ARTICLE
Journal of Cellular Biochemistry 112:1761–1772 (2011)

Journal of Cellular Biochemistry

Transcriptional Regulation of Claudin-18 via Specific Protein Kinase C Signaling Pathways and Modification of DNA Methylation in Human Pancreatic Cancer Cells

Tatsuya Ito,[1,2] Takashi Kojima,[2*] Hiroshi Yamaguchi,[1,2] Daisuke Kyuno,[1,2] Yasutoshi Kimura,[1] Masafumi Imamura,[1] Akira Takasawa,[2] Masaki Murata,[2] Satoshi Tanaka,[2] Koichi Hirata,[1] and Norimasa Sawada[2]

[1]*Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan*
[2]*Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan*

ABSTRACT

Since claudin-18 (Cldn18) is overexpressed in precursor lesion PanIN and pancreatic duct carcinoma, it serves as a diagnostic marker and a target of immunotherapy. The stomach isoform of Cldn18, Cldn18a2 is regulated via a PKC/MAPK/AP-1-dependent pathway in PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated gastric cancer cells. However, little is known about how Cldn18 is regulated, not only in pancreatic duct carcinoma but also in normal human pancreatic duct epithelial cells (HPDE cells). In the present study, four pancreatic cancer cell lines, HPAF-II, HPAC, PANC-1 and BXPC3, and hTERT-HPDE cells in which the hTERT gene was introduced into HPDE cells in primary culture, were treated with TPA. In all human pancreatic cancer cell lines and hTERT-HPDE cells, Cldn18 mRNA indicated as Cldn18a2 was markedly induced by TPA and in well- or moderately differentiated human pancreatic cancer cells HPAF-II and HPAC and hTERT-HPDE cells, the protein was also strongly increased. The upregulation of Cldn18 by TPA in human pancreatic cancer cell lines was prevented by inhibitors of PKCδ, PKCε, and PKCα, whereas the upregulation of Cldn18 by TPA in hTERT-HPDE cells was prevented by inhibitors of PKCδ, PKCθ, and PKCα. Furthermore, a CpG island was identified within the coding sequence of the Cldn18 gene and treatment with the demethylating agent 5-azadeoxycytidine enhanced upregulation of Cldn18 by TPA in HPAF-II and HPAC, but not hTERT-HPDE cells. Our findings suggest that in human pancreatic cancer cells, Cldn18 is primarily regulated at the transcriptional level via specific PKC signaling pathways and modified by DNA methylation. J. Cell. Biochem. 112: 1761–1772, 2011.

KEY WORDS: CLAUDIN-18; HUMAN PANCREATIC CANCER; NORMAL HUMAN PANCREATIC DUCT EPITHELIAL CELLS; TIGHT JUNCTIONS; PKC; DNA METHYLATION

Pancreatic cancer, which has a strong invasive capacity with frequent metastasis and recurrence, is known as one of the most malignant human diseases. It is the fourth leading cause of cancer death in the United States and its death rate has not decreased over the past few decades [Jemal et al., 2010]. Thus, there is an urgent need to develop novel diagnostic and therapeutic strategies to reduce the mortality of pancreatic cancer patients. Recently, it was found that in several human cancers, including pancreatic cancer, some tight junction protein claudins are abnormally regulated and therefore promising molecular targets for diagnosis and therapy [Michl et al., 2003; Karanjawala et al., 2008].

Tight junctions are the most apical components of intercellular junctional complexes. They inhibit solute and water flow through the paracellular space (termed the "barrier" function) [Schneeberger and Lynch, 1992; Gumbiner, 1993]. They also separate the apical from the basolateral cell surface domains to establish cell polarity (termed the "fence" function) [VanMeer et al., 1986; Cereijido et al., 1998]. Recent evidence suggests that tight junctions also participate in signal transduction mechanisms that regulate epithelial cell proliferation, gene expression, differentiation, and morphogenesis [Matter and Balda, 2003]. The tight junction is formed by not only the integral membrane proteins claudins, occludin, and JAMs, but

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Suhara Memorial Foundation; Grant sponsor: The Pancreas Research Foundation of Japan; Grant sponsor: The Ministry of Education, Culture, Sports, Science, and Technology; Grant sponsor: The Ministry of Health, Labour and Welfare of Japan.

*Correspondence to: Takashi Kojima, PhD, Department of Pathology, Sapporo Medical University School of Medicine, S1, W17, Sapporo 060-8556, Japan. E-mail: ktakashi@sapmed.ac.jp

Received 20 February 2011; Accepted 23 February 2011 • DOI 10.1002/jcb.23095 • © 2011 Wiley-Liss, Inc.
Published online 4 March 2011 in Wiley Online Library (wileyonlinelibrary.com).

also many peripheral membrane proteins [Tsukita et al., 2001; Sawada et al., 2003; Schneeberger and Lynch, 2004]. These tight junction proteins are regulated by various cytokines and growth factors via distinct signal transduction pathways [González-Mariscal et al., 2008; Kojima et al., 2009].

Claudin-18 (Cldn18) is in the claudin family, which consists of at least 24 members. It has two alternatively spliced variants, Cldn18a1 and Cldn18a2, which are highly expressed in the lung and stomach, respectively [Yano et al., 2008]. Furthermore, a PKC/MAPK/AP-1-dependent pathway regulates Cldn18a2 expression in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated gastric cancer cells [Yano et al., 2008]. Cldn18a2 is activated in a wide range of human malignant tumors, including gastric, esophageal, pancreatic, lung, and ovarian cancers, and can be specifically targeted with monoclonal antibodies [Sahin et al., 2008]. Cldn18 is highly expressed in pancreatic intraepithelial neoplasia, including precursor lesion PanIN and pancreatic duct carcinoma, and serves as a diagnostic marker [Karanjawala et al., 2008]. However, little is known about how Cldn18 is regulated, not only in pancreatic duct carcinoma but also in normal human pancreatic duct epithelial cells (HPDE cells).

On the other hand, protein kinase C (PKC) is a family of serine–threonine kinases and regulates various cellular functions, including adhesion, secretion, proliferation, differentiation, and apoptosis [Mackay and Twelves, 2007]. It is commonly dysregulated in cancers of the prostate, breast, colon, pancreas, liver, and kidney [Ali et al., 2009]. It is also reported that levels of PKCα, PKCβ1, and PKCδ are higher in pancreatic cancer, whereas that of PKCε is higher in normal tissue [El-Rayes et al., 2008]. We previously found that, in hTERT-HPDE cells in which the hTERT gene was introduced into HPDE cells in primary culture, some tight junction proteins, including Cldn4, were regulated via PKCα and PKCδ [Yamaguchi et al., 2010]. Furthermore, in pancreatic cancer cell line HPAC, tricellulin, which is localized at tricellular tight junctions, is in part regulated via PKCδ and PKCε pathways [Kojima et al., 2010].

In the present study, to investigate how Cldn18 expression is regulated, human pancreatic cell lines HPAF-II, HPAC, PANC-1 and BXPC3, and hTERT-HPDE cells in which the hTERT gene was introduced into HPDE cells in primary culture [Yamaguchi et al., 2010], were treated with the PKC activator TPA or the demethylating agent 5-azadeoxycytidine (5-aza-CdR). Here we present data showing that in well- and moderately differentiated human pancreatic cancer cells, Cldn18 expression is transcriptionally upregulated by TPA via specific PKC signaling pathways and further enhanced by 5-aza-CdR. The regulation of Cldn18 in the pancreatic cancers in part differed from that of normal HPDE cells with regard to the types of PKC isoforms.

MATERIALS AND METHODS

ANTIBODIES, ACTIVATORS, AND INHIBITORS

Rabbit polyclonal anti-occludin, anti-Cldn1, anti-Cldn4, anti-Cldn7, anti-Cldn18 (C-terminal), and anti-phospho-threonine antibodies were obtained from Zymed Laboratories (San Francisco, CA). A rabbit polyclonal anti-phospho-panPKC antibody was obtained from Cell Signaling (Beverly, MA). Rabbit polyclonal anti-panPKC and anti-actin antibodies, TPA, and 5-azadeoxycytidine (5-aza-CdR) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO). Alexa 488 (green)-conjugated anti-rabbit IgG and Alexa594 (red)-conjugated anti-mouse IgG antibodies were purchased from Molecular Probes, Inc. (Eugene, OR). A pan-PKC inhibitor (GF109203X), PKCδ inhibitor (rottlerin), PKCε inhibitor (PKCε translocation inhibitor peptide), PKCθ inhibitor (myristoylated PKCθ pseudosubstrate peptide inhibitor), and PKCα inhibitor (Gö6976) were purchased from Calbiochem–Novabiochem Corporation (San Diego, CA).

