

# Role of ROCK/NF- $\kappa$ B/AQP8 signaling in ethanol-induced intestinal epithelial barrier dysfunction

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**Abstract.** The present study aimed to investigate the signaling pathways and the underlying molecular mechanisms involved in ethanol-induced intestinal epithelial barrier (IEB) dysfunction. Therefore, an *in vitro* experimental model of IEB was established using an ethanol-treated Caco-2 intestinal epithelial cell monolayer. The results confirmed that Rho-associated kinases (ROCKs), namely ROCK1 and ROCK2, were involved in the underlying pathway of ethanol-induced IEB dysfunction. Ethanol exposure significantly increased the expression of both ROCK isoforms and the activity of nuclear factor  $\kappa$ B (NF- $\kappa$ B). Furthermore, ROCK1- and ROCK2-specific small interfering RNAs (siRNAs), and the NF- $\kappa$ B inhibitor ammonium pyrrolidine dithiocarbamate partially inhibited transepithelial electrical resistance in Caco-2 cells in an *in vitro* IEB model. In addition, ROCK1- and ROCK2-specific siRNAs inhibited the activity of NF- $\kappa$ B, thereby downregulating the expression of aquaporin 8 (AQP8). Taken together, the results of the present study suggested that ROCK1/ROCK2-mediated activation of NF- $\kappa$ B and upregulation of AQP8 expression levels may represent a novel mechanism of ethanol-induced impairment of IEB function.

## Introduction

The human body has multiple barriers between the internal and external environment, including the gastrointestinal

barrier, which serves key functions. The intestinal epithelial barrier (IEB) is the line of defense in the intestine that prevents harmful substances from passing through the intestinal epithelium and entering other tissues, organs or the blood stream. The intestinal immune system serves a vital role in maintaining body health; however, it may be dysregulated in response to various diseases (1,2).

Rho is a small G protein with GTPase activity. Rho-associated kinase (ROCK) acts as a downstream effector of Rho signaling and belongs to the serine/threonine protein kinase family (3). The ROCK catalytic center is exposed and activated when the Rho-binding domain binds to Rho-GTP (4). In addition, our previous study using Caco-2 cells *in vitro* demonstrated that Rho A is involved in ethanol-induced IEB permeability increase and inhibits tight junction protein (5). The Rho effector molecule ROCK can affect the function of IEB by adjusting the function of tight junctions (6). Dysfunction can occur when IEB is disabled. Based on this research, it was hypothesized that ROCK may participate in the IEB dysfunction process via a signaling pathway.

Currently, two Rho subtypes have been identified, namely ROCK1 and ROCK2. Both isoforms share an amino acid sequence homology of 64-65%, with the highest homology (80-92%) within the kinase domain (7). Although the two ROCK isoforms are structurally similar, they may have different functions. ROCK1 is associated with cell migration, whereas ROCK2 is associated with vimentin and actin tension fibers (8,9). Therefore, it was hypothesized that they serve different roles in IEB dysfunction.

Nuclear factor  $\kappa$ B (NF- $\kappa$ B) is an important transcription factor regulating the expression of several inflammatory factors. It has been suggested that ROCK activates the NF- $\kappa$ B signaling pathway (10). Thus, ROCK may accelerate the development and progression of IEB dysfunction by activating NF- $\kappa$ B. ROCK may also activate NF- $\kappa$ B p65 by downregulating NF- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ) expression, which in turn mediates the transcription of multiple inflammatory factors.

Aquaporins (AQPs), a family of homologous water transporters in mammals, mainly mediate the passive trans-biofilm transport of free water and regulate water absorption (11). To date, 13 members of the AQP family have been identified (AQP 0-12) (12). Previous studies have shown that AQP8 is downregulated in the intestinal epithelial cells and is associated with water transport in the human colon (13-15).

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*Abbreviations:* AQP8, aquaporin 8; EMSA, electrophoretic mobility shift assay; I $\kappa$ B $\alpha$ , NF- $\kappa$ B inhibitor  $\alpha$ ; IEB, intestinal epithelial barrier; NF- $\kappa$ B, nuclear factor  $\kappa$ B; TEER, transepithelial electrical resistance; RIPK1, receptor interacting protein kinase 1; ROCK, Rho-associated kinase; RT-qPCR, reverse transcription-quantitative PCR

*Key words:* ROCK, NF- $\kappa$ B, AQP8, ethanol, IEB

The Caco-2 cell model was first proposed by Hidalgo *et al.* (16) in 1989. Under normal cell culture conditions, Caco-2 cells can differentiate into a polar monolayer on the porous membrane after 21 days and express certain structural and functional characteristics of intestinal epithelial cells. Since then, a number of studies have used this model. Nighot *et al.* (17) used a Caco-2 monolayer model system to investigate the role of autophagy in regulating the function of the intestinal tight junction barrier. The Caco-2 cell model was also used by Lin *et al.* (18) and Elamin *et al.* (19) to study factors that affect intestinal epithelial barrier function. Therefore, the Caco-2 cell model was selected as a normal human intestinal epithelial cell model in the present study.

Therefore, the aim of the present study was to investigate whether AQP8 was involved in the downstream mechanisms of the ROCK/NF- $\kappa$ B pathway in IEB dysfunction using Caco-2 cell model.

## Materials and methods

**Caco-2 monolayer cell culture.** The human colon adenocarcinoma cell line Caco-2 was purchased from the American Type Culture Collection. Caco-2 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences) at 37°C with 5% CO<sub>2</sub> atmosphere. When Caco-2 cells had grown to 80% confluence, they were plated onto Transwell filters (Corning Inc.) and the medium was changed every two days. The cells were visually monitored using an inverted microscope and epithelial resistance measurements at 21 days.

**Measurement of transepithelial electrical resistance (TEER).** TEER values were measured at when the monolayer formed at 21 days, as previously described (20). The TEER of Caco-2 cell monolayers was measured using a Millicell<sup>®</sup>-electrical resistance system (EMD Millipore) at 37°C (21). The electrical resistance was expressed in ohm ( $\Omega$ )•cm<sup>2</sup> using the surface area of the Transwell insert.

**Gene silencing using small interfering RNA (siRNA).** For knockdown experiments, Caco-2 cells were transfected with 100 pmol ROCK1- and ROCK2-specific siRNAs (Shanghai GenePharma Co., Ltd.), and 100 pmol inducible control siRNA (Shanghai GenePharma Co., Ltd.) using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 24 h. Subsequent experiments were performed 48 h after transfection. The ROCK1 and ROCK2 siRNA sequences are listed in Table I.

**Experimental groups and cell treatment.** Our previous study demonstrated that following 21 days of cell cultivation, TEER values were significantly increased and stabilized compared with day 0 (20). This finding confirmed the successful establishment of the monolayer epithelial cell model. For the ethanol-treatment group, ethanol was added to the culture medium at a concentration of 5% (v/v) for 1 h. ROCK1 and ROCK2 siRNA with high transfection efficiency, as determined by preliminary experiments (Figs. 1 and 2), were selected to transfect Caco-2 monolayer cells. Subsequently, ammonium pyrrolidine dithiocarbamate (PDTC; 100  $\mu$ M), an

Table I. Sequences of siRNA.

