

行政院國家科學委員會專題研究計畫 期中進度報告

磷酸化酪胺酸的訊息傳遞對角膜內皮細胞生理機制的影響

(1/3)

計畫類別：個別型計畫

計畫編號：NSC93-2314-B-002-091-

執行期間：93年08月01日至94年07月31日

執行單位：國立臺灣大學醫學院眼科

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 94 年 5 月 3 日

# 磷酸化酪氨酸的訊息傳遞對角膜內細胞生理機制的影響

## ---第一年精簡報告

### **Abstract**

**Purpose:** Contact inhibition is an important mechanism for maintaining corneal endothelium in a non-replicative state. Protein tyrosine phosphatases (PTPs) play a role in regulating the integrity of cell-cell contacts, differentiation, and growth. In this study, we aimed to evaluate whether phosphatases are involved in the maintenance of contact-dependent inhibition of proliferation in corneal endothelial cells and to identify candidate PTPs that are expressed in these cells and might be involved in regulation of contact inhibition.

**Methods:** Confluent cultures of rat corneal endothelial cells or endothelium in *ex vivo* corneas were treated with the general phosphatase inhibitor, sodium orthovanadate (SOV). Immunocytochemistry (ICC) evaluated the effect of SOV on cell-cell contacts by staining for ZO-1, and on cell cycle progression by staining for Ki67. Transverse sections of rat cornea and cultured rat corneal endothelial cells were used to test for expression of the candidate PTPs: PTP-mu, PTP-LAR, PTP1B, SHP-1, SHP-2, and PTEN using ICC and either Western blots or RT-PCR.

**Results:** ZO-1 staining demonstrated that SOV induced a time-dependent release of cell-cell contacts in confluent cultures of corneal endothelial cells and in the endothelium of *ex vivo* corneas. Staining for Ki67 indicated that SOV promoted limited cell cycle progression in the absence of serum. PTP-mu, PTP1B, SHP-1,

SHP-2, and PTEN, but not PTP-LAR, were expressed in rat corneal endothelial cells *in situ* and in culture. The subcellular location of PTP-mu and PTP1B differed in subconfluent and confluent cells, while that of SHP-1, SHP-2, and PTEN was similar, regardless of confluent status. Western blots confirmed the expression of PTP1B, SHP-1, SHP-2, and PTEN. RT-PCR confirmed expression of PTP-mu mRNA.

**Conclusion:** Phosphatases are involved in regulation of junctional integrity and of cell proliferation in corneal endothelial cells. PTP-mu, PTP1B, SHP-1, SHP-2, and PTEN are expressed in rat corneal endothelium and may be involved in regulation of contact inhibition in these normally non-proliferating cells.

## **2. Materials and Methods**

### ***2.1. Antibodies and Reagents***

Rabbit-anti-human SHP-1, rabbit-anti-human SHP-2, goat-anti-rat PTP-LAR, mouse-anti-human PTEN, horseradish peroxidase-conjugated donkey-anti-rabbit IgG, goat-anti-mouse IgG, and donkey-anti-goat IgG, as well as blocking peptides specific for SHP-1, SHP-2, PTP-LAR and PTEN, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-human-PTP1B was obtained from Oncogene Sciences (Cambridge, MA). Polyclonal rabbit-anti-human zonula occludins-1 (ZO-1) and mouse-anti-human Ki67 were purchased from Zymed Laboratories (San Francisco, CA). Monoclonal antibody against the intracellular (SK7) domains of human PTP-mu was kindly provided by Dr. Susan M. Brady-Kalnay (Case Western Reserve University, Cleveland, OH). Rabbit anti-human non-muscle myosin was obtained from Biomedical Technologies, Inc. (Stoughton, MA). Sodium orthovanadate (SOV), ethylene glycol-bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (disodium EDTA), and 100X antibiotic/antimycotic solution were from Sigma (St. Louis, MO). FITC-conjugated donkey anti-rabbit, anti-mouse, and anti-goat secondary antibodies and donkey serum were purchased from Jackson ImmunoResearch (West Grove, PA). Mounting medium containing propidium iodide (PI) was purchased from Vector Laboratories (Burlingame, CA). Medium-199 and gentamicin were obtained from Invitrogen/Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Bovine serum albumin (BSA) was purchased from Fisher Scientific (Pittsburgh, PA).

## ***2.2. Culture of Rat Corneal Endothelial Cells***

Corneas were obtained from adult male Sprague-Dawley rats, which were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Endothelial cells were grown in explant culture as previously described (Chen et al., 1999). To remove the epithelial cell layers, corneas were incubated for 1 hr at 37°C in 2.5 mM EDTA to disrupt hemidesmosomes, followed by gentle removal of the epithelium. For explant culture of the endothelium, corneas were cut in half and placed endothelium-side down in a 6-well tissue culture plate. Pieces were allowed to attach to the culture plate for approximately 5 min, after which 1 drop of culture medium was placed over the tissue. Culture medium consisted of Medium-199, 50 µg/ml gentamicin, and 10% FBS. Corneal pieces were incubated overnight at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. On the following day, 1 ml culture medium was gently added and cultures were incubated undisturbed. After 5 days, 2 ml of medium were added per well. Medium was changed every other day thereafter. After approximately 10 days, when a sufficient number of endothelial cells had migrated off the cornea, the corneal pieces were carefully removed, and the remaining endothelial cells were grown to confluence. Primary cultured cells were then trypsinized, resuspended in culture medium, and seeded into 2-well chamber slides or into T75 flasks at a density of  $2.5 \times 10^5$  cells per flask.