CULTURES OF CELL LINES AND TREATMENT

Human pancreatic cancer cell lines PANC-1, HPAF-II, BXPC-3, and HPAC were purchased from ATCC (Manassas, VA). PANC-1 cells and HPAC cells were maintained with DMEM (Sigma–Aldrich, Inc.) supplemented with 10% dialyzed fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). HPAF-II cells were maintained with DMEM containing 10% FBS, and supplemented with 0.1 mM nonessential amino acids (Sigma–Aldrich, Inc.) and 1 mM sodium pyruvate (Sigma–Aldrich, Inc.). BXPC-3 was maintained with RPMI-1640 (Sigma–Aldrich, Inc.) supplemented with 10% FBS. The medium for all cell lines contained 100 U/ml penicillin and 100 μg/ml streptomycin and all cells were plated on 35- and 60-mm culture dishes (Corning Glass Works, Corning, NY) that were coated with rat tail collagen (500 μg of dried tendon/ml in 0.1% acetic acid), and incubated in a humidified 5% CO_2 incubator at 37°C.

All cell lines were treated with 1–100 nM TPA. The HPAF-II cells and HPAC cells were pretreated with the inhibitors 10 μM GF109203X, 1 μM rottlerin, 10 μM PKCε translocation inhibitor peptide, 10 μM myristoylated PKCθ pseudosubstrate peptide inhibitor, and 1 μM Gö6976 30 min before treatment with 100 nM TPA. The HPAF-II cells and HPAC cells were pretreated with 5 or 15 μM 5-aza-CdR for 4 days before treatment with 100 nM TPA for 24 h.

ISOLATION AND CULTURE OF HUMAN PANCREATIC DUCT EPITHELIAL (HPDE) CELLS

Human pancreatic tissues were obtained from patients with pancreatic or biliary tract diseases who underwent pancreatic resection in the Sapporo Medical University Hospital. Informed consent was obtained from all patients, and the study was approved by the ethics committee of Sapporo Medical University. Human pancreatic tissues were minced into pieces 2–3 mm^3 in volume and washed with phosphate-buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza Walkersville, Walkersville, MD) three times. These minced tissues were suspended in 10 ml of Hanks' balanced salt solution with 0.5 μg/ml DNase I and 0.04 mg/ml Liberase Blenzyme 3 (Roche, Basel, Switzerland) and then incubated with bubbling of mixed O_2 gas containing 5.2% CO_2 at 37°C for 10 min. The dissociated tissues were subsequently filtrated with 300-μm mesh followed by filtration with 70-μm mesh (Cell Strainer; BD Biosciences, San Jose, CA). After centrifugation at 1,000*g* for 4 min, isolated cells were cultured in bronchial epithelial basal medium (BEBM; Lonza Walkersville) containing 10% FBS (CCB, Nichirei Bioscience, Tokyo, Japan) and supplemented with BEGM® SingleQuots® (Lonza Walkersville), including 0.4% bovine

pituitary extract, 0.1% insulin, 0.1% hydrocortisone, 0.1% gentamicin, amphotericin-B (GA-1000), 0.1% retinoic acid, 0.1% transferrin, 0.1% triiodothyronine, and 0.1% epinephrine, 0.1% human epidermal growth factor, 100 U/ml penicillin, and 100 μg/ml streptomycin on 60-mm culture dishes (Corning Life Science, Acton, MA), coated with rat tail collagen (500 μg of dried tendon/ml of 0.1% acetic acid). Following the above protocol, tissue dissociation and cell isolation were repeated for the same sample seven times at the maximum. The cells were placed in a humidified 5% CO_2:95% air incubator at 37°C.

The retroviral vector BABE-hygro-hTERT (kindly provided by Dr. Robert Weinberg) was used. The viral supernatant was produced from an ecotropic packaging cell line by transfection of plasmid DNA as previously reported [Kawano et al., 2003]. The packaging cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. At 24 h after plating on 60-mm dishes, HPDE cells in primary culture were exposed to the viral supernatant containing the retrovirus overnight. After being washed with serum-free BEBM medium, the hTERT-transfected HPDE (hTERT-HPDE) cells were cultured in serum-free BEBM medium supplemented with the above-mentioned factors and 2.5 μg/ml amphotericin-B. The hTERT-HPDE cells became confluent on the 60-mm culture dishes in 2–3 weeks and the first passage was done using 0.05% trypsin–EDTA (Sigma–Aldrich, Inc.) in 60-mm culture dishes.

The hTERT-HPDE cells were treated with 1–100 nM TPA. Some cells were pretreated with the inhibitors 10 μM GF109203X, 1 μM rottlerin, 10 μM PKCε translocation inhibitor peptide, 10 μM myristoylated PKCθ pseudosubstrate peptide inhibitor, and 1 μM Gö6976 30 min before treatment with 100 nM TPA. Some cells were pretreated with 5 or 15 μM 5-aza-CdR for 4 days before treatment with 100 nM TPA for 24 h.

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical analysis was performed to evaluate the expression and distribution of Cldn18 in normal pancreatic tissues, well- and poor-differentiated pancreatic cancer tissues. Deparaffinized tissue sections were immersed in 10 mmol/L of sodium citrate buffer (pH 6.0) and autoclaved for antigen retrieval. Endogenous peroxidase activity was blocked using methanol containing 0.03% H_2O_2. After incubation with blocking buffer (0.01 mol/L PBS containing 5% bovine serous albumin, Sigma Co, Tokyo, Japan), the sections were incubated with a rabbit polyclonal Cldn18 antibody (1:100 dilution) at 4°C. After a gentle rinsing with 0.05 mol/L of Tris–HCl, the sections were incubated with biotinylated secondary antibody (LSAB2 Kit, Dako A/S, Copenhagen, Denmark) for 30 min. Next, the sections were incubated with horseradish peroxidase-conjugated streptavidin reagent (LSAB2 Kit, Dako A/S) for 30 min. Chromogenic fixation was performed for 5 min in a solution of 3-amino-9-ethyl carbazole substrate chromogen (Dako A/S). The sections were counterstained with Meyer's hematoxylin.

RNA ISOLATION, REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS AND REAL-TIME PCR ANALYSIS

Total RNA was extracted and purified using TRIzol (Invitrogen). One microgram of total RNA was reverse-transcribed into cDNA using a mixture of oligo (dT) and Superscript II reverse transcriptase according to the manufacturer's recommendations (Invitrogen). Synthesis of each cDNA was performed in a total volume of 20 μl for 50 min at 42°C and terminated by incubation for 15 min at 70°C. PCR was performed in a 20-μl total mixture containing 100 pM primer pairs, 1.0 μl of the 20-μl total RT product, PCR buffer, dNTPs, and Taq DNA polymerase according to the manufacturer's recommendations (Takara, Kyoto, Japan). Amplifications were for 25–35 cycles depending on the PCR primer pair 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. Seven microliters of the total 20-μl PCR product was analyzed by 1% agarose gel electrophoresis with ethidium bromide staining and standardized using a GeneRuler™ 100 bp DNA ladder (Fermentas, ON, Canada). The PCR primers used for Cldn1, 4, 7, 18, 18a1, 18a2, occludin, and glutaraldehyde-3-phosphate dehydrogenase (G3PDH) are indicated in Table I.

Real-time PCR detection was performed using a TaqMan Gene Expression Assay kit with a StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The amount of 18S ribosomal RNA (rRNA) (Hs99999901) mRNA in each sample was used to standardize the quantity of the following mRNA: Cldn18 (Hs00221623). The relative mRNA-expression levels between the control and treated samples were calculated by the difference of the threshold cycle (comparative C_T [$\triangle\triangle C_T$] method) and presented as the average of triplicate experiments with a 95% confidence interval.

DETECTION OF Cldn18 GENE METHYLATION

The Cldn18 genomic sequence was searched for the presence of CpG islands using CpG Island Searcher (http://www.cpgislands.com) [Takai and Jones, 2003]. Genomic DNA was extracted from tissues or primary tumor cells using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) and 1 μg of genomic DNA was modified with sodium

TABLE I. Primers for RT-PCR

Gene	Forward primer	Reverse primer	Product size (bp)
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452
Claudin-1	GCTGCTGGGTTTCATCCTG	CACATAGTCTTTCCCACTAGAAG	619
Claudin-4	AGCCTTCCAGGTCCTCAACT	AGCAGCGAGTAGAAG	249
Claudin-7	AGGCATAATTTTCATCGTGG	GAGTTGGACTTAGGGTAAGAGCG	252
Claudin-18	TTCCATCCCAGTACCAAAGC	CCGTTCTTTCCCCAGACATA	226
Claudin-18a1	TCCACCACCACATGCCAAGTG	GTGTACATGTTAGCTGTGGAC	464
Claudin-18a2	TGGCTCTGTGTCGACACTGTG	GTGTACATGTTAGCTGTGGAC	495
Occludin	TCAGGGAATATCCACCTATCACTTCAG	CATCAGCAGCAGCCATGTACTCTTCAC	189

bisulfite using a CpGenome DNA Modification kit (Chemicon, Temecula). Two methylation-specific PCR (MSP) reactions were then performed on each sodium bisulfite-treated genomic DNA sample, using primers specific for the methylated DNA [5'-TATTTTCGG-TAGTAGGAGGGC-3' (MF), 5'-ATATCCATCCCGATAACCG-3' (MR)], or the unmethylated DNA [5'-TTATATTTTTGGTAGTAGGAGGGT-3' (UMF), 5'-ACATATCCATCCCAATAACCAC-3' (UMR)]. Primers were designed using MethPrimer (http://itsa.ucsf.edu/~urolab/methprimer) [Li and Dahiya, 2002]. MSP conditions were for 40 cycles depending on the primer pair with cycle times of 15 s at 96°C, 30 s at 55°C, and 60 s at 72°C using Taq DNA polymerase according to the manufacturer's recommendations.

