

**PKC enhances tight junction barrier function of human nasal epithelial  
cells in primary culture by transcriptional regulation**

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**Running title:** PKC enhances barrier function in nasal epithelial cells

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**Abbreviations:** protein kinase C, PKC; 12-O-tetradecanoylphorbol-13-acetate, TPA;  
myristoylated alanine-rich C kinase substrate, MARCKS; mitogen-activated protein kinase,  
MAPK; transepithelial electrical resistance, TER; growth factor receptor, EGFR

## Abstract

The epithelium of upper respiratory tissues such as human nasal mucosa forms a continuous barrier via tight junctions, which is in part thought to be regulated through a protein kinase C (PKC) signaling pathway. To investigate the mechanisms of the regulation of PKC-mediated tight junction barrier function of human nasal epithelium in detail, primary human nasal epithelial cells were treated with the PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA). In primary human nasal epithelial cells, treatment with TPA led to not only activation of phosphorylation of PKC, myristoylated alanine-rich C kinase substrate (MARCKS) and mitogen-activated protein kinase (MAPK) but also expression of novel PKC- $\delta$ , PKC- $\theta$  and PKC- $\epsilon$ . Treatment with TPA increased transepithelial electrical resistance (TER), with tight junction barrier function more than four-fold that of the control, together with upregulation of tight junction proteins, occludin, ZO-1, ZO-2 and claudin-1 at the transcriptional level. Furthermore, it affected the subcellular localization of the tight junction proteins and the numbers of tight junction strands. The upregulation of barrier function and tight junction proteins was prevented by a panPKC inhibitor, and the inhibitors of PKC- $\delta$  and PKC- $\theta$  but not PKC- $\epsilon$ . In primary human nasal epithelial cells, transcriptional factors GATA-3 and -6 were detected by RT-PCR. The knockdown of GATA-3 using RNAi resulted in inhibition of upregulation of ZO-1 and ZO-2 by treatment with TPA. These results suggest that TPA induced PKC signaling enhances the barrier function of human nasal epithelial cells via transcriptional upregulation of tight junction proteins and the mechanisms may contribute to a drug delivery system.

## Introduction

The epithelial barrier of the upper respiratory tract, which is the first site of exposure to inhaled antigens, plays a crucial role in host defense in terms of innate immunity. The epithelium of the upper respiratory tract such as that of the nasal mucosa forms a continuous barrier against a wide variety of exogenous antigens (Herard et al., 1996; Van Kempen et al., 2000). The epithelial barrier is regulated in large part by the apicalmost intercellular junctions, referred to as tight junctions (Takano et al., 2005; Kurose et al., 2007; Koizumi et al., 2007).

Tight junctions, the most apical component of intercellular junctional complexes, separate the apical from the basolateral cell surface domains to establish cell polarity (performing the function of a fence). Tight junctions also possess a barrier function, inhibiting the flow of solutes and water through the paracellular space (Gumbiner et al., 1993). They form a particular netlike meshwork of fibrils created by the integral membrane proteins occludin and claudin, and members of the Ig superfamilies JAM and CAR (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004). Several peripheral membrane proteins, ZO-1, ZO-2, ZO-3, 7H6 antigen, cingulin, symplekin, Rab3B, Ras target AF-6, and ASIP, an atypical protein kinase C-interacting protein, have been reported (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004). More recently, tricellulin was identified at tricellular contacts where there are three epithelial cells and it has a barrier function (Ikenouchi et al., 2005). Furthermore, ZO-1 and ZO-2 can independently determine whether and where claudins are polymerized (Umeda et al., 2006).

Protein kinase C (PKC) is a family of serine-threonine kinases known to regulate epithelial barrier function (Andreeva et al., 2001; Seth et al., 2007; Tsukamoto and Nigam, 1999). PKC has been shown to induce both assembly and disassembly of tight junctions

depending on the cell type and conditions of activation (Andreeva et al., 2001; Stuart and Nigam, 1995). The activation of PKC causes an increase in permeability in the renal epithelial cell lines LLC-PK1 and MDCK (Ellis et al., 1991; Clarke et al., 2000), whereas it causes a decrease in permeability in the human colon carcinoma cell line HT29 (Sjo et al., 2003). Bryostatin enhances tight junction barrier function in T84 through a PKC signaling pathway (Yoo et al., 2003). PKC appears to regulate the subcellular localization, phosphorylation states, and transcription of several tight junction-associated proteins (Banan et al., 2005), although the isozyme specificity has not been clearly elucidated. At least 11 different isozymes of PKC are known. These can be subdivided in three classes according to their responsiveness to activators (Newton, 1997). The classic or conventional (cPKC) isozymes ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ) are both  $\text{Ca}^{2+}$ - and diacylglycerol (DAG) dependent. The novel (nPKC) isozymes ( $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\eta$ , and  $\mu$ ) are  $\text{Ca}^{2+}$  independent but DAG dependent. The atypical (aPKC) isozymes ( $\iota/\lambda$  and  $\zeta$ ) are neither  $\text{Ca}^{2+}$  nor DAG dependent. In the human intestinal epithelial cell lines HT-29 and Caco-2, stimulation with TLR2 ligands leads to activation of specific PKC isoforms PKC- $\alpha$  and PKC- $\delta$  and enhances barrier function through translocation of ZO-1 on activation (Cario et al., 2004).

Furthermore, activation of PKC by 12-O-tetradecanoylphorbol-13-acetate (TPA) causes increases in transcription of occludin, ZO-1, and claudin-1 in T84 cells and melanoma cells (Weiler et al., 2005; Leotlela et al., 2007). The claudin-2 promoter is activated by CDX2, HNF-1 $\alpha$  and GATA-4 in a cooperative manner (Escaffit et al., 2005). Although activation of PKC exerts its effect directly at the transcriptional level, the responsible transcription factors related to PKC activation remain unknown.

We previously reported that in the epithelium of human nasal mucosa from patients with allergic rhinitis, occludin, JAM-A, ZO-1 and claudin-1, -4, -7, -8, -12, -13, and -14 were

detected together with continuous tight junction strands that formed well-developed networks (Takano et al., 2005). However, the detailed mechanisms of regulation of tight junctions in human nasal epithelial cells remain unclear. In this study, to investigate the mechanisms of regulation of tight junctions through a PKC signaling pathway in primary cultures of human nasal epithelial cells were treated with TPA as a PKC activator. We found that short treatment with TPA greatly enhanced epithelial barrier function together with an increase in expression of occludin, ZO-1, ZO-2 and claudin-1 at the transcriptional level. When we focused on the transcriptional factor GATA-family to investigate the transcriptional mechanisms, upregulation of ZO-1 and ZO-2 by treatment with TPA was regulated by GATA-3 via a PKC signaling pathway.

## **Materials and methods**

### **Antibodies and inhibitors**

Rabbit polyclonal anti-occludin, anti-ZO-1, anti-ZO-2, anti-claudin-1, anti-claudin-7, and mouse monoclonal anti-occludin and anti-claudin-4 antibodies were obtained from Zymed Laboratories (San Francisco, CA). Rabbit polyclonal anti-phospho-panPKC, anti-phospho-MARCKS, and anti-phospho-MAPK (Thr202/Tyr204) and mouse monoclonal anti-phospho-threonine antibodies were obtained from Cell Signaling (Beverly, MA). Rabbit polyclonal anti-PKC- $\alpha$ , anti-PKC- $\gamma$ , anti-PKC- $\delta$ , anti-PKC- $\theta$ , anti-PKC- $\epsilon$  and mouse monoclonal anti-phospho-serine antibodies were obtained from BD Biosciences Pharmingen (San Diego, DA). Rabbit polyclonal anti-ERK1/2 antibodies were obtained from Promega (Madison, WI.). Rabbit polyclonal anti-actin was obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal anti-panPKC and mouse anti-GATA-3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

PKC inhibitor GF109203X, MAPK inhibitors PD98059 and U0126, p38 MAPK inhibitor SB203580, PI3K inhibitor LY294002 and epidermal growth factor receptor (EGFR) inhibitor PD153035, PKC- $\delta$  inhibitor rottlerin, PKC- $\theta$  inhibitor myristoylated PKC- $\theta$  pseudosubstrate peptide inhibitor, and PKC- $\varepsilon$  inhibitor PKC- $\varepsilon$  translocation inhibitor peptide were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA).

### **Human nasal mucosa tissues**

Human nasal mucosa tissues were obtained from patients with hypertrophic rhinitis and chronic sinusitis who underwent inferior nasal turbinectomy. Informed consent was obtained from all patients, and the study was approved by the ethics committee of Sapporo Medical University, the Sapporo Hospital of the Hokkaido Railway Company and the KKR Sapporo Medical Center Tonan Hospital.