Gene	Sequence (5'→3')
ROCK1 siRNA-1	F: UCCACCAGGAAGGUUAUAUGTT R: CAUAUACCUUCCUGGUGGATT
ROCK1 siRNA-2	F: GCCUGAUACAUGCUGCUGTT R: CAGCAGCAUGUUAUCAGGCTT
ROCK1 siRNA-3	F: GGCAUGGUACGAUGUGAUATT R: UAUCACAUCGUACCAUGCCCTT
ROCK2 siRNA-1	F: AUCAGAGGUCUACAGAUGATT R: UCAUCUGUAGACCUCUGAUTT
ROCK2 siRNA-2	F: CUCAGCAGUGACAUAGACATT R: UGUCUAUGUCACUGCUGAGTT
ROCK2 siRNA-3	F: GUGACUCUCAUCUUGUAGTT R: CUACAAGAUGGAGAGUCACTT
Control siRNA	F: UUCUCCGAACGUGUCACGUTT R: ACGUGACACGUUCGGAGAATT

F, forward; R, reverse; ROCK, Rho-associated kinase; siRNA, small interfering RNA.

NF- $\kappa$ B inhibitor, was added to Transwell chamber medium. The upper chamber medium was removed after 30 min and then ethanol was added. The cells were incubated with DAPI (1:5; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 10 min. Finally, the glass slides were observed in all seven groups with three visual fields in each group using immunofluorescence microscopy (Olympus Corporation).

**Reverse transcription-quantitative PCR (RT-qPCR).** Following Caco-2 cell transfection, the expression levels of ROCK1, ROCK2, NF- $\kappa$ B and AQP8 were determined using RT-qPCR. Briefly, total RNA was extracted from Caco-2 cells with the TRIpure reagent (BioTeke Corporation) and the purity and concentration were measured. Subsequently, RNA was reverse transcribed into cDNA using an RT-PCR kit (v3.0; Takara Bio, Inc.) in a final volume of 20  $\mu$ l. PCR amplification was performed using cDNA as a template in 50  $\mu$ l reactions. PCR was performed using SYBR GREEN master kit (Beijing Solarbio Co., Ltd.) running the thermocycling programs: 94°C for 5 min, then 40 two-step cycles of 72°C for 2.5 min, 40°C for 1.5 min and 25°C for 2 min. The specific PCR primers were designed with the Primer Express v2.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) and synthesized by Sangon Biotechnology Co., Ltd. The primer sequences are listed in Table II. Relative gene expression levels were analyzed using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (22).

**Western blot analysis.** Caco-2 cells were plated onto glass coverslips and cultured until 70% confluency was reached. Subsequently, cells were washed with PBS containing 0.1 mM EDTA (without calcium and magnesium ions), scraped from the slides using a scraper, homogenized in 1 ml lysis buffer A (2 mM EDTA, 10 mM EGTA, 0.4% NaF, 20 mM Tris-HCl, protease inhibitor, phenylmethylsulfonyl fluoride, protease

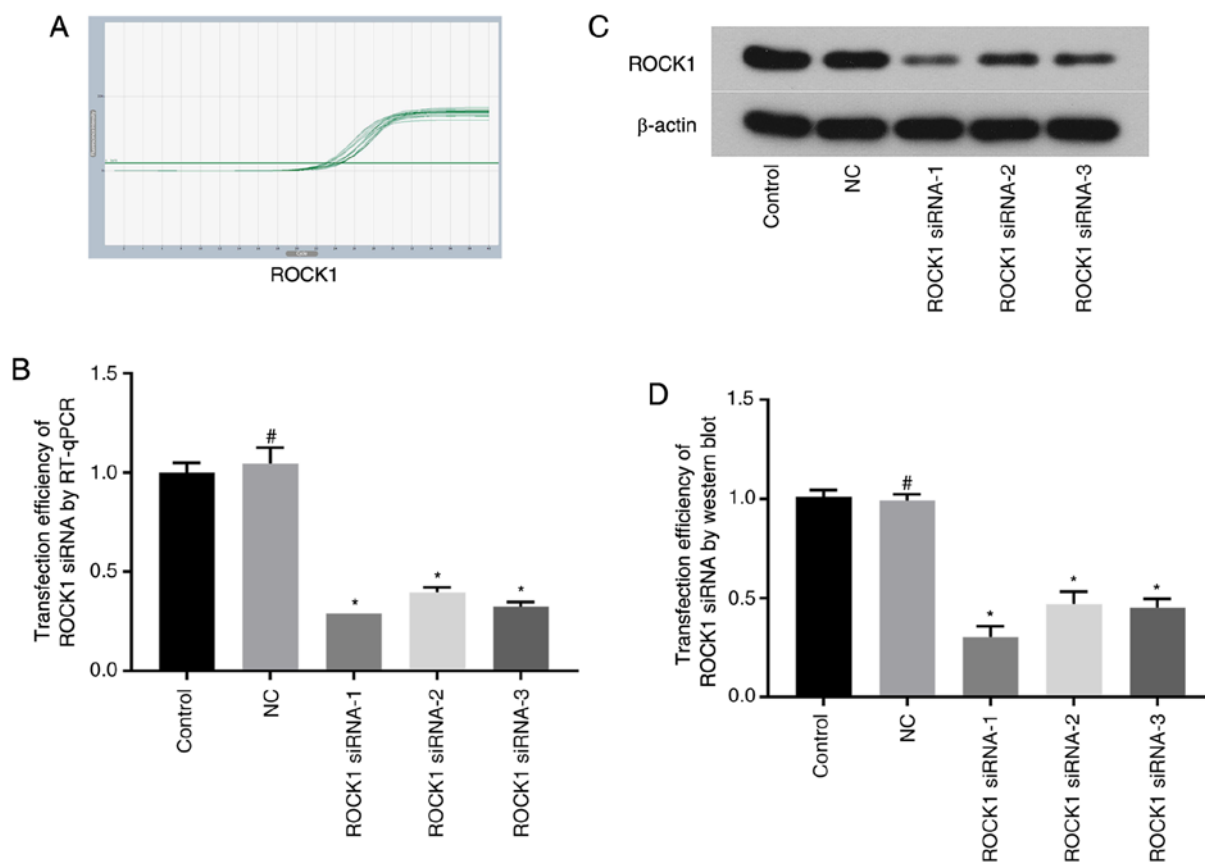


Figure 1. Effects of ROCK1 siRNA. (A) The amplification curve of ROCK1. (B) ROCK1 mRNA expression levels following ROCK1 siRNA transfections. ROCK1 mRNA expression was significantly decreased with the reduction in expression up to 71% in the ROCK1 siRNA-1 group compared with the untreated control. (C) Representative western blot and (D) semi-quantification of ROCK1 protein expression following ROCK1 siRNA transfections. ROCK1 protein expression was significantly decreased in the ROCK1 siRNA-1 group, with the reduction in expression up to 70% compared with the control. All results are expressed as the mean  $\pm$  SD; n=3; \*P<0.01, #P>0.05 vs. Control. NC, negative control; ROCK, Rho-associated kinase; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA.