WESTERN BLOT ANALYSIS

For Western blotting of total cell lysates, the dishes were washed with PBS and 300 μl of sample buffer (1 mM $NaHCO_3$ and 2 mM phenylmethylsulfonylfluoride) was added to 60-mm culture dishes. The cells were scraped and collected in microcentrifuge tubes and then sonicated for 10 s. The protein concentrations of samples were determined using a BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL). Aliquots of 15 μg of protein/lane for each sample were separated by electrophoresis in 4/20% SDS–polyacrylamide gels (Cosmo Bio Co., Tokyo, Japan). After electrophoretic transfer to nitrocellulose membranes (Immobilon; Millipore, Billerica, MA), the membranes were saturated with blocking buffer (Tris-buffered saline [TBS] with 0.1% Tween-20 and 4% skim milk) for 30 min at room temperature and incubated with the following polyclonal antibodies: anti-occludin, anti-Cldn1, anti-Cldn4, anti-Cldn7, anti-Cldn18, anti-phospho-panPKC, anti-panPKC, and anti-actin (1:1,000) for 1 h at room temperature. Then the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Dako A/S) at room temperature for 1 h. The immunoreactive bands were detected using an ECL Western blotting analysis system (GE Healthcare, Little Chalfont, UK).

IMMUNOCYTOSTAINING

The cells were grown on 35-mm glass base dishes (Iwaki, Chiba, Japan) coated with rat tail collagen and incubated with 10% FBS. Then they were fixed with cold acetone and ethanol (1:1) at 20°C for 10 min. After rinsing in PBS, the sections and the cells were incubated with polyclonal anti-Cldn18 (1:100) or monoclonal anti-Cldn4 (1:100) antibodies at room temperature (RT) for 1 h and then with Alexa 488 (green)-conjugated anti-rabbit IgG or Alexa 584 (red)-conjugated anti-mouse IgG antibodies (1:200) at RT for 1 h. DAPI (Sigma–Aldrich, Inc.) was used for counterstaining of nuclei in the cells. The specimens were examined using an epifluorescence microscope (Olympus, Tokyo, Japan) and a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

DATA ANALYSIS

Signals were quantified using Scion Image Beta 4.02 Win (Scion Co.; Frederick, MA). Each set of results shown is representative of at least three separate experiments. Results are given as means ± SE. Differences between groups were tested by ANOVA followed by a post hoc test and an unpaired two-tailed Student's *t*-test and considered to be significant when $P < 0.05$.

RESULTS

EXPRESSION AND DISTRIBUTION OF Cldn18 IN NORMAL PANCREATIC DUCTS, WELL- AND POOR-DIFFERENTIATED PANCREATIC DUCT CARCINOMAS

Cldn18 is highly expressed in pancreatic intraepithelial neoplasia, including precursor lesion PanIN and pancreatic duct carcinoma, and serves as a diagnostic marker [Karanjawala et al., 2008]. In the present study, we examined expression and distribution of Cldn18 in normal pancreatic ducts, well- and poor-differentiated pancreatic duct carcinomas. In well-differentiated pancreatic duct carcinomas, Cldn18 was highly expressed and localized at the membranes, whereas in normal pancreatic ducts and poor-differentiated pancreatic duct carcinomas, Cldn18-immunoreactivity was not observed (Fig. 1).

EXPRESSION PATTERNS OF TIGHT JUNCTION PROTEINS IN HUMAN PANCREATIC CANCER CELL LINES

To study regulation of Cldn18 in human pancreatic cancer cells, we first investigated the expression patterns of tight junction proteins in human pancreatic cancer cell lines PANC-1, HPAF-II, BXPC-3, and HPAC. PANC-1 and BXPC-3 are models of poorly differentiated pancreatic cancer, and HPAF-II and HPAC are models of well- or moderately differentiated cancers [Deer et al., 2010]. All the cells had a small cobblestone appearance in phase-contrast images (Fig. 2A). In Western blots, the Cldn18 protein level was low in all pancreatic cell lines (Fig. 2B). Cldn1, Cldn4, Cldn7, and occludin proteins were detected, while the level of Cldn7 in PANC-1 cells and occludin in BXPC3 cells were low compared to other tight junction proteins. In RT-PCR, Cldn18 mRNA was detected only in HPAC cells, whereas mRNAs of other tight junction molecules were detected in all the cells (Fig. 2C).

UPREGULATION OF Cldn18 mRNA IN HUMAN PANCREATIC CANCER CELL LINES AFTER TREATMENT WITH TPA

Cldn18 has two alternatively spliced variants, Cldn18a1 and Cldn18a2, which are highly expressed in the lung and stomach, respectively [Yano et al., 2008]. Furthermore, Cldn18a2 expression is enhanced by treatment with TPA in human gastric cancer cells [Yano et al., 2008].

To investigate whether Cldn18 mRNA was induced by treatment with TPA in human pancreatic cancer cells, the cell lines were treated with 100 nM TPA for 24 h. In RT-PCR, the mRNAs of Cldn18 and Cldn18a2, but not Cldn18a1, were increased in all TPA-treated cells compared to the control (Fig. 2D). In real-time PCR, Cldn18 mRNA was markedly increased in all TPA-treated cells compared to the control (61.5 ± 12.5-fold in PANC-1, 83.3 ± 13.5-fold in HPAF-II, 60.2 ± 15.8-fold in BXPC3, and 79.3 ± 29-fold in HPAC) (Fig. 2E).

UPREGULATION OF Cldn18 PROTEIN IN HUMAN PANCREATIC CANCER CELL LINES AFTER TREATMENT WITH TPA

To investigate whether Cldn18 protein was induced by treatment with TPA in human pancreatic cancer cells, the cell lines were treated with 1–100 nM TPA. In Western blots, Cldn18 protein was markedly increased from 10 nM TPA in HPAF-II cells and HPAC cells, whereas it was slightly increased at 100 nM TPA in PANC-1 cells and BXPC3 cells (Fig. 3A). When HPAF-II cells and HPAC cells were treated with 100 nM TPA from 1 to 24 h, an increase of Cldn18

