Regular Article

ZO-2 Suppresses Cell Migration Mediated by a Reduction in Matrix Metalloproteinase 2 in Claudin-18-Expressing Lung Adenocarcinoma A549 Cells

Risa Akizuki, Hiroaki Eguchi, Satoshi Endo, Toshiyuki Matsunaga, and Akira Ikari*

Laboratory of Biochemistry, Department of Biopharmaceutical Sciences, Gifu Pharmaceutical University, 1–25–4 Daigaku-nishi, Gifu 501–1196, Japan.

Received August 30, 2018; accepted November 14, 2018

Abnormal expression of the tight junctional components claudins (CLDNs) is observed in various malignant tissues. We reported recently that CLDN18 expression is down-regulated in human lung adenocarcinoma tissues. In the present study, we investigated the biological functions of CLDN18 using lung adenocarcinoma A549 cells. Microarray analysis showed that CLDN18 increases zonula occludens (ZO)-2 expression in A549 cells. The ectopic expression of CLDN18 increased nuclear ZO-2 levels, which were inhibited by *N*-[2-[[3-(4-bromophenyl)-2-propen-1-yl]amino]ethyl]5-isoquinolinesulfonamide (H-89), a nonspecific protein kinase A (PKA) inhibitor, but not by a PKA inhibitor 14-22 amide. In addition, dibutyryl cyclic adenosine monophosphate, an analogue of PKA, did not increase ZO-2 levels. These results suggest that H-89 sensitive factors without PKA are involved in the CLDN18-induced elevation of ZO-2. The cell cycle was affected by neither ZO-2 knockdown in CLDN18-expressing A549 (CLDN18/A549) cells nor ZO-2 overexpression in A549 cells, suggesting that ZO-2 does not play an important role in the regulation of cell proliferation. The introduction of ZO-2 small interfering RNA (siRNA) into CLDN18/A549 cells increased migration, the expression and activity of matrix metalloproteinase 2 (MMP2), and the reporter activity of an MMP2 promoter construct. Furthermore, H-89 enhanced both mRNA levels and reporter activity of MMP2 in CLDN18/A549 cells. These results suggested that a reduction in CLDN18-dependent ZO-2 expression enhances MMP2 expression in lung adenocarcinoma cells, resulting in the promotion of the cell migration. CLDN18 may be a novel marker for metastasis in lung adenocarcinoma.

Key words adenocarcinoma; claudin; lung; matrix metalloproteinase

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide. Most lung cancer patients remain asymptomatic and lack common early symptoms until they present with advanced disease.¹⁾ Therefore, lung cancer is usually discovered and diagnosed at an advanced stage. The incidence and mortality rates of lung cancer are the highest among malignant tumors.^{2,3)} Despite the emergence of targeted therapies and immunotherapies, the 5-year relative survival rate for lung cancer patients remains lower than 18%.⁴⁾ Novel biomarkers for the early detection of lung cancer will be important for improving lung cancer survival.

Epithelial cells form cell–cell contacts through tight junctions (TJs), adherent junctions, and gap junctions. TJs are composed of claudins (CLDNs), which are tetraspan proteins with two extracellular loops, and scaffolding proteins including zonula occludens (ZO)-1 and ZO-2. CLDNs comprise a family of over 20 members, and homo- or heterophilic interactions of CLDNs can confer different paracellular ion permeabilities in epithelial cells.⁵⁾ ZO proteins link claudins to the actin-based cytoskeleton and make belt-like TJs.⁶⁾ The biological functions of ZO-1 are well-characterized,⁷⁾ but those of ZO-2 are still to be fully elucidated.

CLDN1, 3, 4, 5, 7, and 18 are expressed in normal lung tissues.^{8,9)} We reported recently that the expression level of CLDN18 in lung cancer was lower than that in normal tissues.¹⁰⁾ CLDN18 down-regulates the proliferation and motility

of lung adenocarcinoma A549 cells. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is partly involved in the regulation of cell proliferation and migration. Phosphoinositide-dependent kinase 1 (PDK1) is a PI3K-dependent kinase that phosphorylates and activates several members of the AGC kinase family including Akt.¹¹⁾ The activation of PI3K induces the nuclear localization of PDK1, leading to the suppression of p27^{Kip1}, which functions as an inhibitory factor of cell cycle G1-S progression.¹²⁾ CLDN18 inhibits the phosphorylation and nuclear localization of PDK1, leading to the promotion of cell proliferation. In contrast, the molecular mechanism of migration has not been revealed in detail.

Cell migration is essential for tumor metastasis. The activity of cell migration is up-regulated by the expression of matrix metalloproteinases (MMP) and inversely down-regulated by tissue inhibitor of metalloproteinase (TIMP).^{13,14)} The rates of positive MMP2 and MMP9 staining in lung adenocarcinoma are 55.5 and 61.8%, respectively.¹⁵⁾ The expression levels of MMP2 and MMP9 are positively correlated with migration in A549 cells.¹⁶⁾ Both MMP2 and MMP9 are gelatinases that can degrade non-fibrillar collagens and type IV collagen, which are the major structural components of the extracellular matrix.¹⁷⁾ TIMP binds to MMP and inhibits the activities.

