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A simple reporter assay for screening claudin-4 modulators

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ABSTRACT

Claudin-4, a member of a tetra-transmembrane protein family that comprises 27 members, is a key functional and structural component of the tight junction-seal in mucosal epithelium. Modulation of the claudin-4-barrier for drug absorption is now of research interest. Disruption of the claudin-4-seal occurs during inflammation. Therefore, claudin-4 modulators (repressors and inducers) are promising candidates for drug development. However, claudin-4 modulators have never been fully developed. Here, we attempted to design a screening system for claudin-4 modulators by using a reporter assay. We prepared a plasmid vector coding a claudin-4 promoter-driven luciferase gene and established stable reporter gene-expressing cells. We identified thiabendazole, carotene and curcumin as claudin-4 inducers, and potassium carbonate as a claudin-4 repressor by using the reporter cells. They also increased or decreased, respectively, the integrity of the tight junction-seal in Caco-2 cells. This simple reporter system will be a powerful tool for the development of claudin-4 modulators.

1. Introduction

Tight junctions (TJs), the most apical components of intercellular junctional complexes, function as fences that maintain cellular polarity and provide a barrier to regulate intercellular permeability of epithelia [1,2]. Disruption of cellular polarity and the TJ-seal is frequently observed during carcinogenesis and inflammation [3]. Modulation of TJ-seals for drug absorption is now of research interest [4,5]. A series of studies has revealed that TJs are composed of transmembrane proteins (such as occludin and claudins), junction adhesion proteins, and cytoplasmic scaffolding proteins, including ZO-1, ZO-2, and ZO-3 (see reviews [6–8]). Of these, claudins are thought to be the main structural and functional components of TJs.

Claudins, tetra-transmembrane proteins with a molecular mass of approximately 23 kDa, comprise a multigene family containing over 20 members [8]. The barrier-function and the expression patterns of claudin members differ among tissues [6,8,9]. Claudin-1-, -5-, and -11-deficient mice show dysfunction of the epidermal barrier, blood–brain barrier, and blood–testis barrier, respectively [10–12]. The expression levels and the barrier-functions of claudins are often altered in various cancer cells; they can be down-regulated or up-regulated, depending on the type of cancer [13]. Changes in claudin expression have also been observed in the mucosal epithelium under inflammatory conditions [14]. Claudins are thus potent targets for drug development, such as drug delivery, anti-cancer agents, and anti-inflammatory agents.

Since claudins play a role in TJ-seals, modulation of the claudin-barrier is a potent strategy for drug absorption. The carboxyl-terminus of *Clostridium perfringens* enterotoxin (C-CPE) is a modulator of the claudin-barrier [15]. Treatment of cells with C-CPE causes a decrease in claudin-4 proteins in TJs, followed by an enhancement of the paracellular transport of solutes without causing cytotoxicity [15]. C-CPE also enhances jejunal, nasal, and pulmonary absorption of drugs [16]. Thus, proof-of-concept for claudin-targeted drug absorption has been demonstrated. A decrease in claudin-4 in the intestinal epithelium often occurs in colitis [17]. Down-regulation of claudin-4 is also observed in some cancer cells [18]. Induction of claudin-4 is involved in the chemo-preventive effect of nonsteroidal anti-inflammatory drugs [19]. A modulator of claudin-4 expression would therefore be a potent molecule for claudin-targeted drug absorption and drug development for some inflammatory diseases and cancers. However, an effective system to screen for claudin modulators is lacking.

Here, we developed a simple system to monitor claudin-4 expression using a reporter gene, and we screened chemical claudin-4 modulators.

Abbreviations: TJs, tight junctions; C-CPE, the carboxyl terminus of *Clostridium perfringens* enterotoxin; TGF-β, transforming growth factor-β; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative PCR; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TER, transepithelial electric resistance.

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2. Materials and methods

2.1. Reagents and cells

Recombinant human transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) were purchased from R&D systems (Minneapolis, MN) and Peprotech Inc. (Rocky Hill, NJ), respectively. The recombinant proteins were dissolved in water and stored at −80 °C before use. Phorbol 12-myristate 13-acetate (PMA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C before use. List of the chemicals used in this study for screening for claudin-4 modulator is shown in Table 1. All reagents were of research grade.

MCF-7, and Caco-2 cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C. MCF-7 cells were obtained from the RIKEN cell bank (Ibaragi, Japan). Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells stably expressing snail or HRasV12 were prepared by infection with a recombinant retroviral vector coding for snail or HRasV12 gene.