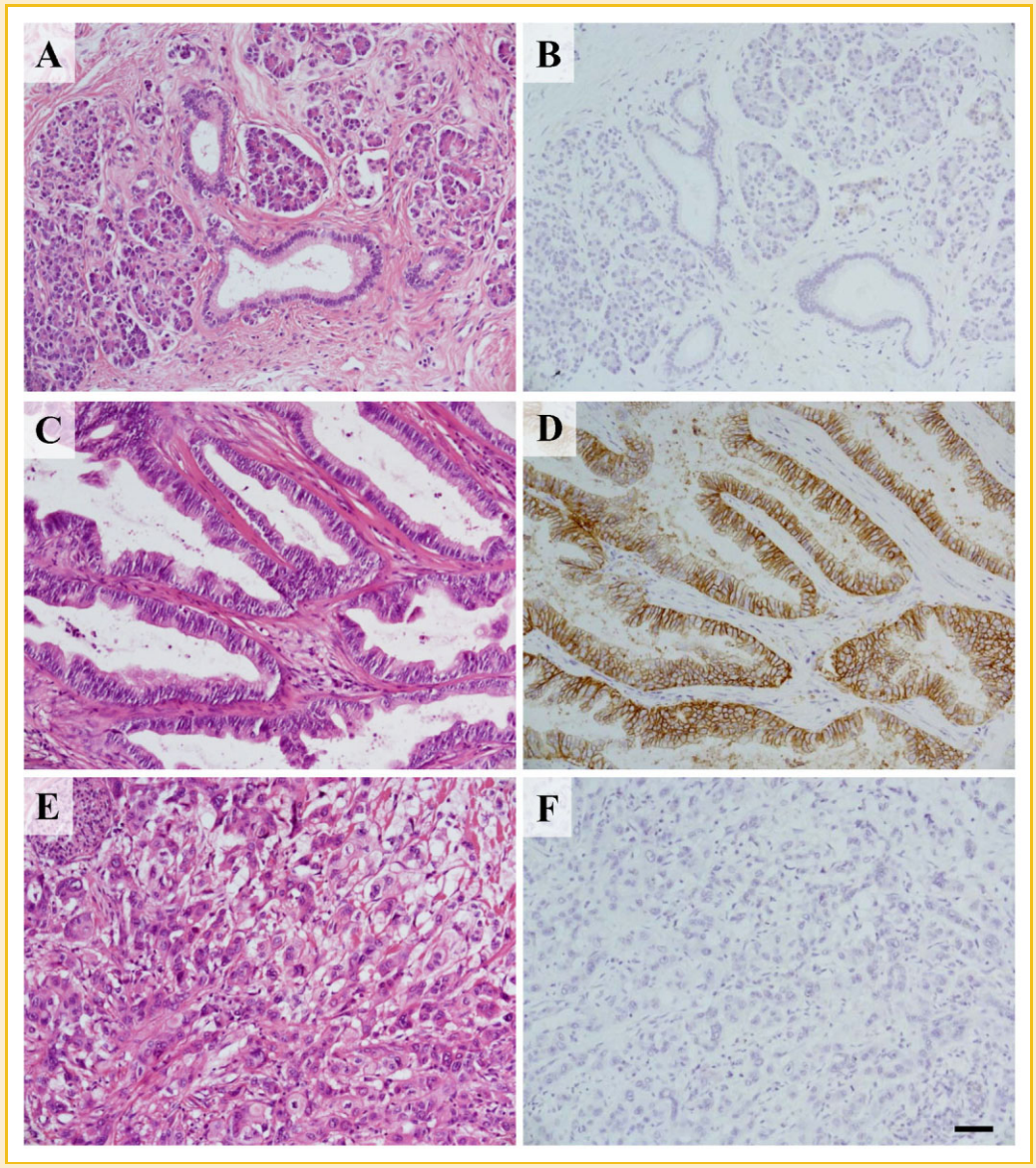

Fig. 1. Hematoxylin–eosin staining (A,C,E) and immunohistochemical staining (B,D,F) for Cldn18 in normal pancreatic ducts (A,B), well (C,D) and poorly (E,F) differentiated pancreatic duct carcinomas. Bar: 50 μm.

protein was observed at 24 h, whereas phospho-panPKC was increased from 1 h and then decreased from 8 h (Fig. 3B).

panPKC INHIBITOR PREVENTS UPREGULATION OF Cldn18 IN HPAF-II CELLS AND HPAC CELLS AFTER TREATMENT WITH TPA

To investigate whether upregulation of Cldn18 induced by TPA was regulated via a direct PKC pathway, HPAF-II cells and HPAC cells were pretreated with panPKC inhibitor 10 μM GF109203X (GF) 30 min before treatment with 100 nM TPA for 24 h. In RT-PCR, upregulation of Cldn18a2 mRNA in HPAF-II cells and HPAF cells caused by treatment with TPA was inhibited by GF (Fig. 3C). In real-time PCR, marked upregulation of Cldn18 mRNA in HPAF-II cells and HPAF cells induced by treatment with TPA was inhibited by GF (Fig. 3D).

Immunostaining showed that Cldn18-positive cells appeared in HPAF-II cells and HPAF cells after treatment with TPA and disappeared when they were pretreated with GF (Fig. 4A). Cldn18-positive cells that appeared in HPAF-II cells and HPAF cells by treatment with TPA were larger in phase-contrast images (Fig. 4B). In confocal laser scanning microscopic images of CLdn18 and Cldn4 in HPAF-II after treatment with TPA, Cldn18 was localized at the apicalmost and basolateral membranes as well as Cldn4 (Fig. 4B,C).

UPREGULATION OF Cldn18 IN hTERT-HPDE CELLS AFTER TREATMENT WITH TPA

To investigate the physiological regulation of Cldn18 expression in normal human pancreatic ductal epithelial cells, hTERT-HPDE cells were treated with TPA as in the experiments using HPAF-II and

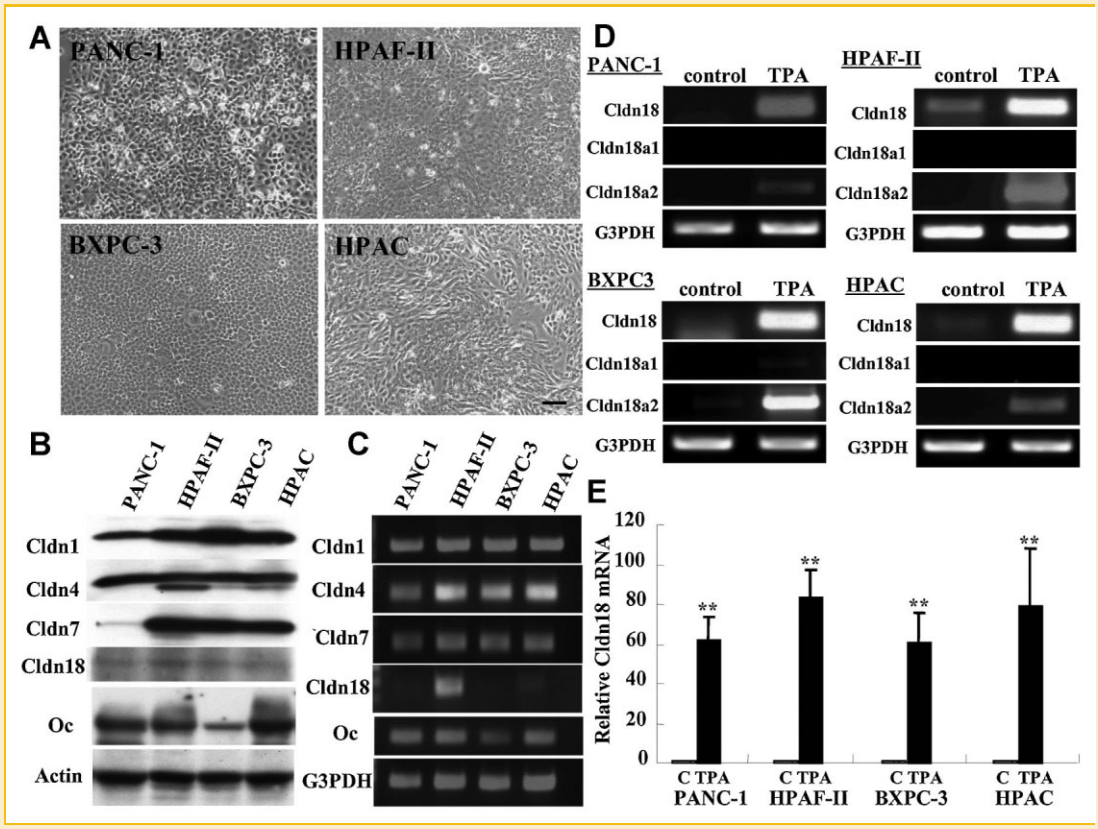

Fig. 2. Expression patterns of tight junction molecules in human pancreatic cancer cell lines PANC-1, HPAF-II, BXPC3, and HPAC. A: Phase-contrast images of human pancreatic cancer cells. Bar: 80 μm. B: Western blotting for Cldn1, 4, 7, 18, and occludin in pancreatic cancer cells. C: RT-PCR for Cldn1, 4, 7, 18, and occludin in pancreatic cancer cells. Upregulation of Cldn18 mRNA in pancreatic cancer cells after treatment with TPA. D: RT-PCR for Cldn18, 18a1, and 18a2 in pancreatic cancer cells after treatment with 100 nM TPA. E: Real-time PCR for Cldn18 in pancreatic cancer cells after treatment with 100 nM TPA. $^{**}P < 0.01$ versus control.

HPAC cells. When hTERT-HPDE cells were treated with 1–100 nM TPA for 24 h, upregulation of Cldn18 protein was observed from 10 nM TPA in Western blots (Fig. 5A). When hTERT-HPDE cells were treated with 100 nM TPA from 1 to 24 h, upregulation of Cldn18 protein was observed from 12 h (Fig. 5A). In RT-PCR, mRNA of Cldn18 and Cldn18a2 were increased by treatment with 100 nM TPA for 24 h, and the upregulation was prevented by GF (Fig. 5B,C). In real-time PCR, Cldn18 mRNA was increased in TPA-treated cells 85.5 ± 13.5-fold compared to the control (Fig. 5C). Immunostaining showed that large Cldn18-positive cells appeared among TPA-treated cells, and disappeared when the cells were pretreated with GF (Fig. 5D).

THE EFFECTS OF INHIBITORS OF PKC ISOFORMS ON UPREGULATION OF Cldn18 IN HPAF-II CELLS, HPAC CELLS, AND hTERT-HPDE CELLS AFTER TREATMENT WITH TPA

To further investigate which PKC isoforms played key roles in the upregulation of Cldn18 in pancreatic cancer cells and normal pancreatic ductal epithelial cells after treatment with TPA, HPAF-II cells, HPAC cells, and hTERT-HPDE cells were pretreated with pan-PKC inhibitor GF109203X (10 μM), PKCδ inhibitor rottlerin (1 μM), PKCε inhibitor PKCε translocation inhibitor peptide (10 μM), PKCθ inhibitor myristoylated PKCθ pseudosubstrate peptide inhibitor (10 μM), and PKCα inhibitor Gö6976 (1 μM) at 30 min before treatment with 100 nM TPA for 24 h. In HPAF-II cells, upregulation of Cldn18 protein by TPA was inhibited by inhibitors of panPKC, PKCδ, PKCε, and PKCα (Fig. 6A). In HPAC cells, upregulation of Cldn18 protein by TPA was inhibited by inhibitors of panPKC, PKCε, and PKCα (Fig. 6B). In hTERT-HPDE cells, upregulation of Cldn18 protein by TPA was inhibited by inhibitors of panPKC, PKCδ, PKCθ, and PKCα (Fig. 6C).