inhibitor cocktail, pH 7.5) and centrifuged at 10,000  $\times$  g at 4°C for 10 min. Finally, the supernatant containing the total cellular proteins was collected. Equal amounts of proteins (40  $\mu$ g) were separated by SDS-PAGE on 8% polyacrylamide gels. After transferring the gel to PVDF membrane, 5% (M/V) skimmed milk powder was used for blocking at 37°C for 1 h. The membrane was incubated with the primary antibodies against ROCK1, ROCK2, NF- $\kappa$ B p65 (all 1:500; cat. nos. WL01761, WL00550 and WL01980, respectively; all Wanleibio Co., Ltd.) and AQP8 (1:1,000; AB2768409; ABclonal Biotech Co., Ltd.) overnight at 4°C. Then, the membranes were incubated with  $\beta$ -actin antibody (1:1,000; cat. no. WL01845; Wanleibio Co., Ltd.) and Histone H3 antibody (1:1,000; cat. no. WL0984a; Wanleibio Co., Ltd.) overnight at 4°C. Subsequently, proteins were incubated with a secondary antibody (anti-rabbit; 1:5,000; cat. no. WLA023; Wanleibio Co., Ltd.) at 37°C for 45 min. The protein bands were visualized using a Gel Imaging Analysis System (cat. no. WD-9413B; Beijing Liuyi Biotechnology Co., Ltd.), and the integrated density values (IDV) for each protein were calculated using the Gel-Pro-Analyzer (v6.0; Media Cybernetics, Inc.) and were normalized to the IDV of  $\beta$ -actin.

**Electrophoretic mobility shift assay (EMSA).** EMSA binding reactions were performed using a Lighshift Chemiluminescent EMSA kit (Pierce; Thermo Fisher

Table II. Primer sequences for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'→3')
ROCK1	F: AGGTTAGGGCGAAATGGT
	R: GAATGTTTCTCCTCCTCCTT
ROCK2	F: CAACTGTGAGGCTTGATGA
	R: CAACCGACTAACCCACTTCT
NF- $\kappa$ B p65	F: TGCCGAGTGAACCGAAAC
	R: TGGAGACACGCACAGGAG
AQP8	F: TGAGAATGGGACGGACACTG
	R: AGTACGGGAGGAGCATCACC
$\beta$ -actin	F: ACCCTGAAGTACCCCATCGA
	R: CAAACATGATCTGGGTCATCT

AQP8, aquaporin 8; F, forward; NF- $\kappa$ B, nuclear factor  $\kappa$ B; R, reverse; ROCK, Rho-associated kinase.

Scientific, Inc.). The double-stranded oligonucleotide probe (5'-AGTTGAGGGGACTTCCAGGC-3') (Sangon Biotechnology Co., Ltd) was labeled with biotin. Nuclear proteins were extracted using a Nuclear and Cytoplasmic

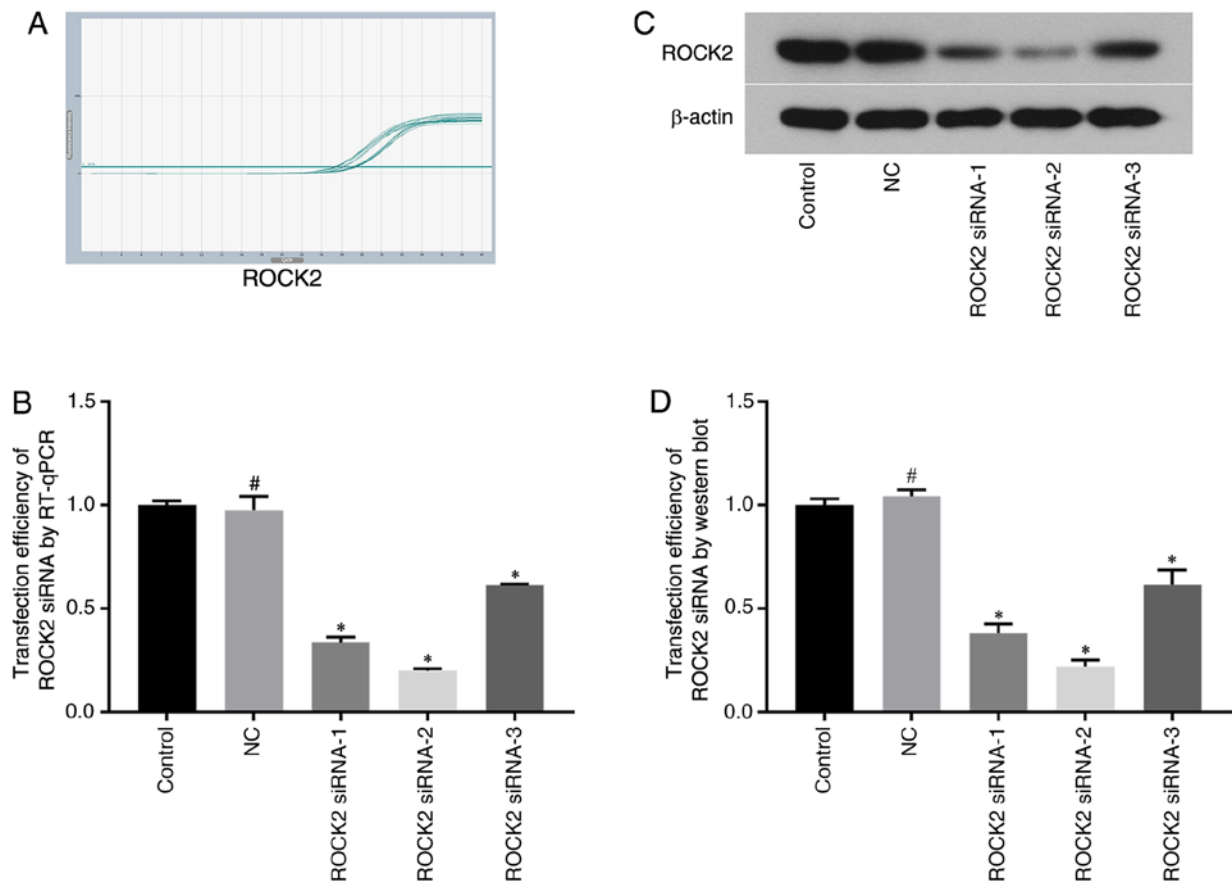


Figure 2. Effects of ROCK2 siRNA. (A) The amplification curve of ROCK2. (B) ROCK2 mRNA expression levels following ROCK2 siRNA. ROCK2 mRNA expression was significantly decreased with the reduction in expression up to 80% in the ROCK2 siRNA-2 group compared with the control. (C) Representative western blot and (D) semi-quantification of ROCK2 protein expression levels following ROCK2 siRNA transfection. ROCK2 protein expression was significantly decreased in ROCK2 siRNA-2 group, with the reduction in expression up to 78% compared with the control. All results are expressed as the mean  $\pm$  SD; n=3; \*P<0.01, #P>0.05 vs. Control. NC, negative control; ROCK, Rho-associated kinase; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA.