## ***2.3. Immunocytochemical Localization***

For immunocytochemical (ICC) analysis of cultured cells, cells were seeded onto chamber slides and either used as a non-confluent culture or grown to confluence. After washing three times with phosphate-buffered saline (PBS), cells were fixed in methanol at -20°C for 15 min followed by a further wash with PBS. The fixed cells were then permeabilized with 1% Triton X-100 in PBS for 10 min. After a PBS wash,

cells were pre-incubated for 10 min in 2% BSA in PBS (5% donkey serum when goat antibody was used) to block non-specific binding, and then incubated with primary antibody at room temperature for 2 hrs. Concentrations of primary antibodies were as follows: anti-ZO-1=1:150; anti-Ki67=1:100; anti-PTP-mu=1:400; anti-PTP-LAR=1:200; anti-PTP1B=1:100; anti-SHP-1=1:400; anti-SHP-2=1:400; and anti-PTEN=1:100. According to the suppliers, all primary antibodies used in these studies were cross-reactive with the appropriate rat antigen. Following primary antibody incubation, cells were washed with PBS, incubated for 10 min with blocking buffer, and then incubated for 1 hr with a 1:200 dilution of the appropriate FITC-conjugated secondary antibody. To assess specificity of the staining, cells were either processed without primary antibody or incubated with primary antibody that had been pre-absorbed overnight with a 5X concentration of blocking peptide. Cells were then washed with PBS and mounted in medium containing propidium iodide (PI) for visualization of nuclei. All experiments were repeated three times to ensure consistent results. Immunolocalization in the endothelium of *ex vivo* corneas was the same as in cultured cells except that 4% BSA in PBS was used in the blocking buffer. (I will insert a description of our new confocal microscope.)

ICC studies were also conducted using transverse sections of rat cornea. Corneas were obtained from adult male Sprague-Dawley rats. Corneas were fixed, sectioned, and immunostained according to published methods (Joyce et al, 1998). The localization pattern in both cultured cells and transverse sections was observed by fluorescence microscopy using an Eclipse E800 Nikon Microscope with a VFM Epi-Fluorescence Attachment (Nikon Inc., Melville, NY) equipped with a Spot digital camera and Spot version 1.1 CE software (Diagnostic Instruments, Sterling Heights, MI). Experiments were conducted using tissue sections from three different rat corneas to ensure consistent results.

#### ***2.4. Protein Extraction and Immunoblotting***

Cultured cells grown in T75 flasks were trypsinized, suspended in buffer containing 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma), and homogenized. Equal amounts of extracted protein were loaded on 4-12% polyacrylamide gels (Invitrogen, Carlsbad, CA) for SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). Non-specific binding was blocked by incubation overnight at 4°C with 5% nonfat dry milk in PBS. Membranes were then incubated at room temperature for 2 hrs with anti-PTP-LAR, anti-PTP-1B, anti-SHP-1, anti-SHP-2 or anti-PTEN primary antibodies at the same dilutions indicated for ICC, with or without pre-incubation with blocking peptide. Anti-non-muscle myosin was used as a positive control for the immunoblot. Membranes were washed three times with the blocking buffer and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at a final dilution of 1:10,000. After final washes with 0.1% Triton X-100 in PBS, signal was detected by enhanced chemiluminescence following manufacturer's instructions (Pierce, Rockford, IL) and exposed to autoradiographic film. All experiments were repeated three times to ensure consistent results.

#### ***2.5. RT-PCR of PTP-mu***

For RT-PCR studies, total RNA was prepared from confluent cultures of rat corneal endothelial cells according to the manufacturer's directions (TRIzol; Gibco, Gaithersburg, MD). cDNA was prepared from 1 µg total RNA by reverse transcription in a volume of 20 µl using reagents from a commercially available kit (Promega, Pittsburgh, PA). Primers specific for PTP-mu were designed using MacVector 5.0

software (Oxford Molecular Group, Oxford, UK). Primer sequences were based on human PTP-mu sequences, since no sequence of rat PTP-mu was available in the GenBank database. In the region chosen for synthesis of the primers, mouse PTP-mu sequences only differed from the human sequence by one nucleotide, suggesting that these primers would recognize rat PTP-mu sequences. The primer pairs used were: upstream sequence, 5'-TCCTATTACCCAGATGAAACCCAC-3'; downstream sequence, 5'-CCACTTCCACAAGATTGGTCACC-3'. PCR was performed in a reaction mixture containing 1 µg cDNA and 2.5 µM each of the upstream and downstream primers, plus reagents from a commercially available kit (Invitrogen). Specificity and yield of the PCR products were enhanced using the hot-start approach (Erlich et al., 1991). PCR was performed for 40 cycles in a thermal cycler. Cycle conditions included denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 mins. A 5-min extension was added at the end of the 40 cycles of PCR. PCR products and 100-bp DNA ladder molecular weight markers were electrophoresed in 1.5% agarose gels containing 0.5 µg/ml ethidium bromide and photographed. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA) acted as a positive control for the PCR. Negative control samples consisted of the PCR reaction mixture, including primers, but without cDNA. To ensure that the total RNA samples were not contaminated with genomic DNA, a negative control using 1 µg total RNA was substituted for cDNA in the PCR reaction mixture, along with 2.5 µM each of upstream and downstream G3PDH primers. All experiments were repeated three times to ensure consistent results.