REPRESSION OF Cldn18 IN HPAC CELLS BUT NOT hTERT-HPDE CELLS IS DEPENDENT ON PROMOTER DNA METHYLATION

A CpG island was identified within the coding sequence of the Cldn18 gene. To investigate whether Cldn18 expression was dependent on promoter DNA methylation in pancreatic cancer cells and normal pancreatic ductal epithelial cells, HPAC cells, and hTERT-HPDE cells were pretreated with the demethylating agent 5-aza-CdR at 5 or 15 μM for 96 h before treatment with or without 100 nM TPA for 24 h. In Western blots, Cldn18 protein induced by TPA in HPAC cells was significantly increased by 5-aza-CdR in a dose-dependent manner (Fig. 7A,B). In RT-PCR and real-time PCR, Cldn18 mRNA in HPAC cells treated with or without TPA was significantly increased by 5-aza-CdR in a dose-dependent manner (Fig. 7C,D). Furthermore, we performed MSP analysis on DNA from

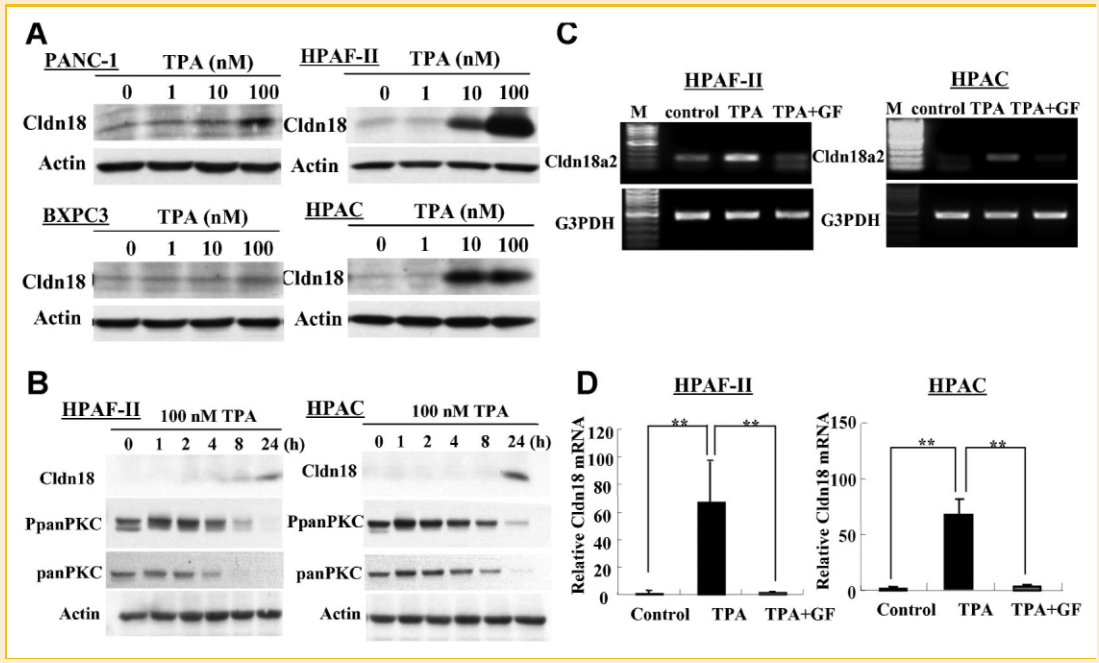

Fig. 3. Upregulation of Cldn18 protein and the phosphorylation in pancreatic cancer cells after treatment with TPA. A: Western blotting for Cldn18 in pancreatic cancer cells after treatment with 0–100 nM TPA for 24 h. B: Western blotting for Cldn18, phospho-panPKC, and PKC in HPAF-II and HPAC cells from 1 to 24 h after treatment with 100 nM TPA. C: RT-PCR for Cldn18a2 in HPAF-II cells and HPAC cells pretreated with 10 μM GF109203X 30 min before treatment with 100 nM TPA for 24 h. D: Real-time PCR for Cldn18 in HPAF-II cells and HPAC cells pretreated with 10 μM GF109203X 30 min before treatment with 100 nM TPA for 24 h. $^{**}P < 0.01$ versus control or TPA.

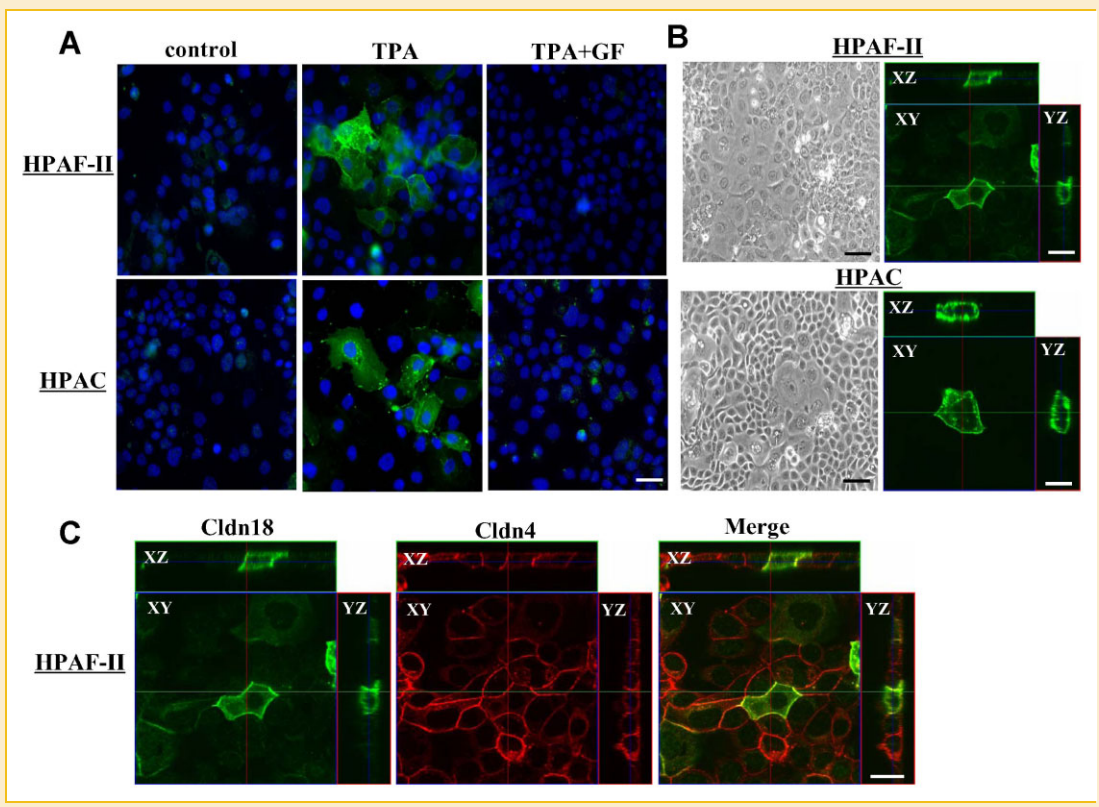

Fig. 4. Appearance of Cldn18-positive cells in HPAF-II and HPAC cells after treatment with TPA. A: Immunostaining for Cldn18 in HPAF-II cells and HPAC cells at 24 h after treatment with 100 nM TPA. Bar: 20 μm. B: Phase-contrast and confocal laser microscopic images of Cldn18 in HPAF-II cells and HPAC cells at 24 h after treatment with 100 nM TPA. Bars: 20 μm. C: Confocal laser microscopic images of Cldn18 and Cldn4 in HPAF-II cells at 24 h after treatment with 100 nM TPA. Bars: 20 μm.