### **Isolation and cell culture**

Human nasal mucosa tissues were minced into pieces 2-3 mm<sup>3</sup> in volume and washed with phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Clonetics Corp. San Diego, CA) four times. These tissue specimens were suspended in 10 ml of dispersing solution with 0.5  $\mu$ g/ml DNase I (Sigma) and 0.08 mg/ml Liberase Blenzyme 3 (Roche, Basel, Switzerland) in PBS and then incubated at 37°C for 20 min. The dissociated specimens were subsequently filtrated with 300  $\mu$ m mesh followed by filtration with 40  $\mu$ m mesh. After centrifugation at 1000  $\times$  g for 4 min, the cells were cultured in serum-free bronchial epithelial growth medium (BEBM, Clonetics Corp. San Diego, CA) supplemented with 0.5  $\mu$ g/ml hydrocortisone, 5  $\mu$ g/ml insulin, 10  $\mu$ g/ml transferrin, 0.5  $\mu$ g/ml epinephrine, 6.5  $\mu$ g/ml triiodothyronine, 50  $\mu$ g/ml gentamycin, 50  $\mu$ g/ml amphotericin B, 0.1

ng/ml retinoic acid, and 0.5 ng/ml epidermal growth factor (Clonetics Corp.), bovine pituitary extract (1% vol/vol, Pel-Freez Biologicals, Rogers, AR), 100 U/ml penicillin and 100 µg/ml streptomycin. The isolated human nasal epithelial cells were plated with modified BEBM medium containing 10% fetal bovine serum (FBS) (Cansera International, Ontario, Canada) on 35-mm or 60-mm culture dishes (Corning Glass Works, Corning, NY, USA), which were coated with rat tail collagen (500 µg dried tendon/ml 0.1% acetic acid) in a humidified, 5% CO<sub>2</sub>:95% air incubator at 37°C (Kurose et al., 2007; Koizumi et al., 2007). The 1st-passaged cells using 0.05% trypsin-EDTA (Sigma) were used at day 7 after plating for the experiments.

The cells were treated with 10 or 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma) until 6 h. Some cells were treated with 2 µM GF109203, 10 µM PD98059, 20 µM U0126, 2 µM PD153035, 10 µM SB203580, 10 µM LY294002, 15 µM rottlerin, 5 µM myristoylated PKC-θ pseudosubstrate peptide inhibitor, or 10 µM PKC-ε translocation inhibitor peptide for 2 h before treatment with 100 nM TPA.

### **Transfection with siRNA**

Stealth siRNA duplex oligonucleotides against human GATA-3 were synthesized by Invitrogen (Carlsbad, CA). The sequences were as follows: GATA-3 RNAi, sense (5'-AUAUUGUGAAGCUUGUAGUAGAGCC-3') and antisense (5'-GGCUCUACUACAAGCUUCACAAUAU-3'). The cells were passaged at subconfluence and plated 1 day before transfection. The cells were transfected with 100 pM siRNA of GATA-3 using lipofectamine RNAiMAX (Invitrogen). The cells at 48 h after transfection were treated with 100 nM TPA for 1 h and examined for Western blot and RT-PCR analyses.

### **Measurement of transepithelial electrical resistance (TER)**

The cells were cultured to confluence on inner chambers of 12 mm Transwell inserts with 0.4  $\mu\text{m}$  pore-size filters. TER was measured using an EVOM voltameter with an ENDOHM-12 (World Precision Instruments) on a heating plate (Fine, Tokyo, Japan) adjusted to 37°C. The values are expressed in standard units of ohms per square centimeter and presented as the mean  $\pm$  SD. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

### **Measurement of permeability**

To determine the paracellular flux, the cells were cultured on 12 mm Transwell, 0.4  $\mu\text{m}$  pore-size filters (Corning Inc.), and then FITC-labeled dextran (MW: 70 kDa)-containing medium was added to the inner chamber. Samples were collected from the outer chamber at 15, 30, 60 and 120 min and were measured with a Wallac 1420 multilabeled counter (PerkinElmer, Turku, Finland).

### **Western blot analysis**

The cells grown on the inner chambers were scraped in 300  $\mu\text{l}$  of buffer (1 mM  $\text{NaHCO}_3$  and 2 mM phenylmethylsulfonyl fluoride), collected in microcentrifuge tubes and then sonicated for 10 s. The protein concentrations of the samples were determined using a BCA Protein Assay Reagent Kit (Pierce Chemical Co, Rockford, IL, USA). Aliquots of 15  $\mu\text{g}$  protein/lane for each sample were separated by electrophoresis in 4/20% SDS polyacrylamide gels (Daiichi Pure Chemicals Co., Tokyo, Japan). After electrophoretic transfer to a nitrocellulose membrane (Immobilon; Millipore), the membrane was saturated for 30 min at room temperature with blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20 and 4% skim milk) and incubated with anti-phospho-panPKC, anti-phospho-MARCKs,

anti-phospho-MAPK, anti-ERK1/2, anti-PKC- $\alpha$ , anti-PKC- $\gamma$ , anti-PKC- $\delta$ , anti-PKC- $\theta$ , anti-PKC- $\epsilon$ , anti-occludin, anti-ZO-1, anti-ZO-2, anti-claudin-1, anti-claudin-4, anti-claudin-7, anti-GATA3, and anti-actin antibodies at room temperature for 1 h. The membrane was incubated with HRP-conjugated anti-rabbit or mouse IgG (DAKO, Copenhagen, Denmark) at room temperature for 1 h. The immunoreactive bands were detected using an ECL Western blotting system (Amersham Corp., Buckinghamshire, UK).

### **Immunoprecipitation**

The dishes were washed with PBS twice, and 300  $\mu$ l of NP-40 lysis buffer (50 mM Tris-HCl, 2% NP-40, 0.25 mM Na-deoxycholate, 150 mM NaCl, 2 mM EGTA, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, and 2 mM PMSF) was added to 60 mm dishes. The cells were scraped and collected in microcentrifuge tubes and then sonicated for 10 s. Cell lysates were incubated with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden) for 1 h at 4°C and then clarified by centrifugation at 15,000  $\times$ g for 10 min. The supernatants were incubated with the polyclonal anti-occludin antibody bound to protein A-Sepharose CL-4B overnight at 4°C. After incubation, immunoprecipitates were washed extensively with the same lysis buffer and were subjected to Western blot analysis using anti-phospho-serine and anti-phospho-threonine antibodies.

### **RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis**

Total RNA was extracted and purified using Trizol (Invitrogen). One microgram of total RNA was reverse transcribed (RT) into cDNA using a mixture of oligo (dT) and Superscript<sup>TM</sup> II RTase under the recommended conditions (Invitrogen). Synthesis of each cDNA was performed in a total volume of 20  $\mu$ l for 50 min at 42°C and terminated by

incubation for 15 min at 70°C. PCR containing 100 pM primer pairs and 1.0 ml of the 20 ml total RT reaction mixture was performed in 20 ml of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5mM MgCl<sub>2</sub>, 0.4 mM dNTPs, and 0.5 U of Taq DNA polymerase (Takara), employing 25 or 30 cycles with cycle times of 15 s at 96°C, 30 s at 55°C, and 60 s at 72°C. Final elongation time was 7 min at 72°C. Ten microliters of the 20 µl total PCR reaction mixture was analyzed in 1% agarose gel after staining with ethidium bromide. The PCR primers used to detect occludin, ZO-1, ZO-2, claudin-1, -4, -7, GATA-1, -2, -3, -4, -5, -6, and glucose-3-phosphate dehydrogenase (G3PDH) are indicated in Table 1.

### **Immunocytochemistry**

The cells grown on inner chambers were fixed with cold absolute acetone or a 1:1 ethanol and acetone mixture at -20°C for 10 min. The cells were stained with anti-occludin, anti-ZO-1, anti-ZO-2 and anti-claudin-1 antibodies overnight at 4°C. Alexa 488 (green)-conjugated anti-rabbit IgG and Alexa 592 (red)-conjugated anti-mouse IgG (Molecular Probes Inc., Eugene, OR) were used as secondary antibodies. The specimens were examined and photographed with an Axioskop 2 plus microscope (Carl Zeiss, Germany) and confocal laser scanning microscope (MRC 1024; Bio-Rad, Hercules, CA). Phase-contrast photomicrographs were taken with a Zeiss Axiovert 200 inverted microscope.

### **Freeze-fracture analysis**

For freeze-fracture experiments, primary cultured human nasal epithelial cells grown on 60 mm dishes were centrifuged into pellets and then immersed in 40% glycerin solution after fixation in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.3). The specimens were mounted on a copper stage, frozen in liquid nitrogen, fractured at -150°C to -160°C, and

replicated by platinum/carbon from an electron beam gun positioned at a 45° angle, followed by carbon applied from overhead in a JFD-7000 freeze-fracture device (JEOL, Tokyo, Japan). After the replicas were thawed, they were floated on filtered 10% sodium hypochlorite solution for 10 min in a Teflon dish. Replicas were washed in distilled water for 30 min, mounted on copper grids, and examined at 80 kV using a JEOL 1200EX transmission electron microscope (JEOL, Tokyo, Japan). Morphometrical analysis was performed on freeze-fracture replica images of tight junctions, which were printed at a final magnification of 20,000×. The mean number of tight junction strands was determined by taking numerous counts along a line drawn perpendicular to the junctional axis at 200 nm intervals (Stevenson et al. 1988).

### **Data analysis**

Signals were quantified using Scion Image Beta 4.02 Win (Scion Corporation, Frederick, MI, USA). Each set of results shown is representative of three separate experiments. Results are given as means ± SEM. Differences between groups were tested by the two-tailed Student's t test for unpaired data.

### **Results**

#### **TPA enhances tight junction barrier function in primary cultures of human nasal epithelial cells**

PKC is a family of serine-threonine kinases known to regulate epithelial barrier function (Andreeva et al., 2001; Seth et al., 2007; Tsukamoto and Nigam, 1999). To investigate effects of the PKC activator TPA on tight junction barrier function of human nasal epithelial cells, primary cultures of human nasal epithelial cells at day 7 after plating were

treated with 10 or 100 nM TPA and then examined for TER and paracellular flux of FITC-labeled dextran (MW: 70 kDa).

In primary human nasal epithelial cells cultured with 10% FBS at day 7 after plating, the maximum value of TER was  $200 \pm 20 \Omega\text{cm}^2$ . The TER was markedly increased beginning from 1 h after treatment with 10 or 100 nM TPA in a dose-dependent manner (Fig. 1A). The TER values from 2 h to 6 h after treatment with 100 nM TPA were more than fourfold those of the control (Fig. 1A). The TER value at 24 h after treatment with 10 or 100 nM TPA recovered to the level of the control (data not shown).