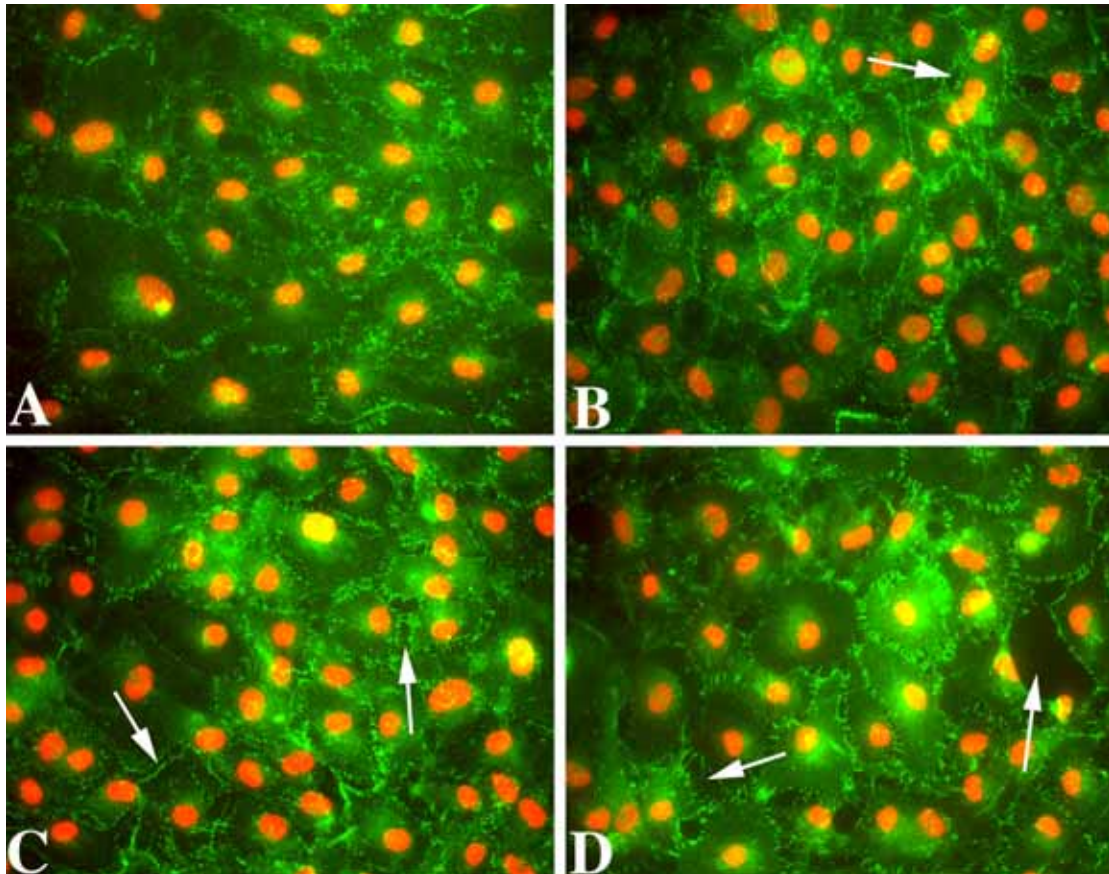
### **3. Results**

#### ***3.1. Phosphatase Inhibition Disrupts Cell-Cell Junctions in Confluent Rat Corneal***



### ***Endothelial Cells and in the Endothelium of Ex Vivo Corneas***

The phosphatase inhibitor, SOV, disrupted cell-cell junctions and triggered cell cycle entry in contact-inhibited human umbilical vein endothelial cells (Suzuki et al., 2000). To test whether phosphatase inhibition could have similar effects on confluent rat corneal endothelial cells, we first examined the effect of SOV on cell-cell contacts. Cells were grown to confluence and maintained an additional week to ensure the formation of a mature, contact-inhibited monolayer. In a preliminary dose-dependent study, the concentration of 50  $\mu\text{M}$  SOV was found to optimally release cell-cell contacts without inducing cell loss or apoptosis. To investigate time-dependent effects, confluent cultures were incubated for 24, 48, or 72 hrs in 50  $\mu\text{M}$  SOV plus Medium-199 and antibiotics as described in Materials & Methods, then fixed and immunostained for ZO-1, a tight junction-associated protein (Siliciano and Goodenough, 1988), to help visualize cell borders. ZO-1 staining in control confluent cultures appeared as a single, discontinuous linear pattern at cell borders (Fig. 1A), indicating intact cell-cell contacts. Treatment of confluent cultures with SOV induced a time-dependent release of cell-cell contacts, resulting in the appearance of gaps between cells and a gradual loss of monolayer integrity (Fig. 1B-D). Secondary antibody controls were negative, indicating the specificity of the ZO-1 antibody staining (data not shown).