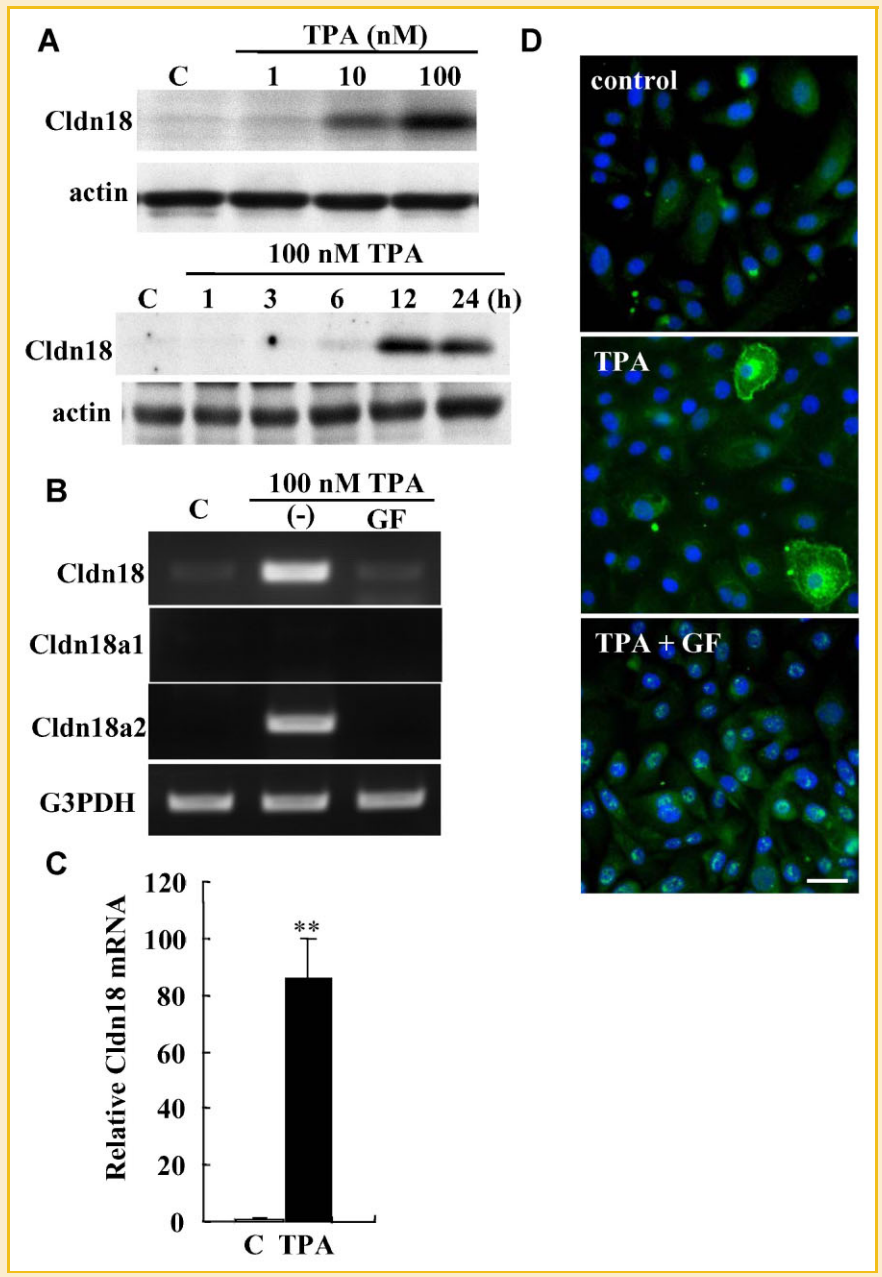

Fig. 5. Upregulation of Cldn18 expression in hTERT-HPDE cells after treatment with TPA. A: Western blotting for Cldn18 in hTERT-HPDE cells from 1 to 24 h after treatment with 1–100 nM TPA. B: RT-PCR for Cldn18 and Cldn18a2 in hTERT-HPDE cells pretreated with 10 μM GF109203X 30 min before treatment with 100 nM TPA for 24 h. C: Real-time PCR for Cldn18 in hTERT-HPDE cells at 24 h after treatment with 100 nM TPA. $^{**}P < 0.01$ versus control. D: Immunostaining for Cldn18 in hTERT-HPDE cells pretreated with 10 μM GF109203X 30 min before treatment with 100 nM TPA for 24 h. Bar: 20 μm.

HPAC cells pretreated with or without 15 μM 5-aza-CdR for 96 h before treatment with or without 100 nM TPA for 24 h. In HPAC cells, both bands of methylation and demethylation were detected, and the demethylated bands in the cells treated with or without 100 nM TPA were significantly increased by 5-aza-CdR (Fig. 7E,F). In hTERT-HPDE cells, no change of Cldn18 protein induced by 100 nM TPA was observed in Western blots after 5 and 15 μM 5-aza-CdR treatments (Fig. 7G,H). In RT-PCR, no change of Cldn18 mRNA in the cells treated with or without TPA was caused by 15 μM 5-aza-CdR (Fig. 7I,J). In MSP analysis bands of demethylation but not methylation were detected in hTERT-HPDE cells and no change of the bands was caused by 15 μM 5-aza-CdR (Fig. 7K,L).

DISCUSSION

In the present study, we demonstrated for the first time that in human pancreatic cancer cells, Cldn18 was regulated at

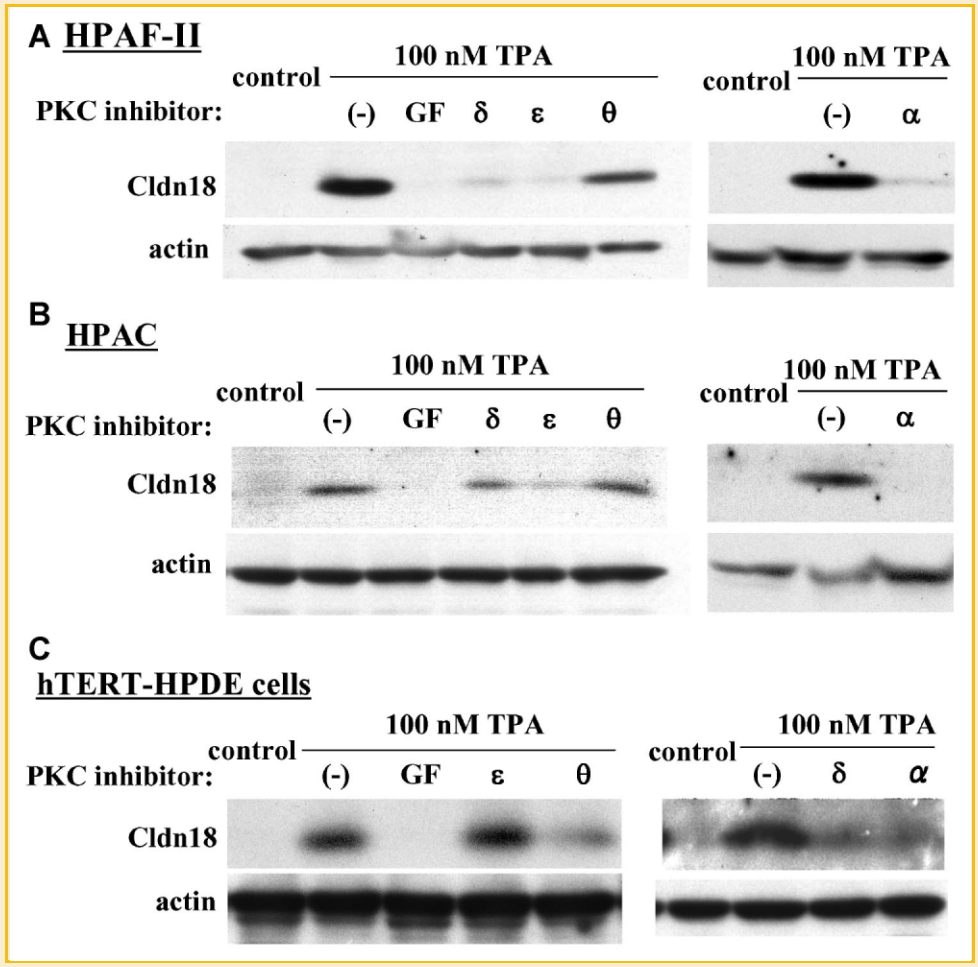

Fig. 6. The effects of inhibitors of PKC isoforms on upregulation of Cldn18 in HPAF-II cells, HPAC cells, and hTERT-HPDE cells after treatment with TPA. A–C: Western blotting for Cldn18 in HPAF-II cells, HPAC cells, and hTERT-HPDE cells pretreated with 10 μM GF109203X as a panPKC inhibitor, 1 μM Gö6976 as a PKCα inhibitor, a 1 μM rottlerin as PKCδ inhibitor, a 10 μM PKCε inhibitor, and a 10 μM PKCθ inhibitor 30 min before treatment with 100 nM TPA.

transcriptional level via specific PKC signaling pathways and modified by DNA methylation. The regulation of Cldn18 in the pancreatic cancers in part differed from that of normal HPDE cells with regard to the types of PKC isoforms.

Claudins represent useful molecular markers for many different cancers because of the high specificity of claudin expression patterns in cancer. In pancreatic cancer, Cldn4 and Cldn18 are highly expressed [Michl et al., 2003; Karanjawala et al., 2008] and are diagnostic or therapeutic targets of monoclonal antibodies against their extracellular loops [Sahin et al., 2008]. In addition, Cldn18 is highly expressed in pancreatic intraepithelial neoplasia, including both precursor lesion PanIN and pancreatic duct carcinoma [Karanjawala et al., 2008]. Cldn18 has two tissue-specific isoforms, Cldn18a1 (lung isoform) and Cldn18a2 (stomach isoform). Cldn18a2 is detected in malignancies of the pancreas, whereas in the normal pancreatic tissues it is not [Sahin et al., 2008]. However, the mechanisms of expression including basal transcriptional regulation, remain to be determined for Cldn18 of pancreatic cancer cells and normal HPDE cells.

