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Increase in claudin-2 expression by an EGFR/MEK/ERK/c-Fos pathway in lung adenocarcinoma A549 cells

Akira Ikari [a,*], Tomonari Sato [a], Ryo Watanabe [a], Yasuhiro Yamazaki [a], Junko Sugatani [a,b]

[a] Department of Pharmaco-Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[b] Global Center of Excellence for Innovation in Human Health Sciences, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

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ABSTRACT

In human adenocarcinoma, claudin-2 expression is higher than that in normal lung tissue, but the regulatory mechanism of its expression has not been clarified. In human adenocarcinoma A549 cells, claudin-2 level time-dependently increased under the control conditions. In contrast, claudin-1 expression remained constant for 24 h. The concentration of epidermal growth factor (EGF) in medium time-dependently increased, which was inhibited by matrix metalloproteinase (MMP) inhibitor II, an inhibitor of MMP-1, 3, 7, and 9. MMP inhibitor II decreased claudin-2 and phosphorylated ERK1/2 (p-ERK1/2) levels, which were recovered by EGF. Both claudin-2 and p-ERK1/2 levels were decreased by EGF neutralizing antibody, EGF receptor (EGFR) siRNA, AG1478, an inhibitor of EGFR, U0126, an inhibitor of MEK, and the exogenous expression of dominant negative-MEK. These results suggest that EGF is secreted from A549 cells by MMP and increases claudin-2 expression mediated via the activation of an EGFR/MEK/ERK pathway. The inhibition of the signaling pathway decreased phosphorylated c-Fos and nuclear c-Fos levels. The introduction of c-Fos siRNA decreased claudin-2 level without affecting claudin-1. The promoter activity of human claudin-2 was decreased by AG1478 and U0126. Furthermore, the activity was decreased by the deletion or mutation of the AP-1 binding site of claudin-2 promoter. Chromatin immunoprecipitation and avidin–biotin conjugated DNA assays showed that c-Fos binds to the AP-1 binding site. We suggest that a secreted EGF up-regulates the transcriptional activity of claudin-2 mediated by the activation of an EGFR/MEK/ERK/c-Fos pathway in A549 cells.

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1. Introduction

Human lung cancer is divided into two classes, small cell and non-small cell lung cancer (NSCLC). NSCLC account for approximately 85% of cases with lung cancer and is the leading cause of cancer-related deaths in the world. More than 50% of NSCLC patients are candidates for systemic treatment with chemotherapy. Epidermal growth factor receptor (EGFR) is more abundantly expressed in lung carcinoma tissue than in adjacent normal tissue [1]. Treatment with the EGFR kinase inhibitors, gefitinib and erlotinib, causes tumor regression in some patients with NSCLC [2]. EGFR-driven cell signaling contributes to the unregulated progression and cancer malignancy. The strong invasive and metastatic characteristics of lung cancer cells are responsible for the relatively high malignancy. The cell–cell adhesion system is involved in the regulation of invasion and metastasis of cancer cells, although the mechanism has not been fully clarified.

The cell–cell adhesion in epithelial and endothelial cells is formed by tight junctions (TJs), adherens junctions, gap junctions, and desmosomes. TJs are located at the apical pole of the lateral membrane and compose a large complex of integral membrane, scaffolding, and signaling proteins [3,4]. TJs separate the apical and basolateral epithelial compartments and regulate the flux of ions and solutes through the paracellular space, cell proliferation, and cell differentiation [5–7]. Claudins and occludin are tetraspanning proteins with cytoplasmic amino and carboxyl termini. The carboxyl terminus has a PDZ-binding motif that can interact with the PDZ domains of scaffolding proteins ZO-1, ZO-2, and ZO-3 [8–10]. Claudins comprise a large family of 27 members that form homo- and heterotypic associations with each other [11–13]. Different combinations of claudins can confer different properties to epithelial cells.

The alteration of claudin expression has been reported in tumors isolated from lung, breast, ovary, pancreas, and colon tissues [14–17]. The expression of certain claudins affects the development as well as invasive and migrative properties of cancer cells. Normal lung epithelia express claudin-1, -3, -4, -5, -7, and -18, but do not express claudin-2, -6, -7, -9, -11, -15, and -16 [18,19]. In patients with lung adenocarcinoma, low claudin-1 expression has shorter overall survival [20]. Claudin-1 overexpression inhibits cell migration, invasion, and metastatic colonization in CL1–5 cells, a human lung adenocarcinoma cell line that lacks endogenous claudin-1 expression. In lung cancer tissue microarrays, claudin-7

Abbreviations: ABCD, avidin–biotin conjugated DNA; CA-MEK, constitutively active-EMK; ChIP, chromatin immunoprecipitation; DN-MEK, dominant negative-MEK; EGF, epidermal growth factor; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; TJs, tight junctions; TGF, transforming growth factor

* Corresponding author. Tel.: +81 54 264 5776; fax: +81 54 264 5773.
E-mail address: ikari@u-shizuoka-ken.ac.jp (A. Ikari).

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expression in lung cancer is either down-regulated or disrupted in its distribution pattern compared to normal tissue [21]. Claudin-7 overexpression inhibits cell migration and invasion in NCI-H1299, a human NSCLC cell line that lacks endogenous claudin-7 expression.

In the present study, we found that claudin-2 expression in human lung adenocarcinoma is higher than that in normal tissue and other lung carcinoma. We and others recently reported that claudin-2 enhances cell migration [22] and colonization [23] in A549 cells derived from human adenocarcinoma. Claudin-2 may be involved in the up-regulation of tumor cell motility. Therefore, we examined the regulatory mechanism of claudin-2 expression using A549 cells. Epidermal growth factor (EGF) is secreted from A549 cells by matrix metalloproteinase (MMP) and the activation of an EGFR/MEK/ERK pathway increases claudin-2 expression. c-Fos, a down-stream target in an EGFR/MEK/ERK pathway, binds to the AP-1 binding site of human claudin-2 promoter and enhances its transcriptional activity. These results indicate that transcriptional activity of claudin-2 is up-regulated by an EGFR/MEK/ERK/c-Fos pathway in A549 cells.

2. Materials and methods

2.1. Materials

The anti-claudin-1 and -2, occludin and ZO-1 antibodies were obtained from Zymed Laboratories (South San Francisco, CA, USA). The anti-E-cadherin antibody was from Becton Dickinson Biosciences (San Jose, CA). The anti-phosphorylated-ERK1/2 (p-ERK1/2), c-Fos, phosphorylated c-Fos (p-c-Fos) and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-ERK1/2 and c-Jun antibodies were from Cell Signaling Technology (Beverly, MA). The anti-phosphorylated c-Jun (p-c-Jun) antibody was from Signalway Antibody (Pearland, TX). The anti-nucleoporin p62 antibody was from Becton Dickinson Biosciences (San Jose, CA). The anti-human EGF antibody was from R&D systems (Minneapolis, MN). Alexa Fluor 488 and 543 conjugated antibodies were from Molecular Probes (Eugene, OR). U0126 was from Sigma-Aldrich (Saint Louis, MO). AG1478 was from Wako Pure Chemical Industries (Osaka, Japan). MMP inhibitor II and MMP-2 inhibitor I were from Merck-Calbiochem (Darmstadt, Germany). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). All other reagents were of the highest grade of purity available.

