

Tight Junction Proteins Claudin-2 and -12 Are Critical for Vitamin D-dependent Ca^{2+} Absorption between Enterocytes

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Submitted September 28, 2007; Revised January 14, 2008; Accepted February 8, 2008
Monitoring Editor: Asma Nusrat

Ca^{2+} is absorbed across intestinal epithelial monolayers via transcellular and paracellular pathways, and an active form of vitamin D_3 , $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25(\text{OH})_2\text{D}_3$], is known to promote intestinal Ca^{2+} absorption. However, the molecules driving the paracellular Ca^{2+} absorption and its vitamin D dependency remain obscure. Because the tight junction proteins claudins are suggested to form paracellular channels for selective ions between neighboring cells, we hypothesized that specific intestinal claudins might facilitate paracellular Ca^{2+} transport and that expression of these claudins could be induced by $1\alpha,25(\text{OH})_2\text{D}_3$. Herein, we show, by using RNA interference and overexpression strategies, that claudin-2 and claudin-12 contribute to Ca^{2+} absorption in intestinal epithelial cells. We also provide evidence showing that expression of claudins-2 and -12 is up-regulated in enterocytes *in vitro* and *in vivo* by $1\alpha,25(\text{OH})_2\text{D}_3$ through the vitamin D receptor. These findings strongly suggest that claudin-2- and/or claudin-12-based tight junctions form paracellular Ca^{2+} channels in intestinal epithelia, and they highlight a novel mechanism behind vitamin D-dependent calcium homeostasis.

INTRODUCTION

Calcium plays a fundamental role in various physiological functions such as bone mineralization, blood coagulation, neuromuscular transmission, and muscle contraction, as well as cell–cell adhesion and intracellular signaling. Ca^{2+} is absorbed in the intestinal mucosa by two distinct routes, the transcellular and paracellular pathways (Bronner *et al.*, 2003; Hoenderop *et al.*, 2005). The transcellular Ca^{2+} transport in the intestine is thought to comprise a multistep process: uptake across the apical plasma membrane via the Ca^{2+} channel protein transient receptor potential vanilloid receptor channel (TRPV)6, intracellular transport by the Ca^{2+} -binding proteins calbindins, and extrusion through plasma membrane Ca^{2+} -ATPase PMCA_{1b} (Hoenderop *et al.*, 2005). In contrast, the molecular basis for paracellular Ca^{2+} absorp-

tion, which occurs throughout the intestine (Bronner *et al.*, 1986), is largely unknown.

One of the most important hormones to enhance intestinal Ca^{2+} absorption is an active form of vitamin D_3 , $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25(\text{OH})_2\text{D}_3$] (Norman, 1990; Hoenderop *et al.*, 2005). Most of its actions are mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily (Mangelsdorf *et al.*, 1995). In fact, VDR knockout (VDRKO) mice exhibit decreased Ca^{2+} uptake in the intestine with a concomitant reduction in expression of some of the aforementioned transcellular Ca^{2+} transport proteins (Yoshizawa *et al.*, 1997; Li *et al.*, 2001; Van Cromphaut *et al.*, 2001; Song *et al.*, 2002), indicating the significance of VDR in transcellular Ca^{2+} absorption. It is also suggested that vitamin D could promote paracellular Ca^{2+} transport across intestinal epithelial cells (Wasserman, 2004). However, it remains obscure which molecules driving paracellular Ca^{2+} absorption are targets for the vitamin D signaling.

Tight junctions are the apical-most constituent of the intercellular junctional complex in mammalian epithelial cell sheets, and they act as a semipermeable barrier to the paracellular transport of ions and solutes (Anderson and Cereijido, 2001). Among molecular components of tight junctions, claudins (Cldns) are the major transmembrane proteins, and they consist of 24 members of a gene family (Furuse *et al.*, 1998; Tsukita *et al.*, 2001). In addition, distinct sets of claudins are generally expressed in a cell- and tissue-specific manner. Recent studies have disclosed that claudins

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-09-0973>) on February 20, 2008.

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Abbreviations used: Cldn, claudin; EBP50, ezrin/radixin/moesin-binding phosphoprotein 50; FHHNC, familial hypomagnesemia with hypercalciuria and nephrocalcinosis; KO, knockout; PCLN-1, paracellin-1; siRNA, small interfering RNA; TER, transepithelial electrical resistance. VDR, vitamin D receptor; WT, wild type.

are the major determinant of the barrier function of tight junctions. Importantly, the first extracellular loop, in which there is a wide variation in the position and number of charged amino acids depending on each claudin, is shown to create paracellular pores (channels) for cations or anions between neighboring cells (Van Itallie and Anderson, 2006). Along this line, we assumed that the specific species of intestinal claudins might contribute to paracellular Ca²⁺ transport. We also hypothesized that expression of these claudins could be up-regulated by VDR. To examine this assumption, we compared the expression of putative cation-permissive claudins in the intestine of VDRKO mice with that of wild-type (WT) mice, and we used RNA interference (RNAi) and overexpression approaches in the vitamin D-responsive intestinal cell line Caco-2. We report here that expression of claudins-2 and -12 seems to be activated by VDR *in vivo* and *in vitro* and that these two claudins are essential for Ca²⁺ absorption between intestinal epithelial cells, providing a novel mechanism underlying vitamin D-dependent intestinal Ca²⁺ transport.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibodies (pAbs) against Cldn7, Cldn12, and Cldn15 were generated, and the specificity of these antibodies were verified as described previously (Satohisaka *et al.*, 2005; Fujita *et al.*, 2006; Sakai *et al.*, 2007). Preimmune sera from each rabbit before being immunized with antigens were also used as negative controls to confirm the selectivity of these pAbs. A rabbit pAb against Cldn2 were obtained from Immuno-Biological Laboratories (Minneapolis, MN) and Zymed Laboratories (South San Francisco, CA). Mouse monoclonal antibodies (mAbs) against occludin and VDR and rabbit pAbs against actin, ezrin/radixin/moesin-binding phosphoprotein 50 (EBP50), and TRPV6 were purchased from Zymed Laboratories, Santa Cruz Biotechnology (Santa Cruz, CA), Sigma-Aldrich (St. Louis, MO), Affinity BioReagents (Golden, CO), and Alomone Labs (Jerusalem, Israel), respectively. A rat mAb against ezrin were obtained from Sanko Junyaku (Tokyo, Japan). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin (Ig)G (Dako Denmark, Glostrup, Denmark), Alexa Fluor 488 (green)-labeled anti-rabbit IgG (Invitrogen, Carlsbad, CA), Alexa Fluor 594 (red)-labeled anti-mouse IgG (Invitrogen), and fluorescein isothiocyanate-conjugated anti-rat IgG (DakoCytomation).

Animals and Tissue Preparation

VDRKO mice were generated and maintained as reported previously (Yoshizawa *et al.*, 1997). Twelve-week-old wild-type and VDRKO mice were anesthetized with diethyl ether, and specimens of the intestine were obtained. All aspects of the study were approved by the animal use and care committee of Sapporo Medical University School of Medicine (Sapporo, Japan).

Cell Lines and Cell Culture

The human intestinal cell line Caco-2 (clone BBe; CRL-2102) was obtained from American Type Culture Collection. To establish Caco-2 cell lines expressing Cldn2, Cldn7, Cldn12, and Cldn15 (Caco-2: Cldn2, Caco-2: Cldn7, Caco-2: Cldn12 and Caco-2: Cldn15, respectively), cells were transfected with 10 µg of individual expression vectors containing the corresponding mouse claudin cDNAs (Ishizaki *et al.*, 2003; Fujita *et al.*, 2006) along with 1 µg of the puromycin-resistant gene expression vector pHRLpuro1 (Chiba *et al.*, 1997a) by using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocols. Puromycin (5 µg/ml)-resistant clones were screened by immunofluorescence staining and immunoblotting.