In the present study, we found that ZO-2 expression is up-regulated in CLDN18-expressing A549 (CLDN18/A549) cells using microarray analysis. The elevation of ZO-2 by CLDN18 was inhibited by *N*-[2-[[3-(4-bromophenyl)-2-propen-1-yl]amino]ethyl]5-isoquinolinesulfonamide (H-89), a nonspe-

* To whom correspondence should be addressed. e-mail: ikari@gifu-pu.ac.jp

cific protein kinase A (PKA) inhibitor,¹⁸⁾ but not by a PKA inhibitor 14-22 amide. In addition, dibutyryl cyclic adenosine monophosphate (DBcAMP), an analogue of cAMP, did not increase ZO-2 expression in A549 cells. The cell cycle was affected by neither ZO-2 knockdown in CLDN18/A549 cells nor ZO-2 overexpression in A549 cells. The expression of MMP2 and cell migration were enhanced by ZO-2 knockdown in CLDN18/A549 cells. These results indicated that the reduction in CLDN18 expression in lung adenocarcinoma cells may be involved in the elevation of migration mediated by decreasing in ZO-2 expression.

MATERIALS AND METHODS

Materials Rabbit anti-CLDN18 polyclonal antibody was obtained from Zymed Laboratories (South San Francisco, CA, U.S.A.). Goat anti- β -actin polyclonal, goat anti-p-cAMP response element binding protein (CREB) polyclonal, goat anti-ZO-2 polyclonal, and rabbit anti-ZO-2 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit anti-p-Akt (S473) and anti-Akt polyclonal antibodies were from Cell Signaling Technology (Beverly, MA, U.S.A.). Rabbit anti-CREB polyclonal antibody was from GeneTex (Hsinchu, Taiwan). Alexa Fluor-conjugated secondary antibodies were from Thermo Fisher Scientific (Waltham, MA, U.S.A.). DBcAMP, H-89 and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002), and PKA inhibitor 14-22 amide were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Cayman Chemical (Ann Arbor, MI, U.S.A.), Biomol BmbH (Hamburg, Germany), and Calbiochem (San Diego, CA, U.S.A.), respectively. All other reagents were of the highest grade of purity available.

Cell Culture Human lung adenocarcinoma A549 cells were obtained from RIKEN BRC (Ibaraki, Japan) and grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Saint Louis, MO, U.S.A.) supplemented with 5% fetal calf serum (FCS), 0.07 mg/mL penicillin-G potassium, and 0.14 mg/mL streptomycin sulfate in a 5% CO₂ atmosphere at 37°C.

Plasmid DNA Construction and Transfection Human CLDN18 or ZO-2 cDNAs containing the entire open reading frames were amplified by RT-PCR using mRNA from human kidney. The cDNAs were subcloned into the mammalian expression vector, pTRE2-hyg. The primer pairs are described in Table 1. Cells were transfected with mock/pTRE2-hyg, ZO-2/pTRE2-hyg, or CLDN18/pTRE2-hyg vector using Lipofectamine 2000 in accordance with the manufacturer's instructions. Stable clones were screened in the presence of 400 μ g/mL hygromycin. Similarly, cells were transfected with negative small interfering RNA (siRNA) or human ZO-2 siRNA using Lipofectamine 2000.

RNA Isolation and Quantitative RT-PCR Total RNA was isolated from cells using TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). Reverse transcription was carried out with a ReverTra Ace qPCR RT Kit (Toyobo Life Science, Osaka, Japan). Quantitative RT-PCR was performed using a KOD SYBR qPCR Mix (Toyobo Life Science). The reaction conditions were an initial 60 s denaturation at 95°C, followed by 40 cycles of amplification (15 s of denaturation at 95°C and 60 s of extension at 60°C). Primers used for PCR are shown in Table 2. The threshold cycle (Ct)

Table 1. Primers for Cloning

Name	Sequence
CLDN18 sense	5'-CGGGATCCCATGTCCACCACCACATGCC-3'
CLDN18 antisense	5'-CCGATATCTTACACATAGTCGTGCTTGG-3'
ZO-2 sense	5'-GAATTCATGCGCGGTGCGAGGAGAC-3'
ZO-2 antisense	5'-GTCGACCTATAATTCTGTGTCCCG-3'

Table 2. Primers for Real Time PCR

Name	Sequence
ZO-2 sense	5'-GCTAAACGGAACCAAAAGATG-3'
ZO-2 antisense	5'-CCTTTGTCTCATGGTTTGGACA-3'
MMP2 sense	5'-ATGACGATGAGCTATGGACCTT-3'
MMP2 antisense	5'-TCAGTGCAGCTGTTGTACTCT-3'
MMP9 sense	5'-TCTTCCAGTACCGAGAGAAAGC-3'
MMP9 antisense	5'-GTCATAGGTACGTCAGCCCACT-3'
TIMP sense	5'-GAAACTGCAGGATGGACTCTT-3'
TIMP antisense	5'-CCAACAGTGTAGGTCTTGGTGA-3'

for each PCR product was calculated using the instrument's software, and Ct values obtained for ZO-2 were normalized by dividing the Ct values obtained for β -actin. The resulting Δ Ct values were then used to calculate the relative change in mRNA expression as a ratio (*R*) according to the equation, $R = 2^{-(\Delta\text{Ct}(\text{drug treatment}) - \Delta\text{Ct}(\text{control}))}$ or $2^{-(\Delta\text{Ct}(\text{CLDN18/A549}) - \Delta\text{Ct}(\text{mock/A549}))}$.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting Nuclear and cytoplasmic fractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). Nuclear (10 μ g) and cytoplasmic (30 μ g) fractions were applied to SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with each primary antibody (1:1000 dilution) at 4°C for 16 h, followed by a peroxidase-conjugated secondary antibody (1:3000 dilution) at room temperature for 1.5 h. Finally, the blots were incubated in EzWestLumi plus (ATTO Corporation, Tokyo, Japan) or ImmunoStar Basic (Wako Pure Chemical Industries, Ltd.) and scanned with a C-DiGit Blot Scanner (LI-COR Biotechnology, Lincoln, NE, U.S.A.). Band density was quantified using ImageJ software (National Institute of Health software).

