

Upregulation of ZO-1 in Cultured Human Corneal Epithelial Cells by a Peptide (PHSRN) Corresponding to the Second Cell-Binding Site of Fibronectin

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PURPOSE. To investigate the effect of a peptide (PHSRN) corresponding to the second cell-binding site of fibronectin on the expression of ZO-1 in cultured human corneal epithelial (HCE) cells.

METHODS. The effects of the PHSRN peptide on the expression of ZO-1, -2, and -3; claudin; and occludin were determined by reverse transcription-polymerase chain reaction (RT-PCR), immunoblot, and immunofluorescence analyses. Phosphorylation of mitogen-activated protein kinases (MAPKs) and the transcription factor c-Jun was assessed with a multiplex analysis system and immunoblot analysis. The barrier function of cultured HCE cells was evaluated by measurement of transepithelial electrical resistance.

RESULTS. RT-PCR and immunoblot analyses revealed the PHSRN peptide increased the amounts of ZO-1 mRNA and protein in HCE cells in a concentration- and time-dependent manner. The PHSRN peptide had no effect on the expression of ZO-2, ZO-3, claudin, or occludin. Immunofluorescence microscopy showed that the PHSRN peptide did not affect the localization of ZO-1 at the interfaces of neighboring cells. The PHSRN peptide induced the phosphorylation of the MAPKs ERK, p38, and JNK as well as that of c-Jun. The upregulation of ZO-1 expression by the PHSRN peptide was blocked by inhibitors of signaling by ERK (PD098059), p38 (SB203580), or JNK (JNK inhibitor II). The PHSRN peptide had no effect on the transepithelial electrical resistance of cultured HCE cells.

CONCLUSIONS. The PHSRN peptide upregulated the expression of ZO-1 through activation of MAPK signaling pathways in HCE cells. This effect of the PHSRN sequence of fibronectin may contribute to the formation of tight junctions and play an important role in the differentiation of corneal epithelial cells. (*Invest Ophthalmol Vis Sci.* 2009;50:2757-2764) DOI: 10.1167/iovs.08-2341

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Fibronectin, an adhesive glycoprotein of the extracellular matrix, serves as a provisional substrate for the attachment, spreading, and migration of fibroblasts and epithelial cells.¹⁻⁴ We have previously shown that fibronectin plays an important role in corneal wound healing both in vitro and in vivo.⁵⁻⁷ In the injured cornea in vivo, fibronectin appears at the bared stromal surface and provides a matrix for the migration of corneal epithelial cells.⁸ Furthermore, the addition of exogenous fibronectin stimulates corneal epithelial migration both in vitro and in vivo.⁵⁻⁷ In addition to its role as a physical substrate, fibronectin activates intracellular signaling systems through interaction with integrins that function as specific fibronectin receptors.⁹⁻¹²

Fibronectin is a disulfide-linked dimer of 230- to 250-kDa subunits, each of which consists of three types of repeating domains designated type I, II, and III.¹³ Each of these domains mediates specific effects of fibronectin such as those on cell adhesion, migration, proliferation, differentiation, or chemotaxis, as well as those on tissue remodeling and wound healing.^{1,13} The amino acid sequence Arg-Gly-Asp (RGD) located in the 10th type III domain of fibronectin serves as the binding site for integrin $\alpha 5 \beta 1$ expressed on the surface of cells.¹⁴⁻¹⁶ In addition to the RGD sequence, the sequence Pro-His-Ser-Arg-Asn (PHSRN) in the ninth type III domain of fibronectin is thought to function as a second cell-binding site of the protein and to promote the fibronectin-integrin interaction.¹⁷ We recently showed that the PHSRN peptide promotes both migration of the rabbit corneal epithelium in organ culture as well as the closure of rabbit corneal epithelial wounds in vivo.¹⁸

Zonula occludens (ZO)-1 is a component of epithelial tight junctions.¹⁹ The tight-junction complex consists of integral transmembrane proteins such as claudin and occludin, membrane-associated proteins (including ZO-1, -2, and -3), and actin filaments, and it subserves the barrier function of epithelia.¹⁹⁻²³ ZO-1 is expressed in superficial and subsuperficial cell layers of the corneal epithelium and contributes to the barrier function of this epithelium.^{24,25} We recently showed that hypoxia induces a change in the distribution of ZO-1 as well as disruption of barrier function in cultured human corneal epithelial cells.²⁶ These effects of hypoxia were inhibited by keratinocyte growth factor in a manner dependent on signaling by extracellular signal-regulated kinase (ERK). ERK is a member of the mitogen-activated protein kinase (MAPK) family of serine-threonine kinases, which play an important role in the transduction of externally derived signals that regulate the growth and differentiation of various cell types.²⁷⁻³¹

To examine whether the PHSRN sequence of fibronectin affects functions of corneal epithelial cells other than cell migration, we investigated the possible effects of the PHSRN peptide on the barrier function of the corneal epithelium. We thus examined the effects of this peptide on the expression of ZO-1 at the mRNA and protein levels as well as on the localization of ZO-1 in cultured human corneal epithelial cells. In addition, we determined the effects of the PHSRN peptide on

the phosphorylation of the MAPKs ERK, p38, and c-Jun NH₂-terminal kinase (JNK) as well as on that of the transcription factor c-Jun in these cells.

METHODS

Antibodies and Reagents

Rabbit polyclonal antibodies to ZO-1, -2, -3; claudin; or occludin were obtained from Zymed (Carlsbad, CA). Mouse monoclonal antibodies to α -tubulin and a protease inhibitor cocktail were from Sigma-Aldrich (St. Louis, MO). Antibodies to total or phosphorylated forms of ERK1 or -2 (ERK1/2), p38 MAPK, JNK, or c-Jun were obtained from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse immunoglobulin-G or -M were from Promega (Madison, WI). PD098059, SB203580, and JNK inhibitor II were obtained from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM)-F12 mixture (50:50, vol/vol), fetal bovine serum, and Alexa Fluor 488-labeled goat antibodies to rabbit immunoglobulin G were obtained from Invitrogen-Gibco (Rockville, MD), and bovine serum albumin (BSA) was from Nacalai Tesque (Kyoto, Japan). All media and reagents used for cell culture were endotoxin minimized.

Synthetic Peptides

Acetyl-PHSRN-amide and acetyl-NRSHP-amide peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy as described previously.³² The purity and identity of the peptides were confirmed by analytic high-performance liquid chromatography and electrospray-ionization mass spectrometry at the Central Analysis Center, Tokyo University of Pharmacy and Life Science.