Cells were plated in DMEM supplemented with 20% fetal bovine serum (heat-inactivated and charcoal-stripped), 100 U/ml penicillin, and 100 µg/ml streptomycin, and they were grown at 37°C in a humidified 5% CO₂ atmosphere. They were refed every 2 d, and they were exposed to the vehicle or 1α,25(OH)₂D₃ (Sigma-Aldrich).

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For analysis of mRNA expression, total RNA was isolated from the intestine and cells using TRIzol reagent (Invitrogen), and RT-PCR was performed as reported previously (Chiba *et al.*, 1997b, 2006). The PCR primers for cDNA were as follows: mouse VDR (GenBank/EMBL/DDBJ accession no. NM_009504), 5'-AACGCTATGACCTGTGAAGGC-3' (nucleotides [nt] 250-270) and 5'-CCTGTACTTACGTCTGCACGA-3' (nt 612-632); human Cldn2

(GenBank/EMBL/DDBJ accession no. NM_020384), 5'-GCTTCTACTGAGA-GGTCTG-3' (nt 306-324) and 5'-TTCITTCACACATACCCTG-3' (nt 1006-1023); human Cldn7 (GenBank/EMBL/DDBJ accession no. NM_001307), 5'-AGG-CATAATTTTCATCGTGG-3' (nt 792-811) and 5'-GAGTTGGACTTAGGG-TAAGAGCG-3' (nt 1021-1043); human Cldn12 (GenBank/EMBL/DDBJ accession no. NM_012129), 5'-CTCCCCATCTATCTGGGTCA-3' (nt 635-654) and 5'-GGTGGATGGGAGTACAATGG-3' (nt 816-835); human Cldn15 (GenBank/EMBL/DDBJ accession no. NM_014343), 5'-AAATACGGCAGAAA-CGCCTA-3' (nt 915-934) and 5'-CGACTTCCCAAGAGCAGTTC-3' (nt 1109-1128). The primers for mouse claudins and those for the housekeeping gene 36B4 encoding the ribosomal protein were described previously (Kubota *et al.*, 2001; Fujita *et al.*, 2006).

To confirm that amplifications were in the linear range, PCR was performed using three different numbers of cycles between 21 and 36, depending on the gene analyzed. Aliquots of PCR products were loaded onto 2% agarose gel, and then they were analyzed after staining with ethidium bromide. The mRNA signals were quantified using Image 1.62c software (Scion, Frederick, MD).

Gel Electrophoresis and Immunoblotting

The mouse intestinal tissue and Caco-2 cells grown on 60-mm tissue culture plates were washed twice with ice-cold phosphate-buffered saline (PBS), sonicated for 20 s in ice-cold NaHCO₃ buffer (1 mM NaHCO₃ and 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.5), and put on ice for 30 min. They were mixed with 2× SDS sample buffer and boiled for 5 min. Total cell lysates were then resolved by one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Billerica, MA). The membrane was saturated with PBS containing 4% skim milk, and then it was incubated for 1 h at room temperature with primary antibodies in PBS. After rinsing in PBS containing 0.1% Tween 20, the membrane was incubated for 1 h at room temperature with HRP-conjugated anti-rabbit or anti-mouse IgG (diluted 1:1000) in PBS. It was then rinsed again, and finally it was analyzed using an ECL Western blotting detection system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The blots were stripped with Restore Western blot stripping solution (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions, and immunoprobed with an anti-actin antibody. When immunoblotted signals were faint, ECL Advance Western blotting detection system (GE Healthcare) was used according to the manufacturer's recommendations. Signals in immunoblots were quantified using Image 1.62c software.

Immunohistochemistry

Ten-micrometer-thick frozen sections of the mouse intestine were fixed in 95% ethanol for 30 min at 4°C and in 100% acetone for 1 min at room temperature. After being washed three times with PBS, they were incubated for 1 h at room temperature with primary antibodies and rinsed again with PBS, followed by reaction for 1 h at room temperature with appropriate secondary antibodies. For immunohistochemistry of Caco-2 cells, cells grown on coverslips were fixed in 1% formaldehyde in PBS for 10 min. After being washed three times with PBS, they were treated with 0.2% Triton X-100 in PBS for 10 min, rinsed again with PBS, and preincubated in PBS containing 5% skim milk. They were subsequently incubated with primary and secondary antibodies as described above. All samples were examined using a laser-scanning confocal microscope (MRC 1024; Bio-Rad, Hercules, CA) and a PlanApo 60× numerical aperture 1.40 oil immersion objective (Nikon, Tokyo, Japan). Photographs were recorded using a Dell computer (PowerEdge 2200) and OS/2 WARP software (IBM, White Plains, NY), and they were processed with Photoshop 6.0 (Adobe Systems, Mountain View, CA). Observations were made at room temperature.

Measurement of Transepithelial Electrical Resistance (TER) and Calcium Transport Studies

Caco-2 cells were grown on 1.0-cm² polycarbonate Transwell-Clear membranes (0.4-µm pore size; Corning Life Sciences, Acton, MA) coated with rat tail collagen. TER was measured using an EVOM voltohmmeter with ENDOHM-12 (WPI, Sarasota, FL) on a heating plate (FHP-30S; Fine Tokyo, Japan) adjusted to 37°C. The values are expressed in standard units of ohms per square centimeter. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

Calcium transport across Caco-2 monolayers was assessed as reported previously (Giuliano and Wood, 1991). In brief, cells were washed twice with PBS, and transport buffer (140 mM NaCl, 5.8 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM NaH₂PO₄, 1 mM MgCl₂, 1 mM CaCl₂, 25 mM glucose, and 20 mM HEPES, pH 7.4) was poured into the inner and outer chambers. After adding 1 µCi/ml ⁴⁵Ca²⁺ to the insert, cells were incubated at 37°C, and samples were collected from the opposite compartment at 15, 30, 60, and 120 min. Radioactivity of ⁴⁵Ca²⁺ was measured using a scintillation counter (LS6500; Beckman).

Electrophysiological Measurement

For electrophysiological measurements, Caco-2 cells were grown on Snapwell filters (0.4- μ m pore size; Corning Life Sciences) coated with rat tail collagen. The permeabilities of Na⁺ and Cl⁻ across Caco-2 monolayers were determined using an Ussing chamber with an ECV4000 Precision V/I Clamp (WPI) according to the procedure of Hou *et al.* (2005).

RNAi and Transfection

Stealth small interfering RNA (siRNA) duplex oligonucleotides against human Cldn2 and Cldn12 were synthesized by Invitrogen. The sequences were as follows: Cldn2 RNAi #1, sense (5'-AUUUAUCUGUCAGGCACCA-GUGG-3') and antisense (5'-CCACUGGUGCCUGACGAUGAAAU-3'); Cldn2 RNAi #2, sense (5'-AGAAUAAUGCCCAAGUAAAGAGCC-3') and antisense (5'-GGCUCUUUACUUGGGCAUUUUUCU-3'); Cldn2 RNAi #3, sense (5'-AGAGGAUGAUUCCAGCUAUCAGGGA-3') and antisense (5'-UCCUGAUAGCUGGAAUCAUCCUCU-3'); Cldn7 RNAi #1, sense (5'-AU-AGGAGCUAUCUGCCACUGCGGG-3') and antisense (5'-CCCAGUG-GCAGAUGAGCUCCUUAU-3'); Cldn7 RNAi #2, sense (5'-AUUAGGGCUC-GAGUGGCCUGCAAGG-3') and antisense (5'-CCUUGCAGGCCACUCGA-GCCUAAU-3'); Cldn7 RNAi #3, sense (5'-AUACGGGCCUUCUUCACU-UUGUCGU-3') and antisense (5'-ACGACAAAGUGAAGAAGGCCCGUAU-3'); Cldn12 RNAi #1, sense (5'-UAUAAGGGAAGUCAUAAACAGGCC-3') and antisense (5'-GGCCUCGUUUUUAUGACUCCUUUAUA-3'); Cldn12 RNAi #2, sense (5'-AUUUAUUGGCAGAGAGGGCAGAGC-3') and antisense (5'-GCUCUGCCUCUCUGCCAUUGAAAU-3'); and Cldn12 RNAi #3, sense (5'-UUCUGUUUCUGUUGAAUGAUCA-3') and antisense (5'-UGAUCACA-UUCAACAGAAACGAGAA-3'). Cells were transfected with 20 pmol of siRNAs or a Stealth RNAi negative control by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocols, and they were treated 12 h after transfection followed by exposure to 1 α ,25(OH)₂D₃.