Immunocytochemistry Cells were cultured on cover glasses. The cells were fixed with methanol for 10 min at -20°C, and then permeabilized with 0.2% Triton X-100 for 15 min. After blocking with 4% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, the cells were incubated with anti-CLDN18 and anti-ZO-2 antibodies for 16 h at 4°C. They were then incubated with Alexa Fluor 488- and 555-conjugated antibodies for 1.5 h at room temperature. Immunolabelled cells were visualized using an LSM 700 confocal microscope (Carl Zeiss, Germany).

Measurement of cAMP Content Cells were lysed with passive lysis buffer (Promega, Madison, WI, U.S.A.). cAMP content was measured using a Cyclic AMP Select ELISA Kit (Cayman) in accordance with the manufacturer's instructions.

Wound-Healing Assay Before wounding, the cells were allowed to form a confluent monolayer in a 35 mm dish. Cells were wounded with a 200- μ L pipette tip and incubated in fresh DMEM supplemented with 0.5% FCS for the period

indicated. Photographs were taken using an Olympus CKX53 microscope with the digital camera. The cell-free area was measured using ImageJ software. Cell migration was expressed as the percentage of the remaining cell-free area to the initial wound area.

Gelatin Zymography Assay The proteolytic activities of MMP2 and MMP9 were assessed by gelatin zymography as described previously.¹⁹⁾ Bands were visualized by staining with Coomassie Brilliant Blue R250.

Luciferase Reporter Assay The reporter plasmid of MMP2 (MMP2/pGL3) was kindly gifted from Dr. I.O.L. Ng (University of Hong Kong). Cells were transfected with MMP2/pGL3 and *renilla* pRL-TK (Promega) vectors using HilyMax (Dojindo Laboratories, Kumamoto, Japan). The pRL-TK vector was used for normalizing transfection efficiency. After 48 h of transfection, luciferase activity was assessed using Dual-Glo Luciferase Assay System (Promega). The luminescence of *firefly* and *renilla* luciferase was measured using an AB-2270 Luminescencer Octa (Atto Corporation).

Cell Cycle Analysis After 48 h of transfection with negative siRNA, ZO-2 siRNA, mock/pTRE2-hyg vector, of ZO-2/pTRE2-hyg vector, the cells were synchronized by incubation in FCS-free media for 24 h. Then, the cells were incubated in fresh DMEM supplemented with 5% FCS for 24 h. After fixation with 70% ethanol, the cells were incubated with 0.2 mg/mL RNase at 37°C for 30 min. The cells were then incubated with 20 µg/mL propidium iodide at room temperature for 30 min. Each sample was analyzed using a BD FACSCant II Flow Cytometer (BD Biosciences), and the percentages of cells in the G1, S, G2/M phases of the cell cycle were analyzed.

Statistics Results are presented as means ± standard error of the mean (S.E.M.). Differences between groups were analyzed using a one-way ANOVA, and corrections for multiple comparison were made using Tukey's multiple comparison tests. Significant differences were assumed at $p < 0.05$.

RESULTS

Elevation of ZO-2 Expression by CLDN18 Expression

Previously, we reported that the mRNA level of CLDN18 in human lung adenocarcinoma tissues is lower than that in normal tissues.¹⁰⁾ To clarify the role of CLDN18 in lung epithelium, we investigated the effect of CLDN18 expression on gene expression in A549 cells using a microarray analysis. The top 20 genes with increased expression levels are shown in Table 3. The mRNA level of ZO-2 was largely increased by CLDN18 expression. Therefore, we decided to examine the expression mechanisms and biological functions of ZO-2.

Nuclear Localization of ZO-2 Quantitative RT-PCR showed that the mRNA level of ZO-2 is elevated by CLDN18 (Fig. 1A). The subcellular localization of ZO-2 was determined using the nuclear/cytoplasmic fractionated lysates. The distinct nuclear and cytoplasmic fractionations were confirmed using nucleoporin p62, a nuclear marker, and Na⁺/K⁺-ATPase, a plasma membrane marker, respectively (Fig. 1B). The protein level of ZO-2 was increased by CLDN18 and mainly distributed in the nuclear fraction. Immunofluorescence measurements showed that CLDN18 was absent in mock/A549 cells and was distributed in the TJs and nuclei of CLDN18/A549 cells (Fig. 1C). Notably, ZO-2 was highly distributed in the nuclei in

Table 3. Microarray Data

Gene symbol	Ratio
TJP2 (ZO-2)	9.350071
LOC100507501	4.502891
LOC100506720	4.391233
FRMD3	3.947342
LOC100507004	3.929409
ZNF527	3.884490
ZBTB46	3.684900
C1orf88	3.511847
XLOC_002629	3.487093
SPATA24	3.375279
Unknown	3.321997
PPY	3.311119
Unknown	3.288450
Unknown	3.266196
RPS6KA6	3.238346
LOC497256	3.098397
LOC100131234	3.089410
Unknown	3.035871
XLOC_013429	2.999107
KLHK33	2.901231

$$\text{Ratio} = \frac{\text{CLDN18} - \text{normalized value}}{\text{Mock} - \text{normalized value}}$$

CLDN18/A549 cells, whereas it was faintly distributed in the cytosol and TJs in mock cells. In contrast, ZO-2 was mainly localized in the TJs in ZO-2/A549 cells (Fig. 1D). These results indicated that CLDN18 may change not only the expression level of ZO-2, but also its subcellular localization.