2.2. Preparation of a reporter plasmid

Genomic DNA was extracted from MCF-7 cells by using a genomic DNA isolation kit (Sigma–Aldrich, St. Louis, MO). The claudin-4 promoter region was cloned by polymerase chain reaction (PCR) using genomic DNA as a template and paired primers (forward primer, 5′-GCGCTAGCGGTTGCCCCCTGGCCTTAAC-3′; reverse primer, 5′-CGCTCGAGGTCCACGGGAGTTGAGGACC-3′). The resultant fragments (500 bp) were subcloned into the pGV-B2 vector encoding the luciferase gene (Toyobo, Osaka, Japan). The sequence of the claudin-4 promoter region was confirmed.

2.3. A transient expression of transfection snail or HRasV12 gene

Transfection was performed with FuGENE HD (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were seeded onto 24-well plates. When the cells reached to 80% confluent cell density, 20 μl of medium containing 0.6 μl of FuGENE HD and 200 ng of plasmid carrying snail or HRasV12 gene was added to the wells. After 48 h of transfection, the luciferase activity of the cell lysates was measured as described below.

2.4. Luciferase assay

Luciferase activity was measured using a commercial available luciferase assay system (Toyo Ink, Tokyo, Japan). Cells were lysed with a cell lysis reagent, LCβ (Toyo Ink). The cell lysates were then centrifuged at *18,000g* for 5 min. The luciferase activity in the resulting supernatant was measured using a TriStar LB 941 microplate reader (Berthold, Wildbad, Germany).

2.5. Establishment of a stable reporter cell line

MCF-7 cells were transfected with the reporter plasmid and a plasmid carrying the puromycin resistance gene. Stable transfectants were selected in the presence of puromycin.

2.6. Screening for claudin-4 modulators

The clone 35 cells were seeded onto 96-well plates at a density of 4×10^4 cells/well. On the following day, vehicle or compound was added, and the cells were cultured for an additional 24 h. The luciferase activity in the cells was then measured as described above.

2.7. Cytotoxicity assay

Clone 35 cells or Caco-2 cells were seeded onto a 96-well plate at a density of 4×10^4 or 6×10^4 cells/well, respectively. On the following day, cells were treated with chemicals at the indicated periods. The cell viability was measured by using a WST-8 assay kit (Nacalai, Kyoto, Japan).

2.8. Reverse transcription–PCR (RT–PCR) analysis

RT reaction and PCR amplification were performed with a cDNA synthesis kit (Roche, Mannheim, Germany) and ExTaqTM (Takara, Shiga, Japan), respectively, according to the manufacturer's instructions. Briefly, total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA). For reverse transcription, 5 μg of total RNA was used. PCR was performed for 23 cycles for claudin-4 (94 °C for 30 s, 55 °C for 15 s, 72 °C for 30 s) and for 20 cycles for GADPH (94 °C for 30 s, 55 °C for 15 s, 72 °C for 60 s). The PCR products were separated by use of agarose gel electrophoresis and stained with ethidium bromide. The sequences of the primers are as follows: forward primer for claudin-4, 5′-CAACATTGTCACCTCGCAGACCATC-3′; reverse primer for claudin-4, 5′-TATCACCATAAGGCCGGCCAACAG-3′; forward primer for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-TCTTCACCACCATGGAGAAG-3′; reverse primer for GAPDH, 5′-ACCACCTGGTGCTCAGTGTA-3′.

2.9. Quantitative PCR (qPCR) analysis

qPCR was performed with SYBR Premix Ex Taq II (Takara) using an Applied Biosystems StepOne Plus (Applied Biosystems, Foster City, CA). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, GAPDH. The primer sequences used for qPCR were as follows: forward primer for claudin-4, 5′-TTGTCACCTCGCAGACCATC-3′ and reverse primer for claudin-4, 5′-CAGCGAGTCGTACACCTTG-3′; forward primer for GAPDH, 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and reverse primer for GAPDH, 5′-GTGGTCGTTGAGGGCAATG-3′.

2.10. Western blot analysis

Cells were lysed with RIPA buffer (0.15 M NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% protease inhibitor cocktail [Sigma–Aldrich]). The cell lysates were subjected to 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), followed by blotting onto polyvinylidene difluoride membrane. The membranes were incubated with anti-claudin-4 mouse monoclonal (Zymed, South San Francisco, CA) and anti-β-actin mouse monoclonal (Sigma–Aldrich) antibodies, respectively, and subsequently treated with horseradish peroxidase-conjugated anti-mouse IgG (Zymed). The reactive bands were detected by using an enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK).