HCE Cells

An HCE cell line, which was established as previously described by transformation with an SV40-adenovirus recombinant vector,³³ was cultured under 5% CO₂ at 37°C in supplemented hormonal epithelial medium (SHEM), which comprises DMEM-F12 supplemented with 15% heat-inactivated fetal bovine serum, bovine insulin (5 μ g/mL), cholera toxin (0.1 μ g/mL), recombinant human epidermal growth factor (10 ng/mL), and gentamicin (40 μ g/mL). The cells were routinely passaged when they achieved 80% confluence.

Immunoblot Analysis

HCE cells (2×10^5) were seeded in 60-mm culture dishes and cultured in SHEM for 1 day. The medium was then changed to unsupplemented DMEM-F12, and the cells were incubated first for 24 hours alone and then for various times in the presence of various concentrations of PHSRN. The cells were then washed with phosphate-buffered saline (PBS) and lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM NaF, 2 mM Na₃VO₄, 2% SDS, and 1% protease inhibitor cocktail. The lysates (equal amounts of protein) were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane and then exposed consecutively to primary antibodies and horseradish peroxidase-conjugated secondary antibodies, as previously described.³⁴ Immune complexes were detected with the use of enhanced chemiluminescence reagents (GE Health Care, Chalfont, UK). Band intensities were measured by image analysis (Multi Gauge V3; Fuji Film, Tokyo, Japan), and those for ZO-1 were normalized by the corresponding value for α -tubulin.

RT-PCR Analysis

Total RNA was isolated from HCE cells (stimulated as for immunoblot analysis; RNeasy kit; Qiagen, Valencia, CA), and portions (0.5 μ g) of the RNA were subjected to reverse transcription (RT) and polymerase chain reaction (PCR) analysis (One-Step RT-PCR kit based on the Platinum *Taq* system; Invitrogen, Carlsbad, CA). The PCR protocol was

designed to maintain amplification in the exponential phase. The PCR primers (sense and antisense, respectively) were 5'-TGCCATTACACG-GTCTCTG-3' and 5'-GGTTCTGCCTCATCATTTCTC-3' for ZO-1 and 5'-ACCACAGTCCACGCCATCAC-3' and 5'-TCCACCACCCTGTTGCT-GTA-3' for glyceraldehyde-3-phosphate dehydrogenase (G3PDH, internal control). RT and PCR incubations were performed (GeneAmp PCR System 2400-R; Perkin-Elmer, Wellesley, MA). RT was performed at 50°C for 30 minutes, and the PCR cycle comprised incubations at 94°C for 2 minutes, 58°C for 30 seconds, and 72°C for 1 minute. The reaction mixture was finally cooled to 4°C, and the products of amplification were fractionated by electrophoresis on a 1.5% agarose gel and then stained with ethidium bromide.

Immunofluorescence Microscopy

HCE cells (5×10^4) were seeded on coverslips (12 \times 12 mm; Matsunami Glass, Osaka, Japan) and cultured in SHEM for 1 day. The medium was then changed to unsupplemented DMEM-F12, and the cells were incubated first for 24 hours alone and then for 24 hours in the absence or presence of the PHSRN peptide (1 μ g/mL). The cells were fixed with 100% methanol for 5 minutes at room temperature and then incubated with 1% BSA in Ca²⁺- and Mg²⁺-free PBS (PBS(-)) for 1 hour at room temperature. They were then incubated for 2 hours with antibodies to ZO-1 (1:100 dilution in PBS(-) containing 1% BSA), washed with PBS(-), and incubated for 1 hour with Alexa Fluor 488-conjugated secondary antibodies (1:2000 dilution in PBS(-) containing 1% BSA). The cells were finally examined with a laser confocal microscope (LSM5; Carl Zeiss Meditec, Oberkochen, Germany).

Phosphoprotein Assay

The phosphorylation state of MAPKs, c-Jun, TrkA, and Src was analyzed with the use of phosphoprotein assays (Bio-Plex; Bio-Rad, Hercules, CA). In brief, serum-deprived HCE cells were incubated in the presence of the PHSRN peptide (1 μ g/mL) for various times, after which cell lysates were prepared (Bio-Plex Cell Lysis Kit; Bio-Rad). The protein concentration of lysates was adjusted to 600 μ g/mL, and 50 μ L of the lysates were added to the wells of a 96-well plate that had been coated with 50 μ L of beads coupled to antibodies specific for phosphorylated forms of ERK1/2, p38 MAPK, JNK, c-Jun, TrkA, or Src. After incubation for 15 to 18 hours, the wells were processed for detection of the target phosphoproteins (Luminex 100 analyzer and Bio-Plex Manager software; Bio-Rad).

Measurement of Transepithelial Electrical Resistance (TER)

TER was measured as described previously.³⁵ In brief, HCE cells (5×10^4) were seeded in the apical chamber of a cell migration apparatus on a 6.5-mm filter with a pore size of 0.22 μ m (Transwell; Costar, Cambridge, MA). The cells were cultured in SHEM for 4 days to allow establishment of barrier function and were then incubated for 48 hours in DMEM-F12 supplemented with various concentrations of the PHSRN peptide. Resistance was measured with STX-2 electrodes and an EVOM Volt ohmmeter (World Precision Instruments, Sarasota, FL). TER was calculated from the measured resistance and normalized by the area of the monolayer. The background resistance attributable to the filter was subtracted from the TER for the cell monolayer.

Statistical Analysis

Data are presented as the mean \pm SE from at least three independent experiments and were analyzed by Dunnett's multiple-comparison test or the Tukey-Kramer test. $P < 0.05$ was considered statistically significant.

RESULTS

We first examined the effects of the PHSRN peptide on expression of the tight-junction proteins ZO-1, -2, and -3; claudin; and

occludin in HCE cells. Immunoblot analysis revealed that the PHSRN peptide (100, 300, 1000, and 3000 ng/mL) increased the amount of ZO-1 protein in a concentration-dependent manner over 24 hours (Fig. 1A). In contrast, the PHSRN peptide had no effect on the abundance of the other tight-junction proteins in the HCE cells. RT-PCR analysis showed that the PHSRN peptide also increased the amount of ZO-1 mRNA in HCE cells in a concentration-dependent manner over 12 hours (Fig. 1B). The peptide NRSHP (1000 ng/mL), whose sequence is reversed compared with that of PHSRN, had no effect on the abundance of ZO-1 protein or mRNA, indicating that the effects of the PHSRN peptide are specific. We next examined the time course of the effects of the PHSRN peptide (1 μ g/mL) on the amounts of ZO-1 protein and mRNA in HCE cells. The