Decrease in ZO-2 Expression by H-89 in CLDN18/A549 Cells

To clarify the mechanism of ZO-2 elevation, we examined the effect of CLDN18 expression on the activation of intracellular signaling factors. CLDN18 decreased the level of p-Akt without affecting the total amount of Akt (Fig. 2A) as recently reported by our group.¹⁰⁾ LY-294002, which inhibits an upstream regulator PI3K of Akt, decreased p-Akt level, but did not change the mRNA and protein levels of ZO-2 in A549 cells (Figs. 2B, 2D). We searched for another factor and found that the level of p-CREB was increased by CLDN18 expression (Fig. 2E). The intracellular content of cAMP in CLDN18/A549 cells was higher than that in mock/A549 cells (Fig. 2F). The elevation of cAMP may induce the activation of PKA and CREB.²⁰⁾ The p-CREB level was decreased by H-89, a non-specific PKA inhibitor, in CLDN18/A549 cells (Fig. 2G). The expression of ZO-2 was also decreased by H-89 in CLDN18/A549 cells, but not by PKA inhibitor 14-22 amide, a specific inhibitor of PKA (Fig. 2H). In addition, the treatment of A549 cells with DBcAMP, a cAMP analogue, increased p-CREB level, but did not increase the expression level of ZO-2 (Fig. 2I).

Effect of ZO-2 Expression on Cell Proliferation To clarify the biological functions of ZO-2, we investigated the effect of ZO-2 expression on cell proliferation. The introduction of ZO-2 siRNA into CLDN18/A549 cells decreased the protein level of ZO-2 (Fig. 3A). However, the cell populations in G1, S, and G2 phases were not significantly changed by ZO-2 knockdown (Fig. 3C). Similarly, the overexpression of ZO-2 in A549 cells, which was confirmed by Western blot-

Fig. 1. Effect of CLDN18 Expression on the Expression and Subcellular Localization of ZO-2

(A) mRNAs were prepared from mock/A549 and CLDN18/A549 cells. Quantitative RT-PCR was performed using primer pairs for ZO-2 and β -actin. The expression level of ZO-2 was represented relative to the value in mock/A549 cells. (B) Nuclear and cytoplasmic fractions were applied to 7.5 or 10% SDS-PAGE. After Western blotting of ZO-2, nucleoporin p62, and Na^+/K^+ -ATPase (NKA), the expression level of ZO-2 was represented relative to the nuclear level in mock/A549 cells. (C) Mock/A549 and CLDN18/A549 cells were immunostained with rabbit anti-CLDN18 (red), and anti-ZO-2 (green) antibodies. Merged images are shown with DNA stained with DAPI. The right panels show enlarged images. Scale bar indicates 10 μm . (D) Mock/A549 and ZO-2/A549 cells were immunostained with rabbit anti-ZO-2 (green) antibodies and DAPI. $n = 3-4$. ** $p < 0.01$ significantly different from mock. NS, not significantly different. (Color figure can be accessed in the online version.)

ting (Fig. 3B), did not change cell cycle progression (Fig. 3D). In addition, ZO-2 knockdown did not significantly change the populations of the cell cycle in A549 cells (G1 phase: $60.7 \pm 0.3\%$ in negative siRNA and $61.4 \pm 2.3\%$ in ZO-2 siRNA, S phase: $22.1 \pm 0.7\%$ and $21.8 \pm 0.3\%$, and G2/M phases: $17.2 \pm 0.7\%$ and $14.4 \pm 0.4\%$, respectively). These results indicated that ZO-2 may not be directly involved in the regulation of proliferation in A549 cells.

Increases in Cell Migration by ZO-2 Knockdown Cell migration was estimated using a wound-healing assay. The wound area decreased in a time-dependent manner using CLDN18/A549 cells transfected with negative siRNA (Fig. 4A, 4B). The introduction of ZO-2 siRNA enhanced migration in CLDN18/A549 cells. In contrast, ectopic expression of ZO-2 in A549 cells inhibited migration ($14.0 \pm 1.0\%$ in mock/A549 cells vs. $7.2 \pm 0.1\%$ in ZO-2/A549 cells). MMP and TIMP are involved in the regulation of cell migration.^{14,16} So far, we reported that the activities of MMP2 and MMP9 were decreased by CLDN18 expression.¹⁰ The expression levels of MMP2 and MMP9 were decreased similarly to activity by CLDN18 expression (Fig. 4C). ZO-2 siRNA increased the mRNA level of MMP2 without affecting that of TIMP in CLDN18/A549 cells, whereas it decreased that of MMP9 (Fig. 4D). A gelatin zymography assay showed that the activity of MMP2 was increased by ZO-2 knockdown (Fig. 4E). The activity of MMP9 was below the levels of detection. Similarly, the reporter activity of the MMP2 promoter construct was increased by ZO-2 knockdown (Fig. 4F). These results indicated that ZO-2

inhibits migration in A549 cells through downregulation of MMP2. The effect of H-89 on cell migration was investigated because ZO-2 expression was decreased by H-89 as shown in Fig. 2. H-89 increased the mRNA level of MMP2 without affecting MMP9 and TIMP in CLDN18/A549 cells (Fig. 4G). The reporter activity of MMP2 in CLDN18/A549 was lower than that in A549 cells, but it was significantly enhanced by H-89 (Fig. 4H).