The PKC/MAPK/AP-1-dependent pathway regulates Cldn18a2 expression in TPA-treated gastric cancer cells [Yano et al., 2008]. In the present study, to investigate the regulation of Cldn18 and Cldn18a2 in pancreatic cancer cells and normal HPDE cells, various differentiated human pancreatic cancer cell lines and hTERT-HPDE cells, which expressed Cldn18 protein at low levels, were treated with the PKC activator TPA. In all cells examined in this study, mRNAs of Cldn18 or Cldn18a2 were markedly enhanced by treatment with TPA. The upregulation of mRNAs of Cldn18 and Cldn18a2 by TPA was completely prevented by a panPKC inhibitor (Figs. 3 and 5, Supplemental Fig. S1). However, a marked increase of Cldn18 protein by TPA was observed in well- and moderately differentiated cell lines HPAC and HPAF-II as well as in hTERT-HPDE cells, but not in poorly differentiated cell lines PANC-1 and BXPC3. Cldn18 induced by TPA in HPAC and HPAF-II cells was localized at all cytoplasmic membranes, whereas Cldn4 was localized at the apicalmost and basolateral membranes.

In the present study, Cldn18 was highly expressed and localized at the membranes in well-differentiated pancreatic duct carcinomas,

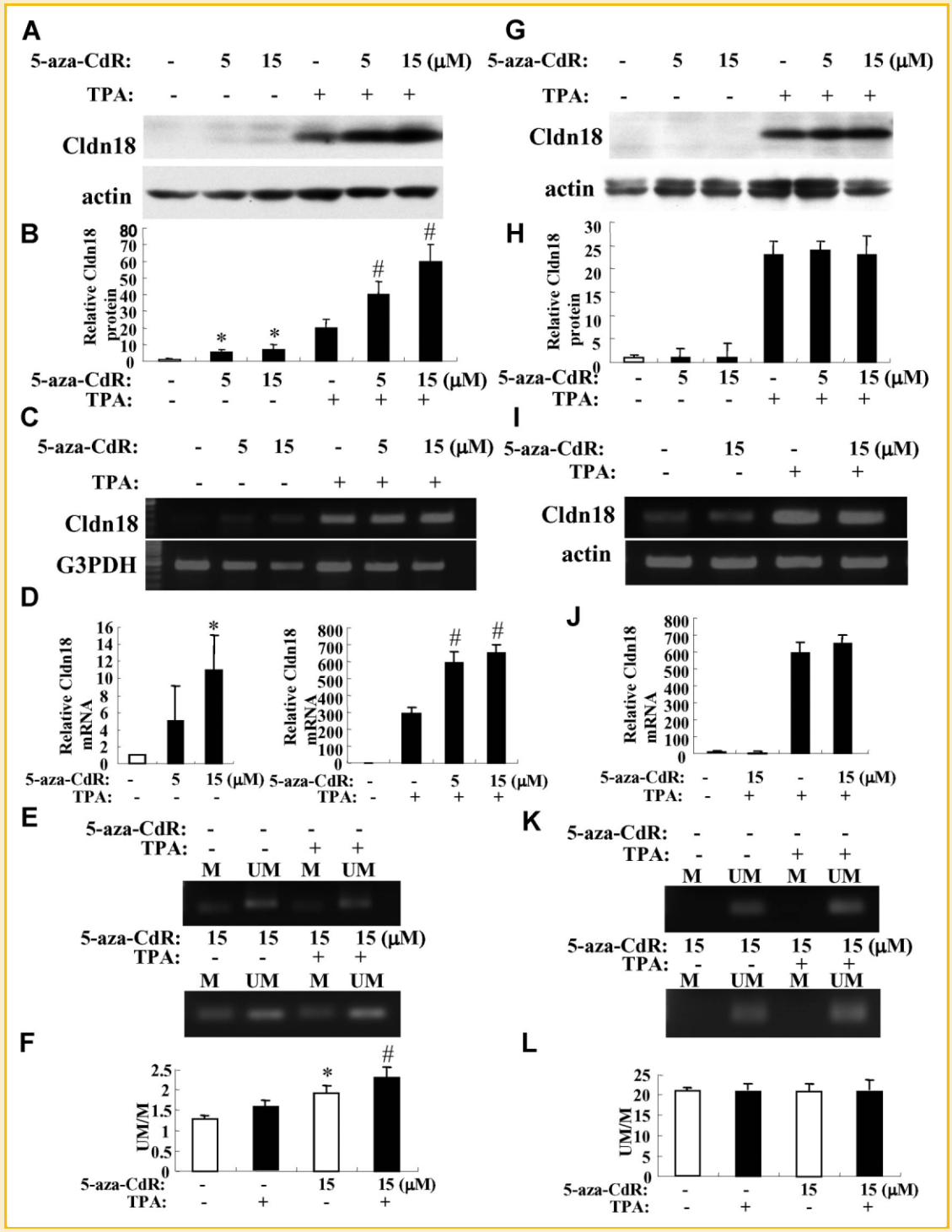

Fig. 7. DNA methylation controls Cldn18 expression in HPAC cells but not hTERT-HPDE cells. A: Western blotting, (C) RT-PCR, and (D) Real-time PCR for Cldn18 in HPAC cells pretreated with 5 and 15 μM 5-aza-CdR for 96 h before treatment with 100 nM TPA. B: The bar graph of A. $^{*}P < 0.05$ versus 5-aza-CdR (−) TPA (−), $^{\#}P < 0.05$ versus 5-aza-CdR (−) TPA (+). E: Methylation-specific PCR (MSP) analysis for Cldn18 in HPAC cells pretreated with 15 μM 5-aza-CdR for 96 h before treatment with 100 nM TPA for 24 h. F: The bar graph of the ratio of unmethylated Cldn18 to methylated Cldn18 (UM/M) from D. $^{*}P < 0.05$ versus 5-aza-CdR (−) TPA (−), $^{\#}P < 0.05$ versus 5-aza-CdR (−) TPA (+). G: Western blotting for Cldn18 in hTERT-HPDE cells pretreated with 5 and 15 μM 5-aza-CdR for 96 h before treatment with 100 nM TPA for 24 h. H: The bar graph of G. $^{*}P < 0.05$ versus 5-aza-CdR (−) TPA (−), $^{\#}P < 0.05$ versus 5-aza-CdR (−) TPA (+). I: RT-PCR, (J) Real-time PCR, and (K) MSP analysis for Cldn18 in hTERT-HPDE cells pretreated with 15 μM 5-aza-CdR for 96 h before treatment with 100 nM TPA for 24 h. L: The bar graph of the ratio of unmethylated Cldn18 to methylated Cldn18 (UM/M) from K.

whereas in normal pancreatic ducts and poorly differentiated pancreatic duct carcinomas, Cldn18-immunoreactivity was not observed. Its expression is most pronounced in well-differentiated pancreatic cancers and Kaplan–Meier analysis showed that the patients whose carcinomas were strongly and diffusely labeled with the antibody to Cldn18 had significantly better survival than those whose carcinomas were weakly labeled or not labeled [Karanjawala et al., 2008]. Cldn4 is highly expressed in many pancreatic carcinomas and its expression decreases the invasiveness and metastatic potential of pancreatic cancer [Michl et al., 2001, 2003]. These findings suggest that an increase in Cldn18 expression in pancreatic cancer cells may be mainly a phenomenon of differentiated and less invasive tumors, like Cldn4 expression, although the biological function of Cldn18 in cancer cells is not precisely known.

At least 12 different isozymes of PKC are known [Newton, 1997] and they appear to regulate the subcellular localization, phosphorylation states, and transcription of several tight junction-associated proteins [Andreeva et al., 2001; Yoo et al., 2003; Banan et al., 2005]. It is also reported that levels of PKCα, PKCβ1, and PKCδ are higher in pancreatic cancer, whereas that of PKCε is higher in normal tissue [El-Rayes et al., 2008]. We previously reported that, in hTERT-HPDE cells after treatment with TPA, a PKCα inhibitor prevented upregulation of Cldn4 and a PKCδ inhibitor prevented upregulation of Cldn7, occludin, ZO-1, and ZO-2 [Yamaguchi et al., 2010]. In the present study, an increase in not only mRNA but also the phosphorylation of threonine of Cldn18 by TPA in HPAC cells was observed to occur via a PKC pathway. Furthermore, the upregulation of Cldn18 by TPA in human pancreatic cancer cell lines was prevented by inhibitors of PKCδ, PKCε, and PKCα, whereas the upregulation of Cldn18 by TPA in hTERT-HPDE cells was prevented by inhibitors of PKCδ, PKCθ, and PKCα. These findings suggested that Cldn18 expression in pancreatic cancer cells was in part controlled by different PKC isozymes than in normal HPDE cells, although PKCα prevented upregulation of Cldn18 in both pancreatic cancer cells and normal cells.