2.11. Transepithelial electric resistance (TER) assay

Caco-2 cells were seeded into Transwell™ chambers (Corning, NY) at a density of 8×10^4 cells/well. On 7 days after the seeding or when TER values reached a plateau, claudin-4 inducers (thiabendazole, carotene, or curcumin) or claudin-4 repressor (potassium carbonate), respectively, was added. The TER values were then monitored at 0, 24, and 48 h using a Millicell-ERS epithelial

Table 1
Chemicals used in this study as screening sources.

Sample number	Sample name	Concentration[a]	Relative luciferase activity[b]
1	Tartrazine	10 mM	1.29
2	Potassium nitrate	1 mM	0.94
3	Potassium carbonate	10 mM	0.56
4	Sodium chlorous	10 mM	0.95
5	Zinc sulfate	0.1 mM	0.95
6	New coccine	0.01 mM	0.98
7	Amaranth (Bordeaux S)	1 mM	1.34
8	Allura red AC	1 mM	1.49
9	Sunset yellow FCF	1 mM	1.59
10	Potassium hydroxide	1 mM	0.83
11	L-ascorbic acid	1 mM	1.02
12	Sodium nitrite	10 mM	0.91
13	Propionic acid	0.0001%	0.82
14	Sodium carbonate	1 mM	0.91
15	Zinc gluconate	0.01%	1.76
16	Benzoic acid	0.01 mM	1.3
17	Sorbic acid	1 mM	1.51
18	Aspartame	1 mM	1.59
19	Dibutylhydroxytoluene	0.01 mM	1.81
20	Allyl isothiocyanate	0.0001%	1.72
21	Saccharin	1 mM	1.5
22	L-Ascorbyl palmitate	1 mM	1.21
23	Hydroxy biphenyl	0.01 mM	1.87
24	Aluminium potassium sulfate	0.1 mM	0.94
25	L-Lysine	10 mM	1.42
26	Calcium pantothenate	10 mM	1.61
27	Carrageenin	0.01 mM	1.56
28	Tartaric acid	1 mM	1.01
29	Sodium acetate	10 mM	1.02
30	Glycine	10 mM	1.68
31	Sodium alginate	10 mM	1.52
32	Ammonium chloride	10 mM	1.91
33	Magnesium sulfate	10 mM	1.56
34	5-Ribonucleotide	0.001 mM	1.15
35	Calcium chloride	1 mM	1.62
36	Valine	10 mM	1.08
37	Erythrosine	0.01 mM	1.22
38	Annatto	0.01 mM	1.96
39	Maltitol	10 mM	1.44
40	Sodium dehydroacetate	1 mM	1.98
41	Nicotinic acid	1 mM	1.55
42	Isoleucine	1 mM	1.06
43	Mannitol	10 mM	1.29
44	Ascorbic acid (Vitamin C)	10 mM	1.17
45	Phenylalanine	1 mM	0.95
46	Gallic acid	0.1 mM	1.41
47	Erythorbic acid (Sodium isoascorbate)	1 mM	1.03
48	Magnesium chloride	0.1%	1.26
49	Cochineal extract	0.1%	1.02
50	Calcium dihydrogen pyrophosphate	1 mM	1.1
51	Calcium citrate	0.01 mM	0.92
52	Polyvinyl acetate	0.1 mM	1.13
53	Fumaric acid	0.01 mM	1.24
54	Sodium methyl *p*-hydroxybenzoate	1 mM	2.04
55	Tocophenol (Vitamin E)	0.0001%	2.14
56	Rennet	0.01%	0.89
57	Ionone	0.01%	1.15
58	Isoeugenol	0.001%	1.15
59	Allyl isosulfocyanate	0.001%	1.06
60	Propylene glycol	0.1%	0.87
61	Ethyl isovalerate	0.001%	0.89
62	Pectin	0.001%	0.98
63	Cysteine	0.01 mM	0.76
64	Tragacanth gum	0.01%	0.83
65	Thiamin	0.1%	1.15
66	Gum arabic	0.01%	0.91
67	Cellulose	0.001%	0.84
68	Thiabendazole	0.1 mM	3.24
69	Isopropyl citrate	10 mM	1.04
70	γ-oryzanol	0.01%	1.02
71	Calcium carbonate	0.001%	0.857
72	Propylene glycol alginate	0.01%	0.87
73	Chlorophyll	0.1%	1.02
74	Sodium chondroitin sulfate	0.1%	1.04

