated kinase-1/2 (ERK-1/2) in both BPH-1 and BHPrE1 cells. Overexpression of snail or slug had no effect on claudin 1 expression. In contrast, PD98059 and U0126, inhibitors of the upstream activator of ERK-1/2 (i.e. MEK-1/2) restored claudin 1 expression level as well as the epithelial barrier.

Conclusion: Our findings suggest that down-regulation of claudin-1 by TGF-β1 acting through the non-canonical MEK-1/2/ERK-1/2 pathway triggers increased prostatic epithelial monolayer permeability *in vitro*. These findings also suggest that elevated TGF-β1 may contribute to claudin 1 down-regulation and compromised epithelial barrier in clinical BPH specimens.

Keywords

BPH; claudin 1; TGF-β1

Introduction

Benign prostatic hyperplasia (BPH) is a common age-related prostatic disease. Although it is in general not lethal, BPH can negatively impact quality of life and is a major cause of lower urinary tract symptoms (LUTS) [1]. With increasing life expectancy, it is expected that BPH incidence and the subsequent costs for treatment and management of BPH will rise, causing an increase in economic burden [2]. Understanding the mechanisms of BPH development and progression is important because it may eventually lead to novel preventive and/or treatment approaches.

Although the etiology of BPH is poorly understood, increased inflammation [3] and changes in epithelial barrier components [4–6] have been postulated as factors potentially contributing to BPH pathogenesis. We previously reported the presence of prostate specific antigen (PSA), which is expressed by prostate luminal epithelial cells, in the stroma of glandular BPH nodules, suggesting that the epithelial barrier in this major type of BPH tissues is compromised [6]. We and others have also reported the down-regulation of E-cadherin, which is important for the maintenance of the epithelial barrier, in glandular BPH nodules [4, 5]. Loss of prostate luminal epithelial barrier integrity through down-regulation of important junction proteins such as E-cadherin could result in the leakage of PSA and other proteins into the stromal compartment, potentially triggering an inflammatory response and contributing to BPH development and progression.

BPH incidence and progression are frequently associated with chronic prostatic inflammation (Reviewed in [7]). Chronic inflammatory infiltrate was identified in 43% of

BPH patients in the multi-center Medical Therapy of Prostatic Symptoms (MTOPS) study [3]. In the MTOPS study, patients with chronic inflammation had higher PSA levels, increased prostate volumes and increased risk of BPH progression [3]. Expression of cytokines and growth factors in the prostate is increased with aging [8], which also suggests a role for inflammation in BPH development [9]. The cytokine transforming growth factor beta 1 (TGF-β1) is a potent regulator of Th17 T cell differentiation and inflammatory response (reviewed in [10]). In the prostate, TGF-β1 regulates ductal morphogenesis during development, as well as proliferation, apoptosis and differentiation (reviewed in [11]), and its expression is increased with age in the prostate [8]. Overexpression of TGF-β1 in the murine prostate induced inflammation and fibrosis [12], and TGF-β1 expression was increased in the luminal epithelium of BPH patients [13, 14]. TGF-β1 has been shown to induce epithelial to mesenchymal transition (EMT), which can disrupt cell-cell adhesions in the epithelial barrier [15], and may play a role in BPH development and progression. Down-regulation of E-cadherin accompanied by an increase in p-Smad 3 and Snail in BPH tissues suggested that TGF-β1 pathway activation plays a role in BPH [4].

In a non-bacterial prostatic inflammation model, prostate inflammation was characterized by an increased in TGF-β1 and a decrease in E-cadherin [16], suggesting that inflammation may contribute to decreased epithelial barrier function in the prostate. We recently reported that TGF-β1 stimulation of benign prostatic cell lines increased epithelial barrier permeability in a dose dependent manner (manuscript in press, Li et al., AJCEU). Although E-cadherin mRNA expression was not altered, tight junction protein claudin 1 mRNA levels and the number of tight junction ‘kiss points’ were significantly decreased following TGF-β1 stimulation of benign prostate epithelial cell lines BHPrE1 and BPH-1. In glandular BPH specimens, mRNA levels of claudin 1, but not E-cadherin, were down-regulated compared to normal adjacent tissues. These observations suggested that the epithelial barrier in glandular BPH is compromised and that inflammatory cytokine TGF-β1 could increase permeability and decrease the formation of tight junctions and expression of claudin 1. However, it was not clear how TGF-β1 increased prostate epithelial monolayer permeability and decreased claudin 1 expression in prostatic epithelial cells.

To provide new insights into the roles of claudin 1 and TGF-β1 in BPH pathogenesis, this study analyzed the expression levels of claudin 1 protein in glandular BPH and normal adjacent tissues, explored the potential function of claudin 1 in maintenance of the prostatic epithelial barrier, and examined the potential mechanisms of TGF-β1 suppression of claudin 1 expression in benign prostate epithelial cell lines BHPrE1 and BPH-1.

Materials and methods

Prostate specimen acquisition

Residual tissue specimens for research were obtained from clinical specimens, under an approved University of Pittsburgh Institutional Review Board protocol through the Pitt BioSpecimen Core at the University of Pittsburgh. BPH tissues were from a total of 22 patients over 60 years of age with clinical symptoms of BPH. The cohort included specimens from 6 patients who were treatment naïve and who also underwent prostatectomy

because of BPH; and 16 patients who were treated for symptomatic BPH by transurethral resection of the prostate. No incidental foci of carcinoma were present in this cohort.

Immunohistochemistry (IHC) and scoring of staining

Slides were deparaffinized in xylene and rehydrated in graded concentrations of ethanol in water, ending with a final rinse in water. Antigen retrieval was performed using a citrate buffer (Dako, Carpinteria, CA) in Decloaking chamber at 123°C. The slides were stained using an Autostainer Plus (Dako) platform with TBST rinse buffer (Dako). The mouse monoclonal anti-Claudin 1 (XX7, Santa Cruz Biotechnology, Dallas, TX, USA) antibody (Table 1) at a 1:100 dilution. Envision + Anti-Mouse HRP polymer (Dako) and the Mouse Signal Stain Boost Detection Kit (Cell Signaling Technology, Beverly, MA) were used for detection. The substrate used was 3,3, diaminobenzidine (Dako). Slides were counterstained with Hematoxylin (Cell Signaling Technology). Immunostained sections were imaged with a Leica DM LB microscope (Leica Microsystems Inc, Bannockburn, IL) equipped with an Imaging Source NII 770 camera (The Imaging Source Europe GmbH, Bremen, Germany) and NIS-Elements Documentation v 4.6 software (Nikon Instruments, Inc., Mellville, NY). All tissues were examined by a board-certified genitourinary pathologist (R.D.) using light microscopy.

Immunostained slides were evaluated quantitatively using the H-Score method by assessing protein expression as a function of staining intensity, where no staining=0, weak=1, moderate=2, strong=3, times the percentage of cells exhibiting each level of intensity as previously [17]. A minimum of 5 fields from each section was analyzed and an average score for each tissue type was calculated for each patient. All scores were reviewed and confirmed by a board-certified genitourinary pathologist (R.D.).

Cell culture

Benign prostatic epithelial cell lines BPH-1 [18] and BHPrE1 [19] were gifts from Dr. Simon Hayward (NorthShore University Health System, Evanston, IL, USA). BPH-1 cells were maintained in RPMI-1640 (10–041, Gibco, Los Angeles, LA, USA) culture medium, supplemented with 10% fetal bovine serum (FBS) was from Atlanta Biologicals (Flowery Branch, GA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (30–002-CI, Gibco), and 29.2 μg/ml L-glutamine (25–015-CI, Corning, Corning, NY). BHPrE1 cells were maintained as described [19], in 50/50 Dulbecco's modified Eagles medium (DMEM)/F12 (10–090-CV, Corning), 1 μg/ml insulin-transferrin-selenium-X (51500056, Invitrogen, Waltham, MA, USA), 0.4% bovine pituitary extract (13028014, Gibco), and 3 ng/ml epidermal growth factor (S0155, Gibco). Cell line HEK-293T was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and was maintained in DMEM (12430–054, Gibco) culture medium, supplemented with 10% FBS, 1% penicillin and streptomycin, 1% L-glutamine. Cells were grown in standard cell culture conditions (5% CO_2, 95% humidity) at 37°C. The 12 mm Transwell® with 0.4 μm Pore Polyester Membrane Inserts (3460) were purchased from Corning (Corning).