Statistical Analysis

All measured values are presented as the mean \pm SD. Statistical significance of differences was evaluated using the unpaired Student's *t* test.

RESULTS

Claudins 2 and 12 Are down-Regulated in the Intestine of VDRKO Mice

Among claudin species that are abundantly expressed in the intestine, Cldn2, Cldn7, Cldn12, and Cldn15 are suggested to act as paracellular channels for cations (Amasheh *et al.*, 2002; Colegio *et al.*, 2002; Van Itallie *et al.*, 2003; Alexandre *et al.*, 2005; Fujita *et al.*, 2006; Hou *et al.*, 2006). Therefore, we first compared, by RT-PCR and Western blot analyses, the mRNA and protein levels of these claudins throughout the intestinal tract in VDRKO mice with those in WT mice. The lack of VDR transcripts and proteins in the intestine of VDRKO mice was confirmed by RT-PCR and immunoblot experiments (Figures 1A and 2, A and C; data not shown). As shown in Figure 1, A and B, expression levels of Cldn3 transcripts were higher in the jejunum in VDRKO mice than in WT mice, in good agreement with the finding that the gene expression of Cldn3 is decreased in the rat small intestine after 1 α ,25(OH)₂D₃ treatment (Kutuzova and Deluca, 2004). In contrast, the mRNA expression of Cldn2 and Cldn12, but not Cldn7 or Cldn15, was reduced in the VDRKO mouse jejunum compared with that of the WT mouse (~3- and 4-fold decreases, respectively). Similarly, levels of Cldn2 and Cldn12 proteins, but not Cldn7 or Cldn15 proteins, were much lower in the VDRKO mouse jejunum than in that of WT mice (Figure 1, C and D) (~3- and 7-fold less, respectively). Cldn2 and Cldn12 were also down-regulated in the duodenum, ileum, and colon of the VDRKO mouse at both mRNA and protein levels (Figure 2, A–D; data not depicted).

We subsequently determined, by immunofluorescent analysis, differences in the distribution of these claudins in the intestinal epithelial cells of WT and VDRKO mice (Figure 3, A–C). In the jejunum, ileum, and colon of WT mice, Cldn2 was localized at the apical-most edges of lateral membranes of crypt epithelia as reported previously (Rahner

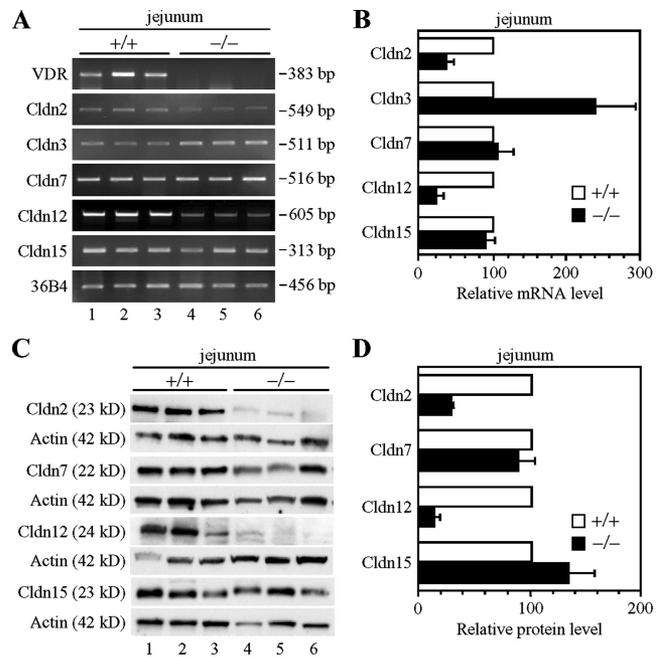


Figure 1. Reduced expression of claudins-2 and -12 in the jejunum of VDRKO mice. (A) Gene expression of Cldns in the jejunum of wild-type (+/+; lanes 1–3) and VDRKO mice (-/-; lanes 4–6) at 12 wk of age. One microgram of total RNA from the jejunum was subjected to RT-PCR analysis for the indicated genes. PCR was performed for 20 (36B4), 21 (Cldn3 and Cldn7), 25 (Cldn15), 28 (Cldn2), 32 (Cldn12), or 36 (VDR) cycles. (B) RT-PCR analysis was performed as described in A for at least five independent experiments. The mRNA levels were normalized to the corresponding 36B4 levels and expressed relative to the amount present in the wild-type mice, which was taken as 100. Values represent the mean \pm SD (error bars; n = 5 for Cldn7 and n = 6 for other Cldns). (C) Expression of Cldn proteins in the jejunum of each genotype at 12 wk of age. Twenty-five micrograms of whole cell extract from the jejunum was separated by SDS-PAGE and immunoblotted with the corresponding antibodies followed by chemiluminescence detection. Each blot was stripped and reimunoprobed with an anti-actin antibody. (D) Western blot analysis was performed as in C for at least four independent experiments. The protein levels are normalized to the corresponding actin levels and expressed relative to the amount present in wild-type mice, which was taken as 100. Values represent the mean \pm SD (error bars; n = 4 for Cldn15, n = 6 for Cldn2 and Cldn7, and n = 7 for Cldn12).

et al., 2001; Holmes *et al.*, 2006). In VDRKO mice, however, it was not detected on the jejunal mucosa and only weakly observed in the ileum and colon compared with WT mice. Cldn12 was expressed at the apical-most sites of lateral membranes of epithelia in the jejunum, ileum, and colon of WT mice as reported previously (Fujita *et al.*, 2006), whereas it was not detectable on intestinal epithelial cells of VDRKO mice. In contrast, the distribution of Cldn7 and Cldn15 in the intestine of VDRKO mice was indistinguishable from that of WT mice (Figure 3A; data not shown). Thus, the expression of both Cldn2 and Cldn12 seemed to be reduced throughout segments of the intestinal tract in VDRKO mice as far as we could determine.

Expression of Claudins-2 and -12 Is Induced by Vitamin D in Caco-2 Cells

We next analyzed the effects of 1 α ,25(OH)₂D₃ on the expression of Cldn2, Cldn7, Cldn12, and Cldn15 in the well-established human cell line Caco-2, because it exhibits a small

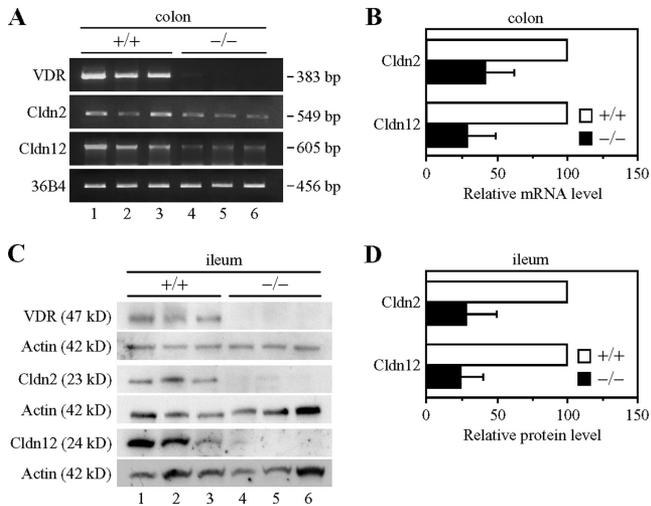


Figure 2. Down-regulation of claudins-2 and -12 in the ileum and colon of VDRKO mice. (A) Gene expression of claudin-2 (Cldn2) and Cldn12 in the colon of wild-type (+/+; lanes 1–3) and VDRKO mice (–/–; lanes 4–6) at 12 wk of age. One microgram of total RNA from the jejunum was subjected to RT-PCR analysis for the indicated genes. PCR was performed for 20 (36B4), 23 (Cldn2), or 36 (Cldn12 and VDR) cycles. (B) RT-PCR analysis was performed as described in A for at least four independent experiments. The mRNA levels are normalized to the corresponding 36B4 levels and expressed relative to the amount present in the wild-type mice, which was taken as 100. Values represent the mean \pm SD (error bars; $n = 4$ for Cldn12 and $n = 6$ for Cldn2). (C) Expression of Cldn2 and Cldn12 proteins in the ileum of each genotype at 12 wk of age. Twenty-five micrograms of whole cell extract from the ileum was separated by SDS-PAGE and immunoblotted with the corresponding antibodies followed by chemiluminescence detection. Each blot was stripped and reimunoprobed with an anti-actin antibody. (D) Western blot analysis was performed as described in C for at least five independent experiments. The protein levels are normalized to the corresponding actin levels and expressed relative to the amount present in wild-type mice, which was taken as 100. Values represent the mean \pm SD (error bars; $n = 5$ for Cldn2 and $n = 8$ for Cldn12).