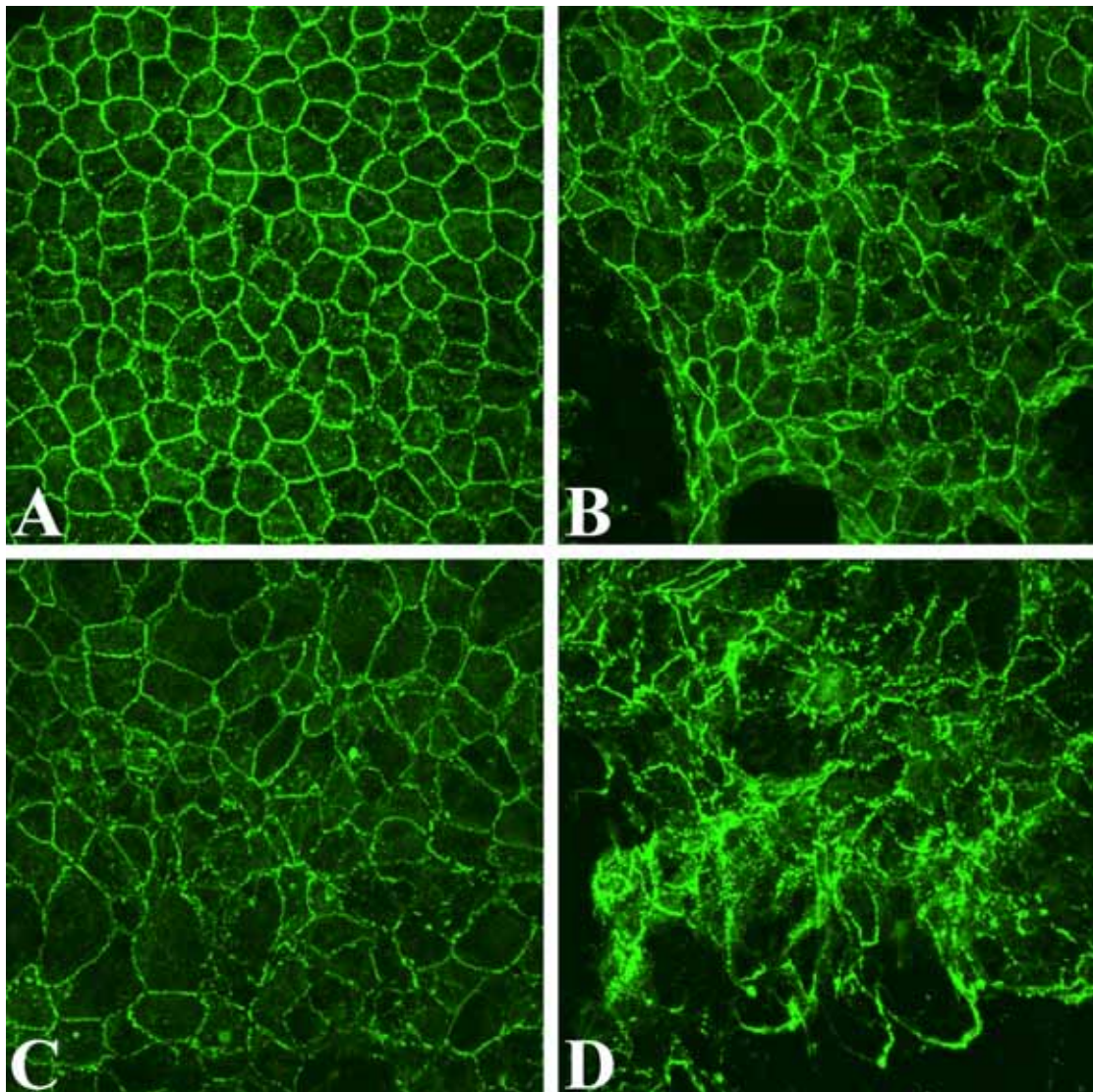


**Figure 1.** Representative images of immunolocalization of ZO-1 in fully confluent cultures of rat corneal endothelial cells. Control cultures were maintained minus FBS (A). Other cultures were incubated in 50  $\mu$ M SOV without FBS for 24 hrs (B), 48 hrs (C), or 72 hrs (D) prior to immunostaining. Arrows in B-D indicate breaks in the confluent monolayer. Green: ZO-1. Red: PI. Original magnification = 40X.

Similar immunolocalization studies were conducted in *ex vivo* rat corneas to determine whether SOV would disrupt the endothelial monolayer *in situ*, thus demonstrating relevance of the results obtained using the cultured cell model.

Figure 2 presents representative confocal microscopic images of ZO-1 staining in rat corneal endothelium treated with 50  $\mu$ M SOV for 24, 48, or 72 hrs. Untreated controls in Fig. 2A demonstrate the discontinuous linear pattern typical for the intact endothelial monolayer. SOV treatment induced a time-dependent loss of cell-cell contacts, a general disruption of monolayer integrity, and loss of normal polygonal

cell shape (Fig. 2B-D), indicating that inhibition of phosphatase activity by SOV disrupts the endothelial monolayer *in situ*, as well as in culture.



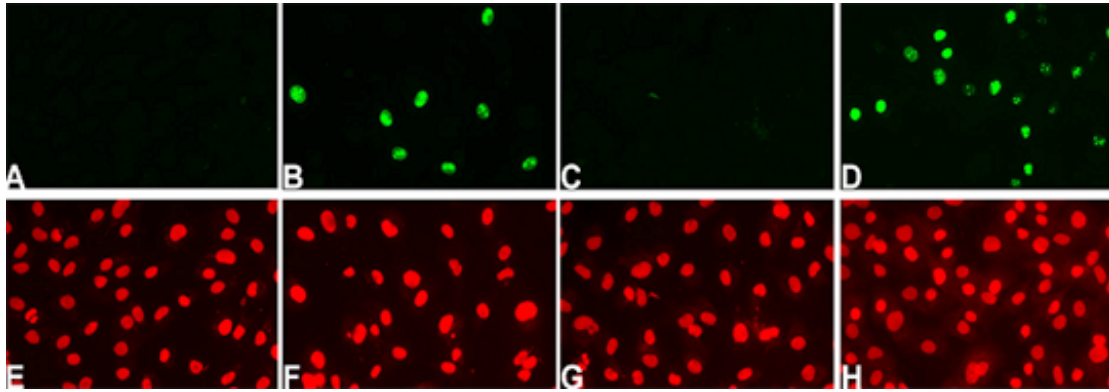
**Figure 2.** Immunolocalization of ZO-1 in the endothelium of *ex vivo* corneas incubated in the absence of SOV (A) and in the presence of 50  $\mu$ M SOV for 24 (B), 48 (C), or 72 hrs (D). PI staining is not shown in order to permit clear visualization of ZO-1. Original magnification: 80 X.