DISCUSSION

We found recently that CLDN18 expression in lung cancer tissues and cell lines is lower than that in normal tissues.¹⁰ Similarly, the attenuation of CLDN18 expression has been reported in gastric cancer.²¹ Of note, a lower CLDN18 level is prominent at the invasive front of submucosal gastric cancer. Zhou *et al.*²² reported that CLDN18 knockout led to lung enlargement, parenchymal expansion, and tumorigenesis in mice. CLDN18 may play an important role in the physiological regulation of lung morphology. In contrast, overexpression of CLDN18 has been reported in human pancreatic cancer²³ and bile duct carcinoma.²⁴ CLDN18 has two splice variants, lung-specific isoform 1 (CLDN18.1) and stomach-specific isoform 2 (CLDN18.2). The function of CLDN18 in tumorigenesis may be different with each isoform.

ZO-2 is localized in the TJs in confluent epithelia, whereas it was detected in the nuclei in sparse proliferating cells.²⁵ Actually, ZO-2 was mainly localized in the TJs of ZO-2/A549

Fig. 2. Decrease in ZO-2 Expression by Treatment with H-89 in CLDN18/A549 Cells

(A) Cytoplasmic fractions of mock/A549 and CLDN18/A549 cells were applied to 10% or 12.5% SDS-PAGE, and blotted with anti-CDLN18, anti-p-Akt, anti-Akt, or anti- β -actin antibodies. (B) A549 cells were incubated in the absence or presence of 10 μ M LY-294002 for 1 h. Cytoplasmic fractions were blotted with anti-p-Akt or anti-Akt antibodies. (C) A549 cells were incubated in the absence or presence of 10 μ M LY-294002 for 6 h. Quantitative RT-PCR was performed using primer pairs for ZO-2 and β -actin. The expression level of ZO-2 is represented relative to the value in control cells. (D) CLDN18/A549 cells were incubated in the absence or presence of 10 μ M LY-294002 for 24 h. After Western blotting of ZO-2 and β -actin, the expression levels in cytoplasmic fractions are represented relative to the values in control cells. (E) Cytoplasmic fractions were blotted with anti-p-CREB or anti-CREB antibodies. The expression level of p-CREB/CREB is represented relative to the values in mock cells. (F) cAMP content was measured using an ELISA kit. (G) CLDN18/A549 cells were incubated in the absence or presence of H-89 for 30 min. Cytoplasmic fractions were blotted with anti-CREB or anti-CREB antibodies. The expression level of p-CREB/CREB is represented relative to the values in control cells. (H) CLDN18/A549 cells were incubated in the absence or presence of 50 μ M H-89 and 10 μ M PKA inhibitor 14-22 amide (PKI) for 24 h. Cytoplasmic fraction was blotted with anti-ZO-2 or anti- β -actin antibodies. (I) A549 cells were incubated in the absence or presence of 500 μ M DBcAMP. Cytoplasmic fractions were blotted with anti-ZO-2, anti-p-CREB, or anti- β -actin antibodies. $n = 3-4$. ** $p < 0.01$ and * $p < 0.05$ significantly different from 0 μ M H-89 or mock cells. NS, not significantly different.

cells under confluent conditions (Fig. 1D). The subcellular localization of ZO-2 is regulated by a nuclear localization signal (NLS) and a nuclear exportation signal (NES). Akt-dependent phosphorylation of SRPK induces the phosphorylation of SR repeats of ZO-2, leading to ZO-2 entry into the nuclei.²⁶⁾ CLDN18 expression decreased p-Akt levels in A549 cells (Fig. 2A). Therefore, it was predicted that ZO-2 is localized in the TJs of CLDN18/A549 cells, but instead it was mainly localized in the nuclei (Figs. 1B, 1C). The phosphorylation of S369 located within the NES sequence of ZO-2 by protein kinase

C ϵ (PKC ϵ) is critical for the exportation of ZO-2 from the nuclei.²⁷⁾ The function of the NLS may be superior compared with that of NES in CLDN18/A549 cells. Another explanation is that ZO-2 forms a complex with LASP-1, a LIM and SH3 domain protein, and translocates to the nuclei in a PKA-dependent manner in breast cancer MDA-MB231 cells.²⁸⁾ Association with other proteins may be necessary for the nuclear localization of ZO-2 in ZO-2/A549 cells.

Intracellular cAMP content is regulated by adenylate cyclase and phosphodiesterase activities.²⁹⁾ cAMP increases

Fig. 3. No Effect of ZO-2 Expression on the Cell Cycle

(A) Cytoplasmic fractions were prepared from the CLDN18/A549 cells transfected with negative or ZO-2 siRNA. (B) Cytoplasmic fractions were prepared from mock/A549 and ZO-2/A549 cells. The aliquots were blotted with anti-ZO-2 or anti- β -actin antibodies. (C and D) The percentages of cells in the G1, S, and G2/M phases were analyzed using a flow cytometer. $n = 4$.

PKA activity and the phosphorylation of PKA substrates including CREB. The intracellular cAMP content and p-CREB level in CLDN18/A549 cells were higher than those in mock/A549 cells, and the phosphorylation of CREB was inhibited by H-89 (Fig. 2). In addition, ZO-2 expression was decreased by H-89 in CLDN18/A549 cells. These results suggested that CLDN18 increased ZO-2 expression mediated by an activation of cAMP/PKA/CREB pathway. However, PKA inhibitor 14-22 amide did not decrease ZO-2 level in CLDN18/A549 cells. In addition, DBcAMP increased p-CREB level in A549 cells, but did not increase ZO-2 level. These results suggested that the cAMP/PKA/CREB pathway is not involved in the elevation of ZO-2 in CLDN18/A549 cells. H-89 can inhibit ribosomal protein s6 kinase, mitogen- and stress-activated kinase 1, and Rho-associated protein kinase II besides PKA.³⁰⁾ Further studies are needed to clarify the molecular mechanism by which CLDN18 increases ZO-2 level in A549 cells.