Human TGF-β1 (8915LC, Cell Signaling Technology), was dissolved according to the manufacturer's recommendation and stored in aliquots at −80°C. For TGF-β1 treatment,

cells were seeded in 60 mm dishes at a concentration of 300,000 cells/well suspended in 3 mL complete culture medium, and dosages (0, 0.2, 1, 5 ng/mL) of TGF-β1 or TGF-β1 (1, 5 ng/mL) with or without 20 μM PD98059 were administered the next day. Briefly, 20 μM PD98059 was added into the wells first, then after 1 hour, the cells were treated with TGF-β1. After 48 h, cells were digested by 0.25% trypsin-EDTA and cell number was determined using a Beckman Z2 coulter counter (Beckman Coulter, Brea, CA, USA). Cells were seeded onto transwell inserts in 12-well plates at a concentration of 150,000 cells suspended in 500 μL medium, the lower chambers were filled with 1.5 mL culture medium. Both upper inserts and lower chambers medium containing TGF-β1 with or without PD98059. Transwells were processed in triplicate. The day when cells were seeded onto transwell inserts was considered Day 0. Medium for each culture condition was replaced every day. From Day 3, TEER was measured every day.

Inhibitors PD98059 (HY-12028) and U0126 (HY-12031) were from MedChemExpress (Monmouth Junction, NJ, USA).

siRNA transfection

The siRNAs targeting claudin 1, snail, slug, and the non-targeting negative control (NC) siRNA (51-01-14-04) were designed and synthesized by IDT (Integrated DNA Technologies, Inc. Illinois USA). Cells were transfected with the oligonucleotide using DharmaFECT 1 transfection reagent (GE Healthcare Life Sciences, Marlborough, MA, USA) following the manufacturer's instructions. The siRNA was used at a concentration of 25 nM. Sequences for siRNA were listed in Table 2. Culture medium was replaced by serum-free medium 1 hour before transfection. Transfection reagents and siRNAs were diluted separately in serum-free medium, incubated for 5 mins and then mixed together for another 15 min incubation at room temperature. The transfection complex was diluted in culture medium and mixed well before placement on cells in culture. Transfection culture medium was replaced by fresh complete medium 24 hours later.

For claudin 1 knockdown in transwell assays, cells were seeded into 60 mm plates at an initial density of 300,000 cells/plate suspended in 3 mL complete culture medium and knockdown of claudin 1 was performed the following day. After 48 h, cells were collected and seeded into transwell inserts as described above. An aliquot of cells was seeded onto 6-well plates and cell lysates were collected the next day for the detection of knockdown efficiency. The day when cells were seeded into transwell inserts was considered Day 0 and medium was replaced every day. From Day 3, TEER was performed every two days. To maintain high knockdown efficiency, claudin 1 knockdown was repeated on Day 3 in inserts.

Plasmids transfection and lenti-viral infection

Plasmids of pLenti6.3-IRES-mCherry-vector, pLenti6.3-IRES-mCherry-snail, pLenti6.3-IRES-mCherry-slug, psPAX2 and pMD2.G were gifts from Binhua Peter Zhou, MD, PhD (College of Medicine, Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY). Transfections were performed in 293T cells using PolyJet In Vitro DNA Transfection Reagent (SignaGen Laboratories, Frederick, MD) according to the manufacturer's instructions. Supernatant was collected at 48 and 72 hours after transfection,

by centrifuging at 1,000 rpm for 5 min then collecting the supernatant, and storing in 1 mL per tubes aliquots, store at −80°C.

For lenti-virus infection, BPH-1 and BHPrE1 were seeded into 6 well plates at a cell density of 200,000/well. The next day, medium was replaced with complete fresh medium plus lentil-viral medium at a ratio of 1:1 mix and incubated for 72 h, then cells were collected for western blot analysis.

Trans-epithelial electrical resistance (TEER) measurement assay

Medium in both upper inserts and lower chambers was replaced by room temperature balanced fresh complete culture medium, with 1.5 mL in lower chambers and 500 μL in upper inserts, respectively. The electrode was sterilized using 75% ethanol and then balanced in room temperature sterilized PBS for 10 min. TEER for each insert was measured by Millicell® ERS-2 voltohmmeter (MERS00002, Millipore, Billerica, MA, USA). TEER ($\Omega \cdot cm^2$) = (R1-R0) × 1.1 cm^2.

FITC-dextran transwell permeability assay

FITC-dextran transwell permeability assays were performed as described previously [5]. Briefly, medium in both upper inserts and lower chambers was aspirated, and the lower chambers were filled with 1.5 mL complete medium while the inserts were filled with 500 μL complete medium in the presence of 50 μg/mL FITC-dextran 70 (46945, Sigma-Aldrich, St. Louis, MO). After 24 hours incubation at 37℃, fluorescence of the medium in the lower chamber were collected and measured by a SpectraMax M2 Microplate Reader (Molecular Devices, San Jose, CA, USA) by 9-points with depth check with excitation at 485 nm and emission at 535 nm as previously [5].

MTT assay

The cells on transwell inserts were incubated with medium containing 0.5 mg/mL of MTT (T-030–1, Goldbio, St. Louis, MO, USA) at 37°C for 4 h, then medium was carefully removed and precipitates were solubilized in 1 mL DMSO. OD value was read by M2 micro-plate reader at wavelength 490 nm.

Western blot

Proteinase and phosphatase inhibitors cocktail were from Sigma-Aldrich (St. Louis, MO, USA). PVDF membrane (1620177), Precision Plus Protein™ Dual Color Standards (1610394) and ECL reagents (1705061) were from Bio-Rad Laboratories (Hercules, CA, USA). Cells treated by siRNA knockdown of claudin 1 or treated with TGF-β (with/without inhibitors) and western blotting was conducted as previously published [20]. GAPDH was probed as loading control for each individual experiment, representative results from at least two experimental replicates are shown. Primary antibodies are listed in Table 1; secondary anti-rabbit and anti-mouse antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Dilutions for primary and secondary antibodies were 1:1000 and 1:5000 respectively. Quantification of band intensity was measured using Adobe PhotoShop v. 21.1.1 (Adobe Systems, San Jose, CA, USA).

qPCR

qPCR analysis was performed on mRNA isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). mRNA was then reverse transcribed to cDNA using Takara reverse transcription reagents (Takara). Samples were analyzed using Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). Each sample was run in triplicate and experiments were performed at least twice. Primer sequences were listed in Table 3.

Statistical methods

Graphs were generated using GraphPad Prism v. 6.0 (GraphPad Software, La Jolla, California, USA). GraphPad Prism 6 or SAS, version 9.4 (SAS) Cay, NC, USA) were used to perform all statistical analyses. Data were expressed as means ± standard deviation (S.D.). Differences between groups were analyzed by Student's *t*-test, One-way ANOVA, and ad hoc multpple comparison tests and statistical significance was defined as $P<0.05$.

Results

Down-regulation of claudin 1 in glandular BPH compared to normal adjacent prostate

To investigate the expression of claudin 1 in glandular BPH, immunohistochemical staining of claudin 1 was performed on prostate tissues from 22 patients. In normal adjacent prostate tissues (NAP), claudin 1 expression was evident as medium intensity staining outlining epithelial cells presumably localized to the plasma membrane, while epithelial cells in BPH tissues were virtually devoid of claudin 1 staining (Fig. 1A). Quantification of immunostaining H-Score showed a statistically significant decrease in claudin 1 staining in BPH compared to normal adjacent prostate (Fig. 1B). Areas of normal adjacent prostate were available in 8 out of the 16 patients subjected to TURP and 6 out of the 6 patients subjected to prostatectomy for BPH.