Protein Extraction kit (Pulilai Gene Technology Co., Ltd.) according to the manufacturer's protocol, rotated at maximum speed every 2 min for a total of 30 min and centrifuged in 10,000  $\times$  g at 4°C for 5 min. The proteins concentration was determined by bicinchoninic acid method. The DNA-nucleoprotein binding reaction using a BCA protein concentration determination kit (A:B=50:1; cat. no. WLA004; Wanleibio Co., Ltd.) was carried out for 20 min at room temperature. The DNA-protein complexes were then separated using a 6% non-denaturing polyacrylamide gel by electrophoresis and transferred onto a nylon membrane. Following transfer, the nylon membrane was exposed to 254 nm UV light to cross-link using an ultraviolet lamp in the darkroom for 15 min. The bands were visualized with enhanced chemiluminescence reagents (cat. no. WLA003; Wanleibio Co., Ltd.). Finally, the membrane was exposed to X-ray film and the binding activity of NF- $\kappa$ B was analyzed using a Gel Imaging Analysis System (cat. no. WD-9413B; Beijing Liuyi Biotechnology Co., Ltd.).

**Statistical analysis.** All data are expressed as mean  $\pm$  SD of at least three independent experiments. Multiple comparisons between groups were performed using one-way ANOVA followed by post hoc analysis (Bonferroni). All statistical analyses were performed with SPSS v22.0 (IBM Corp.). P<0.01

and P<0.05 were considered to indicate a statistically significant difference.

## Results

**Selection of ROCK1/ROCK2 siRNAs and their transfection efficiency by RT-qPCR and western blotting.** The mRNA and protein expression levels of ROCK1 and ROCK2 were measured by RT-qPCR and western blot analysis, respectively, following siRNA transfection. ROCK1 and ROCK2 expressions were reduced following transfections, indicating the transfection was successful. ROCK1 mRNA and protein expression levels were significantly decreased in the ROCK1 siRNA-1 group, with the reduction in expression up to 71 and 70%, respectively (Fig. 1). ROCK2 mRNA and protein expression levels were significantly decreased in ROCK2 siRNA-2 group, with the reduction in expression up to 80 and 78%, respectively (Fig. 2). As the results showed significantly downregulated protein and mRNA expression levels in the ROCK1 siRNA-1 group and ROCK2 siRNA-2 group compared with expressions in the control group, these siRNAs were chosen for use in subsequent experiments.

**Effects of ROCK1 and ROCK2 knockdown or PDTC treatment on ethanol-induced IEB permeability in Caco-2 cells.**

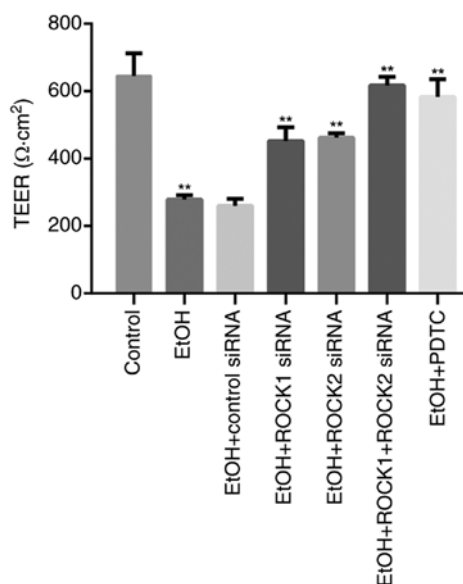


Figure 3. TEER values in Caco-2 cells treated with ethanol, ROCK1 or ROCK2 siRNAs or the NF- $\kappa$ B inhibitor PDTC. All results were compared with the ethanol group and expressed as the mean  $\pm$  SD; n=3; \*\*P<0.01. EtOH, ethanol; PDTC, ammonium pyrrolidine dithiocarbamate ROCK, Rho-associated kinase; siRNA, small interfering RNA; TEER, transepithelial electrical resistance.

Treatment of Caco-2 cells with 5% ethanol for 1 h significantly reduced TEER values compared with the control group (Fig. 3). Furthermore, TEER values were partially recovered in the ethanol + ROCK1 siRNA and in the ethanol + ROCK2 siRNA groups, and significantly affected in the ethanol + ROCK1 + ROCK2 siRNA group. When compared with the ethanol + ROCK1 siRNA (P=0.002) and ethanol + ROCK2 siRNA (P=0.004) groups, TEER values of ethanol + ROCK1 + ROCK2 siRNA group were more significantly recovered. In addition, compared with the ethanol-treated group, treatment of Caco-2 cells with PDTC also restored the decreased TEER values induced by ethanol.

**Effects of ethanol exposure on ROCK isoform expression in Caco-2 cells.** The mRNA and protein expression levels of ROCK1 and ROCK2 in Caco-2 cells from control, ethanol-treated and ROCK siRNA groups were determined by RT-qPCR and western blot analysis, respectively (Fig. 4). The results showed that exposure of intestinal epithelial cells to ethanol significantly increased ROCK1 and ROCK2 mRNA and protein expression in the ethanol-treated group compared with the control group. In addition, ROCK1 and ROCK2 knockdown significantly reduced their respective mRNA expression levels in the ethanol-treated group compared with the ethanol-only group. Furthermore, ethanol-treated cells transfected with the combination of ROCK1 + ROCK2 siRNAs exhibited further reduction of ROCK1 and ROCK2 mRNA and protein expression levels.

**NF- $\kappa$ B activity in Caco-2 cells.** NF- $\kappa$ B p65 mRNA expression levels were determined using RT-qPCR among the different groups. No statistically significant changes were observed in the total NF- $\kappa$ B p65 mRNA expression levels in control, ROCK siRNAs and NF- $\kappa$ B inhibitor-treated groups compared

the with ethanol-treated group (Fig. 5A). However, the intracellular distribution and activity of NF- $\kappa$ B p65 protein was significantly altered in each group. For example, the protein expression of NF- $\kappa$ B p65 was significantly reduced in the cytoplasm of intestinal epithelial cells following ethanol exposure (Fig. 5B); however, the cytoplasmic expression of NF- $\kappa$ B p65 in ethanol-treated cells transfected with ROCK siRNAs, or co-treated with PDTC, was significantly increased compared with expressions in the ethanol-only group (Fig. 5B). Additionally, ethanol exposure significantly upregulated NF- $\kappa$ B p65 in intestinal epithelial cells, whereas it was downregulated in cells transfected with ROCK siRNAs or PDTC-treated cells (Fig. 5C). NF- $\kappa$ B p65 was also significantly increased in cell nuclei in the ethanol-treated group compared with the ethanol-only group (Fig. 5D).