### ***3.2. Phosphatase Inhibition Promotes Limited Cell Cycle Progression in Confluent Rat Corneal Endothelial Cells***

We next tested the ability of SOV to promote cell cycle progression in confluent

cultures of rat corneal endothelial cells. For this study, fully confluent cultures were treated and incubated for 48 hrs as indicated below, then fixed and immunostained for Ki67, a marker of actively cycling cells (Gerdes et al., 1991). Representative images of the results are presented in Figure 3. As expected, confluent cultures maintained for 48 hrs in Medium-199 and antibiotics without FBS did not reveal positive staining for Ki67 (Fig 3A). Positive Ki67 staining was detected in some cells within confluent cultures treated with 50  $\mu$ M SOV (Fig. 3B). We also tested whether release of cell-cell contacts by itself was sufficient to induce cell cycle entry. To answer this question, we compared the effect of SOV and the calcium chelator, EGTA, since chelation of calcium releases cell-cell contacts in corneal endothelium (Stern, 1981; Senoo, 2000b). Confluent cultures were pre-treated for 30 minutes with 5 mM EGTA and then incubated for 48 hrs in the absence or presence of FBS. As seen in Fig. 3C, cultures treated with EGTA in the absence of FBS did not show Ki67-positive cells. As expected, cells did enter the cell cycle when EGTA-treated cultures were incubated in the presence of 10% FBS (Fig. 3D). These results indicate that disruption of cell-cell contacts alone was not sufficient to induce cell cycle entry and that positive Ki67 staining, such as that seen in Fig. 3B, must result from SOV-induced phosphatase inhibition. Ki67 staining patterns change with cell cycle position (Starborg, 1996) and this change in pattern has been used to document cell cycle progression in human corneal endothelial cells in an *ex vivo* cornea wound model (Senoo, 2000a). Interestingly, Ki67 staining patterns in SOV-treated cells (Fig. 3B) appeared to differ from those in cells treated with EGTA in the presence of FBS (Fig. 3D). EGTA-treated cells showed a variety of patterns from G1-phase through mitosis, whereas, a single G1/S-phase pattern was observed in SOV-treated cells.





**Figure 3.** Immunolocalization of Ki67 in confluent cultures of rat corneal endothelial cells incubated minus FBS (A, E); in 50  $\mu$ M SOV alone (B, F); in 5 mM EGTA alone (C, G); or in 5 mM EGTA plus 10% FBS (D, H). Ki67 staining (green) is shown in (A-D); PI (red) in (E-H) shows nuclear staining in the same microscopic field. Original magnification = 400X.

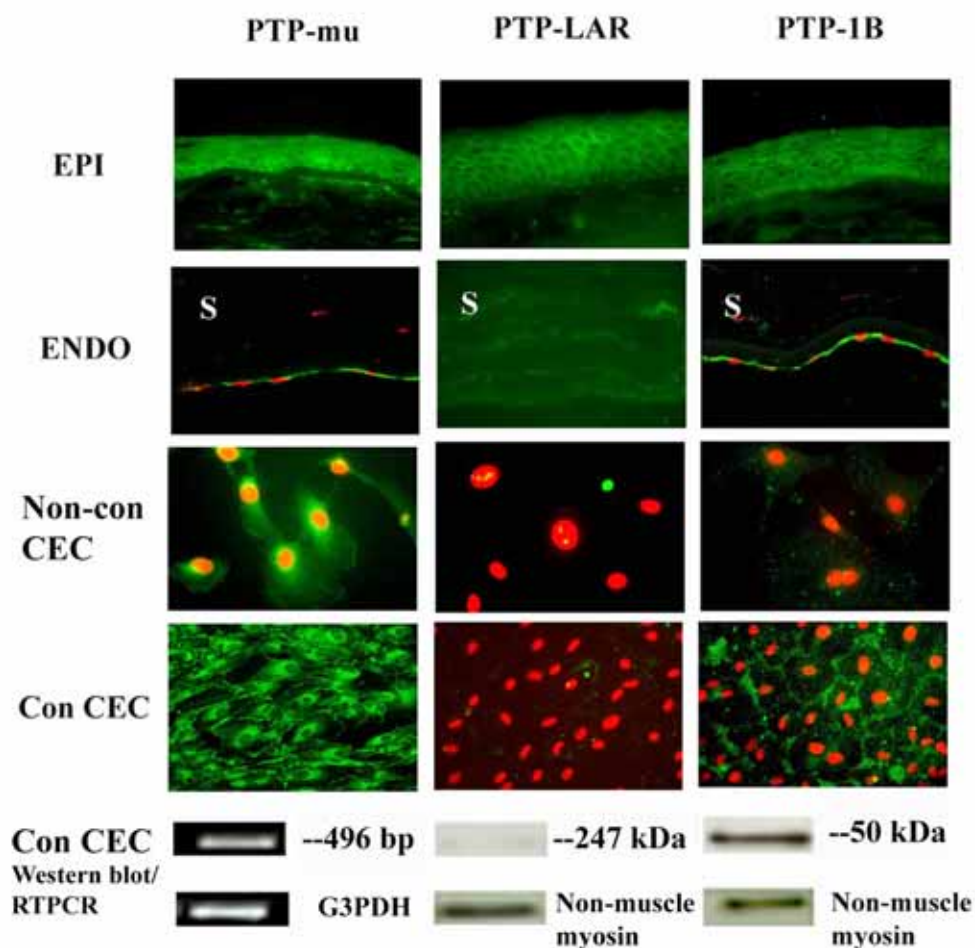
Studies were also conducted in which the endothelium of *ex vivo* corneas was treated with 50  $\mu$ M SOV for 24, 48, and 72 hrs followed by immunostaining for Ki67 or BrdU. Positive Ki67 staining was observed in some endothelial cells in SOV-treated corneas; however, there was no evidence of a time-dependent increase in the relative number of Ki67-positive cells (data not shown). No positive BrdU staining was observed at any time-point. Together, results from both cultured cells and *ex vivo* corneas indicate that SOV is capable of inducing limited G1-phase progression in endothelial cells, but that phosphatase inhibition is not sufficient by itself to induce entry into S-phase of the cell cycle.