Table 1 (*continued*)

Sample number	Sample name	Concentration[a]	Relative luciferase activity[b]
75	Biphenyl	0.1 mM	0.99
76	Sodium cytidylic acid	1 mM	0.77
77	Stevia rebaudiana	0.01%	0.96
78	Calcium stearoyl lactylate	0.01%	0.83
79	Ferrous sulfate	0.1 mM	1.37
80	Calcium sulfate	0.1 mM	0.93
81	Benzoyl peroxide	0.1 mM	1.13
82	Dibenzoyl thiamine	1 mM	0.88
83	Carotene	0.1 mM	2.09
84	Guar gum	0.001%	0.84
85	Xanthan gum	0.001%	0.77
86	Curcumin	0.01 mM	2.0

[a] The chemical concentrations were set at the maximum level to show no cytotoxicity.
[b] The relative luciferase activities were calculated as the ratio of that in the chemical-treated cells to that in the vehicle-treated cells. The treatment period was 24 h.

volt-ohmmeter (Millipore Corporation, Billerica, MA). The TER values were normalized to the area of the Caco-2 cell monolayers, and the TER value of a blank chamber was subtracted.

3. Results

3.1. Preparation of a reporter plasmid encoding a claudin-4-promoter-driven luciferase gene

As a first step toward developing a simple screening system for claudin-4 modulators, we cloned the promoter region of claudin-4. We searched for a region that was highly conserved among animals by using a UCSC Genome Bioinformatics program and cloned a 500 bp fragment corresponding to −293 to +194 bp of the claudin-4 gene. This 500 bp fragment contained various transcription factor-binding sites: an E-box (−276 to −271, −262 to −257, −221 to −216, −19 to −14, +10 to +14), a smad-binding element (SBE; −212 to −209, −103 to −100, −38 to −35), and Sp1 (−66 to −57, −53 to −44) [20,21], indicating that this region is a potent candidate for a regulatory region of claudin-4 expression. We constructed a reporter expression vector, in which the 500 bp fragment was inserted upstream of a luciferase gene Suppl. Fig. 1A). To