When the paracellular flux of FITC-labeled dextran (70 kDa) was measured in the cells at 2 h after treatment with 100 nM TPA, in which the TER was more than fourfold compared to the control, the paracellular flux after treatment with TPA was significantly decreased compared to the control (Fig. 1B). The paracellular flux at 2 h after treatment with 10 nM TPA was also decreased compared to the control and similar to that of 100 nM TPA (supplemental data 1)

### **Increase of tight junction proteins at levels of protein and mRNA by treatment with TPA**

We previously reported expression patterns of tight junction proteins in primary cultures of human nasal epithelial cells using this experiment (Kurose et al., 2007; Koizumi et al., 2007). In primary human nasal epithelial cells at 1 h and 2 h after treatment with 100 nM TPA, in which a marked increase of TER was observed (Fig. 1A), we investigated the changes in expression of tight junction proteins occludin, ZO-1, ZO-2, claudin-1, -4, and -7 using Western blot and RT-PCR analyses. In Western blotting, upregulation in proteins of occludin, ZO-1, ZO-2 and claudin-1, but not claudin-4 and -7, was observed at 1 h and 2 h after treatment with TPA (Fig. 2A, 2C). In RT-PCR, upregulation of mRNAs of occludin, ZO-1,

ZO-2, claudin-1 and -4, but not claudin-7, was observed at 1 h and 2 h after treatment with 100 nM TPA (Fig. 2B, 2D). No changes in expression of claudin-2, -8, -9 and -12 and JAM-A, which were detected in primary cultures of human nasal epithelial cells, were observed (data not shown).

### **Changes in distribution of tight junction proteins by treatment with TPA**

To investigate changes in localization of tight junction proteins in the cells at 2 h after treatment with 100 nM TPA, we performed immunocytochemistry for occludin, ZO-1, ZO-2 and claudin-1. In the control at day 5 after plating, discontinuous lines of occludin, ZO-1 and ZO-2 and weak expression of claudin-1 were observed in some cells (Fig. 3A, 3C, 3E, 3G). In almost cells after treatment with TPA, expression of occludin, ZO-1 and ZO-2 and claudin-1 was observed as continuous wide lines at cell borders (Fig. 3B, 3D, 3F, 3H).

### **Changes of tight junction strands induced by treatment with TPA**

To investigate changes of tight junction strands in the cells at 2 h after treatment with 100 nM TPA, we performed freeze–fracture analysis. In the control, a network composed of several continuous tight junction strands was observed (Fig. 3I). In the cells after treatment with TPA, the mean number of tight junction strands was increased compared to the control and a well-developed network of tight junction strands was observed (Fig. 3J, 3K).

### **Changes of PKC- and MAPK-associated proteins induced by treatment with TPA**

There are at least 11 different isozymes of PKC classified into three broad groups (Newton, 1997). To investigate which specific PKC isozymes affect expression and function of tight junction proteins, we performed Western blotting to examine the expression of five

PKC isoforms PKC- $\alpha$ , PKC- $\gamma$ , PKC- $\delta$ , PKC- $\theta$  and PKC- $\varepsilon$  and the phosphorylation status of PKC, MARCKs, and MAPK in the cells at 1 h and 2 h after treatment with 100 nM TPA. In the cells at 1 h and 2 h after treatment with TPA, increases in expression of PKC- $\delta$ , PKC- $\theta$  and PKC- $\varepsilon$  and activation of phosphorylation of panPKC, MARCKS and MAPK, were observed (Fig. 4B, 4D).

### **PKC inhibitors but not MAPK and EGFR inhibitors prevents increase of barrier function induced by treatment with TPA**

It is known that TPA induces phosphorylation of MAPK through not only phosphorylation of PKC but also EGFR (Barbosa et al., 2003). TPA is involved in multiple signal transduction pathways including JNK and PI3K (Yu et al., 2006; Huang et al., 1997). To investigate which signaling pathways affected the increase of barrier function measured as TER in the cells after treatment with TPA, we used PKC inhibitor GF109203X, MAPK inhibitors PD98059 and U0126, and EGFR inhibitor PD153035. Treatment with GF109203X, but not PD98059, U0126 or PD153035, completely prevented the marked increase of TER values at 2 h after treatment with 100 nM TPA (Fig. 5A, supplemental data 1). Although p38 MAPK inhibitor SB203580 and PI3K inhibitor LY294002 prevented upregulation of TER after treatment with TPA, the effect of the inhibition was weak (Fig. 5B).

### **PKC inhibitor prevents an increase in expression of tight junction proteins by treatment with TPA**

We investigated changes in expression of tight junction proteins and PKC isozymes in the cells treated with the PKC inhibitor GF109203X before pretreatment with TPA. Treatment with GF109203X inhibited upregulation of occludin, ZO-1, ZO-2 and claudin-1 at

the levels of protein and mRNA at 2 h after treatment with 100 nM TPA. (Fig. 6A, 6B, 6C, 6D). Furthermore, the increase of claudin-4 mRNA after treatment with TPA was also inhibited by treatment with GF109203X (Fig. 6B, 6D). Treatment with GF109203X inhibited increases in expression of PKC- $\delta$ , PKC- $\theta$  and PKC- $\epsilon$  at 2 h after treatment with 100 nM TPA (Fig. 7A, 7C).

Furthermore, we investigated changes in expression of tight junction proteins in the cells treated with PKC- $\delta$  inhibitor rottlerin, PKC- $\theta$  inhibitor myristoylated PKC- $\theta$  pseudosubstrate peptide inhibitor, and PKC- $\epsilon$  inhibitor PKC- $\epsilon$  translocation inhibitor peptide before pretreatment with TPA. Treatment with inhibitors of PKC- $\delta$  and PKC- $\theta$  but not PKC- $\epsilon$  prevented upregulation of occludin, ZO-1, ZO-2 and claudin-1 at 2 h after treatment with 100 nM TPA. (Fig. 7B, 7D).

### **Transcriptional factor GATA-3 upregulates tight junction proteins after treatment with TPA**

Tight junction proteins are regulated by various transcription factors in a cooperative manner (Escaffit et al., 2005). Transcriptional factor GATA-3 is associated with differentiation of the luminal cells in the mammary gland (Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007). When we investigated expression of GATA-1, -2, -3, -4, -5 and -6 in primary cultures of human nasal epithelial cells by RT-PCR, mRNAs of GATA-3 and -6 were detected (Fig. 8A). Upregulation of mRNAs of GATA-3 and -6 was observed at 1 h but not 2 h after treatment with 100 nM TPA (Fig. 8A). Treatment with the PKC inhibitor GF109203X prevented upregulation of mRNAs of GATA-3 but not GATA-6 at 1 h after treatment with 100 nM TPA (Fig. 8B).

To investigate whether GATA-3 was an essential transcriptional factor for upregulation of tight junction proteins in the cells after treatment with TPA, GATA-3 was

downregulated by its siRNA of GATA-3 before treatment with 100 nM TPA. Transfection with siRNA of GATA-3 decreased expression of GATA-3 at the levels of protein and mRNA, and prevented upregulation of proteins and mRNAs of ZO-1 and ZO-2 but not occludin and claudin-1 at 1 h after treatment with 100 nM TPA (Fig. 8C, 8D, 8E, 8F).

## Discussion

The PKC family of serine-threonine kinases is known to regulate epithelial barrier function via tight junctions (Andreeva et al., 2001; Seth et al., 2007; Tsukamoto et al., 1999). PKC has been shown to induce both assembly and disassembly of tight junctions depending on the cell type and conditions of activation (Andreeva et al., 2001; Stuart and Nigam, 1995). In this study, TPA-induced nPKC signaling greatly enhanced the barrier function of human nasal epithelial cells in primary culture together with an increase in expression of tight junction-associated proteins, occludin, ZO-1, ZO-2 and claudin-1 at the transcriptional level. The transcriptional upregulation of ZO-1 and ZO-2 was controlled via transcriptional factor GATA-3.

PKC appears to regulate the subcellular localization, phosphorylation states, and transcription of several tight junction-associated proteins, although the PKC isozyme specificity has not been clearly elucidated (Banan et al., 2005). cPKC- $\alpha$  participates in tight junction disassembly, whereas nPKC- $\epsilon$  plays a role in tight junction formation of kidney epithelial cells (Andreeva et al., 2006). In the human intestinal epithelial cell lines HT-29 and Caco-2, stimulation with TLR2 ligands leads to activation of specific PKC isoforms PKC- $\alpha$  and PKC- $\delta$  and enhances barrier function through translocation of ZO-1 on activation (Cario et al., 2004). In the primary human nasal primary cells of the present study, treatment with TPA induced expression of specific nPKC- $\delta$ , - $\epsilon$  and - $\theta$  and greatly enhanced tight junction

barrier function together with increases in expression of occludin, ZO-1, ZO-2 and claudin-1, whereas the pan-PKC inhibitor GF109230X prevented all the changes. Furthermore, treatment with inhibitors of nPKC- $\delta$  and - $\theta$  but not - $\epsilon$  prevented the upregulation of tight junction proteins. Treatment with TPA affected the subcellular localization of the tight junction proteins together with an increase of tight junction strands. These indicate that in human nasal epithelial cells in primary culture, TPA induced nPKC- $\delta$  and - $\theta$  may enhance tight junction barrier function by assembly of tight junctions.