**Ethanol exposure affects AQP8 mRNA and protein expression in Caco-2 cells.** AQP8 mRNA and protein expression levels in the various treatment groups were determined by RT-qPCR and western blot analysis, respectively (Fig. 6). The results demonstrated that exposure of intestinal epithelial cells to ethanol significantly downregulated AQP8 mRNA and protein expression in the ethanol-treated group compared with the control group. ROCK1 and ROCK2 siRNAs, as well as PDTC treatment, partially restored AQP8 mRNA and protein expression levels in the ethanol-treated group compared with the ethanol-only group. Furthermore, AQP8 expression levels were also recovered in ethanol-treated cells transfected with the combination of ROCK1 + ROCK2 siRNAs. The distribution of AQP8 in the IEB model from each group was detected using immunofluorescence (Fig. 7), which showed that the expression level was recovered in the ethanol-treated group compared with the ethanol-only group and that the AQP8 cell distribution pattern was consistent with the aforementioned results.

## Discussion

The effects of Rho/ROCK pathway in IEB dysfunction have already been reported in the literature. Mihaescu *et al* (23), demonstrated that the ROCK signaling pathway was involved in the IEB dysfunction in an experimental C57BL/6J mouse model of radiation enteritis. In addition, our previous studies confirmed that the Rho/ROCK signaling pathway was associated with ethanol-induced IEB dysfunction (5,24). The effects of Rho/ROCK have also been found to be reversed following treatment with the ROCK inhibitor Y-27632 (25).

NF- $\kappa$ B is a nuclear transcription factor that regulates the expression of several genes. In addition, several studies have shown that Rho/ROCK signaling pathway may also activate the NF- $\kappa$ B pathway (26). Segain *et al* (27) and Anwar *et al* (28) demonstrated that Rho/ROCK signaling served a role in the activation of NF- $\kappa$ B in peripheral blood mononuclear cells and endothelial cells, respectively. Other studies in endothelial cells also confirmed that the Rho/ROCK pathway could mediate NF- $\kappa$ B p65 activation (29). Furthermore, Rodriguez *et al* (10) showed that RhoB activated NF- $\kappa$ B via upregulating ROCK1 expression. Therefore, it was hypothesized that ROCK activated NF- $\kappa$ B p65, thereby resulting in IEB dysfunction.

The ROCK protein family comprises two members, ROCK1 and ROCK2. Both members share an overall 64-65% homology

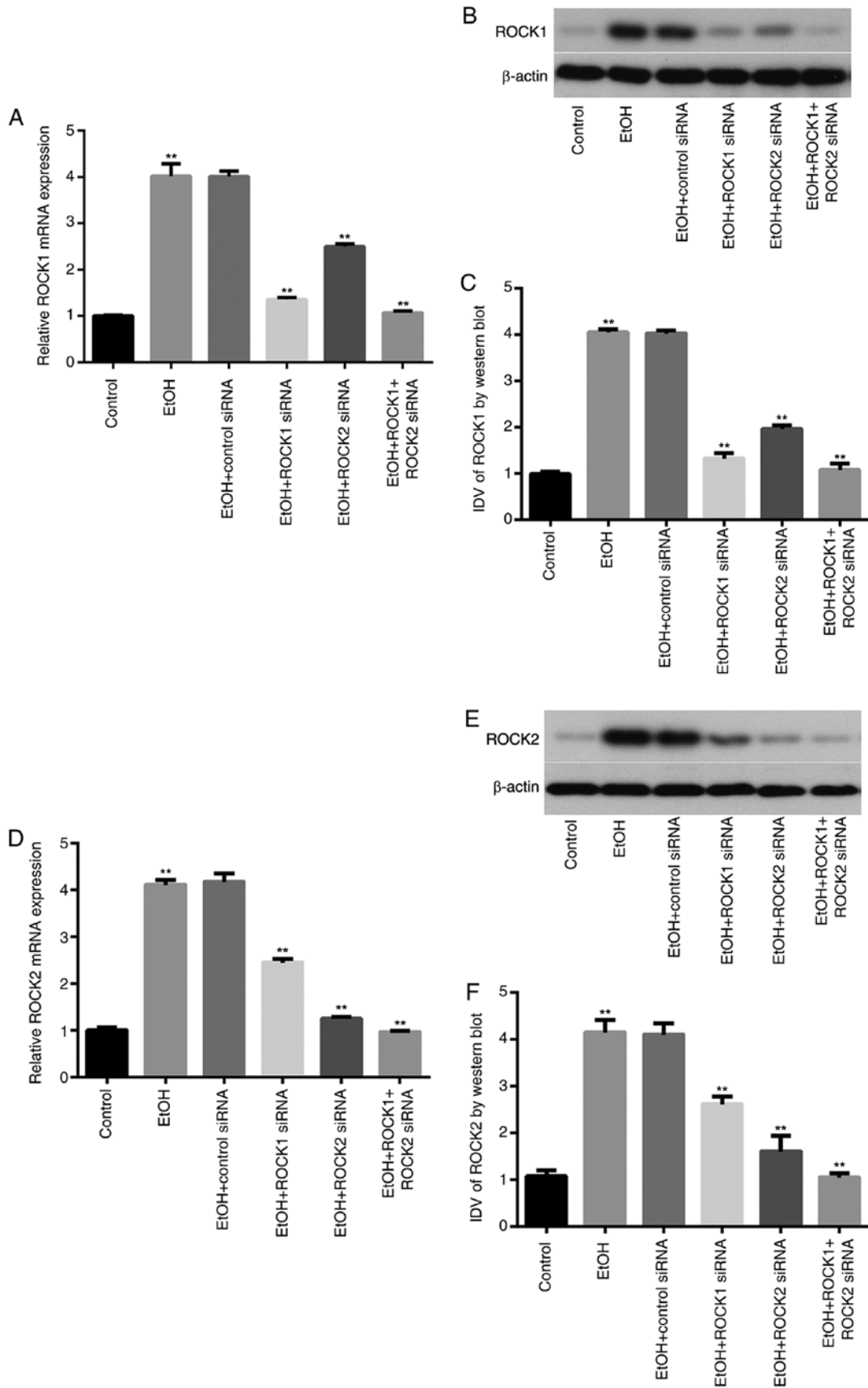


Figure 4. ROCK1 and ROCK2 expression levels in Caco-2 cells treated with ethanol, ROCK1 or ROCK2 siRNAs or the NF- $\kappa$ B inhibitor PDTC. (A) Increased ROCK1 mRNA expression levels in ethanol-treated Caco-2 cells were determined by reverse transcription-quantitative PCR. (B) Representative image and (C) semi-quantification of western blotting showed significantly increased protein expression levels of ROCK1 in ethanol-treated Caco-2 cells. (D) Increased ROCK2 mRNA expression levels in ethanol-treated cultured Caco-2 cells were determined by reverse transcription-quantitative PCR. (E) Representative image and (F) semi-quantification of western blotting showed significantly increased protein expression levels of ROCK2 in ethanol-treated Caco-2 cells. All results were compared with the ethanol group and expressed as the mean  $\pm$  SD; n=3; \*\*P<0.01. EtOH, ethanol; ROCK, Rho-associated kinase; siRNA, small interfering RNA; PDTC, ammonium pyrrolidine dithiocarbamate; IDV, integrated density values.