intestinal phenotype. As expected, the mRNA and protein expression of Cldn2 and Cldn12, but not that of Cldn7 or Cldn15, was induced in the cells by treatment for 48 h with 10^{-7} M $1\alpha,25(\text{OH})_2\text{D}_3$ (~4- to 5-fold and 3- to 4-fold increases in their protein levels, respectively) (Figure 4, A–C). In addition, the levels of Cldn2 and Cldn12 proteins were elevated by $1\alpha,25(\text{OH})_2\text{D}_3$ in time- and dose-dependent manners (Figure 4, D and E). Note that multiple bands were observed in the immunoblots by using the anti-Cldn12 pAb used (also see Figures 6A and 7A), as reported previously for COS-7 cells transfected with the Cldn12 expression vector (Fujita *et al.*, 2006), suggesting that this pAb also recognizes posttranslationally modified Cldn12.

We then examined the localization of Cldn2, Cldn7, Cldn12, and Cldn15 in Caco-2 cells by immunostaining (Figure 4F). Cldn2 and Cldn12 proteins were hardly detected in the cells grown without $1\alpha,25(\text{OH})_2\text{D}_3$, whereas they were concentrated on the cell boundaries in the cells exposed to 10^{-7} M $1\alpha,25(\text{OH})_2\text{D}_3$ for 48 h. By contrast, Cldn7 and Cldn15 were observed along the cell borders regardless of the presence or absence of $1\alpha,25(\text{OH})_2\text{D}_3$. In addition, these four claudin species were colocalized, at least in part, with occludin at tight junctions in Caco-2 cells (Supplemental Figure S1). Hence, expression of Cldn2 and Cldn12 was apparently up-regulated by $1\alpha,25(\text{OH})_2\text{D}_3$ in Caco-2 cells, as in intestinal epithelial cells in mice.

Claudins-2 and -12 Are Required for Vitamin D-induced Calcium Absorption in Caco-2 Cells

Treatment of Caco-2 cells with $1\alpha,25(\text{OH})_2\text{D}_3$ is known not only to decrease TER, an indicator of the paracellular barrier to ion conductance but also to increase Ca²⁺ transport (Giuliano and Wood, 1991; Chirayath *et al.*, 1998; Fleet and Wood, 1999; also see Figure 5, C and D). Therefore, we subsequently verified whether expression of endogenous Cldn2 and/or Cldn12 was required for $1\alpha,25(\text{OH})_2\text{D}_3$ -induced Ca²⁺ absorption. To this end, we used an RNAi strategy to knock down the expression of Cldn2 and Cldn12. Caco-2 cells were transfected with three distinct siRNAs against human Cldn2, those against human Cldn12 or negative control siRNA, and then they were incubated for 12 h after transfection followed by treatment for 48 h with 10^{-7} M $1\alpha,25(\text{OH})_2\text{D}_3$. Western blot and immunofluorescence analyses showed that two different Cldn2 siRNAs (#1 and #3) and a Cldn12 siRNA (#1) effectively diminished the expression of Cldn2 and Cldn12, respectively (Figure 5, A and B; data not shown). Neither Cldn7 nor Cldn15 expression was affected by the Cldn2 or Cldn12 siRNAs, indicating the specific knockdown effects of these siRNAs. Interestingly, when the expression Cldn2 or Cldn12 was suppressed, reduction of TER values by $1\alpha,25(\text{OH})_2\text{D}_3$ was partially but significantly attenuated (Figure 5C and Supplemental Figure S2A). More importantly, the knockdown of Cldn2 or Cldn12 expression resulted in a significant decrease in $1\alpha,25(\text{OH})_2\text{D}_3$ -induced ⁴⁵Ca²⁺ transport (Figure 5D and Supplemental Figure S2B). Note that suppression of Cldn2 or Cldn12 expression did not alter expression of the transcellular Ca²⁺ channel protein TRPV6 or distribution of apical markers (ezrin and EBP50) (Supplemental Figure S3, A and C). By contrast, the specific knockdown of Cldn7 expression did not significantly alter the levels of TER or ⁴⁵Ca²⁺ permeability in Caco-2 cells grown in the presence and absence of $1\alpha,25(\text{OH})_2\text{D}_3$ (Supplemental Figure S4; data not shown). Together, these results indicated that $1\alpha,25(\text{OH})_2\text{D}_3$ induced paracellular Ca²⁺ transport at least in part through the upregulation of both Cldn2 and Cldn12 expression.

Overexpression of Claudins-2 and -12 Results in Enhancement of Calcium Transport across Caco-2 Cells

The question that arises from the aforementioned results is whether expression of exogenous Cldn2 and/or Cldn12 is enough to promote transepithelial Ca²⁺ transport in Caco-2 cells. To address this issue, mouse Cldn2, Cldn7, Cldn12, and Cldn15 cDNAs were introduced into Caco-2 cells along with the puromycin-resistance gene. Cells were then screened by Western blot and immunofluorescence analyses, and stable clones were obtained for Caco-2 cell lines expressing Cldn2, Cldn7, Cldn12, and Cldn15 (Caco-2:Cldn2, Caco-2:Cldn7, Caco-2:Cldn12, and Caco-2:Cldn15, respectively) (Figure 6, A and B, and Supplemental Figure S5A). In Caco-2:Cldn2, Caco-2:Cldn7, and Caco-2:Cldn15 cells, the level of endogenous claudins was not altered compared with that in the mock-transfected Caco-2 cells (Figure 6A; data not shown). Unexpectedly, however, expression of endogenous Cldn2, but not Cldn7 or Cldn15, was slightly increased in Caco-2:Cldn12 cells (Figure 6A; data not shown; see also Figure 7, A and B). Immunofluorescence microscopy revealed that in these Caco-2:Cldn cell lines, cell–cell contact sites were rich in overexpressed Cldn2, Cldn7, Cldn12, and Cldn15 (Figure 6B and Supplemental Figure S5A).

We subsequently measured TER and ⁴⁵Ca²⁺ permeability in control Caco-2 cells and Caco-2:Cldn transfectants.