2.2. Cell culture and transfection

A549, human lung adenocarcinoma, RERF-LC-AI, squamous cells, IA-LM, large cells, and WA-hT, small cells, were obtained from the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Saint Louis, MO) supplemented with 5% fetal calf serum (FCS, HyClone, Logan, UT), 0.07 mg/ml penicillin-G potassium, and 0.14 mg/ml streptomycin sulfate in a 5% CO_2 atmosphere at 37 °C. Growth factors such as EGF and transforming growth factor were not added to the FCS. A549 cells (passages 90–105) were treated with various inhibitors in the FCS-free medium. HA-tagged constitutively active-MEK (CA-MEK) or dominant negative-MEK (DN-MEK) vector (Biomyx, San Diego, CA) was transfected into A549 cells with Lipofectamine 2000 as recommended by the manufacturer.

2.3. Preparation of cell lysates and immunoblotting

Preparation of cell lysates and immunoblotting were performed as described previously [22]. Nuclear protein was isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Rockford, IL).

2.4. Measurement of EGF content

Medium was collected from the cells incubated in the presence and absence of MMP inhibitors. EGF content was measured by Human EGF ELISA kit (Ray Biotech, Norcross, CA) as recommended by the manufacturer.

2.5. RNA isolation and quantitative RT-PCR

Three independent mRNAs of normal lung tissue were purchased from Clontech laboratories (Mountain View, CA), Agilent Technologies (Santa Clara), and BioChain (Hayward, CA). Total RNA was isolated from A549, RERF-LC-AI, IA-LM and WA-hT cells using TRI reagent (Sigma-Aldrich). Reverse transcription was carried out with M-MLV reverse transcriptase (Promega, Madison, WI) and random primers. To compare the expression level of claudin-2 between normal lung and cancer tissues, we obtained Lung Cancer cDNA Array II and V from OriGene (Rockville, MD). Single strand cDNA was amplified by PCR using GoTaq DNA polymerase under the following conditions: denaturation at 94 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 0.5 min; these steps were repeated 30 cycles. Primers used in PCR were 5′-ATGAGGATGGCTGTCATTGG-3′ and 5′-ATTGACTGGGGTCATAGGGT-3′ (claudin-1), 5′-ATTGTGACAGCAGTTGGCTT-3′ and 5′-CTATAGATGTCACACTGGGTGATG-3′ (claudin-2), and 5′-CCTGAGGCACTCTTCCAGCCTT-3′ and 5′-TGCGGATGTCCACGTCACACTTC-3′ (β-actin). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara Bio, Tokyo, Japan). The threshold cycle (Ct) for each PCR product was calculated with the instrument's software, and Ct values obtained for claudin-1 and -2 were normalized by subtracting the ct values obtained for β-actin. The resulting ΔCt values were then used to calculate the relative change in mRNA expression as a ratio (R) according to the equation $R = 2^{-(\Delta Ct(\text{treatment}) - \Delta Ct(\text{control}))}$ or $2^{-(\Delta Ct(\text{cancer}) - \Delta Ct(\text{normal}))}$.

2.6. Confocal microscopy

A549 cells were plated at a confluent density on cover glasses. The cells were fixed with methanol for 10 min at −20 °C, then permeabilized with 0.2% Triton X-100 for 15 min. After blocking with 2% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, the cells were incubated with anti-claudin-2 and ZO-1 antibodies for 16 h at 4 °C. They were then incubated with Alexa Fluor 488 and Alexa Fluor 543-conjugated antibodies for 1 h at room temperature. Immunolabeled cells were visualized on an LSM 510 confocal microscope (Carl Zeiss, Germany). Images were further processed using Adobe Photoshop (Adobe System, San Jose, CA).

2.7. RNA interference

EGFR, c-Fos, and c-Jun siRNAs were produced by Sigma-Aldrich. The oligoribonucleotides were 5′-CAAAGUGUGUAACGGAAUATT-3′ (EGFR siRNA), 5′-CUGUCAACGCGCAGGACUUTT-3′ (c-Fos siRNA) and 5′-CUGAUAAUCCAGUCCAGCATT-3′ (c-Jun siRNA). Cells were transfected with these siRNAs or control siRNA (Fluorescein Conugate)-A (Santa Cruz) using Lipofectamine 2000 as recommended by the manufacturer. Immunoblotting was performed after 48 h of transfection.

2.8. Luciferase reporter assay

The promoter region of the *human claudin-2* gene [NC_000023.10] was subcloned into the pGL4.10(*luc2*) vector (Promega, Madison, WI) upstream of a luciferase reporter gene. The fragment of −1031/+37 was amplified by PCR using a set of primer (sense, 5′-AGCCTGGGTGCCAAAG-3′) and (antisense, 5′-GCTGACTTCTCTCCTCCT-3′). The fragments of −395/+37 and −60/+37 were amplified using primers of (sense, 5′-GTCTGCAGTTTGGCCTC-3′) and (sense, 5′-TTAATCTGGTTTATGG-3′), respectively. A *Renilla* construct, pRL-TK vector (Promega), was used

for normalizing transfection efficiency. Cells were transfected with plasmid DNA using Lipofectamine 2000. After 48 h of transfection, luciferase activity was assessed using the Dual-Glo Luciferase Assay System (Promega). U0126 and AG1478 were added for the final 24 h before the luciferase assay. The luminescence of the *Firefly* and *Renilla* luciferase was measured with a Multilabel Counter 1420 ARVOsx (Perkin Elmer, Wellesley, MA). Relative promoter activity was represented as the fold increase compared to the promoter-less pGL4.10 vector. The mutant of AP-1 binding site (−854/−847) was generated using a Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer pair was 5′-CTGAGATTCCAAAGCATTAAAACAGATACCTGCCTCATGC-3′ (sense) and 5′-GCATGAGGCAG GTATCTGTTTTAATGCTTTGGAATCTCAG-3′ (antisense).