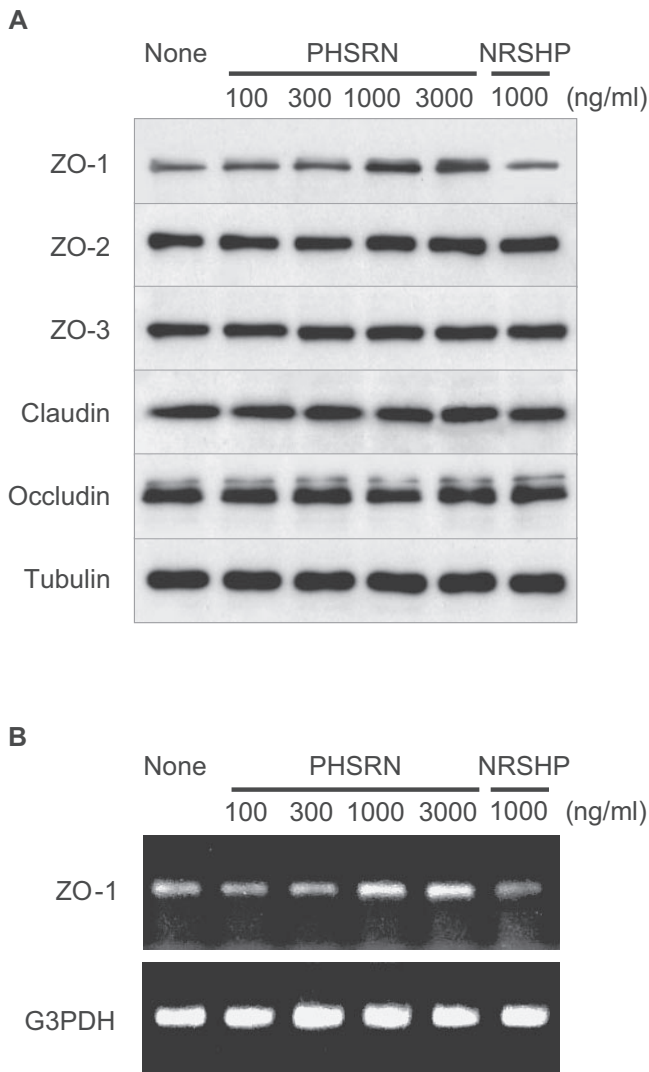


FIGURE 1. Concentration-dependent effects of the PHSRN peptide on the amounts of ZO-1 protein and mRNA in HCE cells. (A) Serum-deprived cells were incubated for 24 hours in the absence or presence of the indicated concentrations of PHSRN or NRSHP peptides, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to ZO-1, -2, -3; claudin; occludin; and α -tubulin (loading control). (B) Serum-deprived cells were incubated for 12 hours in the absence or presence of the indicated concentrations of PHSRN or NRSHP peptides, after which total RNA was isolated from the cells and subjected to RT-PCR analysis of ZO-1 and G3PDH mRNAs. All data are representative of three independent experiments.

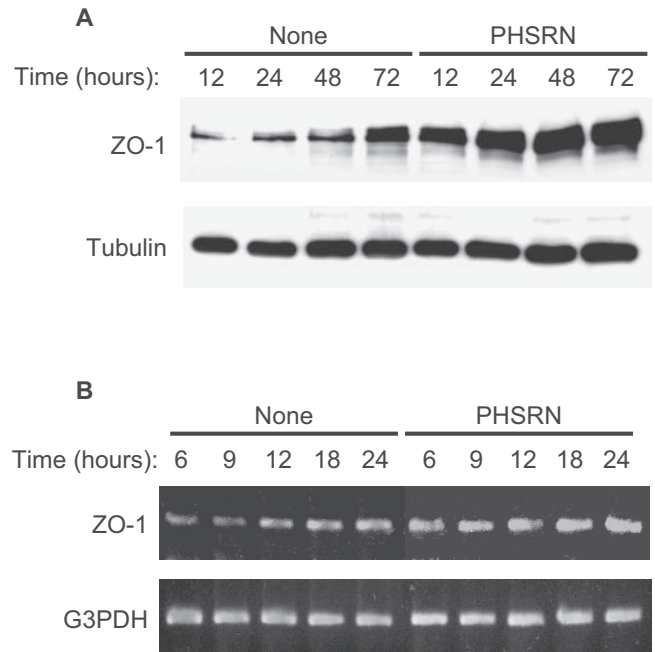


FIGURE 2. Time-dependent effects of the PHSRN peptide on the amounts of ZO-1 protein and mRNA in HCE cells. (A) Serum-deprived cells were incubated for the indicated times in the absence or presence of the PHSRN peptide (1 μ g/mL), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to ZO-1 and to α -tubulin. (B) Serum-deprived cells were incubated for the indicated times in the absence or presence of the PHSRN peptide (1 μ g/mL), after which total RNA was isolated from the cells and subjected to RT-PCR analysis of ZO-1 and G3PDH mRNAs. All data are representative of results in three independent experiments.

abundance of ZO-1 protein (Fig. 2A) and ZO-1 mRNA (Fig. 2B) in these cells increased in a time-dependent manner even in the absence of the PHSRN peptide. However, the amount of ZO-1 protein in cells exposed to the PHSRN peptide was greater than that in control cells at incubation times of 12 to 72 hours, whereas that of ZO-1 mRNA in cells treated with the PHSRN peptide was greater than that in control cells at times of 6 to 24 hours.

To investigate whether the PHSRN peptide might affect the localization of ZO-1 in HCE cells, we performed immunofluorescence microscopy. ZO-1 was localized at the interfaces of adjacent HCE cells under control conditions. Exposure of cells to the PHSRN peptide (1 μ g/mL) for 24 hours did not affect this pattern of ZO-1 distribution but increased the amount of ZO-1 immunoreactivity at the cell surface (Fig. 3). These results thus confirmed that the PHSRN peptide increases the expression of ZO-1 but also indicated that it does not affect the localization of this protein in HCE cells.

To characterize the signaling pathways that mediate the upregulation of ZO-1 expression by the PHSRN peptide in HCE cells, we investigated the possible role of the MAPK family members ERK1/2, p38, and JNK. Detection of the phosphorylated (activated) forms of these MAPKs with the use of a phosphoprotein assay (Bio-Plex; Bio-Rad) revealed that the PHSRN peptide (1 μ g/mL) induced rapid increases in ERK1/2, p38, and JNK phosphorylation, with the maximal \sim 2.9-fold increases apparent at 10, 30, and 10 minutes, respectively (Figs. 4A–C). In contrast, the PHSRN peptide had no effect on the phosphorylation of TrkA or Src (Figs. 4D, 4E). We also examined the effects of the PHSRN peptide on the phosphorylation of ERK1/2, p38 MAPK, and JNK by immunoblot analy-