The correlation between ZO-2 expression and cell proliferation is well understood. ZO-2 enters the nucleus in late G1 phase and inhibits cyclin D1 expression, a putative cell cycle regulator, and proliferation in Madin-Darby canine kidney (MDCK) cells.³¹⁾ ZO-2 was mainly localized in the nuclei in CLDN18/A549 cells (Fig. 1). Our recent results indicated that cell proliferation is suppressed by CLDN18 expression concomitant with inhibition of G1-S progression.¹⁰⁾ These data profoundly suggest that ZO-2 is involved in the downregulation of proliferation in A549 cells. However, the populations of the cell cycle were changed by neither ZO-2 knockdown in

Fig. 4. Suppression of Cell Migration by ZO-2

(A) CLDN18/A549 cells were transfected with negative or ZO-2 siRNA. After wounding, cell images were taken at 0 and 24h. (B) Cell migration into the wound area was represented as the % recovery relative 0h. (C and D) Total RNA was isolated from mock/A549, CLDN18/A549, CLDN18/A549 cells transfected with negative or ZO-2 siRNA. Quantitative RT-PCR was performed using primer pairs for MMP2, MMP9, and TIMP. The expression levels of these mRNAs are represented relative to the value in negative siRNA. (E) After the collection of culture media, aliquots were applied to a SDS-PAGE containing 0.1% gelatin and visualized using Coomassie Brilliant Blue R250. The densities are represented relative to the values in negative siRNA. (F) The reporter activity of MMP2 was measured in negative or ZO-2 siRNA transfected cells. The activity is represented relative to the values in negative siRNA. (G) CLDN18/A549 cells were incubated in the absence or presence of 50 μ M H-89 for 6h. Quantitative RT-PCR was performed using primer pairs for MMP2, MMP9, and TIMP. The expression levels of these mRNAs are represented relative to the value in control cells. (H) The reporter activity of MMP2 was measured in A549, CLDN18/A549, and 50 μ M H-89-treated CLDN18/A549 cells. The activities are represented relative to the value in A549 cells. $n = 3-4$. ** $p < 0.01$ and * $p < 0.05$ significantly different from negative siRNA, control cells, or A549 cells. NS, not significantly different. ## $p < 0.01$ significantly different from A549 cells.

CLDN18/A549 cells nor ZO-2 overexpression in A549 cells (Fig. 3). We suggest that ZO-2 does not play an important role in CLDN18-induced suppression of proliferation in A549 cells.

There are some reports showing that CLDNs expression affects MMP expression. The expression of CLDN1 is positively correlated with MMP2 and MMP9 expression in renal carcinoma cells³²⁾ and endometrial cancer cells.³³⁾ In contrast, CLDN18 is a negatively correlated with MMP2 and MMP9 activities in A549 cells.¹⁰⁾ Similarly, negative correlation between CLDN1 and MMP2 expression was reported in retinoblastoma.³⁴⁾ At present, we do not know how CLDNs up- or down-regulate MMP expression, but ZO-2 may be involved as shown in Fig. 4. ZO-2 siRNA increased MMP2 expression in CLDN18/A549 cells, whereas it decreased MMP9 expression (Figs. 4C, 4D). These data did not coincide with those in CLDN18 overexpressing cells. In addition, H-89 increased MMP2 expression in CLDN18/A549 cells, whereas it did not change MMP9 expression (Fig. 4G). The regulatory mechanism of MMP9 expression may be different in that of MMP2 expression. ZO-2 expression was reduced in bronchopulmonary cancer, leading to an increase in invasive and migrational capacities.³⁵⁾ These data coincided with our data showing that ZO-2 knockdown enhanced cell migration in CLDN18/A549 cells, whereas ZO-2 overexpression showed an inverse effect in A549 cells. ZO-2 forms a complex with a variety of transcriptional factors including c-Jun, c-Fos, C/EBP, and c-Myc,^{36,37)} which are involved in the regulation of MMP2 expression.^{38–40)} We need further studies to understand how ZO-2 suppresses MMP2 expression in lung cells.

In conclusion, CLDN18 increased the expression level and nuclear localization of ZO-2 in A549 cells. p-Akt level were decreased by CLDN18, whereas cAMP and p-CREB levels were increased. The CLDN18-dependent elevation of ZO-2 was inhibited by H-89, but not by PKA inhibitor 14-22 amide. DBcAMP did not elevate ZO-2 expression, suggesting that PKA is not a key regulator of ZO-2. Migration was enhanced by ZO-2 knockdown in CLDN18/A549 cells, but proliferation was not. We suggest that CLDN18 may inhibit abnormal migration in lung epithelial cells, and a low expression level of CLDN18 may be a novel marker of high metastasis potential in lung adenocarcinoma.