### ***3.3. Detection of Candidate PTPs in Rat Corneal Tissue***

Many PTP isoforms have been identified in mammalian cells, but not all tissues express all isoforms. Since phosphatase inhibition by SOV resulted in loss of cell-cell contacts and induced limited cell cycle entry in confluent rat corneal endothelial cells, we attempted to identify specific candidate PTPs known to mediate these functions.

PTP-mu and PTP-LAR (leukocyte antigen-related PTP) are cell contact-associated PTPs. PTP-mu (Brady-Kalnay et al., 1993, 1998) and PTP-LAR (Aicher et al., 1997) are receptor-like transmembrane proteins. PTP1B is a non-receptor protein frequently associated with the endoplasmic reticulum (Haj, 2002) and vesicles subjacent to the plasma membrane. PTP1B appears to help mediate cell adhesion through regulation of cadherin-catenin complexes (Balsamo et al., 1996, 1998; Rhee, et al., 2001; Xu, 2002) and integrin signaling (Liu, 1998), as well as to regulate phosphorylation of the EGF, PDGF, and insulin receptors (Tonks, 2003). We used ICC to determine the relative expression of these PTPs in transverse sections of rat cornea. In Figure 4, we show that PTP-mu, PTP-LAR and PTP1B are expressed at the cell periphery in all layers of the corneal epithelium. Intense staining of PTP-mu was particularly evident in the lateral domains of basal epithelial cells. PTP-mu and PTP1B, but not PTP-LAR, are expressed in rat corneal endothelium. Although no nuclear staining was evident, it was not possible to determine in transverse corneal sections whether PTP-mu or PTP1B were present throughout the cytoplasm or at cell borders. For that reason, the staining patterns were also determined in cultured cells. In non-confluent cultured rat corneal endothelial cells, a predominantly perinuclear distribution of PTP-mu was found. There was no staining for PTP-LAR and little-to-no staining for PTP1B. When the cultured cells became confluent, PTP-mu was localized to both the perinuclear region and the cell periphery. Intense positive staining for PTP1B was observed mainly toward the cell periphery. Controls using secondary antibody alone yielded no positive staining (data not shown). As shown in Figure 4, Western blots confirmed the expression of PTP1B protein and lack of expression of PTP-LAR. Expression of non-muscle myosin was tested in the same blots as a positive control. The PTP-mu monoclonal antibody used in these studies specifically recognized the intracellular domain of PTP-mu (according to Dr. Susan Brady-Kalnay Case Western Reserve

University, Cleveland, OH). Previously this antibody functioned well in immunoblots of an LNCaP prostate carcinoma cell line (Hellberg, 2002); however, it did not work in Western blots of rat corneal endothelial cells. As such, we chose RT-PCR as a method to verify the expression of PTP-mu in rat corneal endothelial cells. As seen in Figure 4, a positive band of the expected size was obtained using primers specific for PTP-mu. No positive bands were observed for the PCR negative controls described in the Materials and Methods section (data not shown). G3PDH, the positive PCR control, yielded a band of the appropriate size.



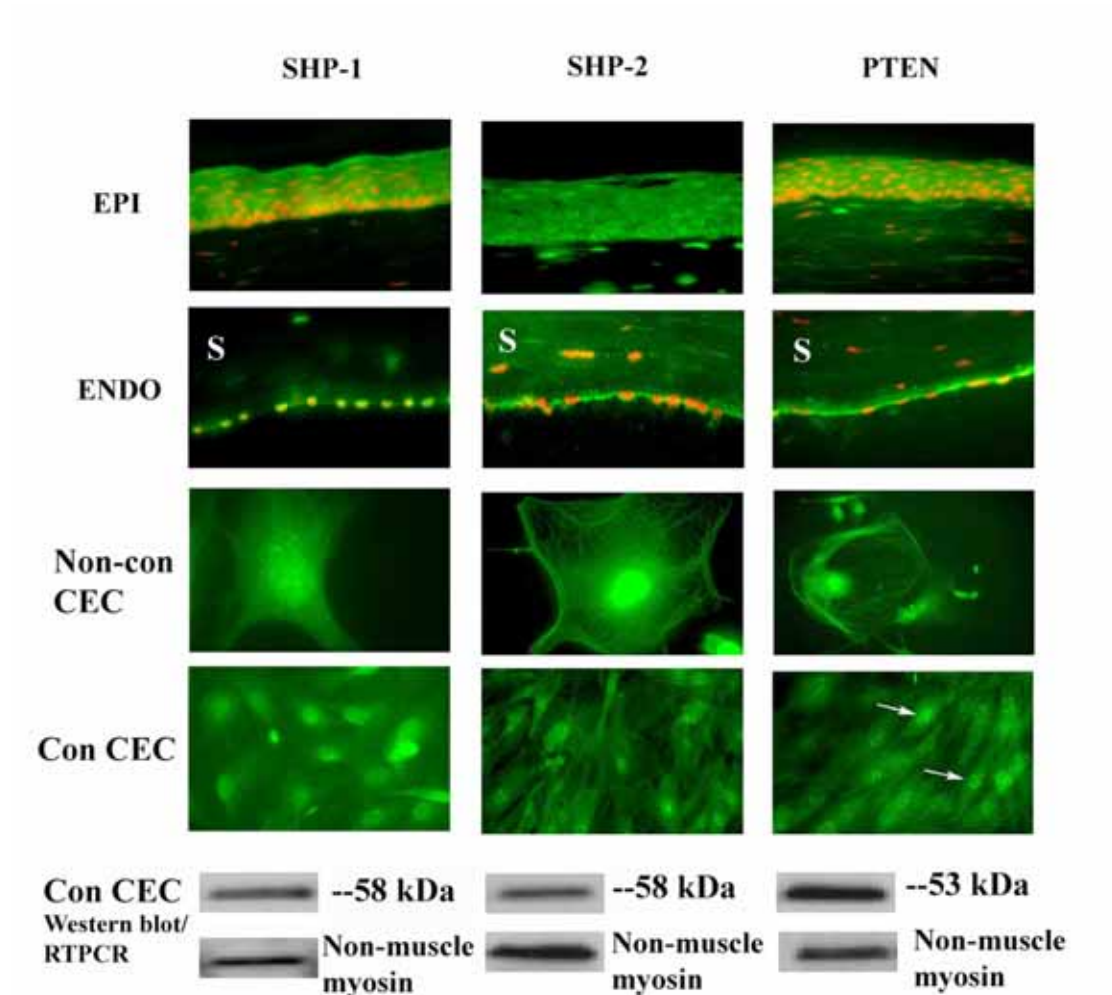
**Figure 4.** Immunolocalization of PTP-mu, PTP-LAR, and PTP1B in rat corneal cells.