It is reported that activation of PKC by TPA causes increases in transcription of occludin, ZO-1, and claudin-1 in T84 cells and melanoma cells (Weiler et al., 2005; Leotlela et al., 2007). In the primary human nasal epithelial cells used in the present study, activation of PKC by TPA increased expression of occludin, ZO-1, -2, and claudin-1 at the transcriptional level. Although activation of PKC exerts its effect on transcription directly, the responsible transcription factors related to PKC activation remain unknown. The claudin-2 promoter is activated by CDX2, HNF-1 $\alpha$  and GATA-4 in a cooperative manner (Escaffit et al., 2005). Tight junctions are regulated via direct repression of the gene expression of claudins/occludin by Snail during epithelial to mesenchymal transition (Ikenouchi et al., 2003). In this study, to investigate the transcriptional mechanisms, we focused on the transcriptional factor GATA family. The GATA family, included in zinc finger transcription factors, has been recognized in the differentiation of the endoderm in several evolutionarily diverse organisms (Reiter et al., 2001). They form an ancient family of transcription factors, that evolved into six related factors in mammals (Clarke and Berg, 1998). GATA-1, -2 and -3 are predominantly associated with the hematopoietic cell lineage, whereas GATA-4, -5 and -6 are mainly associated with development of endodermally derived organs such as the liver, lung, pancreas and heart (Lieuw et al., 1997). More recently, it has been reported that

GATA-3 is associated with differentiation of the luminal cells in the mammary gland not only in embryos but also in adults (Kouros-Mehr et al., 2006; Asselin-Labat et al., 2007). In the primary human nasal epithelial cells of the present study, GATA-3 and -6 were detected by RT-PCR and their expression was increased by treatment with TPA. The PKC inhibitor GF109230X prevented upregulation of GATA-3 but not GATA-6. Furthermore, knockdown of GATA-3 by its RNAi inhibited upregulation of expression of ZO-1 and ZO-2 via the PKC signaling pathway induced by treatment with TPA. These results suggest that in human nasal epithelial cells, GATA-3 is one of the responsible transcription factors related to PKC activation induced by treatment with TPA and may in part regulate ZO-1 and ZO-2. ZO-1 and ZO-2 are membrane-associated guanylate kinase homologues (MAGUKs) that can bind to transmembrane tight junction proteins, occludin, claudins and JAMs, the cytoskeleton and signal transduction molecules (Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004). Furthermore, ZO-1 and ZO-2 are closely associated with polymerization of claudins (Umeda et al., 2006). In this study, it is possible that upregulation of the expression of ZO-1 and ZO-2 might have indirectly affected the expression and localization of occludin and claudin-1.

Although TPA is a typical PKC activator, it induces phosphorylation of MAPK through not only phosphorylation of PKC but also that of EGFR (Barbosa et al., 2003). Furthermore, TPA is involved in multiple signal transduction pathways including JNK and PI3K (Yu et al., 2006; Huang et al., 1997). In primary human nasal epithelial cells, to investigate which signaling pathways mainly affect upregulation of barrier function by treatment with TPA, we used PKC inhibitor GF109203X, MAPK inhibitor PD98059, EGFR inhibitor PD153035, p38MAPK inhibitor SB203580 and PI3K inhibitor LY294002. Treatment with the PKC inhibitor, but not inhibitors of MAPK and EGFR, completely

prevented upregulation of barrier function measured as TER values. Furthermore, inhibitors of p38 MAPK and PI3K also prevented upregulation of barrier function by treatment with TPA, though the effect was weak. These results suggested that the increase of barrier function induced by treatment with TPA in human nasal epithelial cells was directly regulated by a PKC pathway, not via MAPK pathway, and was indirectly controlled by distinct signaling pathways including p38 MAPK and PI3K.

TPA induces the rapid phosphorylation of occludin in MDCK cells cultured in low extracellular calcium medium with concomitant translocation of occludin to the regions of cell-cell contact (Andreeva et al., 2001). Phosphorylation of claudin-4 via PKC- $\epsilon$  by treatment with TPA regulates tight junction barrier function in ovarian cancer cells (Souza et al., 2007). PKC- $\theta$  alters barrier function through changes in phosphorylation and cellular localization of claudin-1 and -4 in Caco-2 (Banan et al., 2005). In the human nasal epithelial cells used in the present study, treatment with TPA slightly increased activities of serine- and threonine-phosphorylation of occludin protein (supplemental data 2). The serine- and threonine-phosphorylation of occludin leads to upregulate barrier function (Andreeva et al., 2001; Seth et al., 2007). In this study, not only an increase in expression of tight junction proteins but also serine- and threonine-phosphorylation of occludin might have contributed to the enhanced tight junction barrier function of human nasal epithelial cells.

PKC regulates a number of fundamental processes such as membrane trafficking, cytoskeletal organization, ion transport, cell growth, and differentiation (Venkatachalam et al., 2004). In the human nasal epithelial cells of the present study, treatment with TPA caused phosphorylation of the endogenous PKC substrate MARCKS, which serves as an actin cross-linking protein (Hartwig et al., 1992), potentially participating in modulation of epithelial integrity. It is possible that PKC-mediated human nasal epithelial barrier

enhancement results from not only upregulation of tight junction proteins but also potential convergence of various signaling interactions and pathways downstream of PKC, which may interact by bridging to the actin cytoskeleton.

In conclusion, the tight junction barrier function of human nasal epithelial cells is in part upregulated by transcriptional factor GATA-3 via an nPKC signaling pathway and the PKC-enhanced epithelial tight junction barrier may play a crucial role in innate immunity against a wide variety of exogenous antigens. The upregulation of human nasal epithelial cells via the nPKC signaling pathway may also be caused by the stimuli such as the cigarette smoke and various dusts (Wyatt et al., 1999; Romberger et al., 2002). Furthermore, the regulation of PKC signaling may contribute to a drug delivery system via human nasal epithelium.

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## References

- Andreeva AY, Krause E, Muller EC, Blasig IE, and Utepbergenov DI (2001) Protein kinase C regulates the phosphorylation and cellular localization of occludin. *J Biol Chem* **276**:38480-38486.
- Andreeva AY, Piontek J, Blasig IE, and Utepbergenov DI (2006) Assembly of tight junction is regulated by the antagonism of conventional and novel protein kinase C isoforms. *Int J Biochem Cell Biol* **38**:222-233.
- Asselin-Labat ML, Sutherland KD, Barker H, Thomas R, Shackleton M, Forrest NC, Hartley L, Robb L, Grosveld FG, van der Wees J, Lindeman GJ, and Visvader JE (2007) Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat Cell Biol* **9**:201-209.
- Banan A, Zhang LJ, Shaikh M, Fields JZ, Choudhary S, Forsyth CB, Farhadi A, and Keshavarzian A (2005) theta Isoform of protein kinase C alters barrier function in intestinal epithelium through modulation of distinct claudin isotypes: a novel mechanism for regulation of permeability. *J Pharmacol Exp Ther* **313**:962-982.
- Barbosa LA, Goto-Silva L, Redondo PA, Oliveira S, Montesano G, De Souza W, and Morgado-Diaz JA (2003) TPA-induced signal transduction: a link between PKC and EGFR signaling modulates the assembly of intercellular junctions in Caco-2 cells. *Cell Tissue Res* **312**:319-331.
- Cario E, Gerken G, and Podolsky DK (2004) Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C. *Gastroenterology* **127**:224-238.
- Clarke ND, and Berg JM (1998) Zinc fingers in *Caenorhabditis elegans*: finding families and probing pathways. *Science* **282**:2018-2022.
- Clarke H, Soler AP, and Mullin JM (2000) Protein kinase C activation leads to

- dephosphorylation of occludin and tight junction permeability increase in LLC-PK1 epithelial cell sheets. *J Cell Sci* **113**:3187-3196.
- Ellis B, Schneeberger EE, and Rabito CA (1991) Cellular variability in the development of tight junctions after activation of protein kinase C. *Am J Physiol* **263**:F293-300.
- Escaffit F, Boudreau F, and Beaulieu JF (2005) Differential expression of claudin-2 along the human intestine: Implication of GATA-4 in the maintenance of claudin-2 in differentiating cells. *J Cell Physiol* **203**:15-26.
- Gumbiner B (1993) Breaking through the tight junction barrier. *J. Cell Biol* **123**:1631-1633.
- Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, and Aderem A (1992) MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* **356**:618-622.
- Herard AL, Zahm JM, Pierrot D, Hinnrasky J, Fuchey C, and Puchelle E (1996) Epithelial barrier integrity during in vitro wound repair of the airway epithelium. *Am J Respir Cell Mol Biol* **15**:624-632
- Huang C, Schmid PC, Ma WY, Schmid HH, and Dong Z (1997) Phosphatidylinositol-3 kinase is necessary for 12-O-tetradecanoylphorbol-13-acetate-induced cell transformation and activated protein 1 activation. *J Biol Chem* **272**:4187-4194.
- Ikenouchi J, Furuse M, Furuse K, Sasaki H, Tsukita S, and Tsukita S (2005) Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells. *J Cell Biol* **171**:939-945.
- Ikenouchi J, Matsuda M, Furuse M, and Tsukita S (2003) Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci* **116**:1959-1967.
- Koizumi JI, Kojima T, Kamekura R, Kurose M, Harimaya A, Murata M, Osanai M, Chiba H, Himi T, and Sawada N (2007) Changes of gap and tight junctions during differentiation of

human nasal epithelial cells using primary human nasal epithelial cells and primary human nasal fibroblast cells in a noncontact coculture system. *J Membr Biol* **218**:1-7.

Kurose M, Kojima T, Koizumi JI, Kamekura R, Ninomiya T, Murata M, Ichimiya S, Osanai M, Chiba H, Himi T, and Sawada N (2007) Induction of claudins in passaged hTERT-transfected human nasal epithelial cells with an extended life span. *Cell Tissue Res* **330**:63-74.