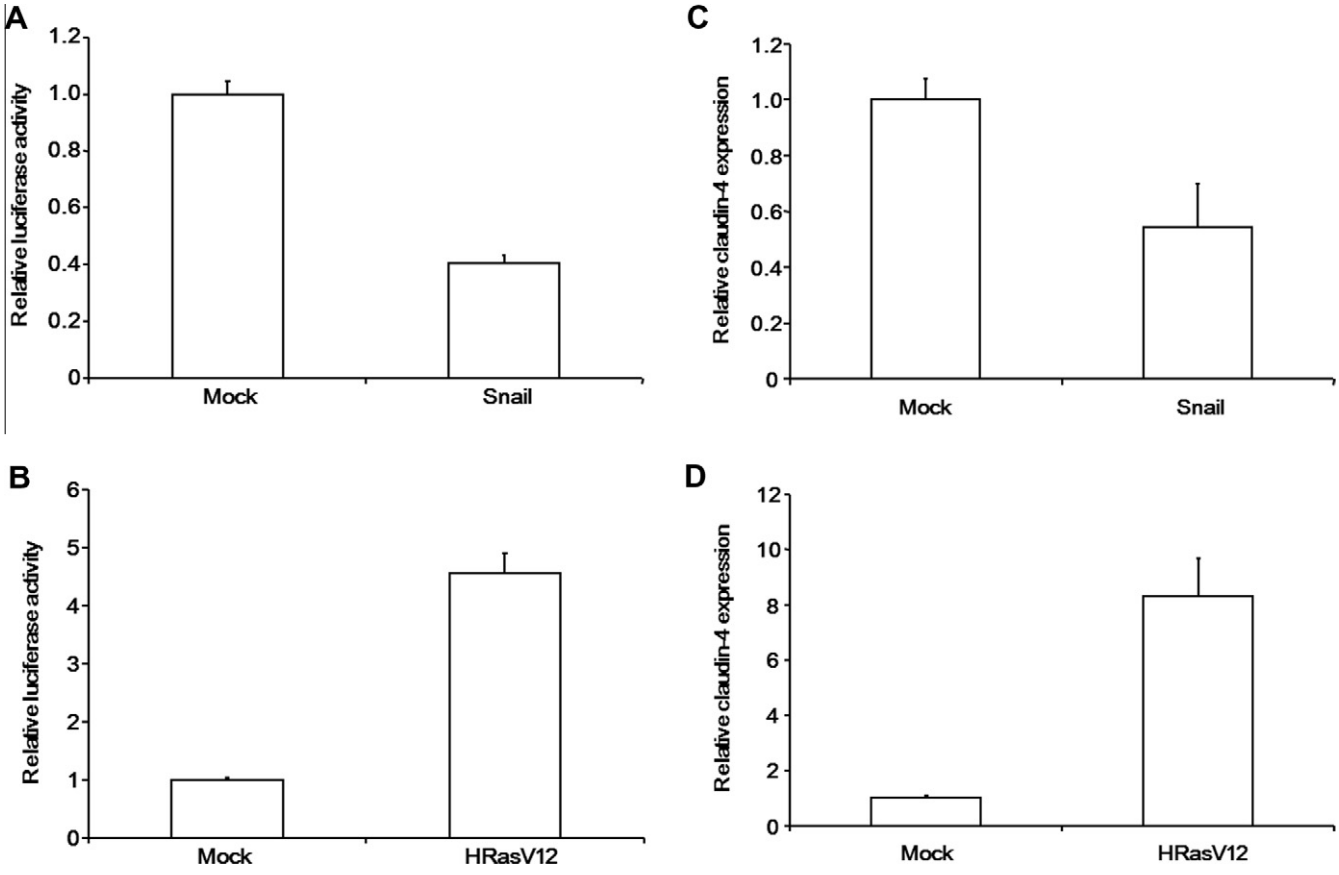

Fig. 1. Preparation of a reporter system monitoring claudin-4 expression. (A, B) Effects of snail and HRasV12 on the luciferase activity in transiently expressing cells. Snail-expressing MCF-7 cells (A) or HRasV12-expressing MCF7 cells (B) were transfected with the claudin-4 reporter plasmid. Two days later, the cells were recovered, and the luciferase activity in the lysates was measured. The data are means ± S.D. ($n = 3$). The results are representative of two independent experiments. (C, D) qPCR analysis of claudin-4 expression in transiently expressing cells. After 2 days of the transfection with the claudin-4 reporter plasmid, total RNA was extracted from snail-expressing MCF-7 cells (C) or HRasV12-expressing MCF-7 cells (D). Expression level of claudin-4 of the transfected cells was quantified by qPCR as described in the Section 2. Claudin-4 expression level was shown as ratio to that of the mock cells. The data are means ± S.D. ($n = 3$). The results are representative of two independent experiments.

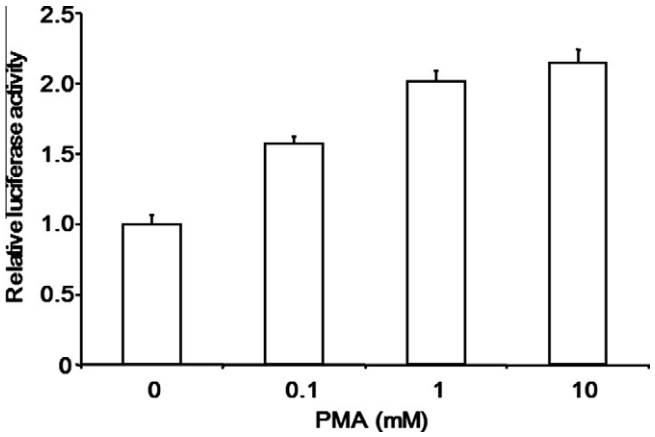

Fig. 2. Effect of PMA on the luciferase activity in clone 35 cells. Clone 35 cells were treated with PMA at the indicated concentrations for 24 h. Luciferase activity in the lysates was measured. The relative luciferase activity is shown as the ratio of the luciferase activity in the treated cells to that of the vehicle-treated cells. The data are means ± S.D. (n = 3). The results are representative of two independent experiments.

evaluate expression of the reporter gene, we checked the endogenous claudin-4 expression level in various cell lines and selected MCF-7, HaCat, HT1080, and SiHa cells, which have different claudin-4 expression levels for our analyses (Suppl. Fig. 1B). We transiently transfected the reporter plasmid into these cell lines and found that the luciferase activity of each was correlated with the endogenous expression level of claudin-4 (Suppl. Fig. 1C). We also investigated expression of the reporter gene in MCF-7 cells stably expressing snail or HRasV12, which suppress or induce claudin-4 expression, respectively [22,23]. Transfection of snail- or HRasV12-expressing MCF-7 cells with the reporter plasmid decreased or increased, respectively, the luciferase activity compared to that of mock-transfected MCF-7 cells (Fig. 1A and B). The difference in luciferase activity paralleled the level of claudin-4 mRNA in the cells (Fig. 1C and D), suggesting that the cloned promoter region was functional.