We previously reported that E-cadherin was down-regulated in BPH tissues compared to NAP [5]. In order to compare the changes observed in E-cadherin to those observed in claudin 1 immunostaining, we calculated the fold-change in H-Score for paired samples from both the current study and the previous study [5]. In the current study, paired data was available for 14 patients with claudin 1 immunostaining. We re-examined the E-cadherin immunostaining data from the previous study [5], and calculated the fold-change in H-Score for paired data from 8 patients (these patients were from a separate cohort than the claudin 1 patients). Claudin 1 immunostaining in BPH had an average H-score ratio to NAP of 0.29 (S.D. = 0.25), while the average E-cadherin BPH:NAP ratio was 0.66 (S.D. = 0.06) (Fig. 1C). The standard deviation in claudin 1 was higher than that of E-cadherin suggesting that claudin 1 immunostaining in BPH compared to normal adjacent prostate is more variable than that of E-cadherin.

Knockdown of claudin 1 increased monolayer permeability in benign prostatic epithelial cell lines

We previously demonstrated that BPH-1 and BHPrE1 cell lines were capable of forming an epithelial barrier when grown as a monolayer on transwell inserts and showed that both cell

lines expressed E-cadherin and claudin 1 [5]. To determine the impact of claudin 1 loss on the prostate epithelial barrier, we performed TEER assays on BPH-1 and BHPrE1 cell lines treated with three different siRNAs targeting claudin 1 and grown as a monolayer on transwell inserts. TEER was decreased is the presence of siRNA targeting claudin 1 by Day 3 in both BPH-1 and BHPrE1 cells (Fig. 2A and B), suggesting that claudin 1 knockdown induced an increase in monolayer permeability. FITC dextran permeability assays showed similar results (Supplemental Fig. S1A and B). These results were similar to the effect of E-cadherin knockdown on monolayer permeability in BPH-1 and BHPrE1 cells [5], indicating claudin 1 is also required for maintaining the barrier integrity of the prostatic epithelial cell monolayer. Claudin 1 knockdown had no effect on cell viability as determined by an MTT assay (Supplemental Fig. S1C).

Since E-cadherin is down-regulated in BPH tissues, we assessed whether claudin 1 knockdown could affect E-cadherin, and N-cadherin, as well as EMT transcription factors snail and slug, which have been shown to repress claudin 1 expression [21, 22]. Claudin 1 knockdown did not affect expression of E-cadherin, N-cadherin, snail, and slug in BPH-1 (Fig. 2C), or in BHPrE-1 (Fig. 2D) cells.

TGF-β1 increased monolayer permeability in benign prostatic epithelial cell lines and inhibited claudin 1 expression in a dose dependent manner

TGF-β1 has been shown to induce EMT, which can disrupt cell-cell adhesions in the epithelial barrier [15]. We recently reported that TGF-β1 increased monolayer permeability and reduced tight junction ‘kiss points’ in BPH-1 and BHPrE1 cells [23]. Furthermore, claudin 1 mRNA was down-regulated in epithelial cell lines following TGF-β1stimulation and was decreased in BPH compared to normal adjacent epithelium of BPH patients [23]. Here, TGF-β1 decreased TEER (Fig. 3A, B), and these results were confirmed by FITC dextran permeability assay (Supplemental Fig. S2A,B). Cell density was not inhibited by TGF-β1 at 0.5, 1 or 2 ng/ml (Supplemental Fig. S2C). TGF-β1 down-regulated claudin 1 expression at the protein level in both BPH-1 and BHPrE1 cell lines, as well as induced EMT-associated signaling including the slight down-regulation of E-cadherin, and the up-regulation of N-cadherin and transcription factors snail and slug (Fig. 3C, D & Supplemental Fig. S3). Taken together, these results suggested that TGF-β1 negatively impacts the prostate epithelial barrier and up-regulates the EMT pathway in a dose-dependent manner in benign prostate epithelial cell lines.

Snail/slug did not regulate claudin 1 expression in benign prostatic epithelial cell lines

Snail and slug were reported to repress the expression of claudin 1, and overexpression of snail and slug decreases TEER in epithelial cells [22], suggesting that TGF-β1 repression of claudin 1 could be directly mediated through snail and/or slug. Therefore, we tested whether claudin 1 could be downregulated by snail and slug in prostatic epithelial cell lines. Overexpression of slug or snail in BPH-1 cells did not alter claudin 1 expression (Fig. 4A). We were unable to overexpress slug in BHPrE1 cells; however, snail overexpression did not alter claudin 1 expression levels, similar to our findings in BPH-1 cells (Fig. 4B).

To further test snail or slug regulation of claudin 1 expression in benign prostatic epithelial cell lines, we used siRNA to knockdown snail or slug in BPH-1 and BHPrE-1 cell lines. The siRNA treated cells were stimulated with TGF-β1 (1 ng/ml) for 48 hours and analyzed for expression of claudin 1, snail and slug. As in previous experiments, TGF-β1 treatment stimulated the expression of both snail and slug in BPH-1 and BHPrE1 cells and down-regulated claudin 1 expression, however no significant changes in claudin 1 expression were observed in response to knockdown of either snail or slug (Fig. 4C, D).

TGF-β1 activated the MAPK pathway in benign prostatic epithelial cell lines

In addition to the canonical TGF-β1 pathway operating through SMADs, TGF-β1 can also activate other signaling cascades, including the MAPK and PI3K pathways [24]. To explore whether MAPK and PI3K pathways could be activated by TGF-β1 in benign prostate epithelial cells, we examined phosphorylation of ERK-1/2, JNK and p38 MAPK in cells treated with increasing doses of TGF-β1 (0 – 2 ng/ml) (Fig. 5). ERK-1/2 phosphorylation (p-ERK) was stimulated in both BPH-1 and BHPrE1 cell lines although much more robustly in BPH-1 cells. JNK phosphorylation (p-JNK) was slightly increased in BPH-1 (Fig. 5 A) but not in BHPrE-1 (Fig. 5B) cells. p38 MAPK phosphorylation (p-p38) was not consistently altered in response to TGF-β1 treatment.

TGF-β1 increased cell monolayer permeability could be blocked by the MEK-1 inhibitor PD98059

To explore the potential relationship between prostate epithelial barrier permeability and MAPK pathways, we tested the ability of several commercially available inhibitors to affect the response of BPH-1 cells to TGF-β1 stimulation (Fig. 5C). Inhibitors of the upstream activator of ERK-1/2 (i.e. MEK-1/2) PD98059 and U0126 were able to abrogate the induction of p-ERK-1/2 and surprisingly also the induction of p-JNK in response to TGF-β1 treatment while PD98059 was able to reverse the TGF-ß1 repression of claudin 1 expression (Fig. 5C, Supplemental Fig. S3). The MEK-1 inhibitor PD98059 could restore claudin 1 expression in both BPH-1 and BHPrE1 cell lines (Supplemental Fig. S4). However, PD98059 did not affect TGF-β1 stimulation of snail and slug (Supplemental Fig. S4). The level of p-AKT^{473} was not altered by TGF-β1, suggesting that the AKT pathway was not influenced by TGF-β1 in prostate epithelial cells (Fig. 5C) and unlikely to mediate TGF-β1 repression of claudin 1 expression and enhancement of the cell monolayer permeability.

We also tested whether PD98059 could reverse the increased monolayer permeability induced by TGF-β1. In both BPH-1 and BHPrE1 cell lines, PD98059 could block epithelial barrier permeability changes induced by TGF-β1 (Fig. 6).