ICC studies of rat corneal sections confirm the expression of PTP-mu, PTP-LAR and PTP1B (green) in corneal epithelium, and PTP-mu and PTP1B (green) in corneal endothelium. PI staining (red) indicates nuclei. RT-PCR confirmed the expression of PTP-mu mRNA, while Western blots confirmed the expression of PTP1B protein and lack of expression of PTP-LAR in cultured corneal endothelial cells. G3PDH acted as the positive control for the RT-PCR. Non-muscle myosin was the positive control for the Western blots. EPI=epithelium, ENDO=endothelium, Non-con CEC=non-confluent rat corneal endothelial cells. Con CEC=confluent corneal endothelial cells. S=stroma. Original magnification = 100X for EPI, 200X for ENDO, 600X for Non-con CEC, 400X for Con CEC.

SHP-1, SHP-2, and PTEN, as well as PTP1B, are growth factor- and cell-cycle associated PTPs (Keilhack et al., 1998; Yu et al., 1998; Besson et al., 1999; Qi et al., 1999; Qu et al., 1999; Ramaswamy et al., 1999; Tonks, 2003). SHP-1 and SHP-2 are cytosolic PTPs that help regulate the function of receptor tyrosine kinases, such as the EGF and PDGF receptors. PTEN is a cytosolic dual-specificity phosphatase, i.e., it regulates serine and threonine, as well as tyrosine phosphorylation. It is also able to dephosphorylate lipids (Machama, 1998; Myers, 1998). Its function, in part, is to regulate signaling via the PI3K/Akt pathway. In Figure 5, we show that SHP-1, SHP-2 and PTEN were expressed in the cytoplasm within the entire rat corneal epithelium. Staining for SHP-1, SHP-2 and PTEN was also seen in the corneal endothelium. In cultured cells, SHP-1, SHP-2 and PTEN antibodies labeled subconfluent and confluent rat corneal endothelial cells, and the staining patterns were similar under both conditions. There was intense nuclear staining with moderate cytoplasmic staining for all three PTPs. The staining was specific for SHP-1, SHP-2, and PTEN, as it was completely blocked when primary antibodies were pre-incubated with the corresponding antigen peptide (data not shown). Western blot analysis confirmed the expression of SHP-1, SHP-2 and PTEN protein in confluent rat corneal endothelial cells. For all three PTPs, positive reaction disappeared after pre-incubating the



primary antibodies with specific blocking peptides (data not shown). As previously, non-muscle myosin acted as a positive control for the Western blot.



**Figure 5.** Expression of SHP-1, SHP-2, and PTEN in rat corneal cells. ICC studies confirm the expression of SHP-1, SHP-2 and PTEN (green) in corneal epithelium, corneal endothelium, confluent and non-confluent rat corneal endothelial cells. PI staining (red) indicates nuclei. Arrows indicate punctate staining in the nucleus of PTEN in confluent corneal endothelial cells. Western blots confirmed the ICC results. Non-muscle myosin was used as a positive control for Western blots in all experiments. EPI=epithelium, ENDO=endothelium, Non-con CEC=non-confluent corneal endothelial cells. Con-CEC=confluent corneal endothelial cells. S=stroma. Original magnification = 100X for EPI, 200X for ENDO, 800X for Non-con CEC, 400X for Con CEC.