3.2. Preparation of a screening system for claudin-4 modulators

We transfected MCF-7 cells with the claudin-4 reporter plasmid and isolated stable transfected clones. We investigated the effect of transient expression of snail and HRasV12 on luciferase activity in these clones and found that several clones showed altered luciferase activity when transfected with the claudin-4 suppressor (snail, Suppl. Fig. 2A) or the claudin-4 inducer (HRasV12, Suppl. Fig. 2B). TGF-β suppresses claudin-4 expression [23], whereas EGF enhances claudin-4 expression [24]. Therefore, we also investigated the effects of TGF-β and EGF on the luciferase activity in the clones (Suppl. Fig. 2C and D, respectively). Since clone 35 showed the best

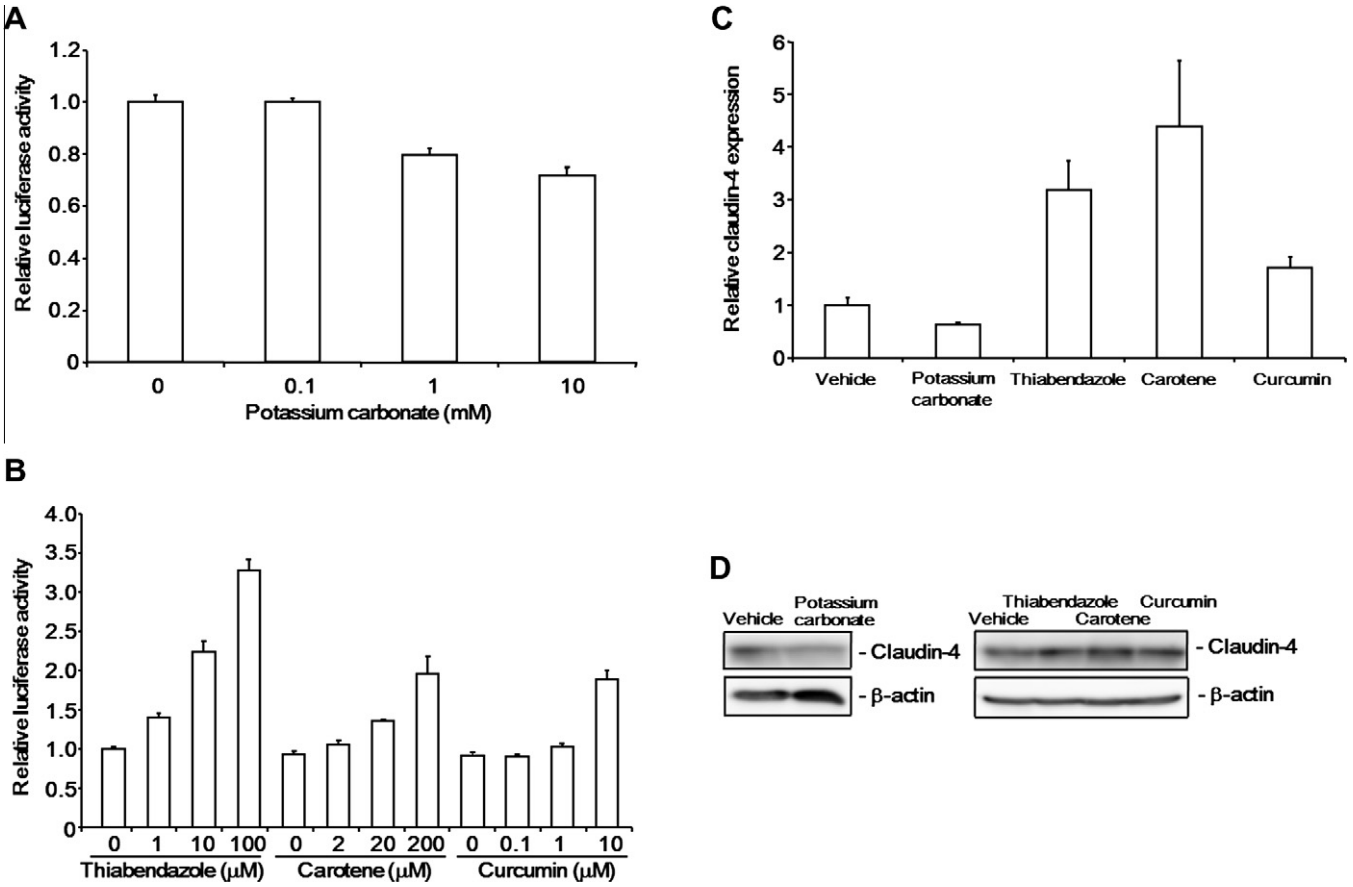

Fig. 3. Screening claudin-4 modulators using the reporter system. (A, B) Dose-dependent effects of the claudin-4 modulator candidates on luciferase expression. Clone 35 cells were treated with potassium carbonate (A), or thiabendazole, carotene, or curcumin (B) at the indicated concentrations for 24 h. Luciferase activity was measured in the lysates. Relative luciferase activity is shown as the ratio of the luciferase activity in the chemical-treated cells to that in the vehicle-treated cells. The data are means ± S.D. (n = 3). The results are representative of three independent experiments. (C, D) Effects of the claudin-4 modulator candidates on claudin-4 mRNA expression (C) and claudin-4 protein (D) levels. Clone 35 cells were treated with potassium carbonate (5 mM), thiabendazole (0.1 mM), carotene (0.2 mM), or curcumin (10 μM) for 24 h (C) or 48 h (D). Total RNA was