2.9. Chromatin immunoprecipitation (ChIP) assay

A549 cells were treated with 1% formaldehyde to crosslink the protein to the DNA. Then, ChIP assay was performed using EZ ChIP Chromatin Immunoprecipitation Kit (Upstate Biotech, Lake Placid, NY) as recommended by the manufacturer's instructions. To co-immunoprecipitate the DNA, c-Fos and c-Jun antibodies were used. The eluted DNA was amplified by PCR using the primer pairs −935S (sense: 5′-CCCTTAGTGTCCTGAATCTTGG-3′) and −782A (antisense: 5′-AAGAGTCTGCCACAGAGGAAAG-3′) or −1538S (sense: 5′-GCCAGAT TGTGGTGGGTTGA-3′) and −1387A (antisense: 5′-AGAGACCAGGTTT TGCCATGT-3′). To confirm the same amounts of chromatins used in immunoprecipitation between groups, input chromatin was also used. The PCR product (154 and 152 bp) was visualized with ethidium bromide after electrophoretic separation on a 2% agarose gel. Immunoprecipitation using goat IgG was carried out as a negative control.

2.10. Avidin–biotin conjugated DNA (ABCD) assay

The following double-stranded oligonucleotides were used. All biotinylated at the 3′-end of the sense strand, wild-type AP-1: 5′-GATTCCAAAGCATTGACTCAGATACCTGCC-3′, and mutant AP-1: 5′-GATTCCAAAGCATTAAAACAGATACCTGCC-3′. An ABCD assay was performed by incubating nuclear extracts derived from A549 cells with double-stranded DNA immobilized on streptavidin agarose in a binding buffer (80 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.1% Triton X-100, 20 mM Hepes-KOH (pH 7.9), a protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride). After 16 h of incubation at 4 °C, the beads were washed four times with the binding buffer and proteins were applied to the SDS-polyacrylamide gel.

2.11. Statistics

Results are presented as means ± S.E.M. Differences between groups were analyzed with a one-way analysis of variance, and corrections for multiple comparison were made using Tukey's multiple comparison test. Comparisons between two groups were made using Student's *t* test. Significant differences were assumed at $P<0.05$.

3. Results

3.1. Elevation of claudin-2 expression in adenocarcinoma

NSCLC is subdivided in adenocarcinoma, squamous carcinoma, and large cell carcinoma [24]. We examined the expression levels of claudins in the normal and lung cancers using Lung Cancer cDNA Array II and V. A total of 78 patients with lung cancer were evaluated in this study. The characteristics of these patients are shown in Table 1. The normal adjacent tissues of 12 were also examined. In human adenocarcinoma, claudin-2 mRNA level was higher than that in normal tissue and cancer tissues including squamous, large, and small cells (Fig. 1A). The patients with less than 1.0 of claudin-2 were 17, whereas the patients with more than 1.0 were 28 (Table 2). Similar to the patients with adenocarcinoma, claudin-2 mRNA level in

Table 1
Characteristics of the patients.

		No. of patients
Gender		
Male		59
Female		31
Normal		12
Cancer types	Stage	
Adenocarcinoma	I	21
	II	8
	III	12
	IV	4
Squamous	I	11
	II	10
	III	4
	IV	1
Large	I	2
	II	1
	III	1
Small	I	1
	III	2

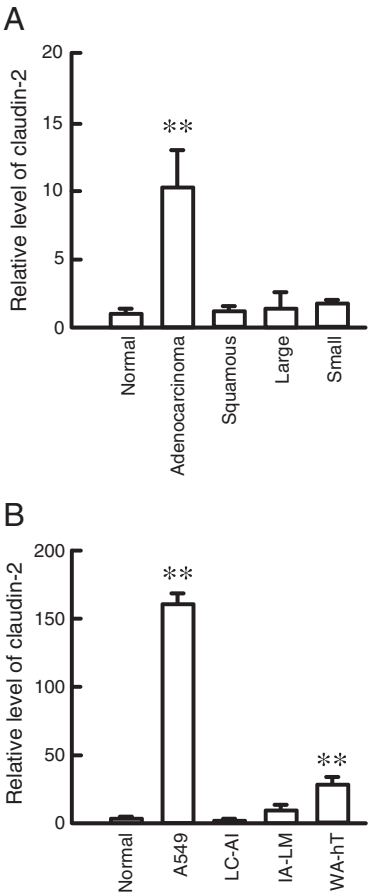

Fig. 1. High levels of claudin-2 in adenocarcinoma and A549 cells. (A) Lung cancer cDNA array II and V contains 12, 45, 26, 4, and 3 samples of normal, adenocarcinoma, squamous, large, and small cells, respectively. Quantitative real-time PCR was performed using primers for claudin-2. β-actin served as an internal control. The expression of claudin-2 mRNA was expressed relative to the value in normal tissue. (B) Three independent mRNAs of normal lung tissue (N) were obtained as described in Materials and methods. A549, RERF-LC-AI (LC-AI), IA-LM, and WA-hT cells were grown to confluence. Total RNA was isolated from these cells and reverse transcribed into cDNA. The expression of claudin-2 mRNA was expressed relative to the value in the normal tissue. ** $P<0.01$ compared with normal tissue.

Table 2
Relative claudin-2 level in the patients with adenocarcinoma.

Relative claudin-2 level	No. of patients
<1.0	17
1.0–5.0	9
5.0<	19

A549 cells was higher than those in normal tissue and other cancer cells (Fig. 1B). Claudin-2 mRNA level in WA-hT cells was also higher than that in normal tissue, but the level was one fifth of A549 cells. These results indicate that claudin-2 expression increases in lung adenocarcinoma.