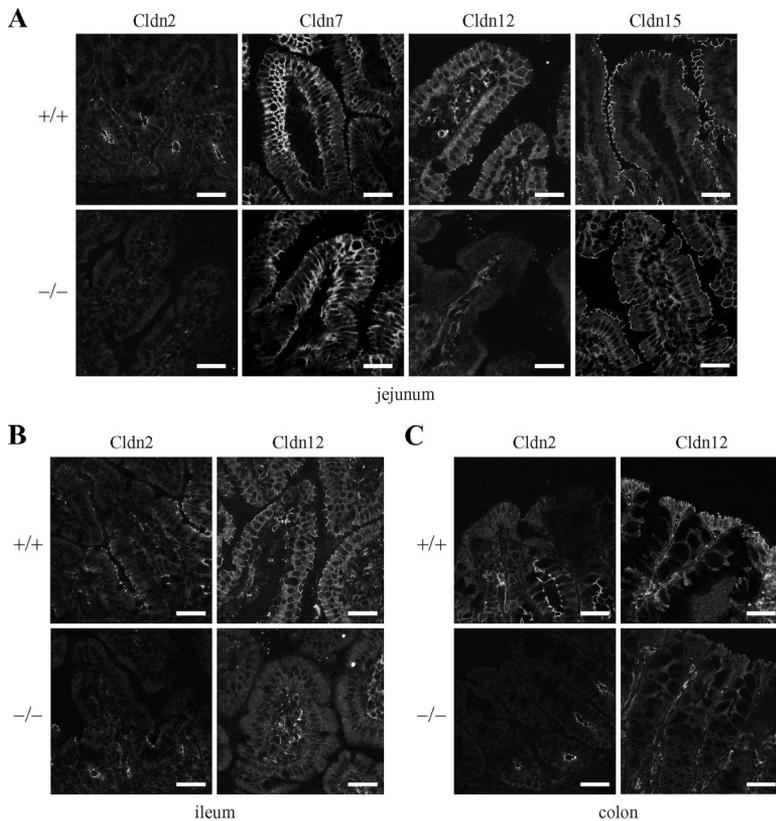


Figure 3. Distribution of claudins in intestinal mucosa of wild-type (+/+) and VDRKO (-/-) mice. Sections of the jejunum (A), ileum (B), and colon (C) of mice at 12 wk of age were subjected to immunostaining with the corresponding antibodies. Bar, 30 μm .

As shown in Figure 6C, the TER values were obviously lower in Caco-2:Cldn2 (clones 1 and 2) and Caco-2:Cldn12 cells (clones 1 and 2) than in control Caco-2 cells (~ 4 -fold and 2-fold decreases, respectively). Conversely, the TER levels were marginally altered in two independent clones of Caco-2:Cldn7 cells, and they were slightly elevated in Caco-2:Cldn15 cells (Figure 6D and Supplemental Figure S5B). Interestingly, the values of $^{45}\text{Ca}^{2+}$ transport were elevated in two different clones of both Caco-2:Cldn2 and Caco-2:Cldn12 cells compared with control cells (an ~ 2 -fold increase) (Figure 6D), although their levels were lower than those in $1\alpha,25(\text{OH})_2\text{D}_3$ -treated Caco-2 cells (see Figure 5D and Supplemental Figure S2B). These results indicated that expression of Cldn2 and/or Cldn12 at least partially replaced the effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on Ca^{2+} transport. In contrast, the levels of $^{45}\text{Ca}^{2+}$ permeability in Caco-2:Cldn7 and Caco-2:Cldn15 cells were indistinguishable from those in control cells (Figure 6D and Supplemental Figure S5C). Neither TRPV6 expression nor localization of apical markers (ezrin and EBP50) in Caco-2 cells was affected by overexpression of Cldn2 or Cldn12 (Supplemental Figure S3, B and D), strongly suggesting that these claudins contribute to paracellular Ca^{2+} transport, but not to transcellular Ca^{2+} flux.

Slight induction of endogenous Cldn2 expression was observed in two independent Caco-2:Cldn12 clones at both mRNA and protein levels (Figure 7, A and B). Therefore, to verify that weak Cldn2 expression could influence the permeability property in Caco-2:Cldn12 cells, we subsequently suppressed endogenous Cldn2 expression by using RNAi (Figure 7C). As shown in Figure 7, D and E, the values of TER and $^{45}\text{Ca}^{2+}$ permeability were not largely altered in Caco-2:Cldn12 cells when Cldn2 expression was knocked down.

Overexpressed Claudin-2, but Not Claudin-12, Promotes Na^+ Permeability across Caco-2 Cells

Because Cldn2 and Cldn12 seemed to participate in Ca^{2+} transport, we next analyzed the permeability of Na^+ and Cl^- in Caco-2:Cldn2 and Caco-2:Cldn12 cells (Figure 8, A and B). Overexpression of Cldn2 in Caco-2 cells enhanced Na^+ permeation (3-fold), but not Cl^- permeation, resulting in a threefold increase of $P_{\text{Na}}/P_{\text{Cl}}$. In contrast, forced expression of Cldn12 did not basically change the permeability of Na^+ or Cl^- in Caco-2 cells. Thus, differences in Na^+ permeability between Caco-2:Cldn2 and Caco-2:Cldn12 cells might contribute to the TER values in these cells (Figure 6C). Nor did suppression of weak induction of endogenous Cldn2 in Caco-2:Cldn12 cells influence Na^+ or Cl^- conductance (Figure 8, C and D).

DISCUSSION

Claudins form the backbone of tight junctions, and they contribute to the barrier properties, including the paracellular charge and size selectivity (Tsukita *et al.*, 2001; Van Itallie and Anderson, 2006). In this study, we found that, among members of the claudin family, Cldn2 and Cldn12 were targets for vitamin D signaling. This was obvious because Cldn2 and Cldn12, but not Cldn7 or Cldn15, were down-regulated in the duodenum, jejunum, ileum, and colon of VDRKO mice, which are known to exhibit reduced Ca^{2+} uptake in the intestine (Van Cromphaut *et al.*, 2001; Song *et al.*, 2002), at both mRNA and protein levels. Our immunofluorescence analysis also showed that signals of these two claudins were decreased or disappeared in intestinal epithelial cells of VDRKO mice. In addition, expression of Cldn2 and Cldn12 was induced by $1\alpha,25(\text{OH})_2\text{D}_3$ treatment in intestinal epithelial cell line Caco-2, further supporting our conclusion.