Discussion

In the current study, immunostaining analysis of specimens from patients with clinical BPH showed a significant decrease in claudin 1 in BPH epithelium relative to normal adjacent epithelium in a cohort of 22 patients with clinical BPH. Claudin 1 knockdown in benign prostatic epithelial cells cultured in transwell resulted in reduced TEER, suggesting a critical role of claudin 1 in maintenance of tight junctions. This study provided evidence that the

inflammatory cytokine TGF-β1 can down-regulate claudin 1 in benign prostatic epithelial cells and showed that MEK-1 inhibitor could reverse TGF-β1 down-regulation of claudin 1 and restore epithelial barrier function in BPH-1 and BHPrE1 cell lines. These findings provide new insights into the mechanisms associated with BPH pathogenesis and potential therapeutic targets for BPH treatment.

Claudin 1 down-regulation appeared to be more dramatic than the downregulation of E-cadherin in BPH. This finding suggests that the mechanisms leading to claudin 1 down-regulation and E-cadherin down-regulation in BPH are different. This is consistent with our observation that inflammatory cytokine TGF-β1 only caused very slight E-cadherin down-regulation in benign prostatic epithelial cells. In contrast, TGF-β1 caused dramatic down-regulation of claudin 1 in benign prostatic cells, suggesting that inflammation may be a major cause of claudin 1 down-regulation. Future studies will be needed to determine if claudin 1 down-regulation correlates with inflammation in human BPH specimens.

The TGF-β1 pathway is activated in BPH [4, 25–27] and may contribute to increased epithelial barrier permeability. The role of TGF-β1 in the maintenance of the epithelial barrier appears to be tissue specific. TGF-β1 plays a critical role in gut mucosal repair and epithelial barrier function by stimulating the expression of tight junction protein claudin 1 [28]. In salivary gland duct, TGF-β1 treatment decreased transepithelial electrical resistance (TEER) [29]. In the prostate, overexpression of TGF-β1 induced inflammation and fibrosis in a mouse model [12], however it is not known how TGF-β1 impacts the prostate epithelial barrier. Our previous study showed that TGF-β1 stimulation increased prostate epithelial monolayer permeability and decreased mRNA expression of claudin 1 in benign prostate epithelial cell lines [23]. This study suggests that TGF-β1 stimulation of prostatic epithelial monolayer permeability is likely mediated through claudin 1 down-regulation because claudin 1 knockdown caused increases in prostatic epithelial monolayer permeability in both BPH-1 and BHPrE1 cell line models.

In our study, knockdown of claudin 1 increased monolayer permeability in benign prostatic epithelial cell lines BHPrE1 and BPH-1 without impacting the expression of E-cadherin, N-cadherin, or EMT transcription factors snail or slug. This suggests that down-regulation of claudin 1 did not affect EMT in prostate epithelial cells. Other studies have shown that claudin 1 overexpression is evident in prostate cancer [30], and that overexpression of E-cadherin (which was not altered by claudin 1 knockdown) down-regulated claudin 1 and inhibited tumor cell migration and invasion [31]. Our studies suggest that in BPH, which is not associated with prostate carcinogenesis, claudin 1 expression is decreased – and thus our experiments focused on the functional consequences of claudin 1 down-regulation and the potential impact of TGF-β1 signaling on the epithelial barrier. It may be that claudin 1 down-regulation also inhibits migration and invasion in prostate cancer cells, this will be an intriguing line of research questions for future studies. Also, EMT signaling did not seem to regulate or mediate TGF-β1 regulation of claudin 1 in prostatic cells. As expected, TGF-β1 stimulation of benign prostatic epithelial cell lines increased snail and slug in our study, but not as dramatically as the downregulation of claudin 1. Overexpression of snail or slug did not affect claudin 1 expression in benign prostatic epithelial cell lines. MEK-1 inhibitor did not impact snail or slug (see Supplemental Fig. S4), while alleviating TGF-β1 enhancement

of prostatic cell monolayer permeability. Thus, TGF-β1 regulation of claudin 1 expression in prostatic epithelial cells appears to be independent of EMT signaling. Altered EMT signaling in BPH may contribute to the disease progression through other mechanisms, independent of claudin 1.

Our studies showed that TGF-β1 activated the MAPK/ERK pathway in benign prostatic epithelial cell lines and TGF-β1 increased cell monolayer permeability could be blocked by MEK-1 inhibitor PD98059. Several studies suggested that ERK signaling is activated in BPH [32–34]. JNK phosphorylation was increased by TGF-β1 as well in BPH-1 cells, but not in BHPrE1 cells (Fig. 5A&5B, Supplemental Fig. S4), suggesting that JNK signaling is unlikely to be a mechanism mediating TGF-β1 repression of claudin 1 expression at least in BHPrE1 cells. Unexpectedly both the MEK-1/2 inhibitor PD98059 and U0126 were able to decrease JNK phosphorylation in both BPH-1 and BHPrE1 cell lines. While chronic activation of JNK has been shown to impact MEK-1/2 and ERK-1/2 activation [35, 36], to our knowledge the impact of MEK-1/2 on JNK phosphorylation under conditions of chronic activation has not been reported. Whether this potentially novel form of crosstalk between the JNK and ERK-1/2 pathways is a unique feature of prostatic epithelial cells or characteristic of other cellular responses to chronic TGF-ß1 stimulation will be of interest to explore in future studies. Nonetheless, our findings suggest that MEK/ERK signaling may play an important role in mediating the impact of inflammatory cytokines, such as TGF-β1, on luminal epithelial layer permeability in glandular BPH. MEK/ERK signaling could represent a therapeutic target for BPH treatment by restoring the prostate epithelial barrier tight junctions.

The cell lines used in this study were two benign prostatic cell lines established from non-cancerous prostatic tissues [18, 19]. The BPH-1 cell line was derived from prostate tissues obtained from a BPH patient through transurethral resection of the prostate [18]. BPH-1 cells express increased levels of estradiol (E2) and aromatase, two factors that have been implicated in BPH progression [37]. The BHPrE1 cell line was derived from benign human prostate [19]. BPH-1 cells do not express AR or PSA [18], while BHPrE1 cells have been shown to express low levels of PSA [19]. In our previous report, we compared the expression of junction proteins in BPH-1 compared to BHPrE1 cell lines. Briefly, BHPrE1 cells have elevated expression of E-cadherin, β-catenin compared to BPH-1; while BPH-1 cells have elevated expression of N-cadherin, TJP1, TJP2 and claudin 1 [5]. The average number of tight junction kiss points in BHPrE1 cells grown in monolayers was higher than that of BPH-1 cells [5]. While both cell lines responded similarly to the knockdown of claudin 1 and TGF-β1 stimulation, the impact on monolayer permeability was more pronounced in BHPrE1 cells. BPH-1 cells displayed a dramatic increase in phosphorylated ERK levels in response to TGF-β1 stimulation compared to the BHPrE1 response, and BPH-1 cells treated with ERK inhibitor also had an increased restoration of claudin 1 expression. Additionally, snail seemed more strongly induced in BHPrE-1 cells while slug was upregulated in BPH-1. It is not clear if these differences are physiologically relevant, and future studies will be required to determine whether BPH-1 or BHPrE1 are more representative of BPH.

Characterization of TGF-β1 and claudin 1 provided insights into how inflammation could influence epithelial barrier in BPH pathogenesis. BPH development and progression have been associated with the presence of chronic prostatic inflammation [38]. However, it is not well understood how chronic inflammation contributes to BPH pathogenesis. Inflammation likely impacts multiple cytokines and chemokines and it will be important to explore other inflammatory proteins, in addition to TGF-β1 that appears to play a role in BPH. Considering the complexity of inflammation and BPH pathogenesis, other inflammatory signaling molecules may affect different junctional proteins in BPH tissues. Inflammation could influence many different pathways and its influence on the luminal epithelial barrier is only one aspect impacted by inflammation. However, increased permeability has been shown to exacerbate inflammation in other organs (Reviewed in [39]). Altered epithelial permeability and subsequent inflammation in the prostate could create a vicious cycle that may speed up BPH pathogenesis.