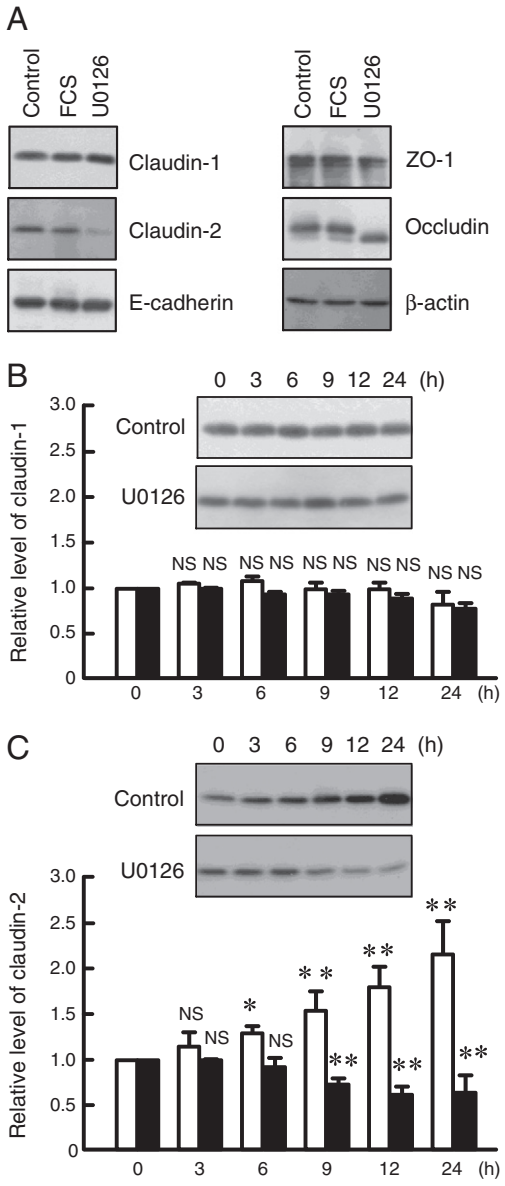

Fig. 2. Decrease in claudin-2 expression by U0126. (A) A549 cells were incubated in the FCS-free medium (control), 5% FCS-containing medium (FCS), or FCS-free medium containing 10 μM U0126 (U0126) for 24 h. Cytoplasmic lysates were immunoblotted with anti-claudin-1, claudin-2, E-cadherin, ZO-1, occludin and β-actin antibodies. (B and C) Cells were incubated in the presence (closed columns) and absence (open columns) of U0126 for the period indicated. Cytoplasmic lysates were immunoblotted with anti-claudin-1 or claudin-2 antibody. The expression of claudin-1 and claudin-2 was expressed relative to the value in 0 h. * $P<0.05$ and ** $P<0.01$ compared with 0 h. NS, $P>0.05$.

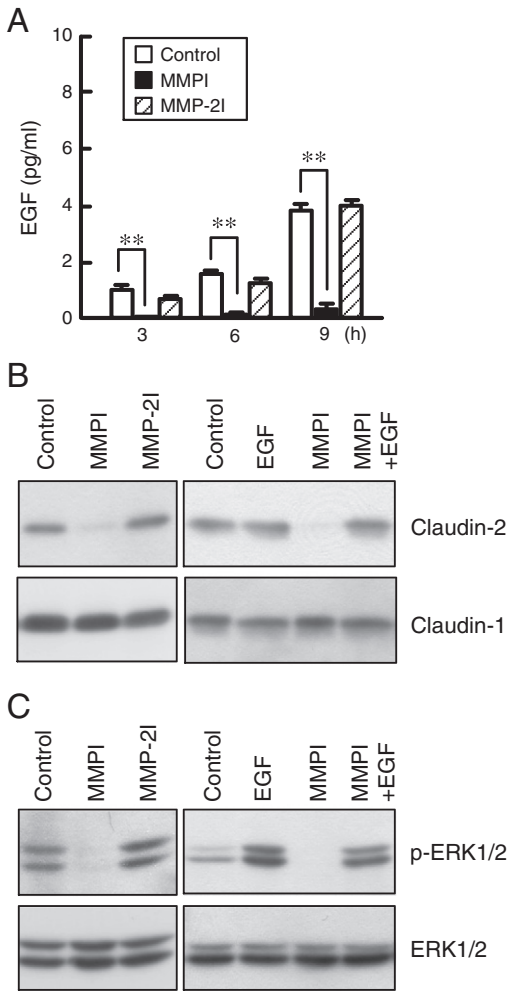

Fig. 3. Decrease in EGF secretion and claudin-2 expression by MMP inhibitor II. (A) Cells were incubated in the presence and absence of 10 μM MMP inhibitor II (MMPI) or 10 μM MMP-2 inhibitor I (MMP-2I) for the period indicated. After being collected the medium, EGF content was measured by the assay kit. (B) Cells were incubated in the absence (control) and presence of MMPI, MMP-2I, 10 ng/ml EGF, or EGF plus MMPI for 24 h. Cytoplasmic lysates were immunoblotted with anti-claudin-1 and claudin-2 antibodies. (C) Cells were incubated in the absence and presence of MMPI, MMP-2I, EGF, or EGF plus MMPI for 30 min. Cytoplasmic lysates were immunoblotted with anti-p-ERK1/2 and ERK1/2 antibodies. ** $P<0.01$ compared with control.

3.2. Decrease in claudin-2 expression by U0126 in adenocarcinoma cell lines

Claudin-2 may be involved in the tumor progression of adenocarcinoma because it enhanced cell migration [22] and colonization [23] in A549 cells. Therefore, we examined what regulatory mechanism is involved in the regulation of claudin-2 expression in A549 cells. Claudin-2 protein was detected in A549 cells, but not in RERF-LC-AI, IA-LM, and WA-hT cells (Supplementary Fig. 1). Claudin-2 level was decreased by U0126, a MEK inhibitor (Fig. 2A). Similarly, U0126 decreased claudin-2 level in lung adenocarcinoma cell lines such as RERF-LC-MA and PC-3 (Supplementary Fig. 2). The levels of claudin-1, E-cadherin, ZO-1, occludin and β-actin were unchanged by U0126. FCS did not affect the levels of these proteins. The band size of occludin was shifted by U0126 because of its de-phosphorylated state [25]. In the absence of FCS, claudin-2 expression increased in a time-dependent manner, which was inhibited by U0126 (Fig. 2C). Claudin-1 expression was constant for 24 h and not inhibited by U0126 (Fig. 2B). These results suggest that some molecules, which activate MEK, increase claudin-2 expression under the present experimental conditions.

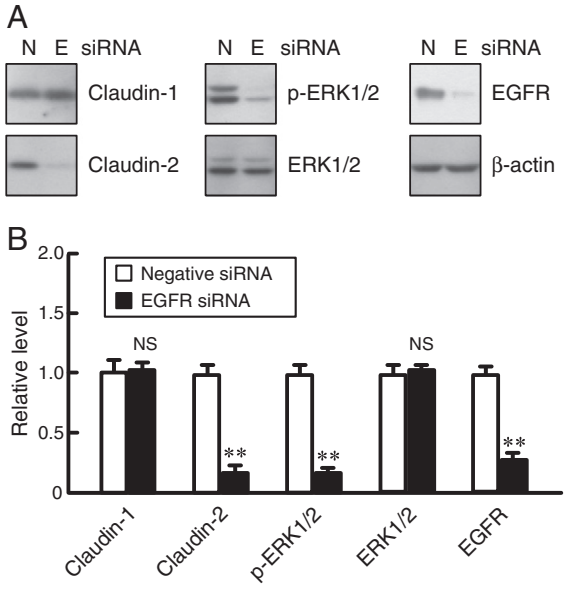

Fig. 4. Decrease in claudin-2 expression by EGFR siRNA. (A) A549 cells were transfected with negative (N) or EGFR (E) siRNA. Cytoplasmic lysates were immunoblotted with anti-claudin-1, claudin-2, pERK1/2, ERK1/2, EGFR and β-actin antibodies. (B) The expression of these proteins was expressed relative to the value in the cells transfected with negative siRNA. ** $P<0.01$ compared with negative siRNA. NS, $P>0.05$.