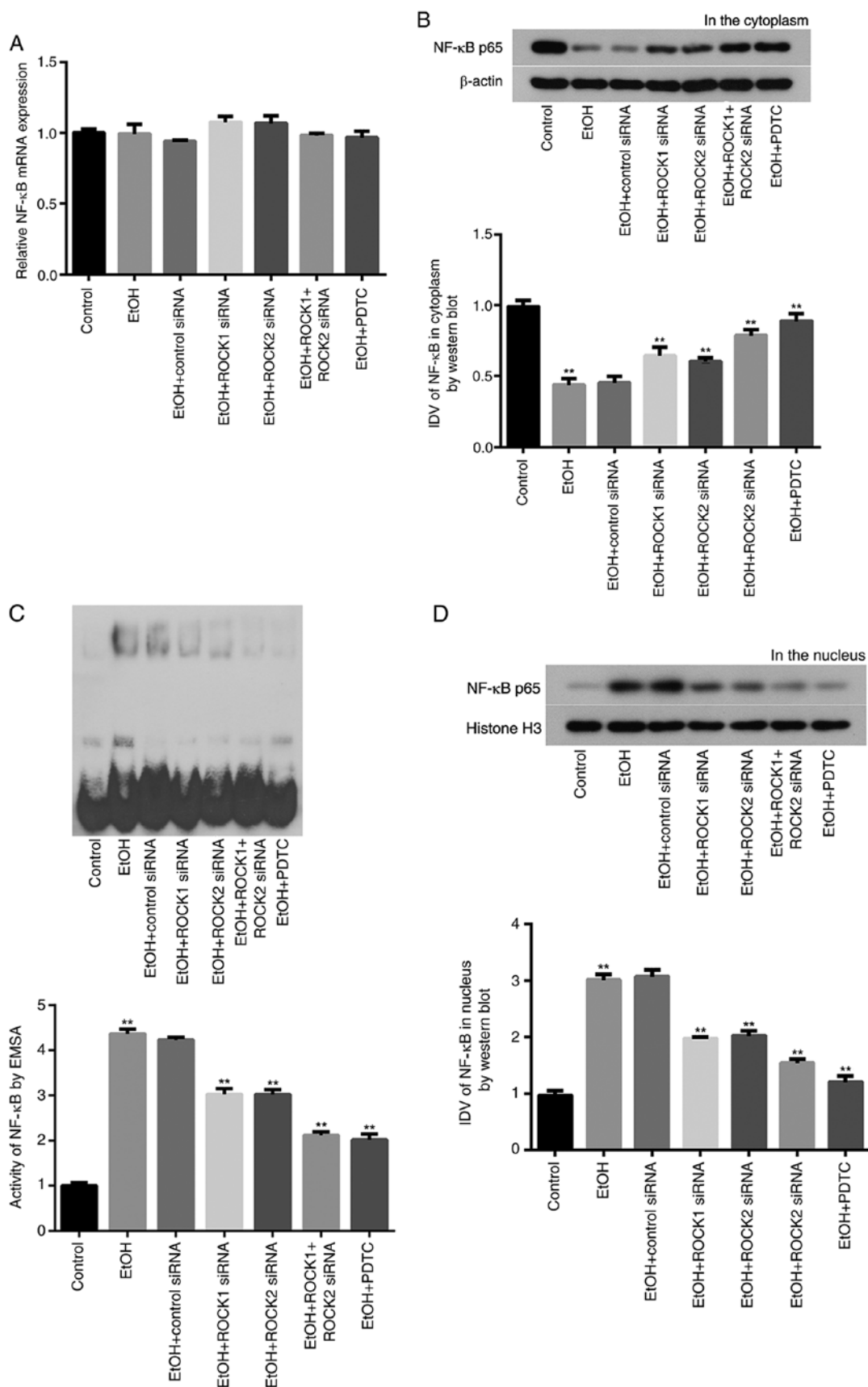


Figure 5. NF-κB p65 expression levels in Caco-2 cells treated with ethanol, ROCK1 or ROCK2 siRNAs or the NF-κB inhibitor PDTC. (A) No statistically significant changes were observed in the total NF-κB p65 mRNA expression in the ethanol, ROCK1 siRNA, ROCK2 siRNA, ROCK1 + ROCK2 siRNAs or PDTC treatment groups. (B) Reduced NF-κB p65 protein expression levels in the cytoplasm of ethanol-treated Caco-2 cells were detected using western blot analysis. (C) Increased activity of NF-κB p65 in cultured ethanol-treated Caco-2 cells was evaluated using EMSA. (D) Increased NF-κB p65 protein expression levels in the nuclei of cultured ethanol-treated Caco-2 cells were detected using western blot analysis. All results were compared with the ethanol group and expressed as mean  $\pm$  SD; n=3; \*\*P<0.01. EtOH, ethanol; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; PDTC, ammonium pyrrolidine dithiocarbamate; ROCK, Rho-associated kinase; siRNA, small interfering RNA; IDV, integrated density values.