On the other hand, in the development and progression of cancer, tumor suppressor genes may be silenced by mechanisms such as methylation. Silencing of the expression of some claudins in several human cancers is correlated with promoter hypermethylation. These include Cldn7 in breast cancer, Cldn4 in bladder cancer, Cldn11 in gastric cancer, and Cldn3 and Cldn4 in ovarian cancer [Kominsky et al., 2003; Boireau et al., 2007; Agarwal et al., 2009; Kwon et al., 2010]. Furthermore, a CpG island is detected within the promoter region of the Cldn18a2 gene, and treatment with the demethylating agent 5-aza-CdR restored the expression in primary cultures prepared from gastric cancer cell line SNU-I [Sahin et al., 2008]. In the present study, treatment with 5-aza-CdR enhanced upregulation of Cldn18 expression by TPA in HPAC cells and HPAF-II cells but not in hTERT-HPDE cells (Fig. 6, Supplemental Fig. S2). MSP analysis of HPAC cells showed bands of both methylation and demethylation of Cldn18, and the demethylated bands were increased by 5-aza-CdR, whereas in hTERT-HPDE cells, only demethylated bands were detected. Furthermore, in MSP analysis of control BXPC3 cells, methylation bands of Cldn18 were stronger than demethylated bands, and the demethylated bands were increased by 5-aza-CdR (Supplemental Fig. S3). These results indicated that alteration of Cldn18 expression was dependent on DNA methylation in pancreatic cancer cells but not normal HPDE cells.

In conclusion, in differentiated human pancreatic cancer cells, Cldn18 is transcriptionally regulated via specific PKC pathways and modified by DNA methylation. Sequence analysis indicates that several other consensus sequence GATA and SOX transcription factors are located in the proximal region of the human Cldn18a2 promoter [Yano et al., 2008]. We previously reported that, in hTERT-HPDE cells, Cldn7 expression was closely associated with the transcription factor ELF3 [Yamaguchi et al., 2010]. The further finding of specific transcriptional factors against Cldn18 in pancreatic cancer are necessary to understand the regulation and as a therapeutic target using a monoclonal antibody.

ACKNOWLEDGMENTS

This work was supported by the Suhara Memorial Foundation, the Pancreas Research Foundation of Japan and Grants-in-Aid from National Project ''knowledge Cluster Initiative'' (2nd stage, ''Sapporo Biocluster Bio-S''), Program for developing a supporting system for upgrading education and research, the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

Agarwal R, Mori Y, Cheng Y, Jin Z, Olaru AV, Hamilton JP, David S, Selaru FM, Yang J, Abraham JM, Morin PJ, Meltzer SJ. 2009. Silencing of claudin-11 is associated with increased invasiveness of gastric cancer cells. PLoS One 4:e8002.

Ali AS, Ali S, El-Rayes BF, Philip PA, Sarkar FH. 2009. Exploitation of protein kinase C: A useful target for cancer therapy. Cancer Treat Rev 35:1–8.

Andreeva AY, Krause E, Müller EC, Blasig IE, Utepbergenov DI. 2001. Protein kinase C regulates the phosphorylation and cellular localization of occludin. J Biol Chem 276:38480–38486.

Banan A, Zhang LJ, Shaikh M, Fields JZ, Choudhary S, Forsyth CB, Farhadi A, 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.

Boireau S, Buchert M, Samuel MS, Pannequin J, Ryan JL, Choquet A, Chapuis H, Rebillard X, Avancés C, Ernst M, Joubert D, Motett N, Hollande F. 2007. DNA-methylation-dependent alterations of claudin-4 expression in human bladder carcinoma. Carcinogenesis 28:246–258.

Cereijido M, Valdés J, Shoshani L, Contreras RG. 1998. Role of tight junctions in establishing and maintaining cell polarity. Annu Rev Physiol 60:161–177.

Deer EL, González-Hernández J, Coursen JD, Shea JE, Ngatia J, Scaife CL, Firpo MA, Mulvihill SJ. 2010. Phenotype and genotype of pancreatic cancer cell lines. Pancreas 39:425–435.

El-Rayes BF, Ali S, Philip PA, Sarkar FH. 2008. Protein kinase C: A target for therapy in pancreatic cancer. Pancreas 36:346–352.

González-Mariscal L, Tapia R, Chamorro D. 2008. Crosstalk of tight junction components with signaling pathways. Biochim Biophys Acta 1778:729–756.

Gumbiner BM. 1993. Breaking through the tight junction barrier. J Cell Biol 123:1631–1633.

Jemal A, Siegel R, Xu J, Ward E. 2010. Cancer statistics. CA Cancer J Clin 60:277–300.

Karanjawala ZE, Illei PB, Ashfaq R, Infante JR, Murphy K, Pandey A, Schulick R, Winter J, Sharma R, Maitra A, Goggins M, Hruban RH. 2008. New markers of pancreatic cancer identified through differential gene expression analyses: Claudin 18 and annexin A8. Am J Surg Pathol 32:188–196.

Kawano Y, Kobune M, Yamaguchi M, Nakamura K, Ito Y, Sasaki K, Takahashi S, Nakamura T, Chiba H, Sato T, Matsunaga T, Azuma H, Ikebuchi K, Ikeda H, Kato J, Niitsu Y, Hamada H. 2003. Ex vivo expansion of human umbilical cord hematopoietic progenitor cells using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human stromal cells. Blood 101:532–540.

Kojima T, Murata M, Yamamoto T, Lan M, Imamura M, Son S, Takano K, Yamaguchi H, Ito T, Tanaka S, Chiba H, Hirata K, Sawada N. 2009. Tight junction proteins and signal transduction pathways in hepatocytes. Histol Histopathol 24:1463–1472.

Kojima T, Fuchimoto J, Yamaguchi H, Ito T, Takasawa A, Ninomiya T, Kikuchi S, Ogasawara N, Ohkuni T, Masaki T, Hirata K, Himi T, Sawada N. 2010. c-Jun N-terminal kinase is largely involved in the regulation of tricellular tight junctions via tricellulin in human pancreatic duct epithelial cells. J Cell Physiol 225:720–733.

Kominsky SL, Argani P, Korz D, Evron E, Reman V, Garrett E, Rein A, Sauter G, Kallioniemi OP, Sukumar S. 2003. Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene 22:2021–2033.

Kwon MJ, Kim SS, Choi YL, Jung HS, Balch C, Kim SH, Song YS, Marquez VE, Nephew KP, Shin YK. 2010. Derepression of CLDN3 and CLDN4 during ovarian tumorigenesis is associated with loss of repressive histone modifications. Carcinogenesis 31:974–983.

Li LC, Dahiya R. 2002. MethPrimer: Designing primers for methylation PCRs. Bioinformatics 18:1427–1431.

Mackay HJ, Twelves CJ. 2007. Targeting the protein kinase C family: Are we there yet? Nat Rev Cancer 7:554–562.

Matter K, Balda MS. 2003. Functional analysis of tight junctions. Methods 30:228–234.

Michl P, Buchholz M, Rolke M, Kunsch S, Löhr M, McClane B, Tsukita S, Leder G, Adler G, Gress TM. 2001. Claudin-4: A new target for pancreatic cancer treatment using Clostridium perfringens enterotoxin. Gastroenterology 121:678–684.

Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, Menke A, Fensterer H, Giehl K, Löhr M, Leder G, Iwamura T, Adler G, Gress TM. 2003. Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. Cancer Res 63:6265–6271.

Newton AC. 1997. Regulation of protein kinase C. Curr Opin Cell Biol 9:161–167.

Sahin U, Koslowski M, Dhaene K, Usener D, Brabdebburg G, Seitz G, Huber C, Türeci Ö. 2008. Claudin-18 splice variant 2 is a pan-cancer target suitable for therapeutic antibody development. Clin Cancer Res 14:7624–7634.

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

Schneeberger EE, Lynch RD. 1992. Structure, function, and regulation of cellular tight junctions. Am J Physiol 262:L647–L661.

Schneeberger EE, Lynch RD. 2004. The tight junction: A multifunctional complex. Am J Physiol Cell Physiol 286:C1213–C1228.

Suzuki M, Kato-Nakano M, Kawamoto S, Furuya A, Abe Y, Misaka H, Kimoto N, Nakamura K, Ohta S, Ando H. 2009. Therapeutic antitumor efficacy of monoclonal antibody against Claudin-4 for pancreatic and ovarian cancers. Cancer Sci 100:1623–1630.

Takai D, Jones PA. 2003. The CpG island searcher: A new WWW resource. In Silico Biol 3:235–240.

Tsukita S, Furuse M, Itoh M. 2001. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2:285–293.

VanMeer G, Gumbiner B, Simons K. 1986. The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. Nature 322: 639–641.

Yamaguchi H, Kojima T, Ito T, Kimura Y, Imamura M, Son S, Koizumi J, Murata M, Nagayama M, Nobuoka T, Tanaka S, Hirata K, Sawada N. 2010. Transcriptional control of tight junction proteins via a protein kinase C signal pathway in human telomerase reverse transcriptase-transfected human pancreatic duct epithelial cells. Am J Pathol 177:698–712.

Yano K, Imaeda T, Niimi T. 2008. Transcriptional activation of the human claudin-18 gene promoter through two AP-1 motifs in PMA-stimulated MKN45 gastric cancer cells. Am J Physiol Gastrointest Liver Physiol 294: G336–G343.

Yoo J, Nichols A, Mammen J, Calvo I, Song JC, Worrell RT, Matlin K, Matthews JB. 2003. Bryostatin-1 enhances barrier function in T84 epithelia through PKC-dependent regulation of tight junction proteins. Am J Physiol Cell Physiol 285:C300–C309.