3.3. Decrease in EGF secretion and claudin-2 expression by MMP inhibitor II

EGF was secreted from A549 cells and increased in a time-dependent manner (Fig. 3A). EGF ligands are synthesized as transmembrane precursors and are cleaved to their active form by metalloproteases [26,27]. We examined the effect of MMP inhibitor II, an inhibitor of MMP-1, -3, -7 and -9, and MMP-2 inhibitor I, an inhibitor of MMP-2, on EGF production, claudin-2 expression, and p-ERK1/2 expression. EGF production was significantly inhibited by MMP inhibitor II, but not by MMP-2 inhibitor I. Similarly, MMP inhibitor II decreased claudin-2 and p-ERK1/2 levels without affecting claudin-1 and ERK1/2 levels (Fig. 3B and C). We examined the effect of MMP-3 and MMP-9 inhibitors on claudin-2 and p-ERK1/2 levels. MMP-9 inhibitor decreased claudin-2 and p-ERK1/2 levels, whereas MMP-3 inhibitor had no effect (Supplementary Fig. 3). These results suggest that MMP-9 is involved in the secretion of EGF, resulting in the elevation of p-ERK1/2 and claudin-2 levels. To clarify the involvement of EGF on the regulation of claudin-2, we examined the effect of EGF on claudin-2 and p-ERK1/2 expression. Both claudin-2 and p-ERK1/2 levels were decreased by MMP inhibitor II, which were recovered by EGF. Furthermore, EGF increased claudin-2 level in a dose-dependent manner (Supplementary Fig. 4). Neither MMP inhibitor II nor EGF affected claudin-1 and p-ERK1/2 levels. These results indicate that EGF is involved in the regulation of claudin-2 expression.

3.4. Decrease in claudin-2 expression by the inhibitors of an EGFR/MEK/ERK pathway

To support the involvement of EGF and its signaling pathway in the regulation of claudin-2 expression, we examined the effects of EGFR siRNA, EGF neutralizing antibody, AG1478, an inhibitor of EGFR, and DN-MEK. EGFR siRNA decreased p-ERK1/2 and claudin-2 levels without affecting ERK1/2 and claudin-1 levels (Fig. 4). Similarly, EGF neutralizing antibody, AG1478, and U0126 decreased p-ERK1/2 and claudin-2 levels without affecting ERK1/2 and claudin-1 (Fig. 5A and B). DN-MEK decreased p-ERK1/2 and claudin-2 levels, whereas CA-MEK did not (Fig. 5C and D). These results indicate that the inhibition of an EGFR/MEK/ERK pathway decreases claduin-2 expression in A549 cells. There raise a possibility that the inhibition of an EGFR/MEK/ERK pathway changes the intracellular distribution of claudin-2. Therefore, we examined the distribution of claudin-2 by immunocytochemistry. Claudin-2 was distributed in both the TJs and the intracellular compartments

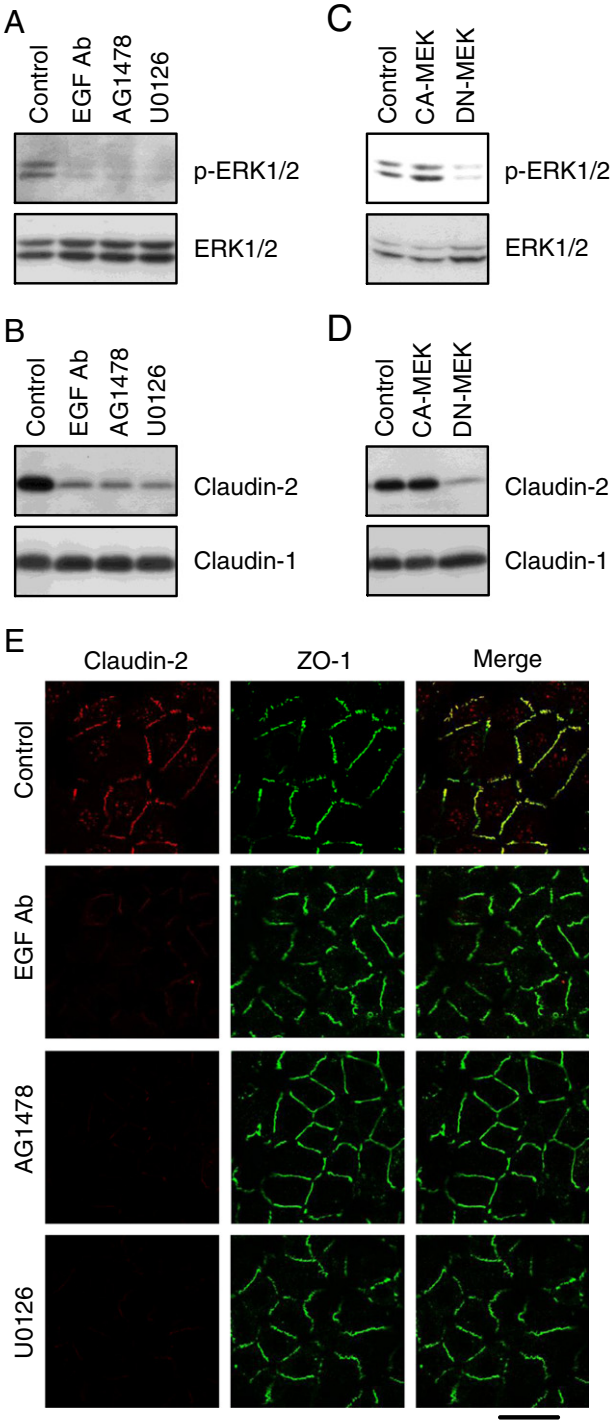

Fig. 5. Decrease in claudin-2 expression by the inhibition of an EGFR/MEK/ERK pathway. (A and B) Cells were incubated in the absence and presence of 1 μg/ml EGF neutralizing antibody (EGF Ab), 10 μM AG1478, or 10 μM U0126 for 30 min (A) or 24 h (B). Cytoplasmic lysates were immunoblotted with anti-p-ERK1/2, ERK1/2, claudin-1, and claudin-2 antibodies. (C and D) Cytoplasmic lysates were isolated from the cells expressing CA-MEK or DN-MEK and was immunoblotted with anti-p-ERK1/2, ERK1/2, claudin-1, and claudin-2 antibodies. (E) Cells were incubated in the absence and presence of EGF neutralizing antibody, AG1478 or U0126 for 24 h. The cells were double stained with anti-claudin-2 (red) and ZO-1 (green) antibodies. The co-localization of claudin-2 and ZO-1 appears yellow in the merged images. The scale bar represents 10 μm.

under the control conditions (Fig. 5E). The red signal of claudin-2 was merged with the green signal of ZO-1. The distribution of ZO-1 was unchanged by EGF neutralizing antibody, AG1478, and U0126. In contrast, the red signal of claudin-2 disappeared from both the TJs and intracellular compartments by these treatments. These results indicate that the inhibition of an EGFR/MEK/ERK pathway decreases claudin-2 expression without affecting its intracellular distribution.