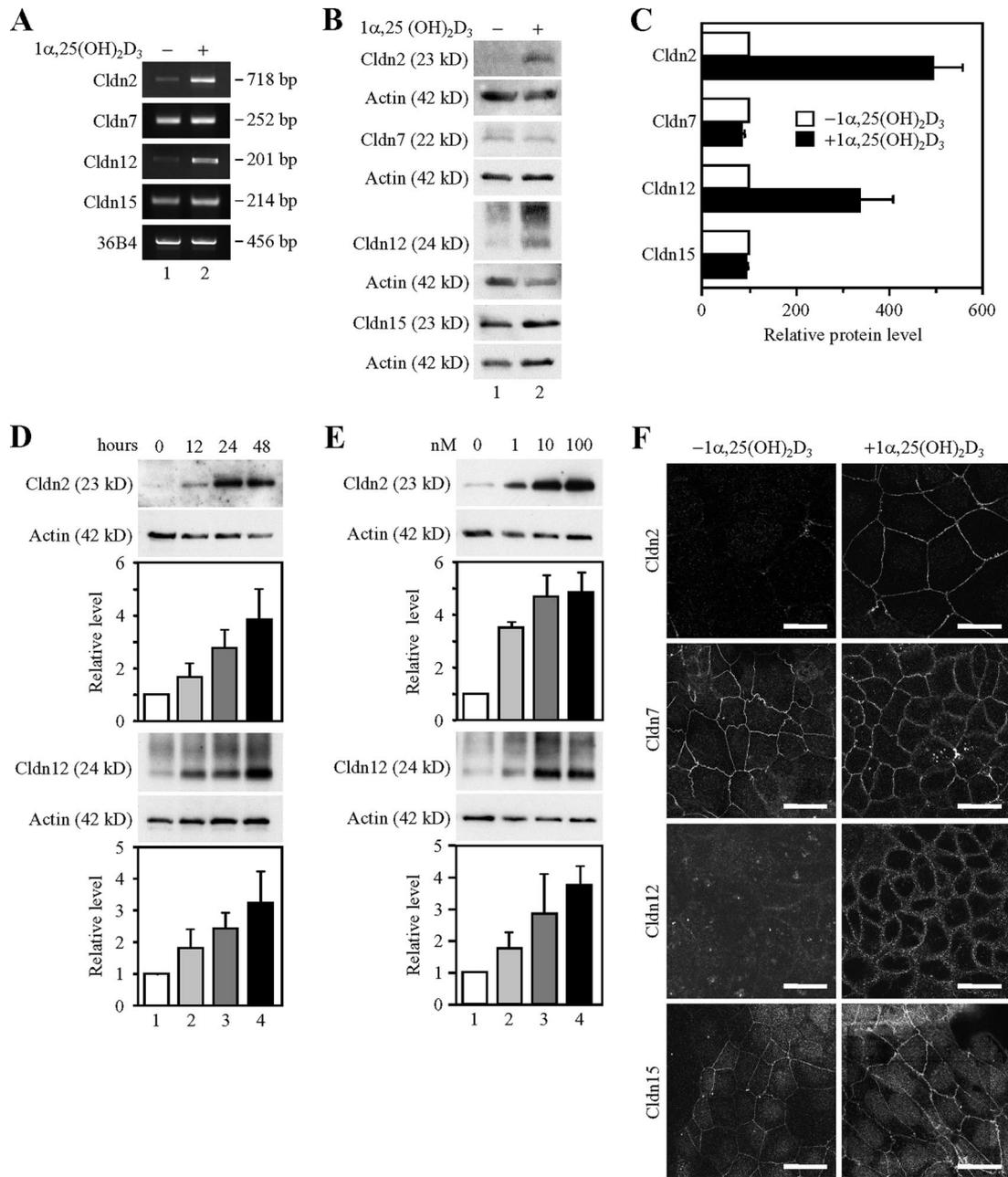


Figure 4. Induction of the expression of claudins-2 and -12 by $1\alpha,25(\text{OH})_2\text{D}_3$ in Caco-2 cells. (A) Expression of Cldn mRNAs in Caco-2 cells treated for 48 h with the vehicle (lane 1) or 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (lane 2). One microgram of total RNA from the cells was subjected to RT-PCR analysis for indicated genes. PCR was performed for 22 (36B4), 30 (Cldn12 and Cldn15) or 32 (Cldn2 and Cldn7) cycles. (B) Expression of Cldn proteins in Caco-2 cells grown for 48 h in the absence (lane 1) or presence of 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (lane 2). Twenty-five micrograms of whole cell extract was separated by SDS-PAGE and immunoblotted with antibodies against the corresponding claudins, followed by chemiluminescence detection. The blots were stripped and immunoprobed with an anti-actin antibody. (C) Western blot analysis was performed as in C for three independent experiments. The protein levels are normalized to the corresponding actin levels and expressed relative to the amount present in the cells cultured without $1\alpha,25(\text{OH})_2\text{D}_3$, which was taken as 100. Values represent the mean \pm SD (error bars; $n = 3$). (D) Expression of Cldn2 and Cldn12 proteins in Caco-2 cells exposed to 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ for 0, 12, 24, and 48 h (lanes 1–4, respectively). Western blot analysis was performed as described in B for three independent experiments, and the protein levels are expressed as described in C (error bars; $n = 3$). (E) Expression of Cldn2 and Cldn12 proteins in Caco-2 cells treated for 48 h with 0, 1, 10, and 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ (lanes 1–4, respectively). Western blot analysis was performed as described in B for three independent experiments, and the protein levels are expressed as described in C (error bars; $n = 3$). (F) Staining pattern of Cldn2, Cldn7, Cldn12, and Cldn15 in Caco-2 cells grown for 48 h in the absence or presence of 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$. Cells were subjected to immunostaining with the corresponding antibodies, and they were observed under a laser-scanning confocal microscope. Bars, 20 μm .

We previously demonstrated that two types of nuclear receptors, retinoid receptors and hepatocyte nuclear receptor 4 α (HNF4 α), triggered expression of Cldn6 and Cldn7 in

embryonal carcinoma cell line F9 (Kubota *et al.*, 2001; Chiba *et al.*, 2003; Satohisa *et al.*, 2005). Subsequently, Battle *et al.* (2006) showed that Cldn1 was absent in hepatocytes in mice

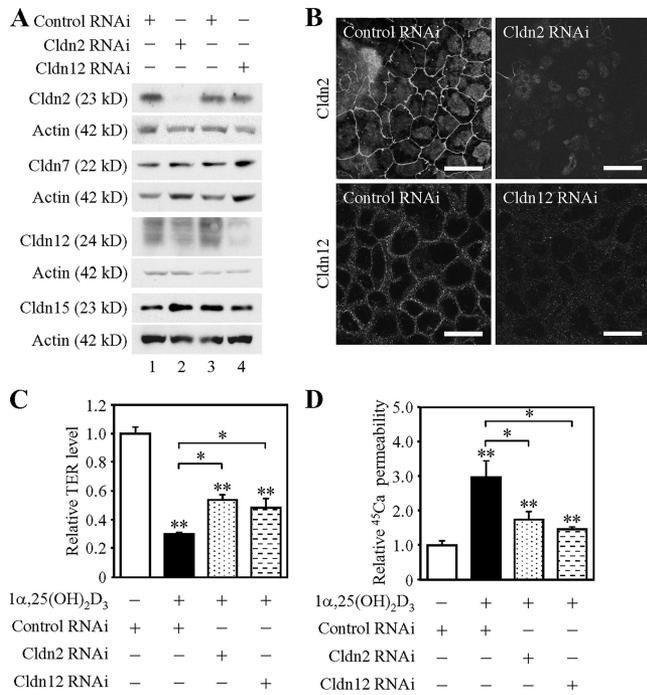


Figure 5. Knockdown of claudins-2 and -12 impairs vitamin D-induced calcium transport across Caco-2 cells. (A) Suppression of claudin-2 (Cldn2) and Cldn12 expression in Caco-2 cells by RNAi. Cells were transfected with negative control siRNA (lanes 1 and 3) or siRNAs against Cldn2 (#1) (lane 2) and Cldn12 (#1) (lane 4), incubated for 12 h after transfection, and then treated for 48 h with 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$. Twenty-five micrograms of whole cell extract from the cells was separated by SDS-PAGE and immunoblotted with the corresponding antibodies, followed by chemiluminescence detection. The blots were stripped and immunoprobed with an anti-actin antibody. (B) Disappearance of Cldn2 and Cldn12 along cell borders in Caco-2 cells by RNAi. Cells were transfected and treated as described in A, subjected to immunostaining with the corresponding antibodies, and observed under a laser-scanning confocal microscope. Bars, 20 μm . (C and D) Suppression of Cldn2 and Cldn12 expression in Caco-2 cells prevents $1\alpha,25(\text{OH})_2\text{D}_3$ -dependent decrease in TER levels and increase in $^{45}\text{Ca}^{2+}$ transport. Cells were transfected and treated as described in A, subjected to measurement of TER (C) and calcium transport studies (D). The values of TER and $^{45}\text{Ca}^{2+}$ permeability are expressed relative to the level in cells transfected with negative control siRNA and grown without $1\alpha,25(\text{OH})_2\text{D}_3$, which was taken as 1, and they represent the mean \pm SD (error bars; $n = 4$). * $p < 0.05$ and ** $p < 0.01$ compared with values of cells grown without $1\alpha,25(\text{OH})_2\text{D}_3$.

with conditional KO of the *HNF4 α* gene. More recently, Gareus *et al.* (2007) found that retinoid receptors and the kinase I κ B kinase complex 1 cooperatively regulated the expression of retinoid target genes, including Cldn23, in keratinocytes. Furthermore, comparison of gene expression profiling between wild-type and Sertoli cell-specific androgen receptor (AR)-knockout mice revealed that AR mediated activation of the Cldn3 expression in Sertoli cells (Meng *et al.*, 2005). Thus, taken collectively with our present work, expression of different claudin species seems to be up-regulated by distinct members of the nuclear receptor superfamily in a cell-specific manner.