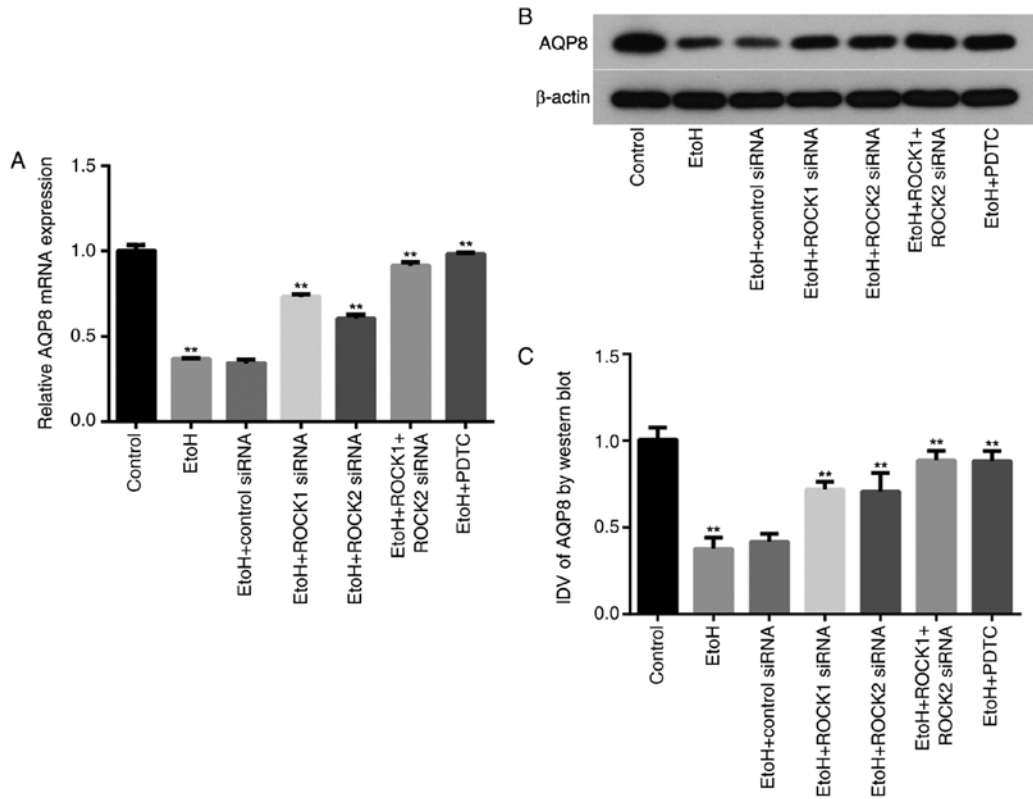


Figure 6. AQP8 expression levels in Caco-2 cells treated with ethanol, ROCK1 or ROCK2 siRNAs or the NF-κB inhibitor PDTC. (A) Reduced AQP8 mRNA expression levels in cultured ethanol-treated Caco-2 cells were detected by reverse transcription-quantitative PCR. (B) Representative image and (C) semi-quantification of western blotting showed significantly reduced AQP8 protein expression levels in ethanol-treated Caco-2 cells. All results were compared with the ethanol group and expressed as the mean ± SD (n=3). \*\*P<0.01. EtoH, ethanol; ROCK, Rho-associated kinase; PDTC, ammonium pyrrolidine dithiocarbamate; AQP8, aquaporin 8; siRNA, small interfering RNA; IDV, integrated density values.

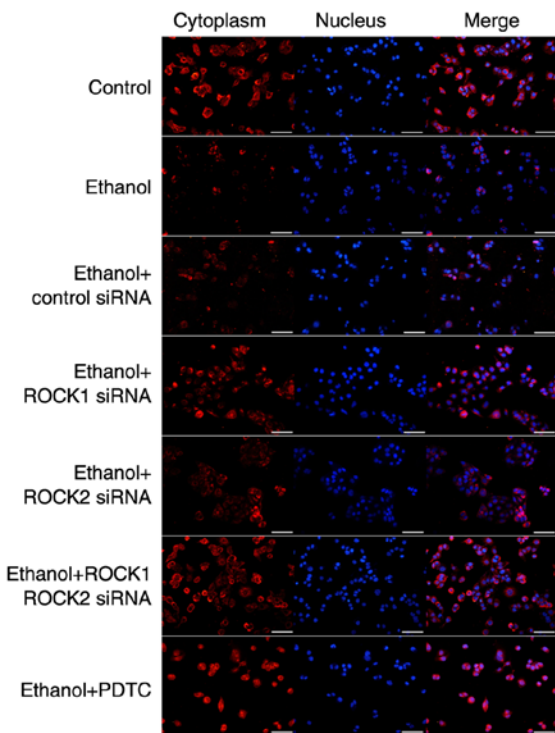


Figure 7. Distribution of AQP8 in the intestinal epithelial barrier model from each group detected using immunofluorescence. Cell nuclei were stained with DAPI. Scale bar, 100 μm. AQP8, aquaporin 8; PDTC, ammonium pyrrolidine dithiocarbamate; ROCK, Rho-associated kinase; siRNA, small interfering RNA.

in amino acid sequence and 80-92% homology in their kinase domains (30). Although ROCK1 and ROCK2 are structurally similar, it is hypothesized that both isoforms may exhibit different functions. However, their functions have not yet been reported. In the present study, ROCK1 and ROCK2 siRNAs and a NF-κB inhibitor, PDTC, were used to investigate the expression levels of ROCK1, ROCK2, NF-κB and AQP8 in Caco-2 cells. Consistent with our previous study (5), the results demonstrated that ethanol treatment reduced the TEER values in the *in vitro* IEB model, which were restored following transfection of cells with ROCK siRNAs and then ethanol treatment, thus suggesting a recovered intestinal epithelial permeability. Furthermore, ROCK siRNAs and PDTC downregulated the expression of NF-κB p65 in the cell nucleus, while its expression was increased in the cytoplasm. This finding indicated that both treatments affected the distribution and activity of NF-κB p65 in Caco-2 cells. In addition, the results revealed that both ROCK isoforms had the same effects on NF-κB expression in ethanol-treated Caco-2 cells.

It has also been reported that NF-κB inhibitors upregulate AQP1, AQP3 and AQP8 expression in irritable bowel syndrome (IBS) (13). This finding suggested that the NF-κB pathway regulated the expression of AQP1, AQP3 and AQP8, and that abnormal water metabolism and intestinal permeability could mediate IBS pathogenesis. However, NF-κB inhibition in IBS model rats did not fully restore the intestinal epithelium (13). Furthermore, Duan *et al* (31) found that the early acute receptor-interacting protein kinase 1



(RIPK1)/NF- $\kappa$ B/AQP8 axis mediated the RIPK1-dependent acinar cell necrosis. Consistent with this, the present study suggested that the NF- $\kappa$ B-mediated regulation of AQP8 expression was likely to be involved in ethanol-induced IEB dysfunction. Following treatment of Caco-2 cells with ROCK siRNAs or PDTC, the expression of AQP8 was significantly increased, indicating that ROCK1, ROCK2 and NF- $\kappa$ B were involved in the regulation of the ethanol-induced increase in IEB permeability. These findings were consistent with the results observed in the IBS model (13). Taken together, the aforementioned results indicated that ROCK may regulate the NF- $\kappa$ B pathway, which in turn mediates the activation AQP8, resulting in the regulation of ethanol-induced IEB dysfunction. However, further research is required to determine the exact molecular mechanism.

There were some limitations in the present study. For example, it could not be verified whether NF- $\kappa$ B was the only intermediate pathway between ROCK and AQP8. In addition, the application of ROCK siRNAs or PDTC did not completely restore the ethanol-induced IEB dysfunction, thus indicating that there are some unknown factors involved in this process. Furthermore, the involvement of a feedback pathway must be investigated. Therefore, the authors aim to explore the role of ROCK/NF- $\kappa$ B/AQP8 signaling pathway and its mechanism of action in future experiments.

In the present study, Caco-2 monolayers were used as a model of the IEB. The study of the role of ROCK and its downstream targets, NF- $\kappa$ B and AQP8, in regulating IEB may lay the foundation for the treatment of ethanol-induced IEB dysfunction.