3.5. Decrease in p-c-Fos level by the inhibition of an EGFR/MEK/ERK pathway

The activation of an EGFR/MEK/ERK pathway is involved in the regulation of transcriptional factors such as AP-1 [28], Egr-1 [29], and Sp-1 [30]. One putative AP-1 binding site was detected in the 5′-flanking region of human claudin-2 by computer analysis using TRANSFAC databases (Supplementary Fig. 5). The AP-1 transcriptional complex is composed of at least two major factors, c-Fos and c-Jun. The p-c-Fos and p-c-Jun were detected under the control conditions (Fig. 6A). EGF neutralizing antibody, AG1478, and U0126 decreased p-c-Fos level, but these did not decrease p-c-Jun level (Fig. 6B). Next, we examined the nuclear distribution of c-Fos and c-Jun. Both c-Fos and c-Jun was detected in the nuclei under the control conditions (Fig. 6C). EGF neutralizing antibody, AG1478, and U0126 decreased c-Fos level in the nuclei without affecting c-Jun (Fig. 6D). These results indicate that EGF neutralizing antibody, AG1478, and U0126 decreased p-c-Fos level and the nuclear distribution of c-Fos without affecting c-Jun.

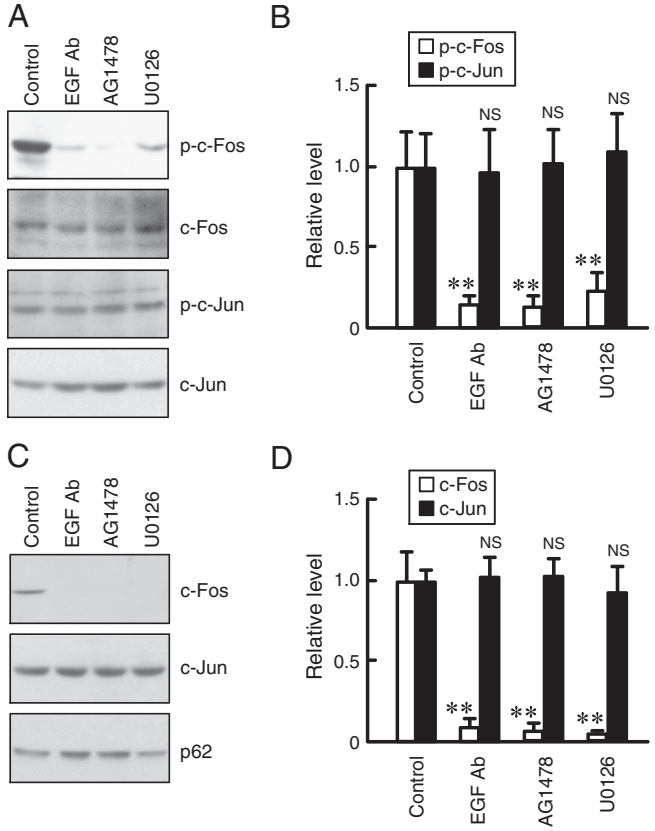

Fig. 6. Effects of EGFR/MEK/ERK pathway inhibitors on the phosphorylation and nuclear distribution of AP-1. Cells were incubated in the absence and presence of 1 μg/ml EGF neutralizing antibody (EGF Ab), 10 μM AG1478, or 10 μM U0126 for 30 min. (A) Cytoplasmic lysates were immunoblotted with anti-p-c-Fos, c-Fos, p-c-Jun, and c-Jun antibodies. (B) The expression of p-c-Fos and p-c-Jun was expressed relative to the value in control. (C) Nuclear fraction was immunoblotted with anti-c-Fos, c-Jun, and nucleoporin p62, a marker of nuclei, antibodies. (D) The expression of c-Fos and c-Jun in the nuclei was expressed relative to the value in control. ** $P<0.01$ compared with control. NS, $P>0.05$.

3.6. Decrease in promoter activity of claudin-2 by AG1478 and U0126

Quantitative real-time PCR showed that the claudin-2 mRNA level is decreased by EGF neutralizing antibody, AG1478, and U0126 (Fig. 7A).