Kouros-Mehr H, Slorach EM, Sternlicht MD, and Werb Z (2006) GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. *Cell* **127**:1041-1055.

Leotlela PD, Wade MS, Duray PH, Rhode MJ, Brown HF, Rosenthal DT, Dissanayake SK, Earley R, Indig FE, Nickoloff BJ, Taub DD, Kallioniemi OP, Meltzer P, Morin PJ, and Weeraratna AT (2007) Claudin-1 overexpression in melanoma is regulated by PKC and contributes to melanoma cell motility. *Oncogene* **26**:3846-3856.

Lieuw KH, Li G, Zhou Y, Grosveld F, and Engel JD (1997) Temporal and spatial control of murine GATA-3 transcription by promoter-proximal regulatory elements. *Dev Biol* **188**:1-16.

Newton AC (1997) Regulation of protein kinase C. *Curr Opin Cell Biol* **9**:161-167.

Reiter JF, Kikuchi Y, and Stainier DY (2001) Multiple roles for Gata5 in zebrafish endoderm formation. *Development* **128**:125-135.

Romberger DJ, Bodlak V, Von Essen SG, Mathisen T, and Wyatt TA (2002) Hog barn dust extract stimulates IL-8 and IL-6 release in human bronchial epithelial cells via PKC activation. *J Appl Physiol* **93**:289-296.

Sawada N, Murata M, Kikuchi K, Osanai M, Tobioka H, Kojima T, and Chiba H (2003) Tight junctions and human diseases. *Med. Electron Microsc* **36**:147-156.

Seth A, Sheth P, Elias BC, and Rao R (2007) Protein phosphatases 2A and 1 interact with

- occludin and negatively regulate the assembly of tight junctions in the CACO-2 cell monolayer. *J Biol Chem* **282**:11487-11498.
- Schneeberger EE, and Lynch RD (2004) The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol* **286**:C1213-1228.
- Sjo A, Magnusson KE, and Peterson KH (2003) Distinct effects of protein kinase C on the barrier function at different developmental stages. *Biosci Rep* **23**:87-102.
- D'Souza T, Indig FE, and Morin PJ (2007) Phosphorylation of claudin-4 by PKCepsilon regulates tight junction barrier function in ovarian cancer cells. *Exp Cell Res* **313**:3364-3375.
- Stevenson BR, Anderson JM, Goodenough DA, and Mooseker MS (1988) Tight junction structure and ZO-1 content are identical in two strains of Madin-Darby canine kidney cells, which differ in transepithelial resistance. *J Cell Biol* **107**:2401-2408
- Stuart RO, and Nigam SK (1995) Regulated assembly of tight junctions by protein kinase C. *Proc Natl Acad Sci USA* **92**:6072-6076.
- Takano K, Kojima T, Go M, Murata M, Ichimiya S, Himi T, and Sawada N (2005) HLA-DR- and CD11c-positive dendritic cells penetrate beyond well-developed epithelial tight junctions in human nasal mucosa of allergic rhinitis. *J Histochem Cytochem* **53**:611-619.
- Tsukamoto T, and Nigam SK (1999) Role of tyrosine phosphorylation in the reassembly of occludin and other tight junction proteins. *Am J Physiol* **276**:F737-750.
- Tsukita S, Furuse M, and Itoh M (2001) Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol* **2**:285-293.
- Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M, and Tsukita S (2006) ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* **126**:741-754.

- van Kempen MJ, Rijkers GT, and Van Cauwenberge PB (2000) The immune response in adenoids and tonsils. *Int Arch Allergy Immunol* **122**:8-19.
- Venkatachalam K, Zheng F, and Gill DL (2004) Control of TRPC and store-operated channels by protein kinase C. *Novartis Found Symp* **258**:172-185
- Weiler F, Marbe T, Scheppach W, and Schaubert J (2005) Influence of protein kinase C on transcription of the tight junction elements ZO-1 and occludin. *J Cell Physiol* **204**:83-86.
- Wyatt TA, Heires AJ, Sanderson SD, and Floreani AA (1999) Protein kinase C activation is required for cigarette smoke-enhanced C5a-mediated release of interleukin-8 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* **21**:283-288.
- Yoo JW, Kim YS, Lee SH, Lee MK, Roh HJ, Jhun BH, Lee CH, and Kim DD (2003) Serially passaged human nasal epithelial cell monolayer for in vitro drug transport studies. *Pharm Res* **20**:1690-1696.
- Yu X, Luo A, Zhou C, Ding F, Wu M, Zhan Q, and Liu Z (2006) Differentiation-associated genes regulated by TPA-induced c-Jun expression via a PKC/JNK pathway in KYSE450 cells. *Biochem Biophys Res Commun* **342**:286-292.

### **Footnotes**

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### Figure legends

**Fig. 1.** Barrier function measured as TER (A) and paracellular flux using FITC-dextran (70 kDa) in primary human nasal epithelial cells treated with TPA. (A): The TER was markedly increased beginning from 1 h to 6 h after treatment with 10 or 100 nM TPA in a dose-dependent manner.  $n=6$ .  $** p < 0.01$  versus control. (B) Paracellular flux of FITC-dextran of 70 kDa was significantly decreased after treatment with 100 nM TPA compared to the control.  $n=6$ .  $* p < 0.05$  versus control.

**Fig. 2.** Western blotting (A) and RT-PCR (B) for occludin, ZO-1, ZO-2, claudin-1, -4, and -7 in primary human nasal epithelial cells at 1 h and 2 h after treatment with 100 nM TPA. (A): Upregulation of proteins of occludin, ZO-1, ZO-2 and claudin-1, but not claudin-4 and -7, is observed at 1 h and 2 h after treatment with 100 nM TPA. (B) Upregulation of mRNAs of occludin, ZO-1, ZO-2, claudin-1 and -4, but not claudin-7, is observed at 1 h and 2 h after treatment with 100 nM TPA. (C, D): The corresponding expression levels are shown as bar graphs.

**Fig. 3.** Immunocytochemistry for occludin (A, B), ZO-1 (C, D), ZO-2 (E, F) and claudin-1 (G, H) in primary human nasal epithelial cells treated with (B, D, F, H) and without (A, C, E, G) TPA. In almost cells after treatment with TPA, expression of occludin, ZO-1 and ZO-2 and claudin-1 is observed as distinct continuous lines at cell borders, whereas in the control, discontinuous lines of occludin, ZO-1 and ZO-2 and weak expression of claudin-1 are observed in some cells. Bar: 20  $\mu\text{m}$  in H. Freeze-fracture replicas of primary human nasal epithelial cells treated with (J) and without (I) TPA. Bar: 50 nm in J. (K): Morphometrical

analysis of tight junction strands is shown as a bar graph. In the cells after treatment with TPA, the numbers of tight junction strands are increased compared to control.

**Fig. 4.** (A) Western blotting for phospho-panPKC, phospho-MARCKS and phospho-MAPK in primary human nasal epithelial cells at 1 h and 2 h after treatment with 100 nM TPA. In the cells at 1 h and 2 h after treatment with TPA, activation of phosphorylation of panPKC, MARCKS and MAPK is observed. (B) Western blotting for PKC- $\alpha$ , PKC- $\gamma$ , PKC- $\delta$ , PKC- $\theta$  and PKC- $\epsilon$  in primary human nasal epithelial cells at 1 h and 2 h after treatment with 100 nM TPA. In the cells at 1 h and 2 h after treatment with TPA, increases in expression of PKC- $\delta$ , PKC- $\theta$  and PKC- $\epsilon$  are observed. (C, D): The corresponding expression levels are shown as bar graphs.

**Fig. 5.** Barrier function measured as TER in primary human nasal epithelial cells treated with inhibitors before treatment with TPA. (A) Treatment with PKC inhibitor GF109203X but not MAPK inhibitor PD98059 or EGFR inhibitor PD153035 prevented an increase of TER values at 2 h after treatment with 100 nM TPA.  $**p < 0.01$  versus control. (B) p38 MAPK inhibitor SB203580 and PI3K inhibitor LY294002 slightly prevented an increase of TER values at 2 h after treatment with 100 nM TPA.

**Fig. 6.** (A) Western blotting for occludin, ZO-1, ZO-2 and claudin-1 in primary human nasal epithelial cells treated with PKC inhibitor GF109203X before treatment with 100 nM TPA. Treatment with GF109203X inhibited upregulation of occludin, ZO-1, ZO-2 and claudin-1 proteins at 2 h after treatment with 100 nM TPA. (B) RT-PCR for occludin, ZO-1, ZO-2 and claudin-1 in primary human nasal epithelial cells treated with PKC inhibitor GF109203X

before treatment with 100 nM TPA. Treatment with GF109203X inhibited upregulation of occludin, ZO-1, ZO-2, claudin-1 and -4 mRNAs at 2 h after treatment with 100 nM TPA. (C, D): The corresponding expression levels are shown as bar graphs.