Cumulatively, these results suggest that claudin 1 down-regulation in BPH could be induced by TGF-β1 through activation of the ERK pathway and that loss of claudin 1 expression could increase the epithelial permeability in prostate tissues. Claudin 1 down-regulation in response to prostatic inflammation could reduce the epithelial barrier, subsequently contributing to a chronic inflammatory response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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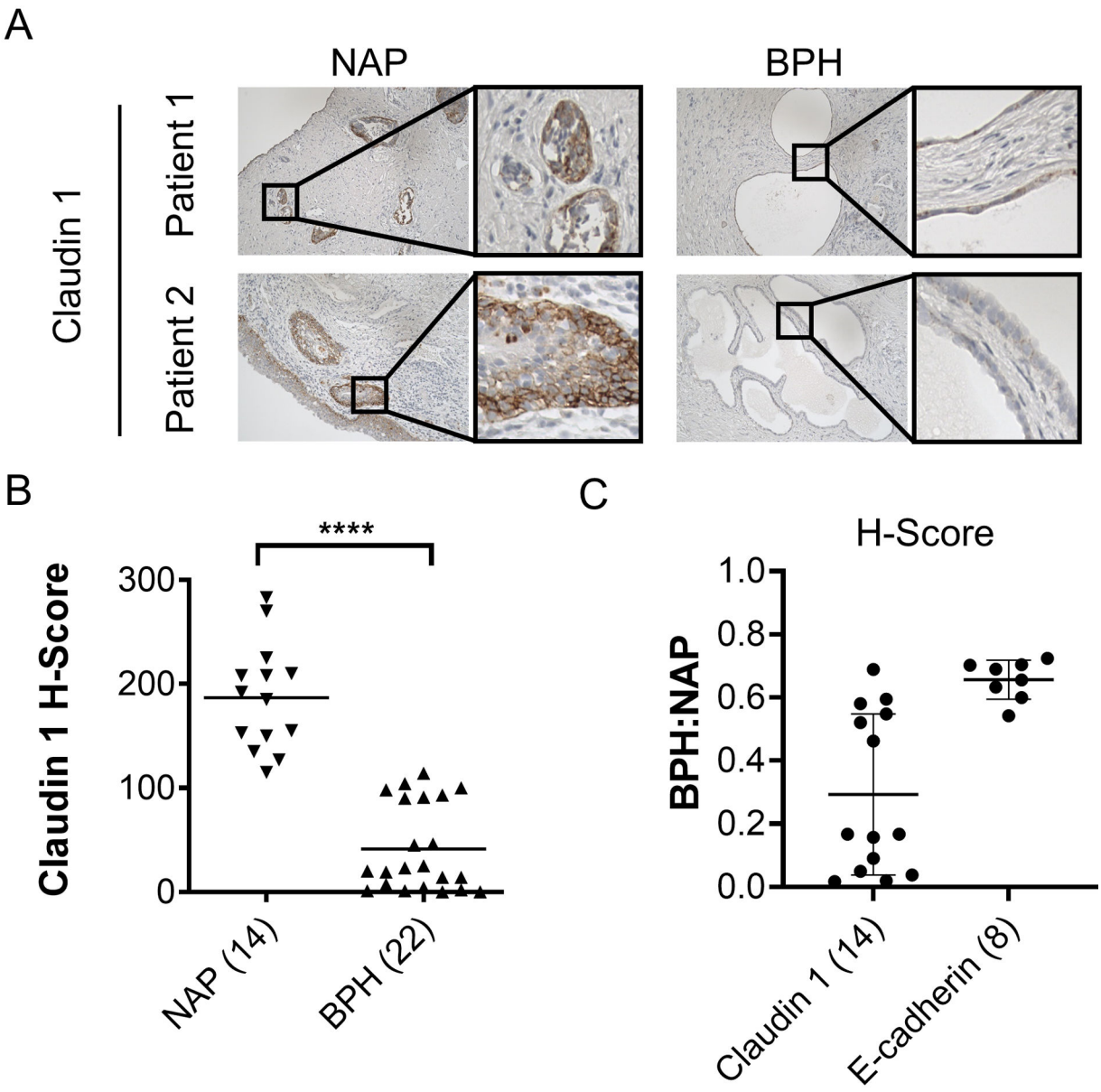

Figure 1.

Immunostaining of claudin 1 expression in BPH specimens. (A) Representative images showing the expression of claudin 1 in normal adjacent prostate (NAP) and BPH area in two individual patients. (B) Quantification of mean claudin 1 staining intensity H-score of claudin 1 in 14 paired (NAP and BPH) plus 8 BPH only specimens from a total of 22 patients. (C) Quantification of Claudin 1 and E-Cadherin BPH:NAP H-Score ratios in paired patient tissues. (D) Quantification of Claudin 1 and E-Cadherin BPH:NAP mRNA ratios in paired patient tissues. Number of patients in parentheses. Data represent mean ± S.D. ****, P<0.0001.

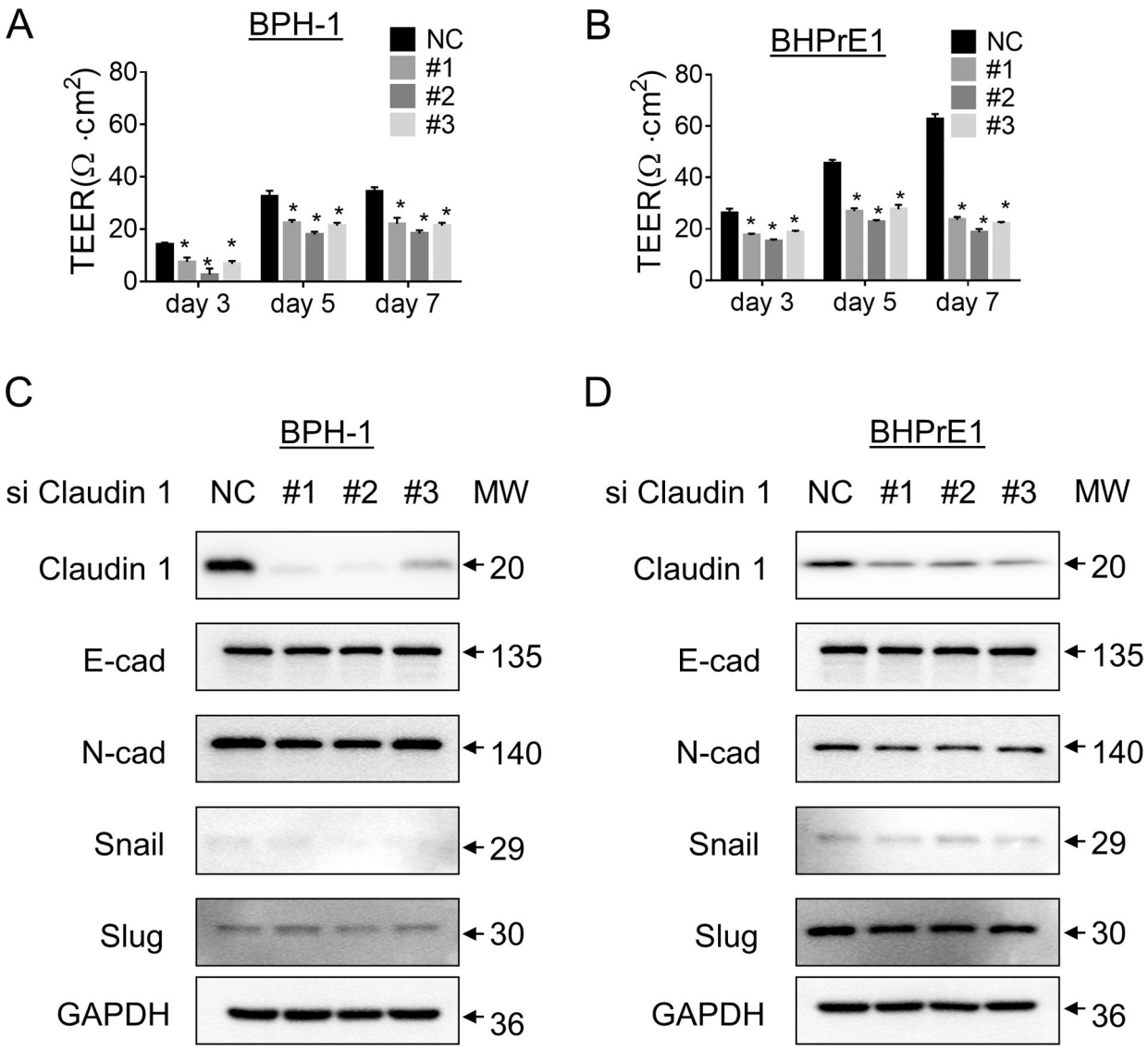

Figure 2.