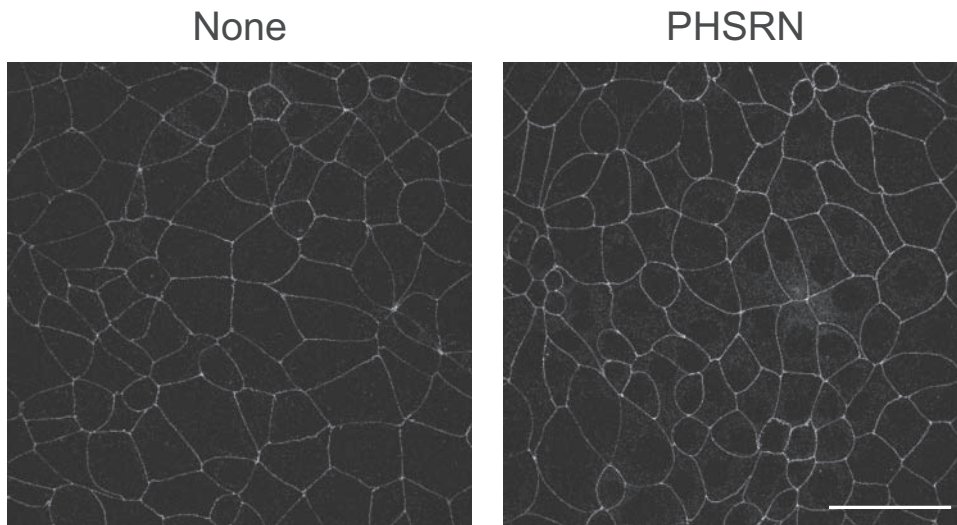


FIGURE 3. Effects of the PHSRN peptide on ZO-1 localization and abundance in HCE cells. Serum-deprived cells were incubated for 24 hours in the absence or presence of the PHSRN peptide (1 $\mu\text{g}/\text{mL}$), after which the cells were fixed and subjected to immunofluorescence analysis with antibodies to ZO-1. Scale bar, 20 μm .

sis. Whereas the PHSRN peptide (1 $\mu\text{g}/\text{mL}$) had no effect on the total amounts of ERK1/2, p38 MAPK, or JNK in HCE cells, it increased the amounts of the phosphorylated forms of these proteins in a time-dependent manner, with the maximum effects being apparent at 10, 30, and 15 minutes, respectively (Fig. 5). These results suggest that the PHSRN peptide activates ERK1/2, p38 MAPK, and JNK signaling pathways in HCE cells.

To investigate whether the upregulation of ZO-1 expression by the PHSRN peptide in HCE cells is mediated by ERK1/2, p38 MAPK, or JNK signaling pathways, we examined the effects of specific inhibitors of these MAPKs. Cells were incubated with 10 μM PD098059 (inhibitor of ERK activation by the upstream kinase MEK), 10 μM SB203580 (inhibitor of p38 MAPK), or 5 μM JNK inhibitor II (inhibitor of JNK) for 1 hour before exposure to the PHSRN peptide (1 $\mu\text{g}/\text{mL}$) for 24 hours. The stimulatory effect of the PHSRN peptide on the expression of ZO-1 protein was inhibited completely or almost completely by PD098059, SB203580, or JNK inhibitor II (Fig. 6). The three MAPK inhibitors had no marked effect on ZO-1 expression in HCE cells incubated in the absence of the PHSRN peptide.

Next, to investigate the possible effect of the PHSRN peptide on the activation status of c-Jun, we examined the phosphorylation of this transcription factor in HCE cells. A phosphoprotein assay showed that the PHSRN peptide (1 $\mu\text{g}/\text{mL}$) induced a rapid increase in the level of c-Jun phosphorylation that reached its maximum at 10 minutes (Fig. 7A). This effect of the PHSRN peptide was confirmed by immunoblot analysis (Fig. 7B), which revealed that the level of c-Jun phosphorylation was increased between 5 and 60 minutes after the onset of exposure to the peptide.

Finally, to determine whether the PHSRN peptide affects the barrier function of HCE cells, we measured the TER of cell monolayers. Neither the PHSRN peptide (100–3000 ng/mL) nor the NRSHP peptide (1000 ng/mL) had an effect on the TER of HCE cells (Fig. 8).

DISCUSSION

We have shown that the PHSRN peptide, which corresponds to the second cell-binding domain of fibronectin, upregulated the expression of ZO-1 at both the mRNA and protein levels, without affecting that of other tight-junction proteins (ZO-2, ZO-3, claudin, or occludin), in cultured human corneal epithelial cells. This effect of the PHSRN peptide appeared to be mediated through the activation of ERK1/2, p38 MAPK,

and JNK. In addition, the peptide induced the activation of c-Jun in HCE cells. However, the PHSRN peptide did not affect the barrier function of HCE cell monolayers, as revealed by measurement of TER. The effect of the PHSRN peptide on the expression of ZO-1 was not mimicked by the control NRSHP peptide, suggesting that this effect was sequence specific.

Fibronectin is a multifunctional extracellular glycoprotein. It is composed of several functional domains that mediate the effects of the protein on cell adhesion, migration, proliferation, differentiation, and chemotaxis as well as its roles in inflammation and wound healing.¹³ Various peptides derived from fibronectin have been shown to exhibit biological activities such as stimulation of cell adhesion, migration, or chemotaxis.^{36,37} The PHSRN sequence of fibronectin is thought to promote cell spreading on fibronectin mediated by the RGD sequence.^{17,38,39} We have previously shown that the PHSRN peptide induces the tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin and the accumulation of F-actin in HCE cells as well as the formation of focal adhesions at the leading edge of these cells.¹⁸ The PHSRN peptide also increased the motility of individual HCE cells without affecting their proliferation, and it promoted the healing of rabbit corneal epithelial wounds *in vivo*.¹⁸ The PHSRN peptide thus appears to mimic various biological activities of fibronectin including stimulation of cell motility,⁴⁰ cell migration,⁶ and wound healing.⁷ However, we previously found that this peptide did not affect adhesion of HCE cells to a fibronectin matrix.¹⁸ We also recently showed that it upregulated the expression of heat shock protein of 70 kDa (HSP70) in HCE cells.³⁴ In the present study, we found that the PHSRN peptide induced upregulation of the expression of ZO-1, a key component of tight junctions, in cultured human corneal epithelial cells without affecting the localization of ZO-1 at the interfaces of neighboring cells.