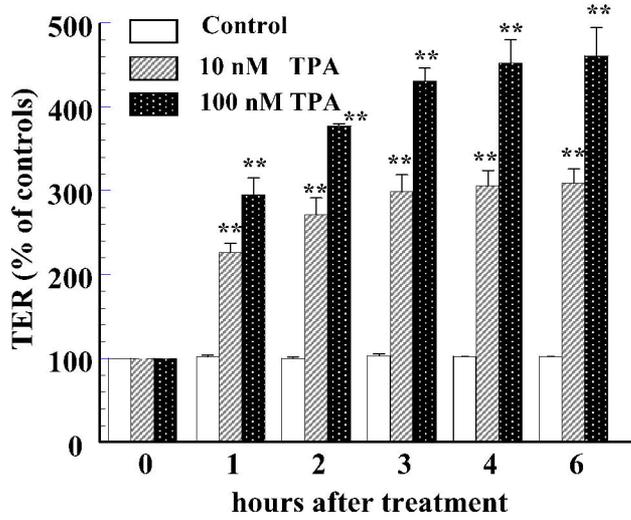
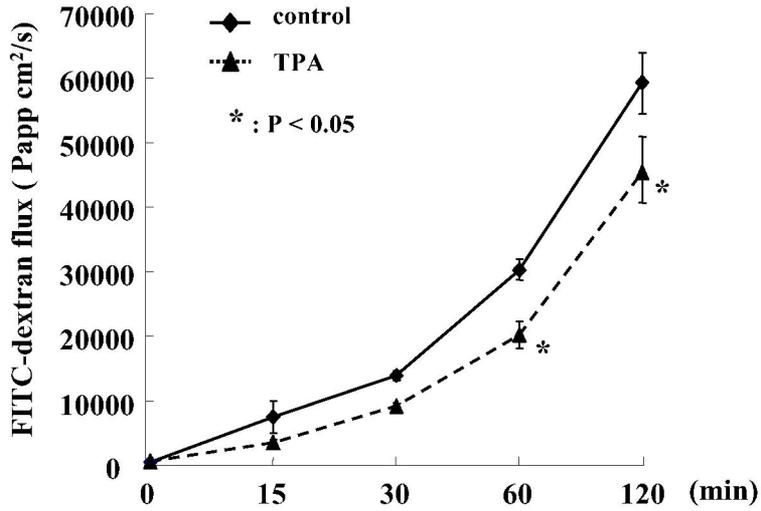
**Fig. 7.** (A) Western blotting for PKC- $\delta$ , - $\theta$  and - $\epsilon$  in primary human nasal epithelial cells treated with PKC inhibitor GF109203X before treatment with 100 nM TPA. Treatment with GF109203X inhibited increases in expression of PKC- $\delta$ , - $\theta$  and - $\epsilon$  at 2 h after treatment with 100 nM TPA. (B) Western blotting for occludin, ZO-1, ZO-2 and claudin-1 in primary human nasal epithelial cells treated with inhibitors of PKC- $\delta$ , - $\theta$  and - $\epsilon$  before treatment with 100 nM TPA. Treatment with inhibitors of PKC- $\delta$ , - $\theta$  but not - $\epsilon$  prevented upregulation of occludin, ZO-1, ZO-2 and claudin-1 proteins at 2 h after treatment with 100 nM TPA. (C, D): The corresponding expression levels are shown as bar graphs.

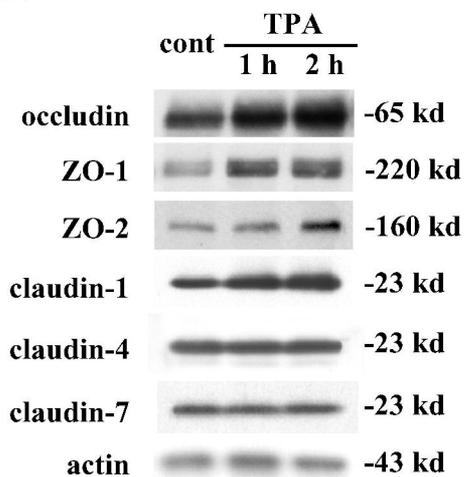
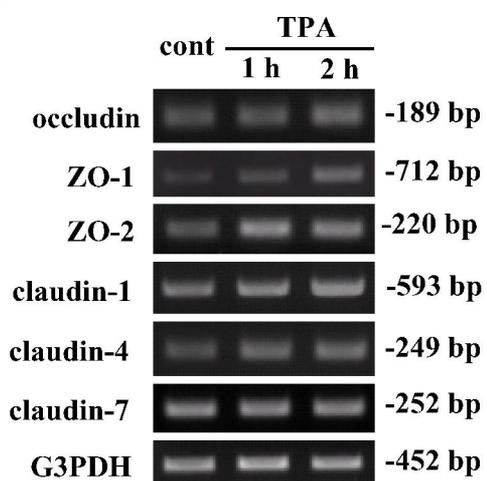
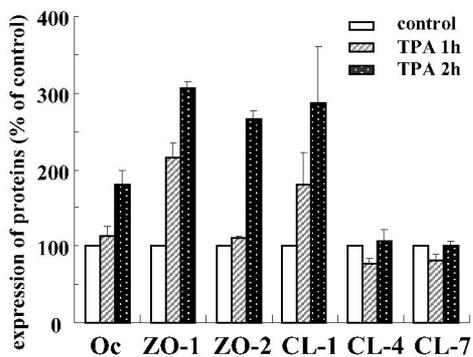
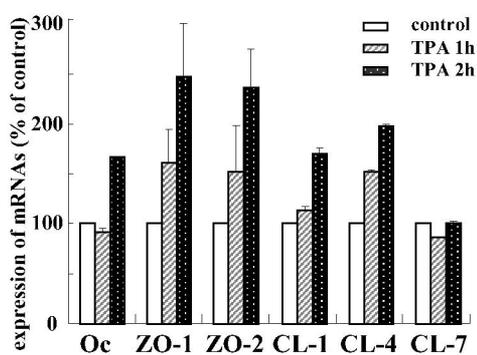
**Fig. 8.** (A) RT-PCR for GATA-1, -2, -3, -4, -5 and -6 in primary human nasal epithelial cells at 1h and 2h after treatment with 100 nM TPA. GATA-3 and -6 mRNAs are detected in primary human nasal epithelial cells and are increased at 1 h treatment with 100 nM TPA. (B) RT-PCR for GATA-3 and -6 in primary human nasal epithelial cells treated with PKC inhibitor GF109203X before treatment with 100 nM TPA. The increase in mRNA of GATA-3 but not GATA-6 at 1 after treatment was inhibited by GF109203X. (C) Western blotting for occludin, ZO-1, ZO-2 and claudin-1 in primary human nasal epithelial cells treated with 100 nM TPA after transfection with or without siRNA of GATA-3. (D) RT-PCR for occludin, ZO-1, ZO-2 and claudin-1 in primary human nasal epithelial cells treated with 100 nM TPA after transfection with or without siRNA of GATA-3. Transfection with siRNA of GATA-3 decreased expression of GATA-3, and prevented upregulation of ZO-1 and ZO-2 but not

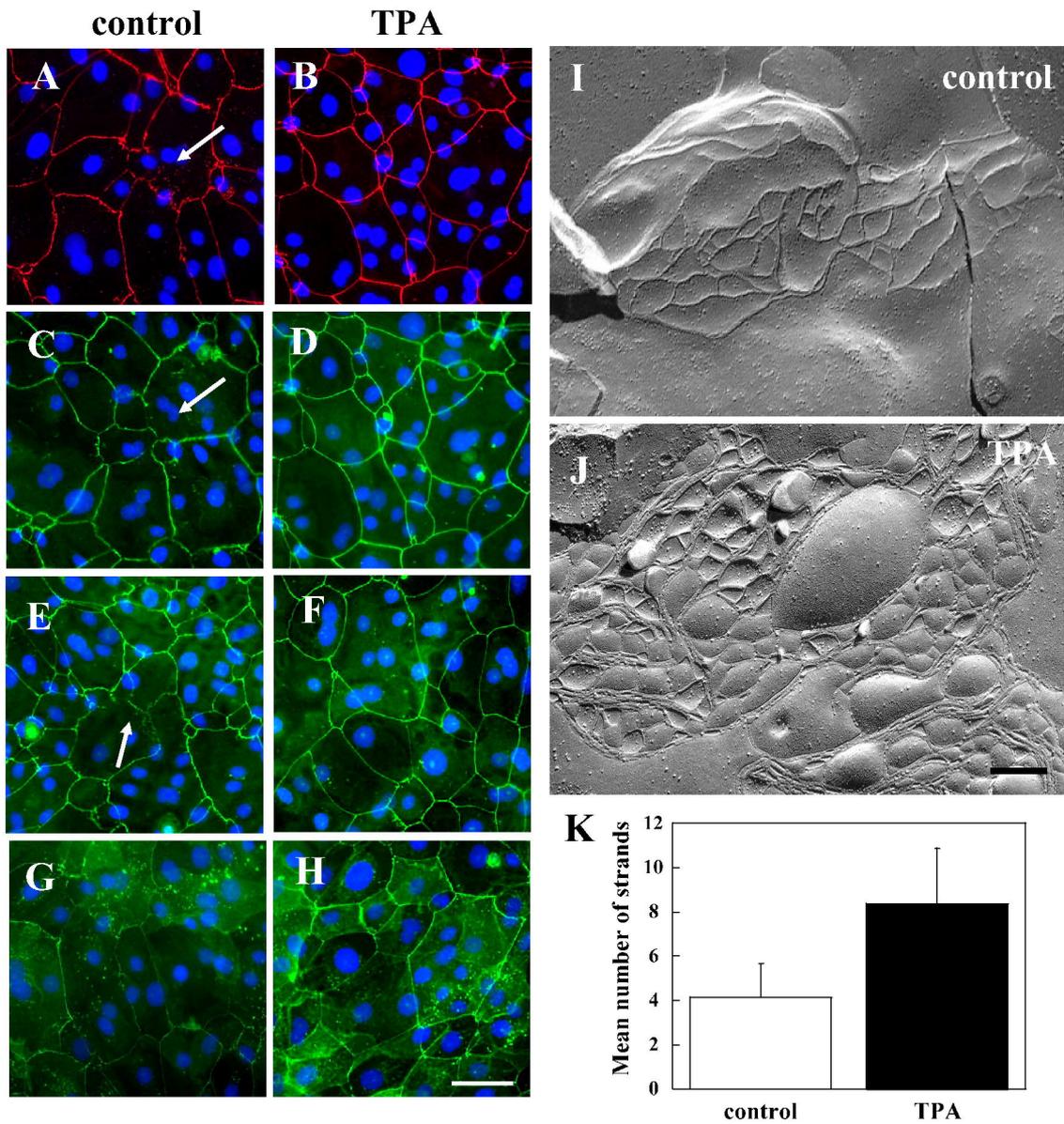
occludin and claudin-1 at 1 h after treatment with 100 nM TPA. (E, F): The corresponding expression levels are shown as bar graphs.