Effect of claudin 1 siRNA knockdown on cell monolayer permeability and expression of junction proteins, snail and slug in benign prostatic epithelial cell lines. Cells were seeded into 60 mm dishes (3×10^5 cells/dish), followed by siRNA knockdown with non-targeting control (NC), or three different siRNAs targeting claudin 1 (#1, #2, #3). Cells were seeded to transwell inserts (1.5×10^5 cells/well) 48 hours after knockdown. (A) BPH-1 cell monolayer permeability measured by TEER on day 3, day 5 and day 7. (B) BHPrE1 cell monolayer permeability measured as in (A). *, $P<0.05$. E and F: The effect of claudin 1 knockdown on the expression of EMT markers and cell junction proteins. Cell lysates were collected after 72 hours siRNA transfection for detection of claudin 1, E-cad, N-cad, snail, and slug by western blot in BPH-1 cells (C) and BHPrE1 cells (D). GAPDH was probed as a loading control for each individual experiment, and representative results from at least two experimental replicates are shown.

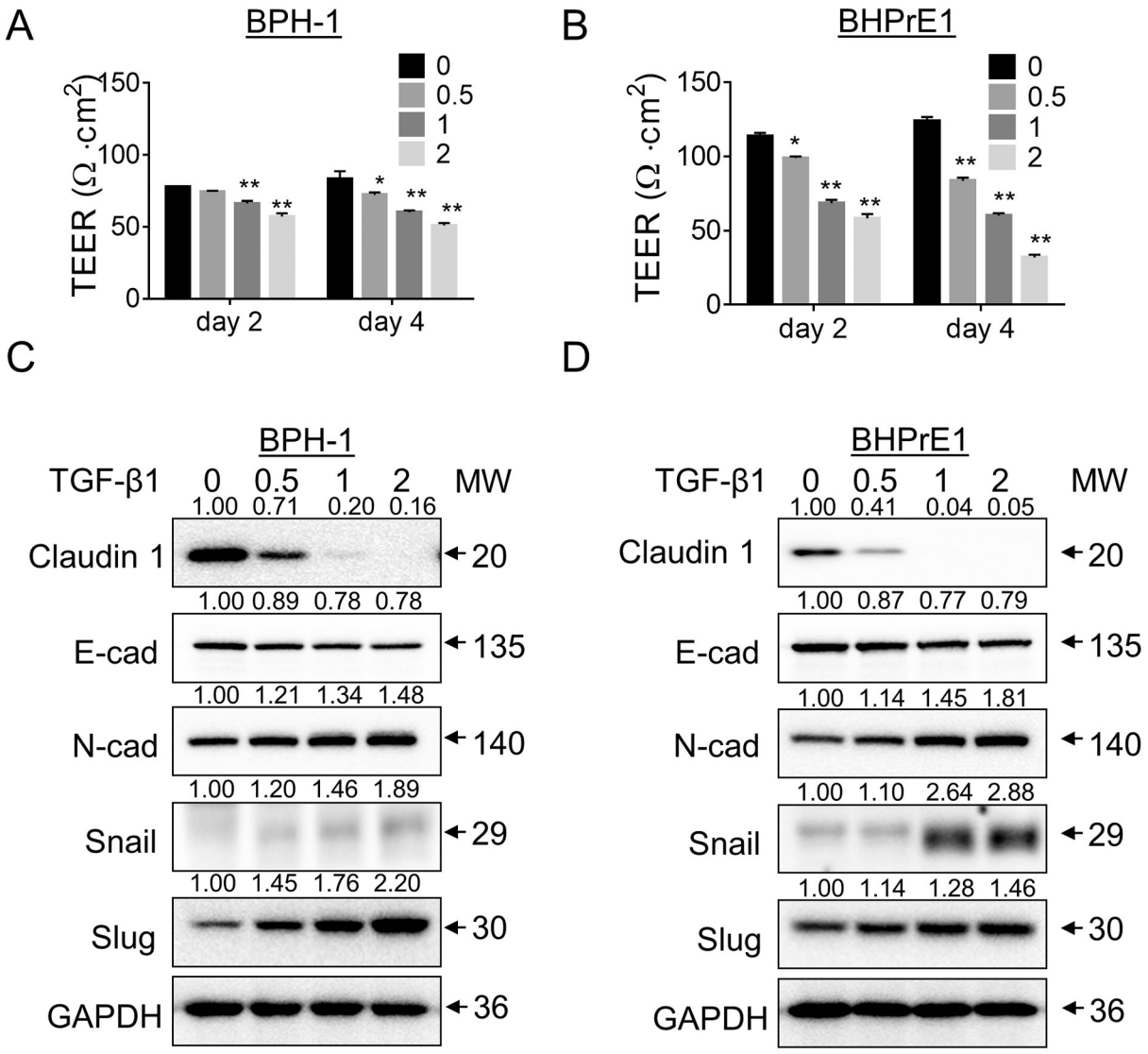

Figure 3.

Effect of TGF-β1 stimulation on monolayer permeability and the expression of claudin 1, E-cad, N-cad, snail, and slug in benign prostatic epithelial cell lines. Cells were seeded to 60 mm dishes (3×10^5 cells/ dish) followed by TGF-β1 treated in 0, 0.5, 1, 2 ng/mL. After 24 hours treatment, cells (1.5×10^5 cells/dish) were seeded to transwell inserts. A and B: Cell monolayer permeability was measured by transepithelial electrical resistance (TEER) in day 2 and day 4 in BPH-1 cells (A) and BHPrE1 cells (B). Error bar represents mean ± S.D. *, P<0.05 and **, P<0.01. (C) and (D): Western blot analysis of claudin 1, E-cad, N-cad, snail and slug in BPH-1 cells (C) and BHPrE1 cells (D) in response to TGF-β1. GAPDH was probed as a loading control for each individual experiment, and representative results from at least two experimental replicates are shown. Band intensity determined using Adobe PhotoShop.