used for qPCR analysis to detect claudin-4 mRNA (C). The relative mRNA expression of claudin-4 normalized to GAPDH expression. The cell lysates were subjected to SDS–PAGE, followed by immunoblotting for claudin-4 (D). GAPDH or β-actin served as loading controls. The result is representative of three independent experiments.

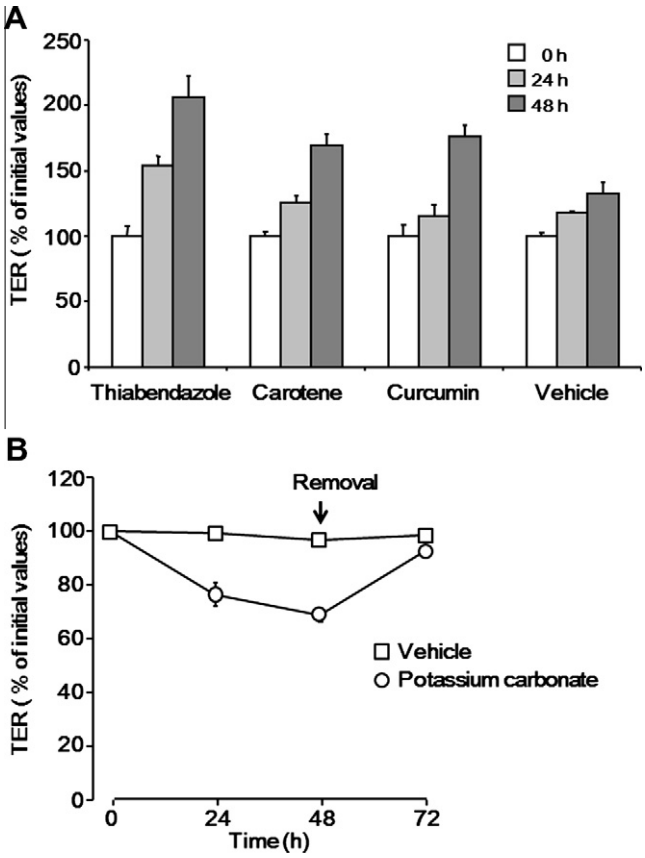

Fig. 4. Effects of claudin-4 modulator on the TJ-barrier in Caco-2 cells. (A) Effect of claudin-4 inducers on the TJ-barrier. Cells were seeded in Transwell™ chambers. Seven days after seeding, the cells were treated with thiabendazole (0.05 mM), carotene (0.2 mM), or curcumin (10 μM). TER values were monitored every 24 h. (B) Effect of a claudin-4 repressor on the TJ-barrier. Cells were seeded in Transwell™ chambers. When the TER values reached a plateau, the TJ-developed cells were treated with potassium carbonate (10 mM). After 48 h of treatment, the medium was replaced with fresh medium. The cells were then cultured for an additional 24 h. TER values were monitored every 24 h. TER values are shown as percentages of the TER values before treatment relative to those in treated cells, as described in the Section 2. The data are means ± S.D. ($n = 3$). These results are representative of three independent experiments.

response to the various claudin-4-modulating treatments, we selected it for further analysis. The clone 35 cells were treated with PMA, which enhances claudin-4 expression [25]. PMA increased luciferase activity in a dose-dependent manner (Fig. 2). These results indicate that clone 35 could be used to screen for modulators of claudin-4 expression.