**Table 1. Primers of PCR**

Gene	forward primer	reverse primer	product size (bp)
occludin	TCAGGGAATATCCACCTATCACTTCAG	CATCAGCAGCAGCCATGTACTCTTCAC	189
ZO-1	CGGTCCTCTGAGCCTGTAAG	GGATCTACATGCGACGACAA	712
ZO-2	CGGATTCCAGACAAGGTGTT	CCTTCAGAGACCCAGACTGC	220
claudin-1	AACGCGGGGCTGCAGCTGTTC	GGATAGGGCCTTGGTCTTGGGT	593
claudin-4	AGCCTTCCAGGTCTCAACT	AGCAGCGAGTCGTACACCTT	249
claudin-7	AGGCATAATTTTCATCGTGG	GAGTTGGACTTAGGGTAAGAGCG	252
GATA-1	TCAATTCAGCAGCCTATTCC	TTCGAGTCTGAATACCATCC	377
GATA-2	TGTTGTGCAAATTGTCAGACG	CATAGGTGCCATGTGTCCAGC	279
GATA-3	AAGTGCATGACTCACTGGAGG	TAGGCTTCATGATACTGCTCC	251
GATA-4	CTGGCCTGTCATCTCACTACG	GGTCCGTGCAGGAATTTGAGG	263
GATA-5	TCGCCAGCACTGACAGCTCAG	TGGTCTGTTCCAGGCTGTTCC	290
GATA-6	TTCTAACTCAGATGATTGCAGC	GCTGCACAAAAGCAGACACG	300
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452

**A****B****Figure 1**

**A****B****C****D****Figure 2**



**Figure 3**

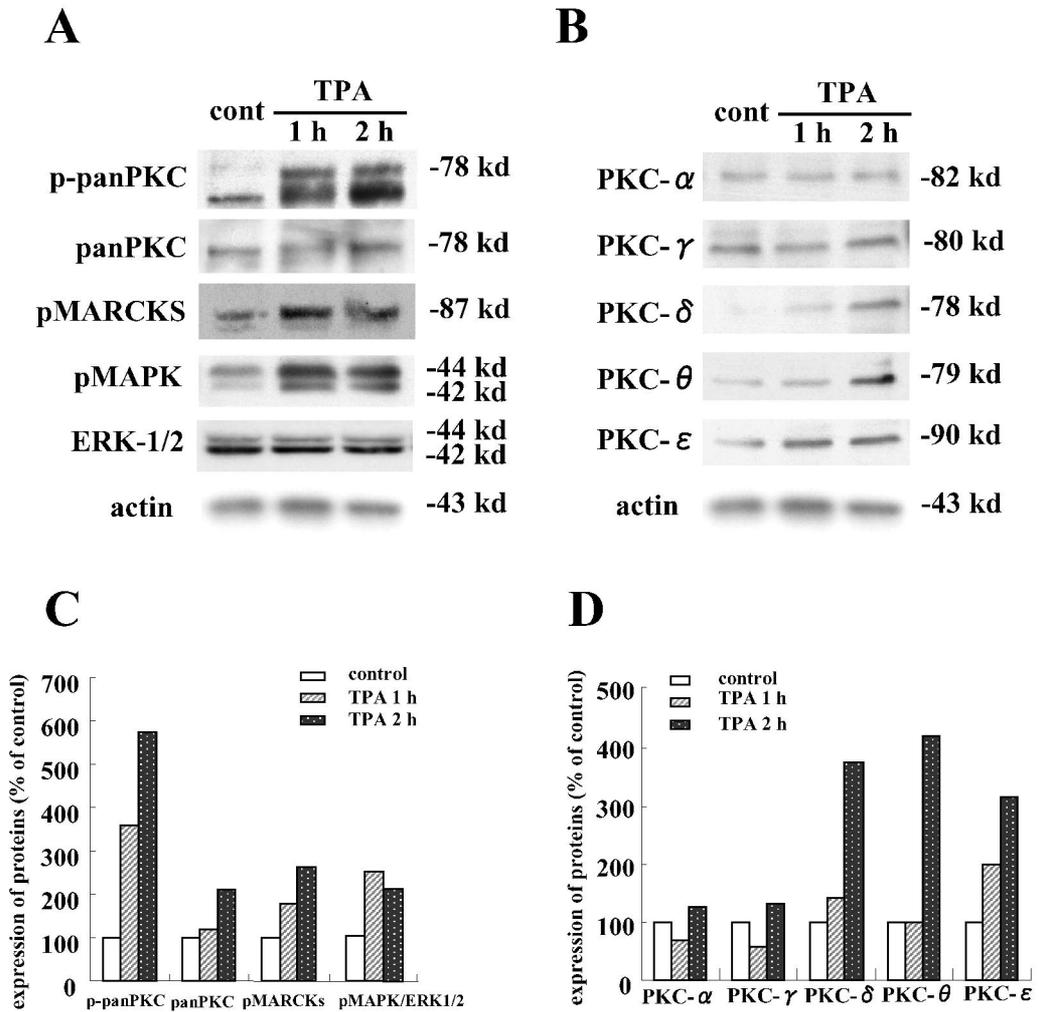
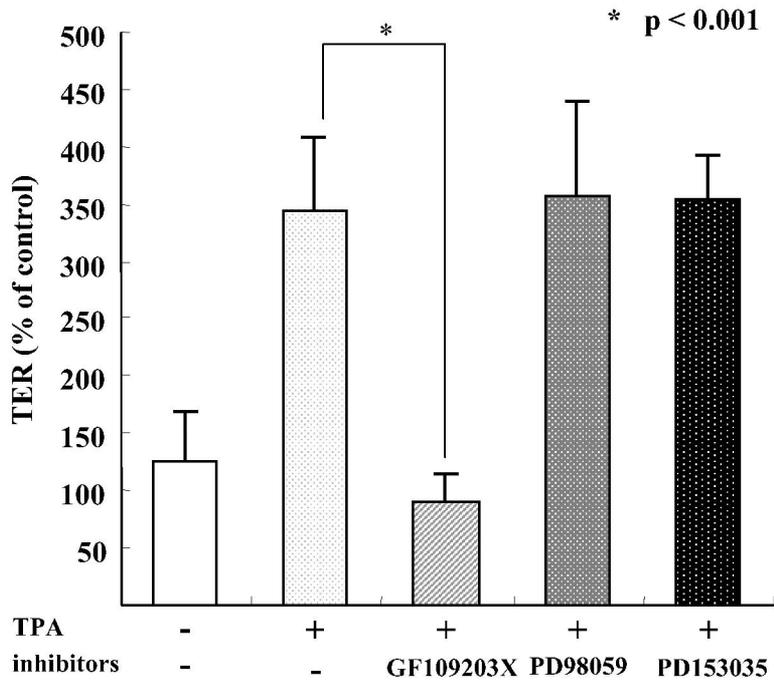
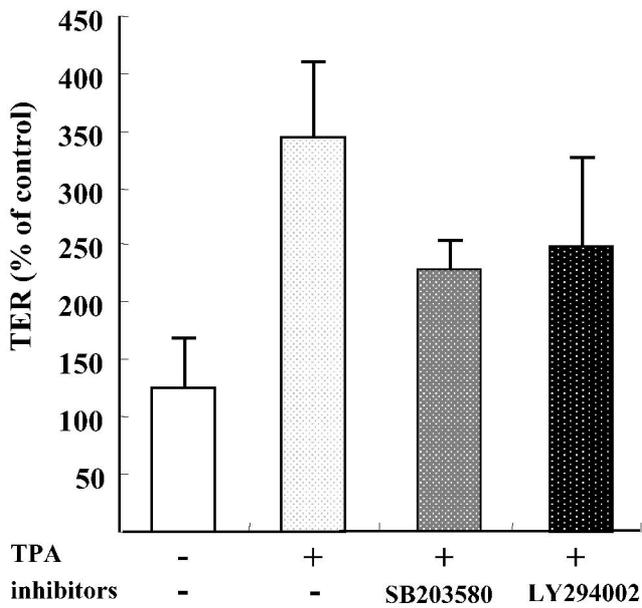