ZO-1 is thought to play an important role in cell differentiation through its associations at sites of cell-cell attachment.⁴¹ The upregulation of ZO-1 by the PHSRN peptide observed in the present study suggests that such regulation of ZO-1 expression in corneal epithelial cells by fibronectin may contribute to reorganization of the corneal epithelium and epithelial cell differentiation. The expression of ZO-1 was previously shown to be reduced only in the leading cells of the migrating corneal epithelium during healing of an epithelial debridement wound,⁴² and ZO-1 was found to be expressed in multiple cell

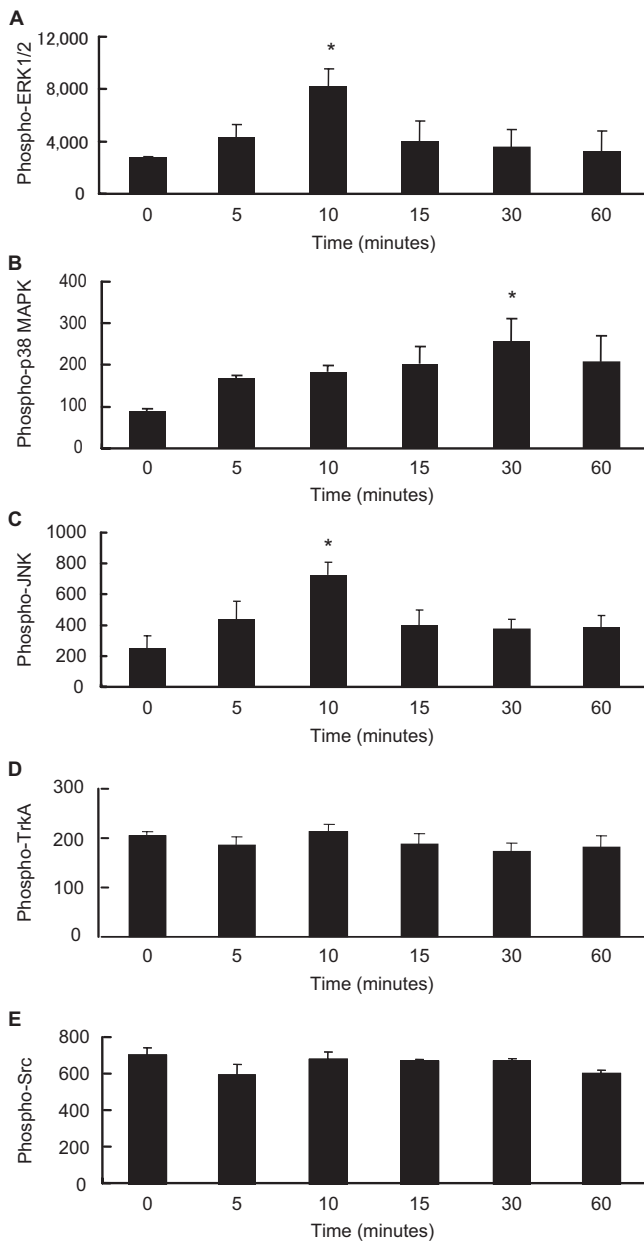


FIGURE 4. Effects of the PHSRN peptide on MAPK phosphorylation in HCE cells as determined by phosphoprotein analysis. Serum-deprived cells were incubated with the PHSRN peptide (1 $\mu\text{g}/\text{mL}$) for the indicated times, after which cell lysates were prepared and subjected to analysis for quantitation of the phosphorylated forms of ERK1 (Thr²⁰², Tyr²⁰⁴) or ERK2 (Thr¹⁸⁵, Tyr¹⁸⁷) (A), p38 MAPK (Thr¹⁸⁰, Tyr¹⁸²) (B), JNK (Thr¹⁸³, Tyr¹⁸⁵) (C), TrkA (Tyr⁴⁹⁰) (D), or Src (Tyr⁴¹⁶) (E). Data are expressed as the fluorescence intensity index and are the mean \pm SE of results in three independent experiments. * $P < 0.05$ (Dunnett's test) versus the value for time 0.

layers in the closed area of corneal epithelial wounds.⁴³ ERK has also been shown to play an important role in the cell migration and proliferation associated with corneal wound healing.⁴⁴ These previous observations and our present results suggest that the stimulation of corneal epithelial wound healing by the PHSRN peptide¹⁸ may be mediated in part by ERK activation and upregulation of ZO-1 expression in corneal epithelial cells, with ZO-1 contributing to the terminal differentiation of these cells after their migration.

Although ZO-1 was identified as a tight junction-associated molecule,¹⁹ evidence suggests that it may not be localized exclusively at tight junctions.⁴⁵ In the corneal epithelium, ZO-1 may thus not only contribute to the formation of tight junctions and the establishment of a barrier to paracellular flow but may also be involved in the cell-cell adhesion of basal and wing epithelial cells and in the anchorage of the adhesion complexes to the actin cytoskeleton.²⁵ It has been proposed that adherens junctions containing ZO-1 and paxillin reinforce attachment of basal cells to wing cells and are regulated by reversible phosphorylation.²⁵ Our previous observation that the PHSRN peptide induced the tyrosine phosphorylation of paxillin in HCE cells¹⁸ and our present results showing that it upregulated the expression of ZO-1 in these cells warrant further investigation of whether this peptide might modulate the ZO-1-paxillin interaction and thereby regulate adherens junctions in the corneal epithelium.

The MAPK cascade is a pivotal intracellular signaling module and is activated by a diverse range of stimuli, including growth factors, chemical agents, osmotic stress, radiation, bacterial infection, and proinflammatory cytokines. We previously showed that the PHSRN peptide stimulates the expression of HSP70 in HCE cells through activation of the p38 MAPK signaling pathway.³⁴ We have now shown that the PHSRN peptide induced the activation of ERK, p38 MAPK, and JNK in HCE cells and that the upregulation of ZO-1 expression by this peptide was blocked by inhibitors of signaling by these MAPKs, suggesting that these kinases mediate the effect of the peptide on ZO-1 expression. The observed activation of the transcription factor c-Jun by the PHSRN peptide may also contribute to the transcriptional activation of the ZO-1 gene. The PHSRN peptide had no effect on signaling by the translational control protein receptor tyrosine kinase TrkA or the nonreceptor tyrosine kinase Src in HCE cells, suggesting that its effects on MAPKs are specific.

Previous studies have implicated MAPK activation in regulation of tight junctions by diverse stimuli. MAPK signaling pathways have been found to modulate paracellular transport through up- or downregulation of tight-junction proteins and consequent changes in the molecular composition of tight-

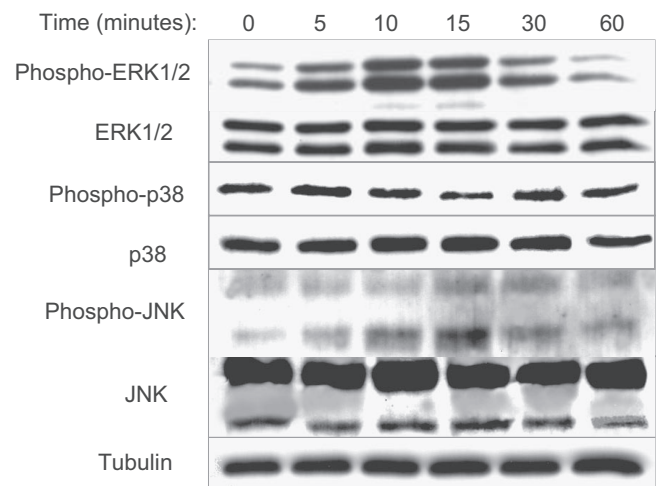


FIGURE 5. Effects of the PHSRN peptide on MAPK phosphorylation in HCE cells as determined by immunoblot analysis. Serum-deprived cells were incubated with the PHSRN peptide (1 $\mu\text{g}/\text{mL}$) for the indicated times, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to total or phosphorylated forms of ERK1/2, p38 MAPK, or JNK as well as with those to α -tubulin. Data are representative of results of three independent experiments.