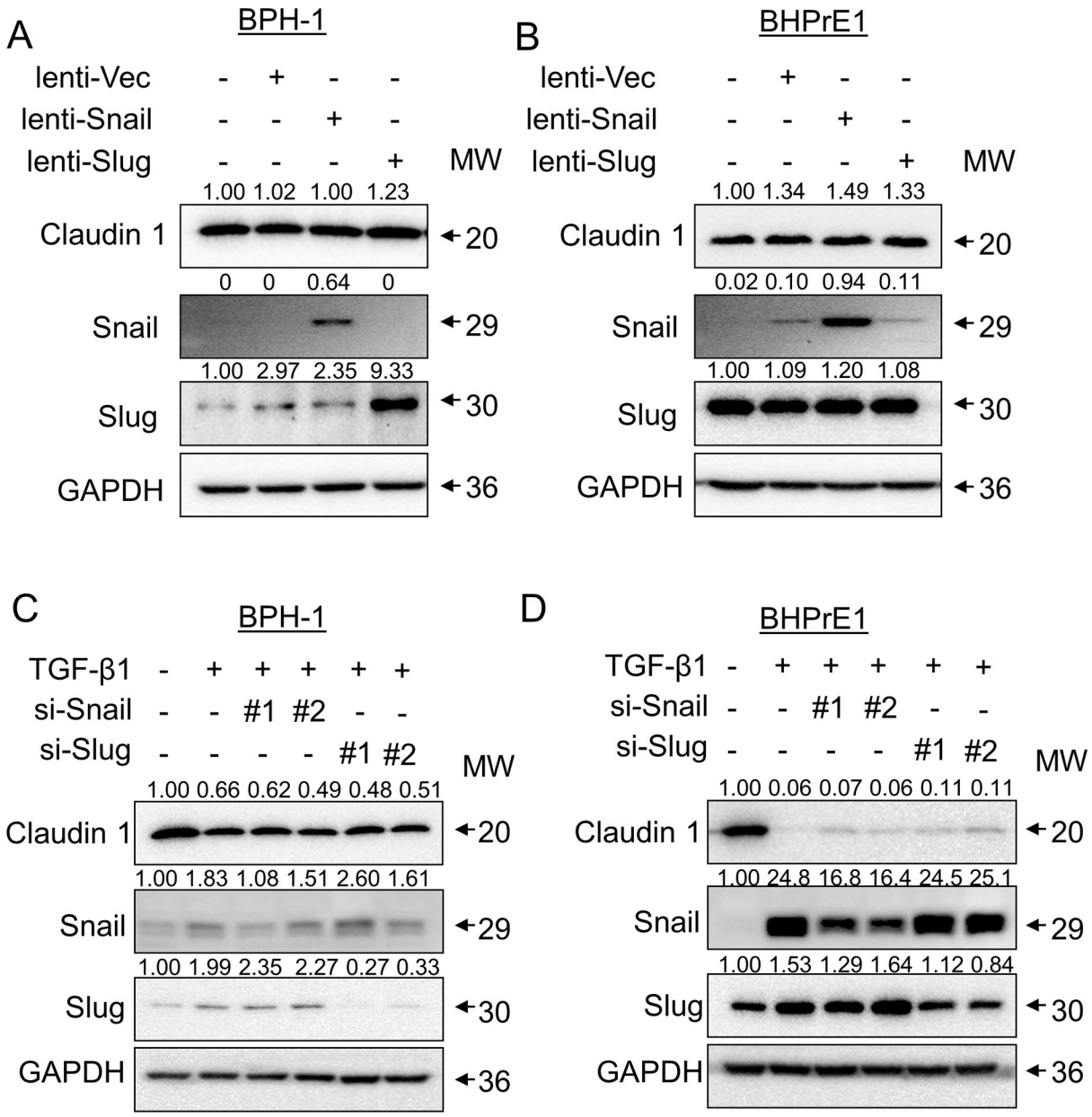

Figure 4.

Effect of snail and slug overexpression and knockdown on claudin 1 expression in benign prostatic epithelial cell lines. A and B: Western blot analysis of BPH-1 cells (A) and BHPrE1 cells (B) overexpressing snail or slug. C and D: Western blot analysis of BPH-1 cells (C) and BHPrE1 cells (D) with knockdown of snail or slug in the presence of 1 ng/mL TGF-β1. Cell lysates were collected for detection of claudin 1, snail and slug by western blot. GAPDH was probed as a loading control.

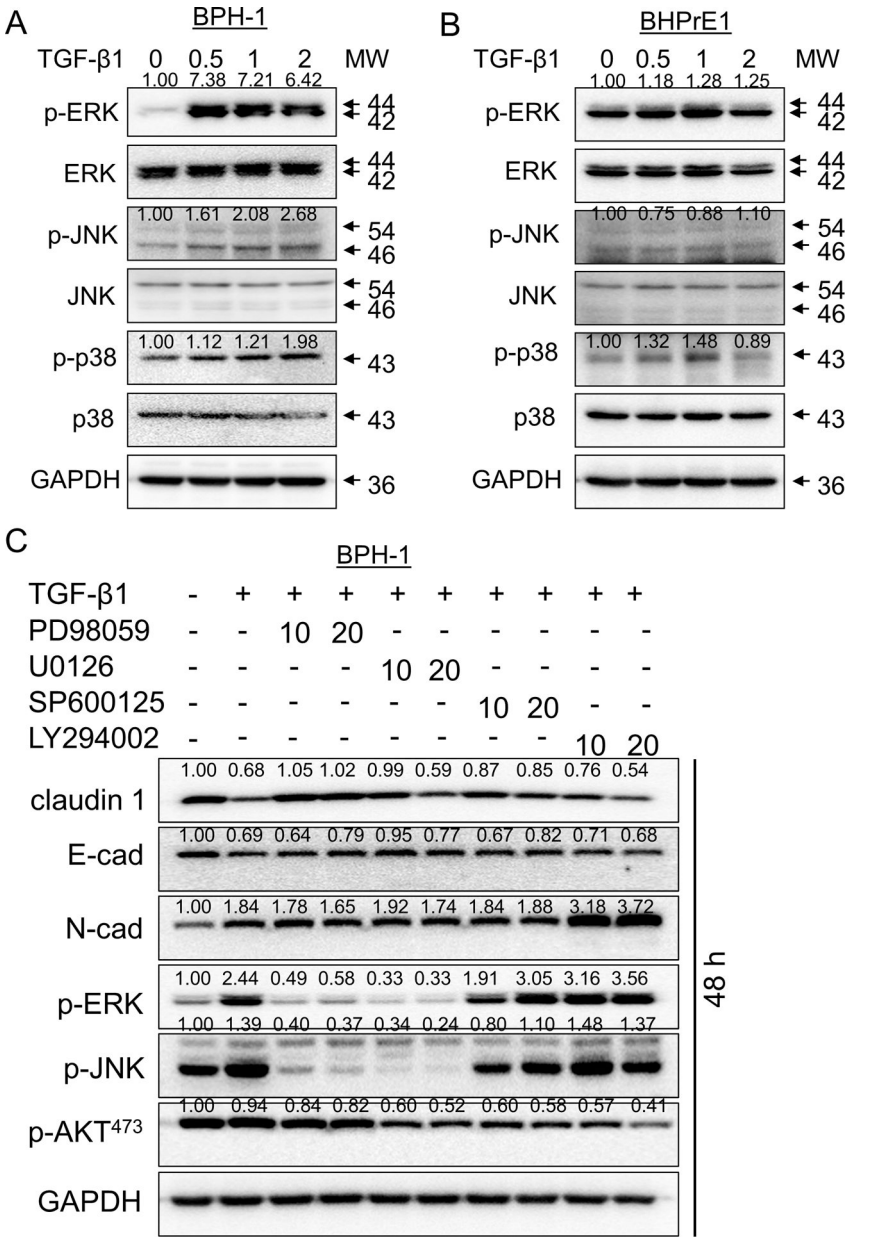

Figure 5.

MAPK/ERK pathway activation by TGF-β1 in benign prostatic epithelial cell lines. A and B: Western blot analysis of indicated signaling proteins in BPH-1 cells (A) and BHPrE1 cells (B) treated with 0, 0.5, 1, 2 ng/mL TGF-β1 for 48 hours. (C) Rescue of claudin 1 expression in BPH-1 cells by MEK-1 inhibitor when cells were treated with TGF-β1. BPH-1 cells were seeded to 6 well plates, treated with 1 ng/mL TGF-β1 together with MEK-1 inhibitor PD98059 (10 and 20 μM) or U0126 (10 and 20 μM) for 48 hours. Cells were pre-treated with each inhibitor for 1 hour before adding TGF-β1. Cell lysates were prepared for detection of p-ERK, p-JNK, p-AKT473 and claudin 1 by western blot. GAPDH was probed as a loading control for each individual experiment. Representative results from at least two experimental replicates are shown.