Figure 4

**A****B****Figure 5**

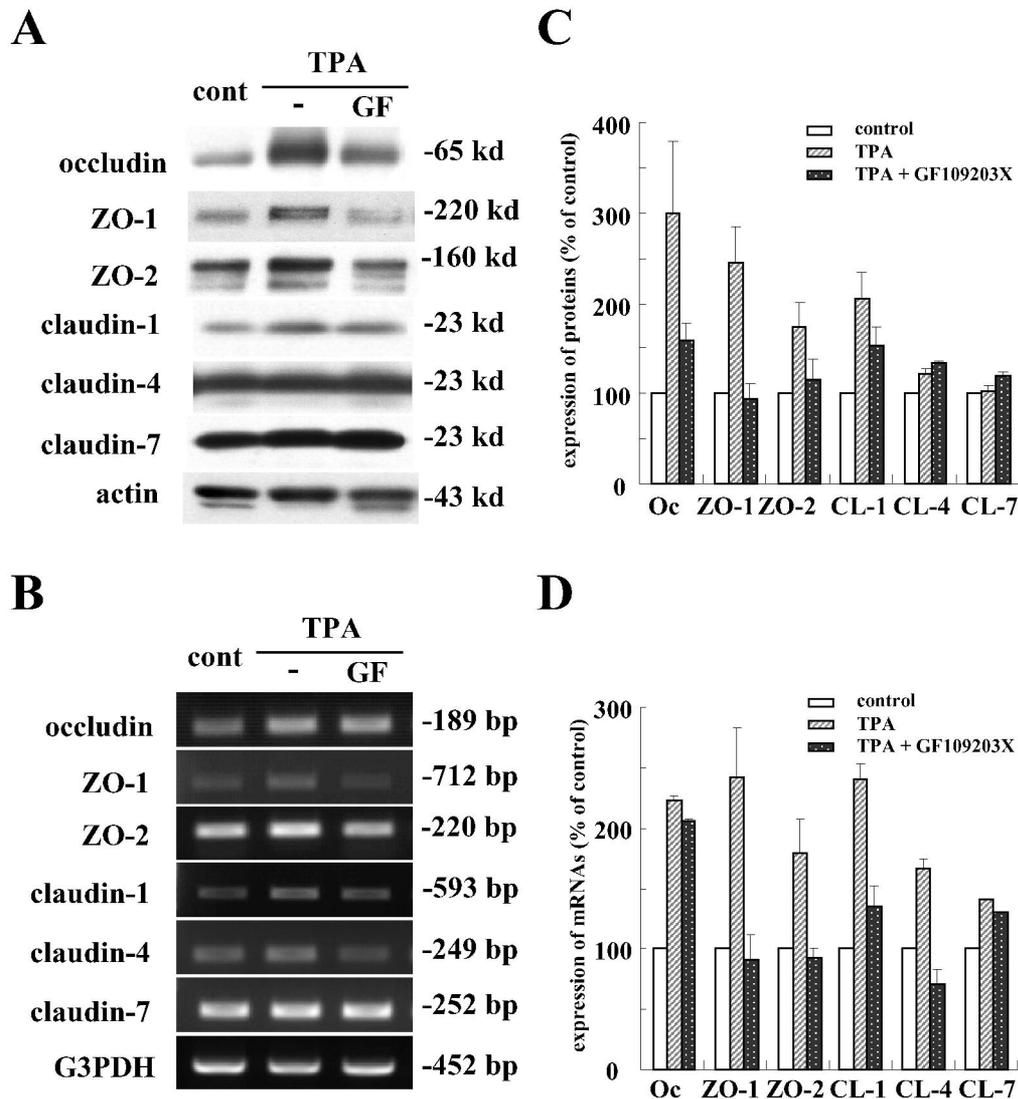


Figure 6

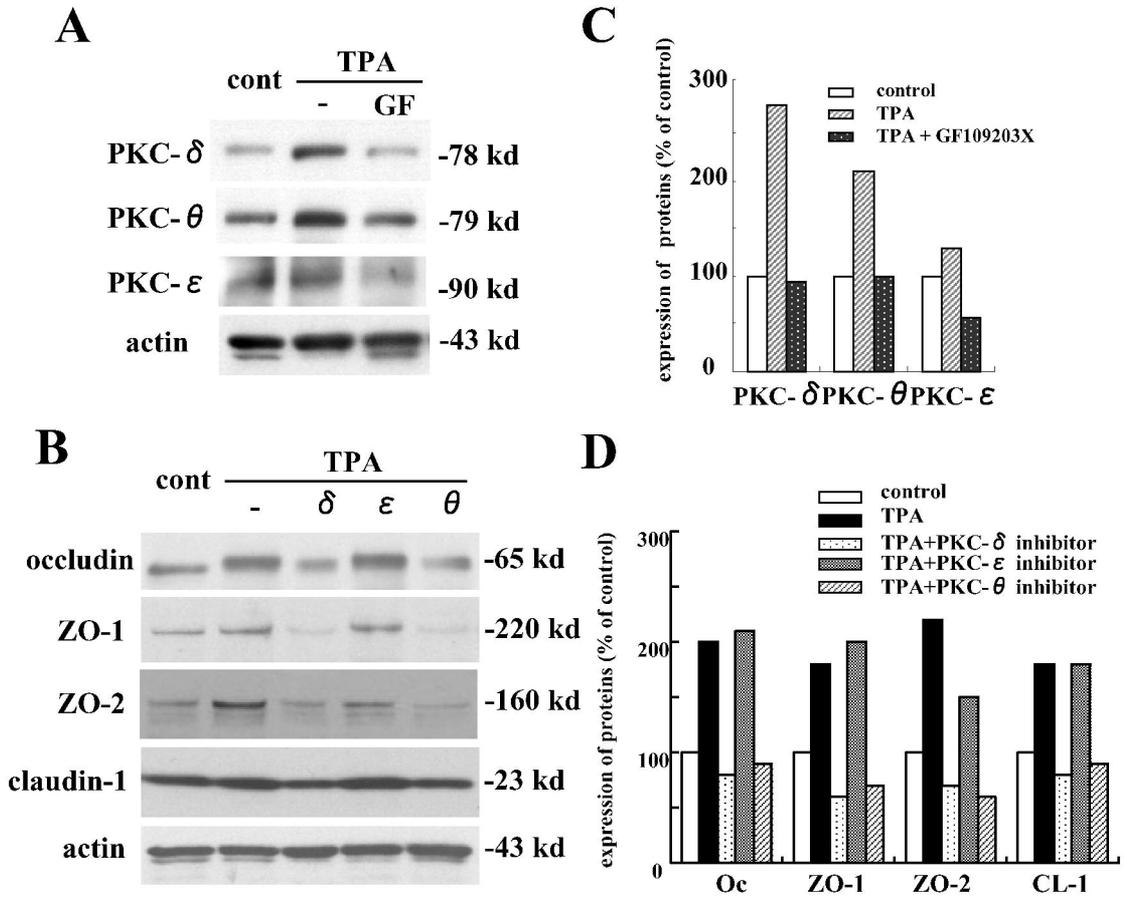
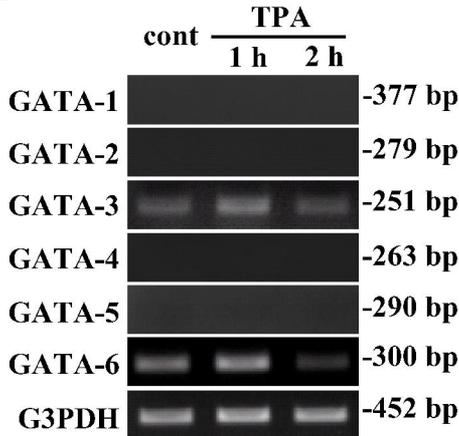
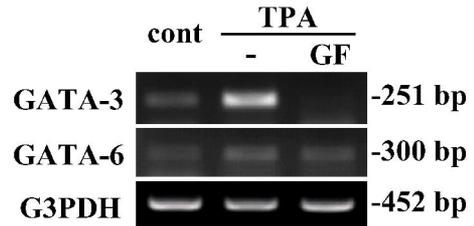
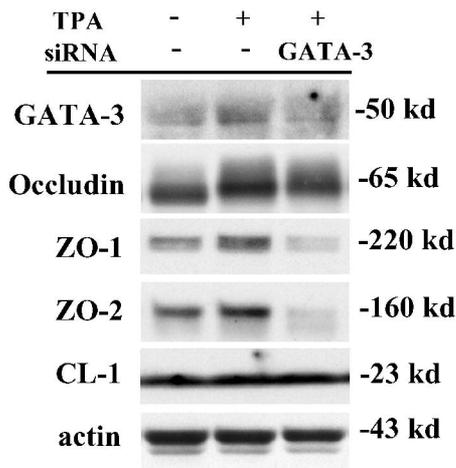
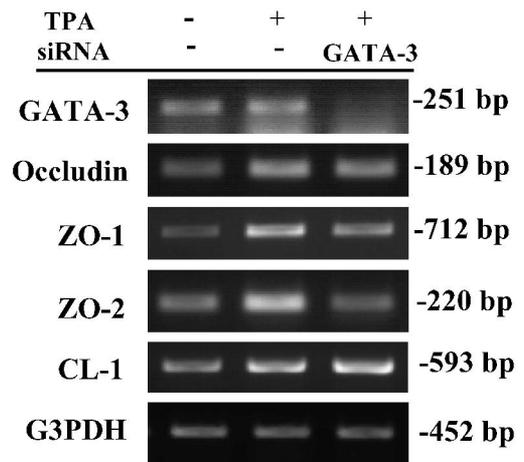
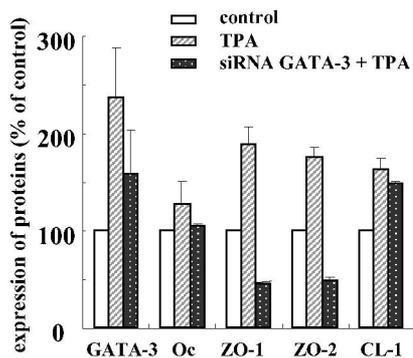
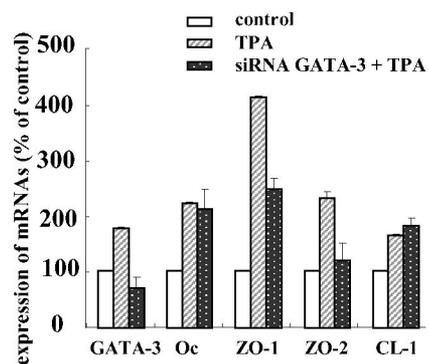


Figure 7

**A****B****C****D****E****F****Figure 8**