The most important conclusion of the present study is that Cldn2 and Cldn12 facilitate the paracellular conductance and Ca^{2+} transport in intestinal epithelial cells. This conclusion was drawn from results using RNAi and overexpression strategies in Caco-2 cells, in which endogenous Cldn2

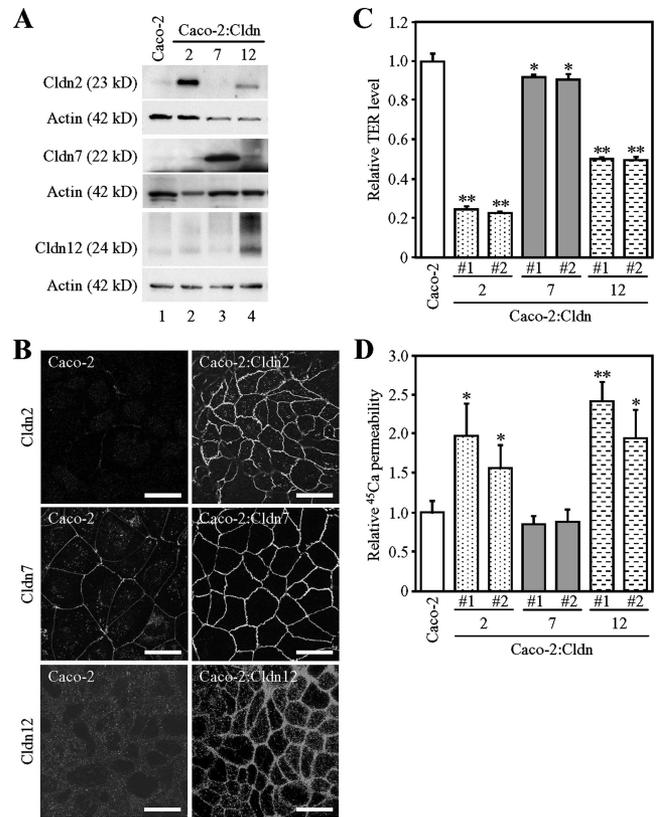


Figure 6. Overexpression of claudins-2 and -12 promotes calcium transport in Caco-2 cells. (A) Expression of claudin-2 (Cldn2), Cldn7, and Cldn12 proteins in Caco-2 and Caco-2:Cldn transfectants. Twenty-five micrograms of whole cell extract from the cells was separated by SDS-PAGE and immunoblotted with antibodies against the corresponding claudins, followed by chemiluminescence detection. The blots were stripped and reimunoprobed with an anti-actin antibody. (B) Staining pattern of Cldn2, Cldn7, and Cldn12 in Caco-2 and Caco-2:Cldn transfectants. Cells were subjected to immunostaining with the corresponding claudin antibodies. Bar, 20 μm . (C and D) Relative levels of TER (C) and $^{45}\text{Ca}^{2+}$ transport (D) in Caco-2 and Caco-2:Cldn transfectants. The values of TER and $^{45}\text{Ca}^{2+}$ permeability in Caco-2:Cldn transfectants (clones #1 and #2) are expressed relative to the level in the mock-transfected cells, which was taken as 1, and they represent the mean \pm SD (error bars; $n = 4$). * $p < 0.05$ and ** $p < 0.01$ compared with values of the mock-transfected cells.

and Cldn12 expression was hardly detected along cell interfaces and was induced upon $1\alpha,25(\text{OH})_2\text{D}_3$ treatment. The suppression of either Cldn2 or Cldn12 expression by RNAi in Caco-2 cells hindered $1\alpha,25(\text{OH})_2\text{D}_3$ -induced passage of electricity and $^{45}\text{Ca}^{2+}$, indicating that these claudin species were at least in part necessary for vitamin D-triggered calcium absorption. Furthermore, overexpression of Cldn2 and Cldn12, but not Cldn7 or Cldn15, led to significant increases in the electrical conductivity and $^{45}\text{Ca}^{2+}$ transport across Caco-2 cells. These results highlighted that Cldn2 and Cldn12 were not only required but also sufficient to promote the paracellular conductance and calcium absorption in intestinal epithelial cells. In this sense, it should be noted that, among the different segments of the intestinal tract, Cldn2 and Cldn12 are most abundantly expressed in the ileum (Fujita *et al.*, 2006; Holmes *et al.*, 2006), where the majority (65–88%) of dietary calcium is absorbed (for review, see Wasserman, 2004). It is also noteworthy that, in both mice and humans, Cldn2 has three negatively charged amino

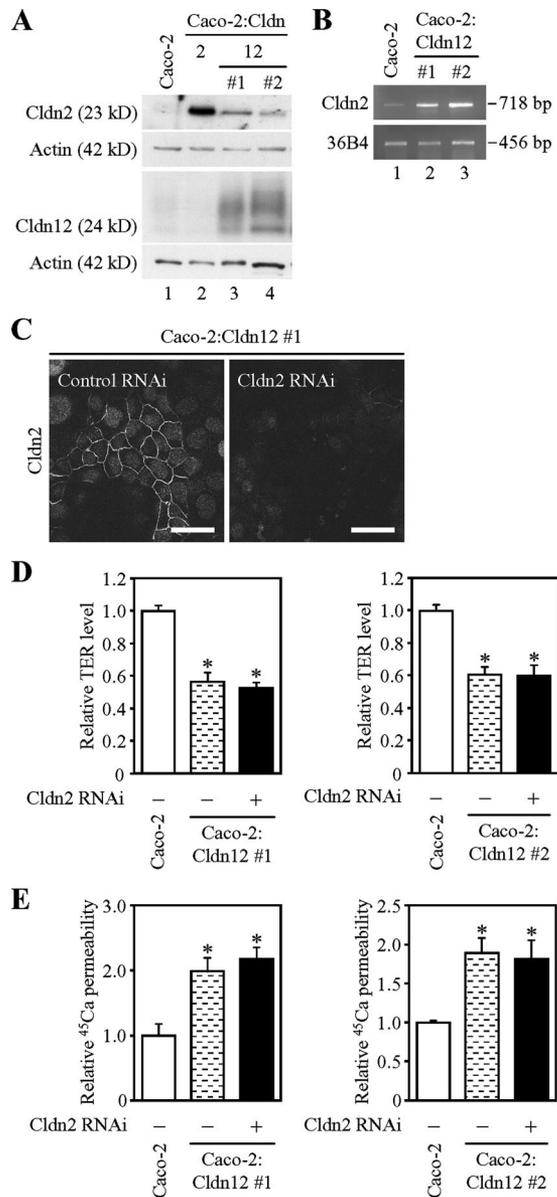


Figure 7. Weak induction of claudin-2 does not contribute to increased calcium transport in Caco-2 cells overexpressing claudin-12. (A and B) Slight induction of endogenous Cldn2 protein (A) and mRNA (B) in Caco-2:Cldn12 cells (clones #1 and #2). (A) Twenty-five micrograms of whole cell extract from the cells was separated by SDS-PAGE and immunoblotted with antibodies against the corresponding claudins, followed by chemiluminescence detection. The blots were stripped and reimmunoprobed with anti-actin antibody. (B) One microgram of total RNA from the cells was subjected to RT-PCR analysis for indicated genes. PCR was performed for 23 (36B4) or 30 (Cldn2) cycles. (C) Knockdown of endogenous Cldn2 expression in Caco-2:Cldn12 cells (clone #1) by RNAi. Cells were transfected with negative control siRNA (left) or siRNA against Cldn2 (#1; right), incubated for 12 h after transfection, and then they were refed and grown for 48 h. They were subjected to immunostaining with the corresponding antibodies, and they were observed under a laser-scanning confocal microscope. Bars, 20 μm. (D and E) Effects of suppressed endogenous Cldn2 on TER (D) and ⁴⁵Ca²⁺ permeability in Caco-2:Cldn12 cells (clones #1 and #2). Cells were transfected and grown as described in C, and then they were subjected to measurement of TER (D) and calcium transport studies (E). The values are expressed relative to the level in Caco-2 cells transfected with negative control siRNA, which was taken as 1, and they represent the mean ± SD (error bars; n = 6 [left] and n = 3 [right]). *p < 0.01 compared with values of the mock-transfected cells.