Taken together, results from the present study suggested that the activation of ROCK/NF- $\kappa$ B/AQP8 signaling pathway in Caco-2 cells may be involved in ethanol-induced IEB dysfunction.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

HZ and JT designed the experiment together. HZ conducted experiments, collected and analyzed data, and wrote the manuscript. XS provided technical support for experiments. JT helped analyze results and gave useful suggestions for modification. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### References

1. Cheru L, Saylor CF and Lo J: Gastrointestinal barrier breakdown and adipose tissue inflammation. *Curr Obes Rep* 8: 165-174, 2019.
2. Hao H, Gokulan K, Piñeiro SA, Williams KM, Yuan Z, Cerniglia CE and Khare S: Effects of Acute and Chronic Exposure to Residual Level Erythromycin on Human Intestinal Epithelium Cell Permeability and Cytotoxicity. *Microorganisms* 7: 7, 2019.
3. Sunico CR, González-Forero D, Domínguez G, García-Verdugo JM and Moreno-López B: Nitric oxide induces pathological synapse loss by a protein kinase G-, Rho kinase-dependent mechanism preceded by myosin light chain phosphorylation. *J Neurosci* 30: 973-984, 2010.
4. Liao JK, Seto M and Noma K: Rho kinase (ROCK) inhibitors. *J Cardiovasc Pharmacol* 50: 17-24, 2007.
5. Tong J, Wang Y, Chang B, Zhang D and Wang B: Evidence for the involvement of RhoA signaling in the ethanol-induced increase in intestinal epithelial barrier permeability. *Int J Mol Sci* 14: 3946-3960, 2013.
6. Zou Y, Ma L, Zhao Y, Zhang S, Zhou C and Cai Y: Inhibition of Rho kinase protects against colitis in mice by attenuating intestinal epithelial barrier dysfunction via MLC and the NF- $\kappa$ B pathway. *Int J Mol Med* 41: 430-438, 2018.
7. Pan P, Shen M, Yu H, Li Y, Li D and Hou T: Advances in the development of Rho-associated protein kinase (ROCK) inhibitors. *Drug Discov Today* 18: 1323-1333, 2013.
8. Bauer PO, Hudec R, Goswami A, Kurosawa M, Matsumoto G, Mikoshiba K and Nukina N: ROCK-phosphorylated vimentin modifies mutant huntingtin aggregation via sequestration of IRBIT. *Mol Neurodegener* 7: 43, 2012.
9. Farber MJ, Rizaldy R and Hildebrand JD: Shroom2 regulates contractility to control endothelial morphogenesis. *Mol Biol Cell* 22: 795-805, 2011.
10. Rodriguez PL, Sahay S, Olabisi OO and Whitehead IP: ROCK I-mediated activation of NF- $\kappa$ B by RhoB. *Cell Signal* 19: 2361-2369, 2007.
11. Verkman AS: Physiological importance of aquaporin water channels. *Ann Med* 34: 192-200, 2002.
12. Wu Q, Yang ZF, Wang KJ, Feng XY, Lv ZJ, Li Y and Jian ZX: AQP8 inhibits colorectal cancer growth and metastasis by down-regulating PI3K/AKT signaling and PCDH7 expression. *Am J Cancer Res* 8: 266-279, 2018.
13. Chao G and Zhang S: Aquaporins 1, 3 and 8 expression in irritable bowel syndrome rats' colon via NF- $\kappa$ B pathway. *Oncotarget* 8: 47175-47183, 2017.
14. Wang JP, Hou XH and Ma RJ: The clinical features and colonic epithelium AQP8 expression in diarrhea-irritable bowel syndrome. *Zhonghua Nei Ke Za Zhi* 45: 1000-1003, 2006 (In Chinese).
15. Escudero-Hernández C, Münch A and Koch S: The water channel aquaporin 8 is a critical regulator of intestinal fluid homeostasis in collagenous colitis. *J Crohn's Colitis*: Feb 4, 2020 (Epub ahead of print).
16. Hidalgo IJ, Raub TJ and Borchardt RT: Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 96: 736-749, 1989.
17. Nighot PK, Hu CA and Ma TY: Autophagy enhances intestinal epithelial tight junction barrier function by targeting claudin-2 protein degradation. *J Biol Chem* 290: 7234-7246, 2015.
18. Lin S, Han Y, Jenkin K, Lee SJ, Sasaki M, Klapproth JM, He P and Yun CC: Lysophosphatidic Acid Receptor 1 Is Important for Intestinal Epithelial Barrier Function and Susceptibility to Colitis. *Am J Pathol* 188: 353-366, 2018.
19. Elamin E, Jonkers D, Juuti-Uusitalo K, van Ijzendoorn S, Troost F, Duimel H, Broers J, Verheyen F, Dekker J and Masclee A: Effects of ethanol and acetaldehyde on tight junction integrity: In vitro study in a three dimensional intestinal epithelial cell culture model. *PLoS One* 7: e35008, 2012.

20. Tong J, Wang Y, Chang B, Zhang D and Wang B: Y-27632 inhibits ethanol-induced increase in intestinal epithelial barrier permeability. *Mol Med Rep* 9: 2357-2361, 2014.
21. Turner JR, Angle JM, Black ED, Joyal JL, Sacks DB and Madara JL: PKC-dependent regulation of transepithelial resistance: Roles of MLC and MLC kinase. *Am J Physiol* 277: C554-C562, 1999.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
23. Mihaescu A, Santén S, Jeppsson B and Thorlacius H: Rho kinase signalling mediates radiation-induced inflammation and intestinal barrier dysfunction. *Br J Surg* 98: 124-131, 2011.
24. Tong J, Wang Y, Chang B, Zhang D, Liu P and Wang B: Activation of RhoA in alcohol-induced intestinal barrier dysfunction. *Inflammation* 36: 750-758, 2013.
25. Narumiya S, Ishizaki T and Uehata M: Use and properties of ROCK-specific inhibitor Y-27632. *Methods Enzymol* 325: 273-284, 2000.
26. Li Z, Gao M, Yang B, Zhang H, Wang K, Liu Z, Xiao X, Yang M: Naringin attenuates MLC phosphorylation and NF-kappaB activation to protect sepsis-induced intestinal injury via RhoA/ROCK pathway. *Biomed Pharmacother* 103: 50-58, 2018.
27. Segain JP, Raingeard de la Blétière D, Sauzeau V, Bourreille A, Hilaret G, Cario-Toumaniantz C, Pacaud P, Galmiche JP and Loirand G: Rho kinase blockade prevents inflammation via nuclear factor kappa B inhibition: Evidence in Crohn's disease and experimental colitis. *Gastroenterology* 124: 1180-1187, 2003.
28. Anwar KN, Fazal F, Malik AB and Rahman A: RhoA/Rho-associated kinase pathway selectively regulates thrombin-induced intercellular adhesion molecule-1 expression in endothelial cells via activation of I kappa B kinase beta and phosphorylation of RelA/p65. *J Immunol* 173: 6965-6972, 2004.
29. Shimada H and Rajagopalan LE: Rho kinase-2 activation in human endothelial cells drives lysophosphatidic acid-mediated expression of cell adhesion molecules via NF-kappaB p65. *J Biol Chem* 285: 12536-12542, 2010.
30. Amano M, Nakayama M and Kaibuchi K: Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. *Cytoskeleton (Hoboken)* 67: 545-554, 2010.
31. Duan PY, Ma Y, Li XN, Qu FZ, Ji L, Guo XY, Zhang WJ, Xiao F, Li L, Hu JS, *et al*: Inhibition of RIPK1-dependent regulated acinar cell necrosis provides protection against acute pancreatitis via the RIPK1/NF- $\kappa$ B/AQP8 pathway. *Exp Mol Med* 51: 1-17, 2019.



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