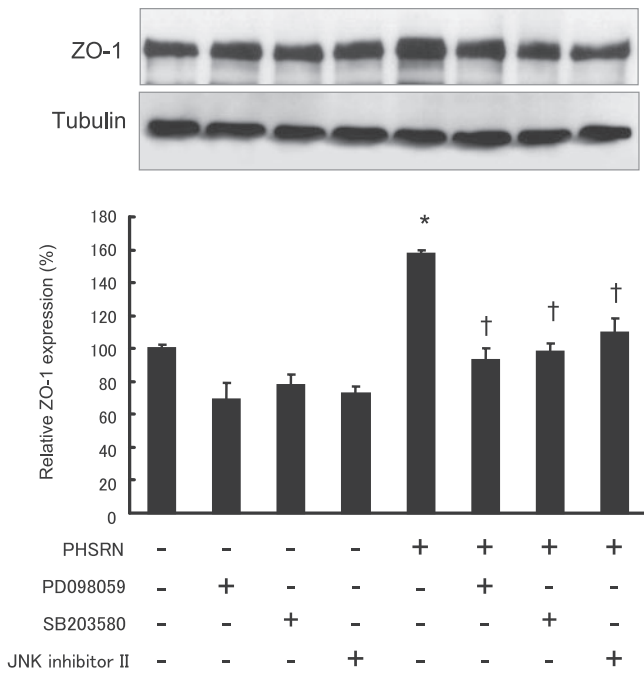


FIGURE 6. Effects of MAPK inhibitors on the upregulation of ZO-1 expression in HCE cells by the PHSRN peptide. Serum-deprived cells were incubated first for 1 hour in the absence or presence of PD098059 (10 μ M), SB203580 (10 μ M), or JNK inhibitor II (5 μ M) and then for 24 hours in the additional absence or presence of the PHSRN peptide (1 μ g/mL). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to ZO-1 and to α -tubulin. *Top*: a representative blot; *bottom*: quantitative analysis of ZO-1 band intensity in blots from four independent experiments. Quantitative data are expressed as the mean \pm SE and are relative to the value for cells incubated without addition. * $P < 0.05$ (Tukey-Kramer test) versus the value for cells incubated without addition; † $P < 0.05$ (Tukey-Kramer test) versus the value for cells incubated with the PHSRN peptide alone.

junction complexes.⁴⁶ The cytokine interleukin-17⁴⁷; various growth factors including transforming growth factor- β ,⁴⁸ epidermal growth factor,⁴⁹ and hepatocyte growth factor,⁵⁰ and bile⁵¹ have been shown to activate ERK1/2 and to increase the barrier function of tight junctions. Thiol compounds also promote tight-junction function by activating ERK1/2, JNK, and p38 MAPK.⁵² In contrast, activation of MAPKs is involved in the disruption of tight junctions triggered by Ras,⁵³ Raf,⁵⁴ alcohol,⁵⁵ bile,⁵⁶ oxidative stress,⁵⁷ metalloproteinases,⁵⁸ CdCl₂,⁵⁹ interferon- γ ,⁶⁰ or growth factors.^{61–65} These findings suggest that activation of MAPK pathways can lead to assembly or disruption of tight junctions depending on the stimulus and cell type. Furthermore, cross talk between protein kinase C (PKC) and MAPK signaling has been shown to regulate tight-junction integrity in HCE cells.⁶⁶ Activation of PKC was thus found to result in a decrease in TER in a manner dependent on MAPK activation.

TER is a sensitive measure of barrier function or tight-junction integrity in epithelial cell monolayers, with the resistance value being inversely proportional to the permeability of tight junctions to inorganic ions.⁶⁷ We have previously shown that the TER of HCE cell monolayers increases in a time-dependent manner during culture.³⁵ Our present results indicate that the PHSRN peptide had no effect on TER in HCE cells. This finding appears consistent with our observation that the PHSRN peptide also did not affect the expression of the tight-junction proteins ZO-2, ZO-3, claudin, or occludin. The up-

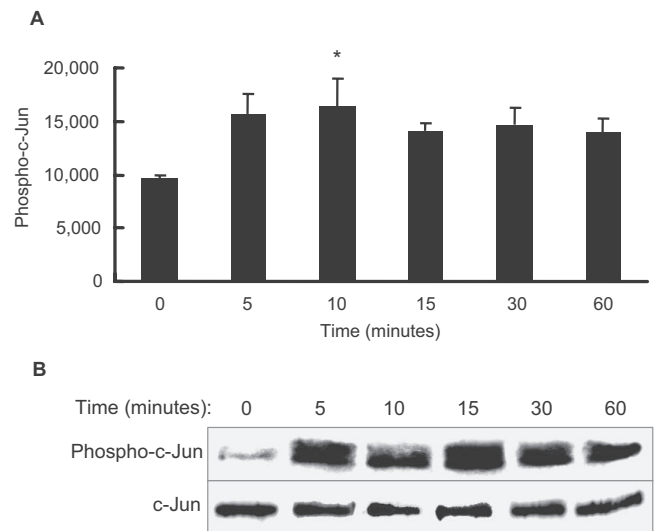


FIGURE 7. Effect of the PHSRN peptide on c-Jun phosphorylation in HCE cells. (A) Serum-deprived cells were incubated with the PHSRN peptide (1 μ g/mL) for the indicated times, after which cell lysates were prepared and subjected to phosphoprotein analysis for measurement of the Ser⁶³-phosphorylated form of c-Jun. Data are expressed as the fluorescence intensity index and are the mean \pm SE of three independent experiments. * $P < 0.05$ (Dunnett's test) versus the value for time 0. (B) Serum-deprived cells were incubated with the PHSRN peptide (1 μ g/mL) for the indicated times, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to total or phosphorylated forms of c-Jun. Data are representative of three independent experiments.

regulation of ZO-1 expression by the PHSRN peptide is thus probably insufficient to increase the barrier function of HCE cells.

In summary, our results suggest that the PHSRN sequence of fibronectin increases the expression of the tight junction-associated protein ZO-1 in human corneal epithelial cells in a manner dependent on the activation of MAPKs. This effect of the PHSRN sequence may contribute to the formation of tight junctions and play an important role in the differentiation of corneal epithelial cells.