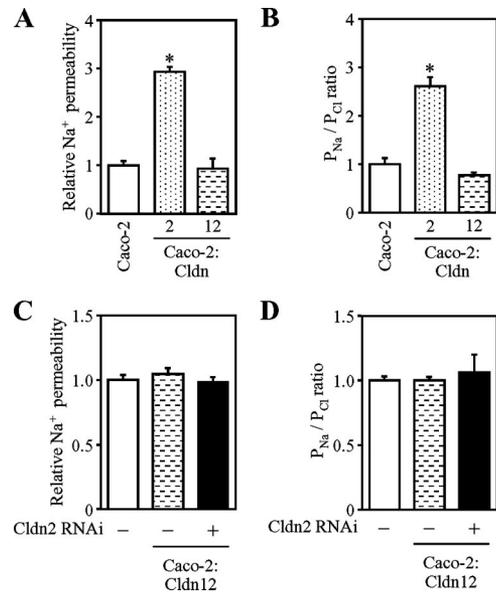


Figure 8. Overexpression of claudin-2, but not claudin-12, increases Na⁺ permeability in Caco-2. (A and B) Na⁺ permeability (A) and relative levels of Na⁺/Cl⁻ permeability (B; P_{Na}/P_{Cl}) in Caco-2, Caco-2:Cldn2 (clone #2) and Caco-2:Cldn12 (clone #1). (C and D) Effects of suppressed endogenous Cldn2 on Na⁺ permeability (C) and Na⁺/Cl⁻ (D) in Caco-2:Cldn12 cells (clones #1). Cells were transfected with negative control siRNA (left) or siRNA against Cldn2 (#1, right), incubated for 12 h after transfection, and then they were refed and grown for 48 h. They were then subjected to the measurement. The values are expressed relative to the level in Caco-2 cells transfected with negative control siRNA, which was taken as 1, and they represent the mean ± SD (error bars; n = 4). *p < 0.01 compared with values of the mock-transfected cells.

acids (positions 53, 65, and 75) within the first extracellular loop (Van Itallie *et al.*, 2003), and that Cldn12 contains four negatively charged residues (positions 62, 66, 71 and 74) in the same domain (Fujita *et al.*, 2006), suggesting that these claudins form paracellular pores for cations. Hence, although a relative contribution of paracellular and transcellular Ca²⁺ transport has not been established (McCormick, 2002; Bronner *et al.*, 2003; Wasserman, 2004; Hoenderop *et al.*, 2005), we propose that Cldn2- and/or Cldn12-based tight junctions create paracellular Ca²⁺ channels in enterocytes to maintain calcium homeostasis together with the transcellular pathway.

Several lines of evidence have suggested that Cldn16/paracellin-1 (PCLN-1) plays a key role in the reabsorption of Mg²⁺ and Ca²⁺. First, positional cloning has identified that familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC), an autosomal recessive disease showing severe Mg²⁺ and Ca²⁺ wasting, is caused by mutations in the *Cldn16/PCLN-1* gene (Simon *et al.*, 1999). It is also known that Cldn16 is highly expressed at tight junctions of epithelial cells in the thick ascending loop of Henle, where the reabsorption of divalent cations occurs (Simon *et al.*, 1999). Second, disease-associated mutations in patients with FHHNC do indeed affect the intracellular traffic and paracellular Mg²⁺ transport functions of Cldn16 (Kausalya *et al.*, 2006). Third, overexpression of Cldn16 enhances Mg²⁺ and Ca²⁺ transport in Madin-Darby canine kidney (MDCK) cells (Ikari *et al.*, 2004, 2006). Fourth, using the pig renal epithelial cell line LLC-PK1 and transgenic mice, Cldn16, which also seems to cause profound effects on permeability of monovalent cations, is shown to drive the reabsorption of Mg²⁺

and Ca^{2+} (Hou *et al.*, 2005, 2007). However, *Cldn16* seems not to be expressed along the intestinal tract (Fujita *et al.*, 2006; Holmes *et al.*, 2006), suggesting that other claudin species may contribute to Ca^{2+} absorption in the intestine. These reports, taken together with our present findings, indicate that distinct claudins, *Cldn2* and *Cldn12* in the intestine and *Cldn16* in the kidney, are most likely involved in paracellular Ca^{2+} transport.

Cldn2 is well known to function as paracellular channel to Na^+ in renal epithelial cells without altering Cl^- conductance. For example, overexpression of *Cldn2* in MDCK strain I cells lacking endogenous *Cldn2* increases paracellular Na^+ permeability and decreases TER (Furuse *et al.*, 2001; Amasheh *et al.*, 2002). Similar effects are observed when *Cldn2* is overexpressed in LLC-PK1 cells expressing little endogenous *Cldn2* (Van Itallie *et al.*, 2003). Conversely, knockdown of endogenous *Cldn2* expression in MDCK strain II cells reduces paracellular Na^+ permeation and elevates TER (Hou *et al.*, 2006). We have revealed that forced expression of *Cldn2* in Caco-2 cells results in enhancement of permeability of Na^+ and Ca^{2+} , indicating that *Cldn2* forms paracellular channel for both cations in intestinal epithelial cells. In contrast, overexpression of *Cldn12* promoted conductance of Ca^{2+} , but not of Na^+ , suggesting that *Cldn12*, unlike *Cldn2* (this study) and *Cldn16* (Hou *et al.*, 2005, 2007), may preferentially serve as a paracellular channel to Ca^{2+} in enterocytes.

Another issue that should be discussed is weak induction of endogenous *Cldn2* expression by *Cldn12*. Overexpression of *Cldn2*, *Cldn7*, and *Cldn15* in Caco-2 cells did not alter the levels of endogenous claudins, in good agreement with previous results using MDCK and LLC-PK1 cells (Furuse *et al.*, 2001; Van Itallie *et al.*, 2003; Alexandre *et al.*, 2005; Hou *et al.*, 2006). In contrast, increased expression of endogenous *Cldn2* was detected in two independent Caco-2:*Cldn12* clones, but not in Caco-2:*Cldn7* and Caco-2:*Cldn15* cells, at both transcription and protein levels. In this regard, it is worth noting that forced expression of *Cldn8* in MDCK strain II cells results in down-regulation of endogenous *Cldn2* protein (Yu *et al.*, 2003). Although it is unclear by which mechanism *Cldn12* activates *Cldn2* expression, we excluded, by RNAi experiments, the possibility that slight induction of *Cldn2* influenced the permeability of both Ca^{2+} and Na^+ as well as TER in Caco-2:*Cldn12* cells.

In conclusion, we have shown that expression of *Cldn2* and *Cldn12* is up-regulated in intestinal epithelial cells by $1\alpha,25(\text{OH})_2\text{D}_3$ via VDR. We also found that these two claudin species acted as paracellular Ca^{2+} channels in intestinal mucosa. These findings identify essential roles of *Cldn2* and *Cldn12* in the vitamin D-dependent intestinal Ca^{2+} absorption, providing new insight into Ca^{2+} homeostasis. Further studies will be required to determine whether expression of *Cldn2* and *Cldn12* genes are directly and/or indirectly regulated by VDR. It will also be important to generate and characterize *Cldn2*- and/or *Cldn12*-deficient mice, as well as to elucidate possible contributions of these claudins to pathological conditions such as intestinal malabsorption and impaired Ca^{2+} absorption. Moreover, it will be interesting to investigate whether the luminal Ca^{2+} concentration in the intestine might affect expression of *Cldn2* and *Cldn12*.

ACKNOWLEDGMENTS

We are grateful to Immuno-Biological Laboratories for cooperation in generation of anti-claudin-7, anti-claudin-12, and anti-claudin-15 antibodies; Dr. T. Onaga for technical advice; N. Sakai, K. Takeda, and M. Nakazawa as well as the staff of the animal facility for technical assistance; and K. Barrymore for help with manuscript.

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Akiyama Foundation, and the Takeda Science Foundation.

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