Acknowledgments This work was supported in part by Grants from the Takeda Science Foundation, Takahashi Sangyo-Keizai Research Foundation, Smoking Research Foundation, and Futaba Electronics Memorial Foundation (to A.I.).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

- Shao Q, Li J, Li F, Wang S, Wang W, Liu S, Zhang Y. Clinical investigation into the initial diagnosis and treatment of 1,168 lung cancer patients. *Oncol. Lett.*, **9**, 563–568 (2015).
- Scheff RJ, Schneider BJ. Non-small-cell lung cancer: treatment of late stage disease: chemotherapeutics and new frontiers. *Semin. Intervent. Radiol.*, **30**, 191–198 (2013).
- Wong MCS, Lao XQ, Ho KF, Goggins WB, Tse SLA. Incidence and mortality of lung cancer: global trends and association with socioeconomic status. *Sci. Rep.*, **7**, 14300 (2017).
- Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J. Clin.*, **66**, 7–30 (2016).
- Turksen K, Troy TC. Barriers built on claudins. *J. Cell Sci.*, **117**, 2435–2447 (2004).
- Tsukita S, Katsuno T, Yamazaki Y, Umeda K, Tamura A, Tsukita S. Roles of ZO-1 and ZO-2 in establishment of the belt-like adherens and tight junctions with paracellular permselective barrier function. *Ann. N. Y. Acad. Sci.*, **1165**, 44–52 (2009).
- Hervé JC, Derangeon M, Sarrouilhe D, Bourmeyster N. Influence of the scaffolding protein Zonula Occludens (ZO) on membrane channels. *Biochim. Biophys. Acta*, **1838**, 595–604 (2014).
- Coyne CB, Gambling TM, Boucher RC, Carson JL, Johnson LG. Role of claudin interactions in airway tight junctional permeability. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **285**, L1166–L1178 (2003).
- Daugherty BL, Mateescu M, Patel AS, Wade K, Kimura S, Gonzales LW, Guttentag S, Ballard PL, Koval M. Developmental regulation of claudin localization by fetal alveolar epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **287**, L1266–L1273 (2004).
- Shimobaba S, Taga S, Akizuki R, Hichino A, Endo S, Matsunaga T, Watanabe R, Yamaguchi M, Yamazaki Y, Sugatani J, Ikari A. Claudin-18 inhibits cell proliferation and motility mediated by inhibition of phosphorylation of PDK1 and Akt in human lung adenocarcinoma A549 cells. *Biochim. Biophys. Acta*, **1863** (6 Pt A), 1170–1178 (2016).
- Williams MR, Arthur JS, Balendran A, van der Kaay J, Poli V, Cohen P, Alessi DR. The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.*, **10**, 439–448 (2000).
- Kikani CK, Verona EV, Ryu J, Shen Y, Ye Q, Zheng L, Qian Z, Sakaue H, Nakamura K, Du J, Ji Q, Ogawa W, Sun LZ, Dong LQ, Liu F. Proliferative and antiapoptotic signaling stimulated by nuclear-localized PDK1 results in oncogenesis. *Sci. Signal.*, **5**, ra80 (2012).
- Gueders MM, Foidart JM, Noel A, Cataldo DD. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung diseases. *Eur. J. Pharmacol.*, **533**, 133–144 (2006).
- Ramer R, Merkord J, Rohde H, Hinz B. Cannabidiol inhibits cancer cell invasion via upregulation of tissue inhibitor of matrix metalloproteinases-1. *Biochem. Pharmacol.*, **79**, 955–966 (2010).
- Cai X, Zhu H, Li Y. PKC ζ , MMP2 and MMP9 expression in lung adenocarcinoma and association with a metastatic phenotype. *Mol. Med. Rep.*, **16**, 8301–8306 (2017).
- Keerathichamroen S, Lirdprapamongkol K, Svasti J. Mechanism of ECM-induced dormancy and chemoresistance in A549 human lung carcinoma cells. *Oncol. Rep.*, **39**, 1765–1774 (2018).
- Crouch E, Sage H, Bornstein P. Structural basis for apparent heterogeneity of collagens in human basement membranes: type IV procollagen contains two distinct chains. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 745–749 (1980).
- Murray AJ. Pharmacological PKA inhibition: all may not be what it seems. *Sci. Signal.*, **1**, re4 (2008).
- Ikari A, Sato T, Takiguchi A, Atomi K, Yamazaki Y, Sugatani J. Claudin-2 knockdown decreases matrix metalloproteinase-9 activity and cell migration via suppression of nuclear Sp1 in A549 cells. *Life Sci.*, **88**, 628–633 (2011).
- Sands WA, Palmer TM. Regulating gene transcription in response to cyclic AMP elevation. *Cell. Signal.*, **20**, 460–466 (2008).
- Oshima T, Shan J, Okugawa T, Chen X, Hori K, Tomita T, Fukui H, Watari J, Miwa H. Down-regulation of claudin-18 is associated with the proliferative and invasive potential of gastric cancer at the invasive front. *PLOS ONE*, **8**, e74757 (2013).
- Zhou B, Flodby P, Luo J, Castillo DR, Liu Y, Yu FX, McConnell A, Varghese B, Li G, Chinge NO, Sunohara M, Koss MN, Elatre W, Conti P, Liebler JM, Yang C, Marconett CN, Laird-Offringa IA, Minoo P, Guan K, Stripp BR, Crandall ED, Borok Z. Claudin-