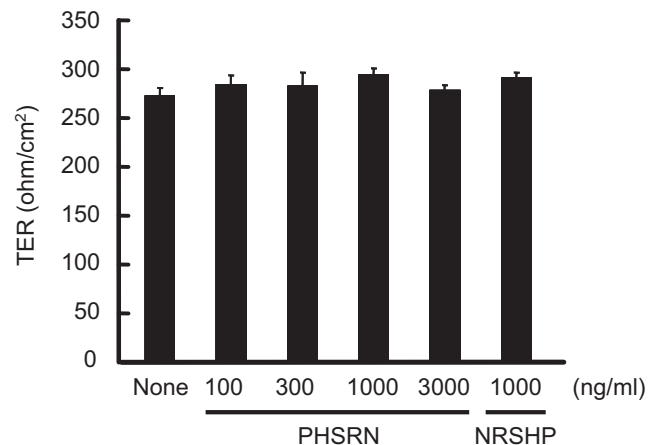


FIGURE 8. Lack of effect of the PHSRN peptide on the barrier function of HCE cells. Cells were cultured on cell migration filters for 4 days and then incubated for 48 hours in serum-free medium containing the indicated concentrations of PHSRN or NRSHP peptides. TER was then determined. Data are the mean \pm SE of results in three independent experiments.

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References

- Grinnell F. Fibronectin and wound healing. *J Cell Biochem.* 1984; 26:107-116.
- Grinnell F, Toda K, Lamke-Seymour C. Reconstitution of human epidermis in vitro is accompanied by transient activation of basal keratinocyte spreading. *Exp Cell Res.* 1987;172:439-449.
- Briggs SL. The role of fibronectin in fibroblast migration during tissue repair. *J Wound Care.* 2005;14:284-287.
- Yamada KM. Fibronectin peptides in cell migration and wound repair. *J Clin Invest.* 2000;105:1507-1509.
- Nishida T, Nakagawa S, Ohashi Y, Awata T, Manabe R. Fibronectin in corneal wound healing: appearance in cultured rabbit cornea. *Jpn J Ophthalmol.* 1982;26:410-415.
- Nishida T, Nakagawa S, Awata T, Ohashi Y, Watanabe K, Manabe R. Fibronectin promotes epithelial migration of cultured rabbit cornea in situ. *J Cell Biol.* 1983;97:1653-1657.
- Nishida T, Nakagawa S, Nishibayashi C, Tanaka H, Manabe R. Fibronectin enhancement of corneal epithelial wound healing of rabbits in vivo. *Arch Ophthalmol.* 1984;102:455-456.
- Suda T, Nishida T, Ohashi Y, Nakagawa S, Manabe R. Fibronectin appears at the site of corneal stromal wound in rabbits. *Curr Eye Res.* 1981;1:553-556.
- Maxfield SR, Moulder K, Koning F, et al. Murine T cells express a cell surface receptor for multiple extracellular matrix proteins: identification and characterization with monoclonal antibodies. *J Exp Med.* 1989;169:2173-2190.
- Peltonen J, Larjava H, Jaakkola S, et al. Localization of integrin receptors for fibronectin, collagen, and laminin in human skin: variable expression in basal and squamous cell carcinomas. *J Clin Invest.* 1989;84:1916-1923.
- Carter WG, Kaur P, Gil SG, Gahr PJ, Wayner EA. Distinct functions for integrins $\alpha 3 \beta 1$ in focal adhesions and $\alpha 6 \beta 4$ /bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J Cell Biol.* 1990;111:3141-3154.
- Carter WG, Wayner EA, Bouchard TS, Kaur P. The role of integrins $\alpha 2 \beta 1$ and $\alpha 3 \beta 1$ in cell-cell and cell-substrate adhesion of human epidermal cells. *J Cell Biol.* 1990;110:1387-1404.
- Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci.* 2002; 115:3861-3863.
- Pytela R, Pierschbacher MD, Ruoslahti E. Identification and isolation of a 140 kD cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 1985;40:191-198.
- Akiyama SK, Yamada SS, Chen WT, Yamada KM. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J Cell Biol.* 1989;109:863-875.
- Yamada KM, Kennedy DW, Yamada SS, Gralnick H, Chen WT, Akiyama SK. Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. *Cancer Res.* 1990;50:4485-4496.
- Aota S, Nomizu M, Yamada KM. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. *J Biol Chem.* 1994;269:24756-24761.
- Kimura K, Hattori A, Usui Y, et al. Stimulation of corneal epithelial migration by a synthetic peptide (PHSRN) corresponding to the second cell-binding site of fibronectin. *Invest Ophthalmol Vis Sci.* 2007;48:1110-1118.
- 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.
- Stevenson BR. Understanding tight junction clinical physiology at the molecular level. *J Clin Invest.* 1999;104:3-4.
- Furuse M, Hirase T, Itoh M, et al. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol.* 1993; 123:1777-1788.
- Tsukita S, Furuse M, Itoh M. Structural and signalling molecules come together at tight junctions. *Curr Opin Cell Biol.* 1999;11: 628-633.
- Fanning AS, Mitic LL, Anderson JM. Transmembrane proteins in the tight junction barrier. *J Am Soc Nephrol.* 1999;10:1337-1345.
- Wang Y, Chen M, Wolosin JM. ZO-1 in corneal epithelium; stratal distribution and synthesis induction by outer cell removal. *Exp Eye Res.* 1993;57:283-292.
- Sugrue SP, Zieske JD. ZO1 in corneal epithelium: association to the zonula occludens and adherens junctions. *Exp Eye Res.* 1997;64: 11-20.
- Teranishi S, Kimura K, Kawamoto K, Nishida T. Protection of human corneal epithelial cells from hypoxia-induced disruption of barrier function by keratinocyte growth factor. *Invest Ophthalmol Vis Sci.* 2008;49:2432-2437.
- Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev.* 2001;22:153-183.
- Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* 2001;81:807-869.
- Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature.* 2001;410:37-40.
- Zhang Y, Dong C. Regulatory mechanisms of mitogen-activated kinase signaling. *Cell Mol Life Sci.* 2007;64:2771-2789.
- Coulombe P, Meloche S. Atypical mitogen-activated protein kinases: structure, regulation and functions. *Biochim Biophys Acta.* 2007;1773:1376-1387.
- Nomizu M, Kim WH, Yamamura K, et al. Identification of cell binding sites in the laminin $\alpha 1$ chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. *J Biol Chem.* 1995;270:20583-20590.
- Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci.* 1995;36:614-621.
- Ko JA, Yanai R, Quan WY, Morishige N, Nishida T. Up-regulation of HSP70 by the fibronectin-derived peptide PHSRN in human corneal epithelial cells. *Biochem Biophys Res Commun.* 2008; 370:424-428.
- Kimura K, Teranishi S, Fukuda K, Kawamoto K, Nishida T. Delayed disruption of barrier function in cultured human corneal epithelial cells induced by tumor necrosis factor- α in a manner dependent on NF- κ B. *Invest Ophthalmol Vis Sci.* 2008;49:565-571.
- Rosso F, Giordano A, Barbarisi M, Barbarisi A. From cell-ECM interactions to tissue engineering. *J Cell Physiol.* 2004;199:174-180.
- Rosso F, Marino G, Giordano A, Barbarisi M, Parmeggiani D, Barbarisi A. Smart materials as scaffolds for tissue engineering. *J Cell Physiol.* 2005;203:465-470.
- Akiyama SK, Olden K, Yamada KM. Fibronectin and integrins in invasion and metastasis. *Cancer Metastasis Rev.* 1995;14:173-189.
- Grant RP, Spitzfaden C, Altroff H, Campbell ID, Mardon HJ. Structural requirements for biological activity of the ninth and tenth FIII domains of human fibronectin. *J Biol Chem.* 1997;272:6159-6166.
- Murakami J, Nishida T, Otori T. Coordinated appearance of $\beta 1$ integrins and fibronectin during corneal wound healing. *J Lab Clin Med.* 1992;120:86-93.
- Rycom SW, Paul D, Goodenough DA. Truncation mutants of the tight junction protein ZO-1 disrupt corneal epithelial cell morphology. *Mol Biol Cell.* 2000;11:1687-1696.
- Danjo Y, Gipson IK. Actin 'purse string' filaments are anchored by E-cadherin-mediated adherens junctions at the leading edge of the epithelial wound, providing coordinated cell movement. *J Cell Sci.* 1998;111:3323-3332.
- Hutcheon AE, Sippel KC, Zieske JD. Examination of the restoration of epithelial barrier function following superficial keratectomy. *Exp Eye Res.* 2007;84:32-38.
- Imayasu M, Shimada S. Phosphorylation of MAP kinase in corneal epithelial cells during wound healing. *Curr Eye Res.* 2003;27:133-141.