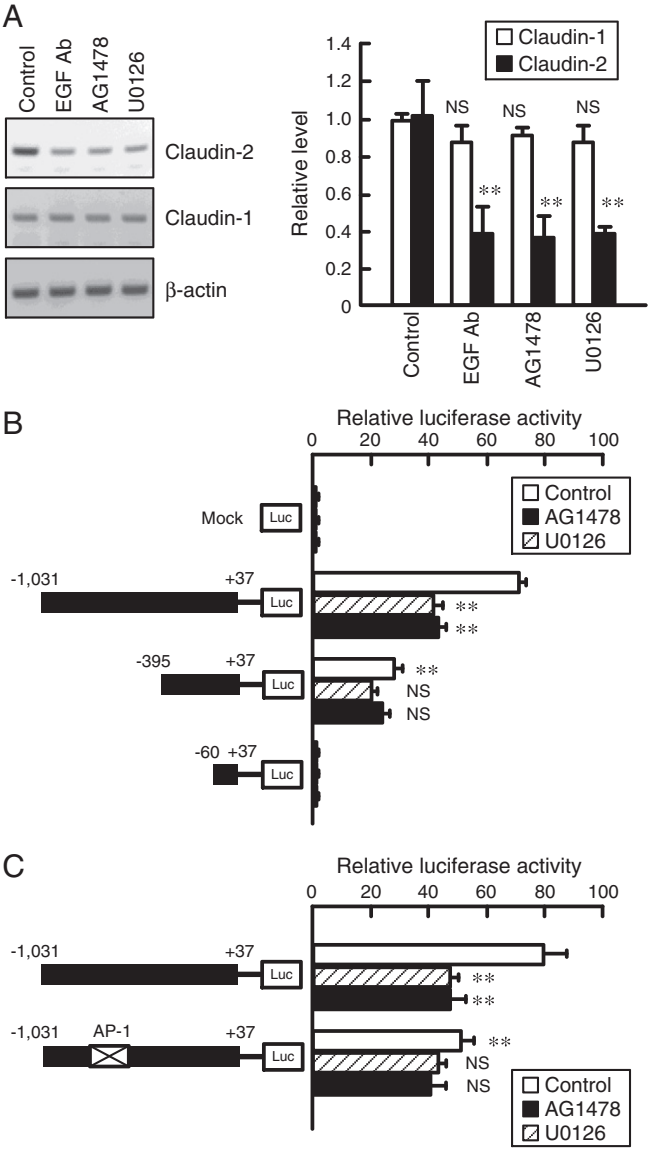

Fig. 7. Effects of EGFR/MEK/ERK pathway inhibitors on mRNA expression and promoter activity of claudin-2. (A) Cells were incubated in the absence and presence of 1 μg/ml EGF neutralizing antibody (EGF Ab), 10 μM AG1478, or 10 μM U0126 for 12 h. After isolating RNA, RT-PCR was performed using the specific primers for claudin-1, claudin-2, and β-actin (Left images). The data of quantitative RT-PCR are shown in the right graph. Claudin-1 and claudin-2 mRNA levels were normalized to β-actin level and represented as the fold increase over control. ** $P<0.01$ compared with control. NS, $P>0.05$. (B) The 5′-flanking regions of human claudin-2 from −1031 to +37, from −395 to +37, and from −60 to +37 were cloned into the promoter-less pGL4.10 vector. Three promoter luciferase constructs were co-transfected with the pRL-TK vector into A549 cells. After 24 h of transfection, the cells were incubated in the absence and presence of 10 μM AG1478 or 10 μM U0126 for 24 h. The relative promoter activity was represented as the fold induction compared to that of the promoter-less pGL4.10 vector (Mock). (C) The 5′-flanking regions of human claudin-2 from −1031 to +37 and mutant of AP-1 binding site were cloned into the promoter-less pGL4.10 vector. After 24 h of transfection, the cells were incubated in the absence and presence of 10 μM AG1478 or 10 μM U0126 for 24 h. Data represent means ± S.E.M. of 3–4 experiments. ** $P<0.01$ compared with control of −1031/+37. NS, $P>0.05$ compared with control of −395/+37.

These results are consistent with those in Western blotting. To examine the effect of AG1478 and U0126 on the transcriptional activity of claudin-2, we measured promoter activity of human claudin-2. The construct of −1031/+37 showed 70-fold promoter activity compared with mock (Fig. 7B). AG1478 and U0126 significantly inhibited the promoter activity. The deletion construct of −395/+37 showed 30-fold promoter activity, which was not significantly inhibited by AG1478 and U0126. The construct of −60/+37 did not show any promoter activity. These results indicate that AG1478- and U0126-sensitive region exists between −1031 and −395. This region contains one putative AP-1 binding site. The promoter activity of the mutant of the putative AP-1 binding site was half of that of wild-type (Fig. 7C). Furthermore, it was not inhibited by AG1478 and U0126. These results indicate that AP-1 binding site is involved in the regulation of promoter activity of claudin-2 and both AG1478 and U0126 inhibits the reaction in AP-1 binding site.

3.7. Interaction of AP-1 with claudin-2 promoter

The introduction of c-Fos or c-Jun siRNA decreased the expression of c-Fos and c-Jun, respectively (Fig. 8A). Both siRNAs decreased claudin-2 expression without affecting claudin-1 expression. In the ChIP assay, a primer pair of –935S/−782A containing AP-1 binding site showed positive PCR signals in the control cells using anti-c-Fos or c-Jun antibody (Fig. 8B). In contrast, a primer pair of −1538S/−1387A, which amplifies a sequence about 500 bases upstream from AP-1 binding site, showed no positive signals. In the U0126- or AG1478-treated cells, no PCR signals were observed using anti-c-Fos or c-Jun antibody. Using goat IgG, neither −935S/−782A nor −1538S/−1387A produced a PCR signal. Next, we performed an ABCD assay using biotin conjugated double-strand oligonucleotides that contain wild-type or mutant AP-1 binding site. Wild-type AP-1 binding site bound to both c-Fos and c-Jun (Fig. 8C). In contrast, mutant AP-1 binding site bound to neither c-Fos nor c-Jun. These results indicate that c-Fos and c-Jun bind to the promoter region containing AP-1 binding site of human claudin-2.

4. Discussion

Claudin-2 mRNA is not expressed in normal lung epithelia [18,19], but its expression is increased in human lung adenocarcinoma [31]. We also found that claudin-2 expression in lung adenocarcinoma and A549 cells is higher than that in normal tissue and other lung carcinomas (Fig. 1). Claudin-2 knockdown decreases cell migration in A549 cells [22], whereas claudin-2 overexpression increases A549 cell colonization [23]. Claudin-2 may be involved in lung adenocarcinoma development. Similar to lung adenocarcinoma, claudin-2 expression increases in colonic [32] and gastric [33] cancers. Forced claudin-2 expression in colon cancer cells increases tumor growth *in vivo*. In contrast, claudin-2 expression decreases in breast carcinoma [34] and prostate adenocarcinoma [35]. These reports suggest that claudin-2 expression may involve organ specificity and increased expression of claudin-2 participates in lung, colonic, and gastric carcinogenesis.

The regulatory mechanism of claudin-2 expression has been clarified using renal and intestinal epithelial cells. Claudin-2 expression is down-regulated by protein kinases including ERK1/2 [36,37], small GTPase RhoA, and c-Jun NH_2-terminal kinase [38] in renal Madin–Darby canine kidney (MDCK) II cells. In contrast, the expression is up-regulated by transcriptional factors including cdx1, cdx2, HNF-1α and GATA-4 in intestinal HT-29, Caco-2, or HIEC-6 cells. It was unknown what transcriptional factors are involved in the regulation of claudin-2 expression and what mechanisms are involved in the regulation of the transcriptional factors of claudin-2 in lung adenocarcinoma. Lung adenocarcinoma shows negative or only focal cdx2 expression [39]. GATA-4 expression is absent in various lung cancer cell lines including A549 cells [40]. Therefore, other transcriptional factors may be involved in the regulation of claudin-2 expression in A549 cells. Peter et al. [23] reported that EGF increases claudin-2 expression in A549 cells, which is inhibited by a MEK inhibitor. However, it is unknown what regulatory mechanism is involved in the basal expression of claudin-2 and what transcriptional factor is involved in the regulation of claudin-2 in A549 cells. Here we found that claudin-2 expression is up-regulated by the secreted