3.3. Screening for claudin-4 modulators

When we eat, fragments of partially digested food, which still have antigenicity, exist in the intestine. This suggests that claudin modulators that tighten TJ-barriers may be contained in food. Therefore, we screened 86 chemicals used as food additives for claudin-4 modulators (Table 1). At first, we checked the cytotoxicity of these compounds in the clone 35 cells (Table 1). Then, we treated the cells with the compounds at non-toxic concentrations and identified the following claudin-4 modulator candidates: potassium carbonate (No. 3), thiabendazole (No. 68), carotene (No. 83), and curcumin (No. 86) (Suppl. Fig. 3). Each chemical modulated luciferase activity in a dose-dependent manner (Fig. 3A and B). qPCR analysis revealed that thiabendazole, carotene, and curcumin increased claudin-4 expression in the clone 35 cells (Fig. 3C), whereas potassium carbonate decreased claudin-4 expression. Similar results were obtained from Western blot analysis of claudin-4 (Fig. 3D).

To test whether the screened compounds also modulated the TJ-barrier, we investigated the effect of the compounds on the TER value, a marker of TJ-integrity, in Caco-2 cell monolayers, which is a popular model for mucosal barrier. Treatment of cells with thiabendazole, carotene, and curcumin increased the TER values (Fig. 4A). In contrast, potassium carbonate decreased the TER value. Moreover, the TER values recovered when the potassium carbonate was removed (Fig. 4B), and treatment with potassium carbonate did not cause cytotoxicity (data not shown). Thus, we successfully identified claudin-4 modulators.

4. Discussion

Claudin-4 inducers have been the focus of attention in drug development to treat inflammatory diseases and cancers [17–19]; however, their development has been slow. Some chemicals that modulate TJ integrity have been identified: glutamine, bryostatin-1, berberine, quercetin, and butyrate [26–30]. Here, we established a simple monitoring system for claudin-4 expression using a reporter gene, luciferase, and successfully identified chemical claudin-4 modulators: one suppressor of claudin-4 expression, potassium carbonate, and three inducers of claudin-4, thiabendazole, carotene, and curcumin.

Curcumin is an active ingredient of the spice turmeric, which is used in curry powders and as a food preservative. It is also used in traditional medicine to treat various inflammatory conditions, such as arthritis, colitis, and hepatitis [31]. Curcumin has various biological activities, such as anti-inflammatory, anti-oxidant, and anti-cancer effects [32]; however, the underlying mechanisms have never been fully understood. Here, we found that curcumin induces claudin-4 expression and increases TJ integrity. This enhancement of TJ integrity by curcumin may be associated with its therapeutic activities.

Carotene is a precursor of vitamin A. Retinoic acid, a metabolite of vitamin A, enhances TJ integrity in epithelial cells accompanied by expression of claudin-1, -4, and occludin [33]. These findings suggest that metabolized β-carotene-activated expression of claudins enhances the epithelial barrier in Caco-2 cells. Retinoic acid is a biologically active regulator of cell differentiation, proliferation, and apoptosis in various cell types [34]. The activities of retinoic acid are mediated by two types of nuclear receptors: retinoic acid receptors and their heterodimeric counterparts, retinoid X receptors [35]. Specific heterodimer-mediated transcriptional activation increases TJ integrity [36]. The increase in claudin-4 expression and TJ integrity induced by carotene may be caused by the formation of the heterodimer, followed by transcriptional activation.

Thiabendazole is used as a broad spectrum anthelmintic in various animal species and is also used to control parasitic infections in humans [37]. It is also used as an anti-fungal agent for the treatment of fruits [38]. Here, we found that thiabendazole increases claudin-4 expression and TJ integrity, but the mechanism for these activities remains unclear.

Our screening system identified a repressor of intestinal epithelial barrier function as well as three enhancers. We showed that potassium carbonate reduces claudin-4 expression and epithelial barrier function in Caco-2 cells without causing cytotoxicity. Potassium carbonate is used as an acidity regulator, and paracellular permeability is sensitive to pH [39]. Thus, potassium carbonate might reduce epithelial barrier integrity by changing the pH.

In conclusion, we developed the simple screening system for claudin-4 modulator, and we identified several claudin-4 modulators, including three inducers and one repressor. The screening system will thus be a tool for the development of claudin-4

modulators, thereby contributing to basic and pharmaceutical researches.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.083.

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