45. Schnabel E, Anderson JM, Farquhar MG. The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium. *J Cell Biol.* 1990;111:1255-1263.
46. Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. *Biochim Biophys Acta.* 2008;1778:729-756.
47. Kinugasa T, Sakaguchi T, Gu X, Reinecker HC. Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology.* 2000;118:1001-1011.
48. Feldman G, Kiely B, Martin N, Ryan G, McMorrow T, Ryan MP. Role for TGF- β in cyclosporine-induced modulation of renal epithelial barrier function. *J Am Soc Nephrol.* 2007;18:1662-1671.
49. Basuroy S, Seth A, Elias B, Naren AP, Rao R. MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. *Biochem J.* 2006;393:69-77.
50. 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.
51. Yang R, Harada T, Li J, et al. Bile modulates intestinal epithelial barrier function via an extracellular signal related kinase 1/2 dependent mechanism. *Intens Care Med.* 2005;31:709-717.
52. Usatyuk PV, Parinandi NL, Natarajan V. Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. *J Biol Chem.* 2006;281:35554-35566.
53. Mullin JM, Leatherman JM, Valenzano MC, et al. Ras mutation impairs epithelial barrier function to a wide range of nonelectrolytes. *Mol Biol Cell.* 2005;16:5538-5550.
54. Lan M, Kojima T, Osanai M, Chiba H, Sawada N. Oncogenic Raf-1 regulates epithelial to mesenchymal transition via distinct signal transduction pathways in an immortalized mouse hepatic cell line. *Carcinogenesis.* 2004;25:2385-2395.
55. Singh AK, Jiang Y, Gupta S, Benhabib E. Effects of chronic ethanol drinking on the blood brain barrier and ensuing neuronal toxicity in alcohol-preferring rats subjected to intraperitoneal LPS injection. *Alcohol Alcohol.* 2007;42:385-399.
56. Alvarez C, Fasano A, Bass BL. Acute effects of bile acids on the pancreatic duct epithelium in vitro. *J Surg Res.* 1998;74:43-46.
57. Krizbai IA, Bauer H, Bresgen N, et al. Effect of oxidative stress on the junctional proteins of cultured cerebral endothelial cells. *Cell Mol Neurobiol.* 2005;25:129-139.
58. Tan X, Egami H, Abe M, Nozawa F, Hirota M, Ogawa M. Involvement of MMP-7 in invasion of pancreatic cancer cells through activation of the EGFR mediated MEK-ERK signal transduction pathway. *J Clin Pathol.* 2005;58:1242-1248.
59. Lui WY, Wong CH, Mruk DD, Cheng CY. TGF- β 3 regulates the blood-testis barrier dynamics via the p38 mitogen activated protein (MAP) kinase pathway: an in vivo study. *Endocrinology.* 2003;144:1139-1142.
60. Patrick DM, Leone AK, Shellenberger JJ, Dudowicz KA, King JM. Proinflammatory cytokines tumor necrosis factor- α and interferon- γ modulate epithelial barrier function in Madin-Darby canine kidney cells through mitogen activated protein kinase signaling. *BMC Physiol.* 2006;6:2.
61. Leung JC, Chan LY, Li FF, et al. Glucose degradation products downregulate ZO-1 expression in human peritoneal mesothelial cells: the role of VEGF. *Nephrol Dial Transplant.* 2005;20:1336-1349.
62. Lui WY, Lee WM, Cheng CY. Transforming growth factor β 3 regulates the dynamics of Sertoli cell tight junctions via the p38 mitogen-activated protein kinase pathway. *Biol Reprod.* 2003;68:1597-1612.
63. Xia W, Mruk DD, Lee WM, Cheng CY. Differential interactions between transforming growth factor- β 3/T β R1, TAB1, and CD2AP disrupt blood-testis barrier and Sertoli-germ cell adhesion. *J Biol Chem.* 2006;281:16799-16813.
64. Miyamoto N, de Kozak Y, Jeanny JC, et al. Placental growth factor-1 and epithelial haemato-retinal barrier breakdown: potential implication in the pathogenesis of diabetic retinopathy. *Diabetologia.* 2007;50:461-470.
65. Grande M, Franzen A, Karlsson JO, Ericson LE, Heldin NE, Nilsson M. Transforming growth factor- β and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes. *J Cell Sci.* 2002;115:4227-4236.
66. Wang Y, Zhang J, Yi XJ, Yu FS. Activation of ERK1/2 MAP kinase pathway induces tight junction disruption in human corneal epithelial cells. *Exp Eye Res.* 2004;78:125-136.
67. Reuss L. Tight junction permeability to ion and water. In: Cerejido M, Anderson J, eds. *Tight Junctions*. Boca Raton, FL: CRC Press; 2001:61-88.