- 18-mediated YAP activity regulates lung stem and progenitor cell homeostasis and tumorigenesis. *J. Clin. Invest.*, **128**, 970–984 (2018).
- 23) Kyuno D, Yamaguchi H, Ito T, Kono T, Kimura Y, Imamura M, Konno T, Hirata K, Sawada N, Kojima T. Targeting tight junctions during epithelial to mesenchymal transition in human pancreatic cancer. *World J. Gastroenterol.*, **20**, 10813–10824 (2014).
 - 24) Takasawa K, Takasawa A, Osanai M, Aoyama T, Ono Y, Kono T, Hirohashi Y, Murata M, Sawada N. Claudin-18 coupled with EGFR/ERK signaling contributes to the malignant potentials of bile duct cancer. *Cancer Lett.*, **403**, 66–73 (2017).
 - 25) Islas S, Vega J, Ponce L, Gonzalez-Mariscal L. Nuclear localization of the tight junction protein ZO-2 in epithelial cells. *Exp. Cell Res.*, **274**, 138–148 (2002).
 - 26) Quiros M, Alarcon L, Ponce A, Giannakouros T, Gonzalez-Mariscal L. The intracellular fate of zonula occludens 2 is regulated by the phosphorylation of SR repeats and the phosphorylation/O-GlcNAcylation of S257. *Mol. Biol. Cell*, **24**, 2528–2543 (2013).
 - 27) Chamorro D, Alarcon L, Ponce A, Tapia R, Gonzalez-Aguilar H, Robles-Flores M, Mejia-Castillo T, Segovia J, Bandala Y, Juaristi E, Gonzalez-Mariscal L. Phosphorylation of zona occludens-2 by protein kinase C epsilon regulates its nuclear exportation. *Mol. Biol. Cell*, **20**, 4120–4129 (2009).
 - 28) Mihlan S, Reiss C, Thalheimer P, Herterich S, Gaetznert S, Kremer-Skothien J, Pavenstadt HJ, Lewandrowski U, Sickmann A, Butt E. Nuclear import of LASP-1 is regulated by phosphorylation and dynamic protein-protein interactions. *Oncogene*, **32**, 2107–2113 (2013).
 - 29) Dessauer CW. Adenylyl cyclase–A-kinase anchoring protein complexes: the next dimension in cAMP signaling. *Mol. Pharmacol.*, **76**, 935–941 (2009).
 - 30) Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.*, **351**, 95–105 (2000).
 - 31) Tapia R, Huerta M, Islas S, Avila-Flores A, Lopez-Bayghen E, Weiske J, Huber O, Gonzalez-Mariscal L. Zona occludens-2 inhibits cyclin D1 expression and cell proliferation and exhibits changes in localization along the cell cycle. *Mol. Biol. Cell*, **20**, 1102–1117 (2009).
 - 32) Ogasawara N, Kudo T, Sato M, Kawasaki Y, Yonezawa S, Takahashi S, Miyagi Y, Natori Y, Sugiyama A. Reduction of Membrane Protein CRIM1 Decreases E-Cadherin and Increases Claudin-1 and MMPs, Enhancing the Migration and Invasion of Renal Carcinoma Cells. *Biol. Pharm. Bull.*, **41**, 604–611 (2018).
 - 33) Shimada H, Satohisa S, Kohno T, Konno T, Takano KI, Takahashi S, Hatakeyama T, Arimoto C, Saito T, Kojima T. Downregulation of lipolysis-stimulated lipoprotein receptor promotes cell invasion via claudin-1-mediated matrix metalloproteinases in human endometrial cancer. *Oncol. Lett.*, **14**, 6776–6782 (2017).
 - 34) Wan WC, Jin XM, Zheng GY, Zhang FY, Lv Y, Zhu Y. How expressions of Claudin-1 and Mmp-2 in retinoblastoma correlate with histological differentiation and optic nerve invasion. *J. Biol. Regul. Homeost. Agents*, **29**, 373–378 (2015).
 - 35) Luczka E, Syne L, Nawrocki-Raby B, Kileztky C, Hunziker W, Birembaut P, Gilles C, Polette M. Regulation of membrane-type 1 matrix metalloproteinase expression by zonula occludens-2 in human lung cancer cells. *Clin. Exp. Metastasis*, **30**, 833–843 (2013).
 - 36) Betanzos A, Huerta M, Lopez-Bayghen E, Azuara E, Amerena J, Gonzalez-Mariscal L. The tight junction protein ZO-2 associates with Jun, Fos and C/EBP transcription factors in epithelial cells. *Exp. Cell Res.*, **292**, 51–66 (2004).
 - 37) Huerta M, Munoz R, Tapia R, Soto-Reyes E, Ramirez L, Recillas-Targa F, Gonzalez-Mariscal L, Lopez-Bayghen E. Cyclin D1 is transcriptionally down-regulated by ZO-2 via an E box and the transcription factor c-Myc. *Mol. Biol. Cell*, **18**, 4826–4836 (2007).
 - 38) Hwang YP, Yun HJ, Choi JH, Han EH, Kim HG, Song GY, Kwon KI, Jeong TC, Jeong HG. Suppression of EGF-induced tumor cell migration and matrix metalloproteinase-9 expression by capsaicin via the inhibition of EGFR-mediated FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 signaling. *Mol. Nutr. Food Res.*, **55**, 594–605 (2011).
 - 39) Pramanik KK, Nagini S, Singh AK, Mishra P, Kashyap T, Nath N, Alam M, Rana A, Mishra R. Glycogen synthase kinase-3beta mediated regulation of matrix metalloproteinase-9 and its involvement in oral squamous cell carcinoma progression and invasion. *Cell Oncol. (Dordr)*, **41**, 47–60 (2018).
 - 40) Staun-Ram E, Goldman S, Shalev E. p53 Mediates epidermal growth factor (EGF) induction of MMP-2 transcription and trophoblast invasion. *Placenta*, **30**, 1029–1036 (2009).