## 4. Discussion

The present study provides evidence that SOV, a general phosphatase inhibitor, can both disrupt junctional integrity and promote limited cell cycle progression in confluent rat corneal endothelial cells both in culture and in *ex vivo* corneas. Results of our studies on cell-cell contact are consistent with those of others in which treatment with SOV interfered with normal cell-cell adhesion (Tsukita et al., 1991; Sorby and Ostman, 1996; Takahashi and Suzuki, 1996; Young et al., 2003). Since a phosphatase inhibitor was capable of disrupting cell-cell junctions, our results suggest that there must be a constitutive phosphatase activity in the confluent corneal endothelial monolayer that helps maintain the integrity of these junctions. Exposure of confluent cells to SOV also promoted limited cell cycle progression in the absence of serum, but was not sufficient to induce S-phase entry. The fact that phosphatase inhibition promoted at least limited cell cycle entry in the absence of serum strongly suggests that constitutive phosphatase activity must help mediate the suppression of cell cycle entry in the endothelial monolayer. We previously demonstrated the importance of cell-cell contacts in mediating inhibition of proliferation in confluent cultures of rat corneal endothelial cells (Joyce, Harris and Mello, 2002) and in *ex vivo* human corneal endothelium (Senoo et al., 2000b). The fact that SOV was able to both alter the integrity of cell-cell contacts and promote limited cell cycle progression strongly suggests that phosphatases are involved in regulating contact-induced inhibition of proliferation in corneal endothelium; however, further study will be needed to confirm this.

It is well known that protein tyrosine phosphorylation and dephosphorylation are important physiological mechanisms that control diverse cellular behaviors, such as cell growth, differentiation, adhesion, and migration (Pallen and Tong, 1991; Brady-Kalnay et al., 1993; Takahashi and Suzuki, 1996; Aicher et al., 1997; Petrone

and Sap, 2000). As such, our intriguing findings led us to initiate studies to identify PTPs expressed in rat corneal endothelial cells. This identification is an important first step in determining how the activity of specific phosphatases contributes to the regulation of contact inhibition in these cells. Our data indicates that PTP-mu, PTP1B, SHP-1, SHP-2, and PTEN are expressed in rat corneal endothelial cells both in culture and *in situ*.

The PTP family can be divided into receptor-like transmembrane proteins, such as PTP-mu, PTP- $\kappa$ , PTP- $\lambda$ , PTP-LAR, PTP $\alpha$  and CD45, and soluble cytosolic proteins, including SHP-1, SHP-2, and PTP1B (Barford et al., 1998). There are also dual-specificity phosphatases, such as PTEN, CDC25, and MAP kinase phosphatase-1, which catalyze the dephosphorylation of both protein serine/threonine and tyrosine kinases (Barford et al., 1998). We report here the identification of candidate PTPs that are members of each of these groups.

The receptor-like transmembrane PTP-mu and the non-receptor phosphatase, PTP1B can interact with proteins that comprise adhesion junctions, such as cadherins and catenins. Previous studies have demonstrated the expression of N-cadherin (Beebe and Coats, 2000; Ickes, et al. 2002) and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -catenin (plakoglobin) (Petroll, et al. 1999) in adhesion junctions of corneal endothelial cells, providing a rationale for examining the expression of these PTPs. PTP-mu interacts with E-, N- and R-cadherin (Brady-Kalnay et al., 1998). PTP1B has been shown to be essential for both N-cadherin and beta-1 integrin-mediated adhesion in some cell types (Balsamo et al., 1996; Balsamo et al., 1998; Pathre et al., 2001; Rhee et al., 2001). Immunolocalization of these PTPs in cross-sections of rat cornea indicated that PTP-mu and PTP1B are expressed in both the epithelium and endothelium, whereas, PTP-LAR was only expressed in the epithelium. Of interest was the observation of differences in the relative localization of PTP-mu and PTP1B in subconfluent and

confluent cells. Their peripheral localization in the intact endothelium suggests that both PTP-mu and PTP1B may be involved in the regulation of the integrity of junctional complexes.

The non-receptor PTPs, SHP-1 and SHP-2, as well as PTP1B, and the dual-specificity phosphatase, PTEN are among phosphatases known to help regulate proliferation in other cell types. Several positive growth factors, such as EGF, FGF, and PDGF, can trigger proliferation in corneal endothelial cells (Gospodarowicz et al., 1977; Kamiyama et al., 1995). SHP-1, SHP-2, and PTP1B help regulate the function of various growth factor receptors (Keilhack et al., 1998; Yu et al., 1998; Qi et al., 1999; Qu et al., 1999; Tonks, 2003). SHP-1 and PTP1B are generally considered negative regulators of cell signaling, while SHP-2 is largely considered a positive signal transducer (Marrero et al., 1998; Haj, 2002). PTEN has been shown to negatively regulate cell cycle progression through the PI3K/Akt pathway (Li et al., 1997; Besson et al., 1999; Paramio et al., 1999; Ramaswamy et al., 1999). All four of these PTPs were expressed in rat corneal endothelium *in situ* and in culture. Unlike with PTP-mu and PTP1B, SHP-1, SHP-2, and PTEN did not demonstrate a significant change in localization based on the state of confluence of the endothelial monolayer. The specific function of these PTPs in corneal endothelial cells needs to be examined.

To the best of our knowledge, this is the first study regarding phosphatase function and PTP expression in corneal endothelium. In summary, the results of our studies suggest that phosphatases play an important role in regulating the integrity of cell-cell junctions and cell proliferation in this tissue. We have identified five phosphatases, PTP-mu, PTP1B, SHP-1, SHP-2, and PTEN, that are expressed in rat corneal endothelium. These are most likely not the only phosphatases expressed in this tissue and their specific function in the endothelium remains to be elucidated. Regardless, this study has opened a fruitful avenue for future investigation and may

lead to a greater understanding of the specific mechanisms underlying regulation of contact-dependent inhibition in corneal endothelium.

## **ACKNOWLEDGEMENTS**

The authors wish to thank Ms. Rebecca Ickes for excellent technical assistance in early phases of this project.

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