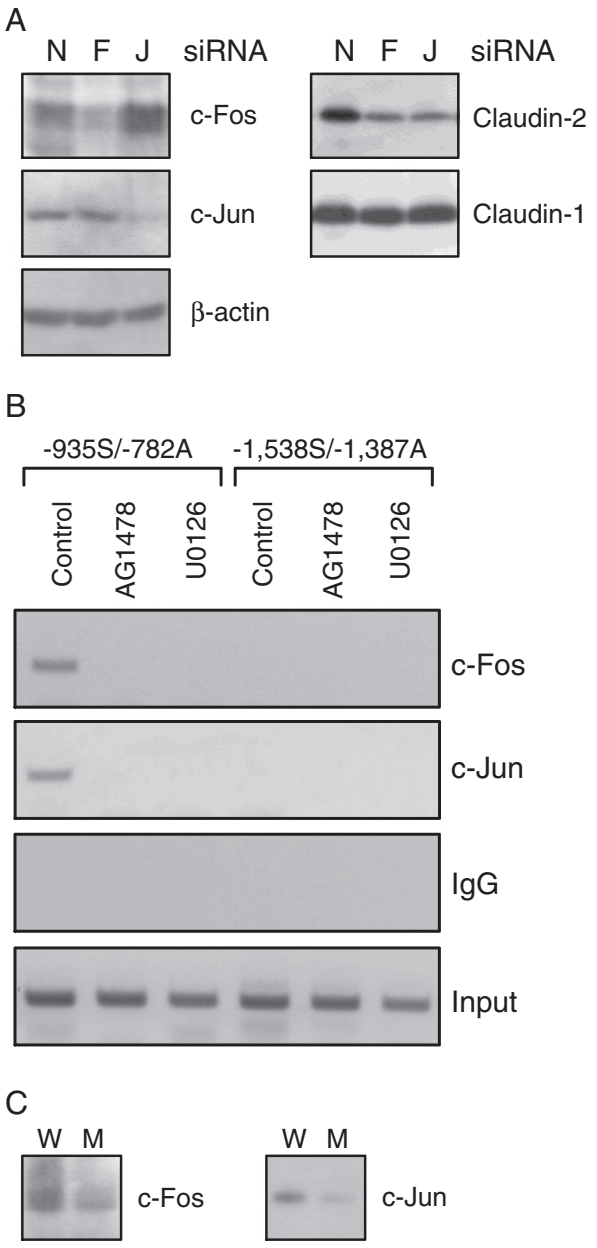

Fig. 8. Association of c-Fos and c-Jun with putative AP-1 binding site of claudin-2 promoter region. (A) Nuclear proteins were prepared from the cells expressing negative (N), c-Fos (F), or c-Jun (J) siRNA and immunoblotted with anti-c-Fos, c-Jun, claudin-1, claudin-2, and β-actin antibodies. (B) Genomic DNA was immunoprecipitated with anti-c-Fos or c-Jun antibody. Immunoprecipitation using goat IgG was carried out as a negative control. After immunoprecipitation, the 5′-flanking region of human claudin-2 was amplified by PCR using primers pairs of −935S/−782A or −1538S/−1387A. The PCR products were analyzed on an agarose gel. To confirm that the same amounts of chromatin were used for the immunoprecipitation in each group, input chromatin was also used. (C) Nuclear proteins were immunoprecipitated with streptavidin agarose and biotinylated double-stranded DNA containing wild-type (W) or mutant (M) of AP-1 binding site. The immune pellets were immunoblotted with anti-c-Fos and c-Jun antibodies.

EGF and its transcriptional activity is activated by a EGFR/MEK/ERK/c-Fos pathway. The activation of MEK have opposite effects on claudin-2 expression in A549 and MDCK II cells. The elucidation of the regulatory mechanism of EGF on claudin-2 expression in MDCK II cells may be also useful to clarify the mechanism of lung adenocarcinoma.

Lung cancer cells secrete various growth factors including EGF, transforming growth factor (TGF) α, TGF β-2, and insulin-like growth factor, and basic fibroblast growth factor [41,42]. The EGF precursor (pro-EGF) is present as its membrane associated high molecular weight precursor exposed at the cell surface. In various epithelial cells, different processing of pro-EGF is involved in the release of soluble EGF. EGF is secreted by a serine protease in the salivary gland [43], mammary gland [44] and the kidney, and zinc dependent metalloprotease in CHO and HMEC cells [26,27]. Heparin-binding EGF is secreted by MMP in lung A431 cells [45], but it was unknown whether EGF is secreted by same MMP in A549 cells. We found that EGF is secreted from A549 cells by MMP, resulting in the activation of an EGFR/MEK/ERK pathway and the increase in claudin-2 expression. Claudin-2 expression was decreased by MMP-9 inhibitor, but not by MMP-3 inhibitor (Supplementary Fig. 3) and serine protease inhibitor (data not shown). We do not know whether MMP-1 and -7 are involved in the secretion of EGF in A549 cells, but we suggest that EGF may be secreted by MMP-9, resulting in the up-regulation of claudin-2 expression.

The deletion or mutation of putative AP-1 binding site decreased the promoter activity of human claudin-2 to the same extent as in the inhibitors of an EGFR/MEK/ERK pathway. AP-1 functions as a dimer in the form of c-Fos/c-Jun or c-Jun/c-Jun. The nuclear localization of c-Fos was decreased by EGF antibody, AG1478, and U0126, whereas that of c-Jun was unaffected by these drugs (Fig. 6C and D). However, the involvement of c-Jun was demonstrated by three experiments. (1) c-Jun siRNA decreased claudin-2 expression similar to c-Fos siRNA (Fig. 8A). (2) ChIP assay showed that c-Fos and c-Jun are bound to the promoter region containing AP-1-binding site under the control conditions, and the association was inhibited by AG1478 and U0126 (Fig. 8B). (3) ABCD assay showed that c-Jun is bound to wild-type AP-1 binding site, whereas it is not bound to mutant AP-1 binding site (Fig. 8C). We suggest that the dimer of c-Fos/c-Jun is bound to AP-1 binding site of human claudin-2 promoter and increases claudin-2 expression.

Vicent et al. [46] reported that there is a strong statistical correlation between nuclear and cytoplasmic p-ERK1/2 staining and advanced stages of NSCLC. Patients with a positive p-ERK1/2 cytoplasmic staining had a lower survival. Furthermore, c-Fos and c-Jun expressions are down-regulated in long-term survivors with NSCLC compared to those in short-term survivors [47]. Our data showed that claudin-2 expression is up-regulated by p-ERK1/2, c-Fos, and c-Jun. Claudin-2 may affect survivorship of NSCLC.

In conclusion, we found that claudin-2 expression in lung adenocarcinoma is higher than that in normal tissue and lung cancers including squamous, large, and small cells. EGF was secreted from A549 cells by MMP and increased claudin-2 expression mediated via the activation of an MEK/ERK/c-Fos pathway. Claudin-2 up-regulates cell migration and colonization in A549 cells. Claudin-2 may become a novel marker for lung adenocarcinoma and its regulatory pathway may become a novel target for anticancer drugs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.04.005.

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