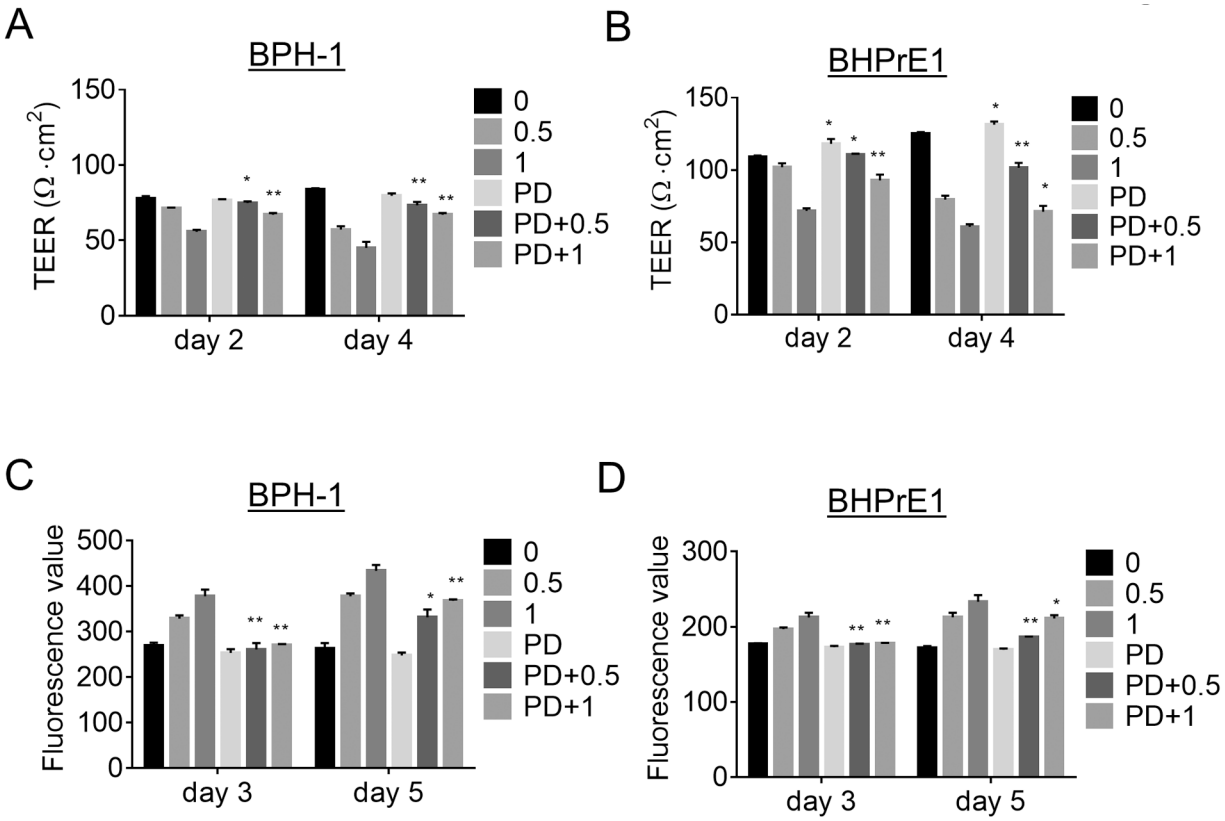

Figure 6.

Effect of ERK inhibition on benign prostate epithelial monolayer permeability following TGF-β1 treatment. TEER measurement in BPH-1 cells (A) and BHPrE1 cells (B) treated with indicated doses of TGF-β1 in the presence or absence of PD98059. (C) Monolayer permeability was measured by FITC-dextran 70 kDa permeability assay in BPH-1 cells, and (D) BHPrE1 cells. Cells were seeded into 60 mm dishes, treated by TGF-β1 in 0.5 and 1 ng/mL with or without ERK inhibitor PD98059 in 20 μM. Cells were pre-treated with PD98059 for 1 hour before addition of TGF-β1. After 24 hours treatment, cells (1.5×10^5 cells/well) were seeded to transwell inserts and continued culture and TEER measurement as described in Materials and Methods. Results shown as mean ± S.D. *, $P<0.05$ and **, $P<0.01$.

Table 1.

Antibodies used for Western blot

Antibody	Clone, Cat#	Source
Claudin 1	XX7, sc-81796	Santa Cruz Biotechnology
E-cadherin	3195	Cell Signaling Technology
N-cadherin	14215	Cell Signaling Technology
Snail	3879	Cell Signaling Technology
Slug	9585	Cell Signaling Technology
p-ERK	4370	Cell Signaling Technology
ERK	4695	Cell Signaling Technology
p-JNK	9255	Cell Signaling Technology
JNK	9252	Cell Signaling Technology
p-p38	4511	Cell Signaling Technology
p38	8690	Cell Signaling Technology
p-Akt	4060	Cell Signaling Technology
GAPDH	sc-47724	Santa Cruz Biotechnology

Table 2.

Sequences for siRNA.

Gene	Primer Sequence
CLDN1 #1	Forward: 5′- rCrArUrGrUrGrGrArUrArUrCrArGrUrUrArCrCrArArArCAT-3′ Reverse: 5′- rArUrGrUrUrUrGrGrUrArArCrUrGrArUrArUrCrCrArCrArUrGrGrA-3′
CLDN1 #2	Forward: 5′- rArGrCrArUrGrGrUrArUrGrGrCrArArUrArGrArArUrCrGTT-3′ Reverse: 5′- rArArCrGrArUrUrCrUrArUrUrGrCrCrArUrArCrCrArUrGrCrUrGrU-3′
CLDN1 #3	Forward: 5′- rArCrArCrCrArArGrGrCrCrCrUrArUrCrCrArArArArCrCTG-3′ Reverse: 5′- rCrArGrGrUrUrUrUrGrGrArUrArGrGrGrCrCrUrUrGrGrUrGrUrUrG-3′)
SNAIL #1	Forward: 5′-rArArArGrGrUrArCrArCrUrGrGrUrArUrUrUrArUrArUrUrUrC-3′ Reverse: 5′-rGrArArArUrArUrArArArUrArCrCrArGrUrGrUrArCrCrUrUrUrArA-3′
SNAIL #2	Forward: 5′-rCrArGrArUrGrUrCrArArGrArArGrUrArCrCrArGrUrGrCrCrA-3′ Reverse: 5′-rUrGrGrCrArCrUrGrGrUrArCrUrUrCrUrUrGrArCrArUrCrUrG-3′)
SLUG #1	Forward: 5′-rArCrUrGrArGrUrGrArCrGrCrArArUrCrArArUrGrUrUrUrArC-3′ Reverse: 5′-rGrUrArArArCrArUrUrGrArUrUrGrCrGrUrCrArCrUrCrArGrUrGrU-3′
SLUG #2	Forward: 5′-rCrArUrUrArGrUrGrArUrGrArArGrArGrGrArArArGrArCrUrA-3′ Reverse: 5′-rUrArGrUrCrUrUrUrCrCrUrCrUrUrCrArUrCrArCrUrArArUrG-3′

Table 3.

Primers for qPCR.

Gene	Forward	Reverse
CLDN1	5’-CCTCCTGGGAGTGATAGCAAT-3’	5’-GGCAACTAAAATAGCCAGACCT-3’
Snail	5’-TCGGAAGCCTAACTACAGCGA-3’	5’-AGATGAGCATTGGCAGCGAG-3’
Slug	5’-CGAACTGGACACACATACAGTG-3’	5’-CTGAGGATCTCTGGTTGTGGT-3’
GAPDH	5’-CGACCACTTTGTCAAGCTCA-3’	5’-AGGGGAGATTCAGTGTGGTG-3’