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Claudin-1 inhibits human parainfluenza virus type 2 dissemination

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ABSTRACT

Tight junctions enable epithelial cells to form physical barriers that act as an innate immune defense against respiratory infection. However, the involvement of tight junction molecules in paramyxovirus infections, which include various respiratory pathogens, has not been examined in detail. Human parainfluenza virus type 2 (hPIV2) infects airway epithelial cells and causes respiratory illness. In the present study, we found that hPIV2 infection of cultured cells induces expression of claudin-1 (CLDN1), an essential component of tight junctions. This induction seemed to be intrinsically restricted by V, an accessory protein that modulates various host responses, to enable efficient virus propagation. By generating CLDN1 over-expressing and knockout cell lines, we showed that CLDN1 is involved in the restriction of hPIV2 spread via cell-to-cell contact. Taken together, we identified CLDN1 an inhibitory factor for hPIV2 dissemination, and that its V protein acts to counter this.

1. Introduction

Human parainfluenza virus type 2 (hPIV2) is a respiratory pathogen that causes croup and bronchiolitis in infants and young children (Hall, 2001; Henrickson, 2003). hPIV2 belongs to the genus *Rubulavirus* in the family *Paramyxoviridae* (Lamb and Parks, 2013). Viruses belonging to this family include other important human respiratory pathogens, such as hPIV1, hPIV3, hPIV4 and measles virus (MeV). The hPIV2 genome consists of a single negative-strand RNA that encodes 7 viral proteins from 6 genes; the nucleocapsid (NP), phospho- (P), V, matrix (M), hemagglutinin-neuraminidase (HN), fusion (F) and large (L) proteins. NP, P and L proteins, as well as genome RNA, form the ribonucleoprotein complex that is responsible for transcription and replication. P protein and V protein are both produced from the P gene that contains a gene editing signal. These two proteins share an N-terminal domain, but have distinct C-terminal domains due to mRNA editing (Kolakofsky et al., 2005; Ohgimoto et al., 1990). In contrast to P protein that is an essential component for viral polymerase, V protein works as an accessory factor to facilitate viral growth efficiency by associating with various host proteins. V protein interacts with multiple intracellular sensor molecules and signal transduction proteins; melanoma differentiation-associated protein 5 (MDA-5), laboratory of genetics and physiology 2 (LGP2), tumor necrosis factor receptor-associated factor 6 (TRAF6) and signal transducer and activator of transcriptions (STATs) to prevent the interferon (IFN)-mediated antiviral response (Andrejeva et al., 2004; Childs et al., 2012; Kitagawa et al., 2013; Nishio et al., 2001, 2005). Moreover, it interacts with ALG-2 interacting protein 1/ALG2-interacting protein X (AIP1/Alix), a membrane protein required for the budding of progeny virions (Nishio et al., 2007). V protein also interacts with and antagonizes tetherin, an antiviral membrane protein prevents the release of enveloped viruses from the cell surface (Ohta et al., 2015). These interactions are mediated via the C-terminal V-specific region, which include unique motifs containing seven Cys, and three Trp residues.

Tight junctions form an intercellular barrier between epithelial cells, and act as an innate immune mechanism that can impede invading pathogens. The compartmentalization between apical and basolateral membrane domains of epithelial cells maintains cell polarity, and tight junctions have also been implicated in signal transduction and regulation of paracellular permeability (Colpitts and Baumert, 2016; Mineta et al., 2011). Tight junctions are formed by various integral membrane proteins, including occludin, junctional adhesion molecules (JAMs) and the claudin family (Furuse et al., 1993, 1998; Martìn-Padura et al., 1998; Bazzoni, 2003). The claudin family consists of at least 27 members. They have four transmembrane domains and two extracellular loops, and are solely responsible for forming tight junction strands (Furuse et al., 1998; Kojima et al., 2013).

Claudin family proteins have been implicated in the infection of several human pathogens. Claudin-1 (CLDN1) plays a critical role in the entry process of hepatitis C virus (HCV). The envelope glycoprotein of HCV interacts with CLDN1 that acts as a co-receptor (Evans et al., 2007), and CLDN1 is involved in both cell-free and cell-to-cell

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transmission of HCV (Brimacombe et al., 2011). Moreover, CLDN6 and CLDN9 function as a co-receptor for HCV in a virus genotype–dependent manner (Meertens et al., 2008; Haid et al., 2014; Zheng et al., 2007). CLDN1 was shown to be involved in dengue virus (DENV) entry (Che et al., 2013; Gao et al., 2010). It has been proposed that DENV prM protein binds to CLDN1, and CLDN1 knockout and knockdown inhibit DENV infection in hepatoma cells. However, the involvement of tight junction molecules and respiratory viruses, many of which are paramyxoviruses, has not been investigated in detail. In the present study, we examine the correlation between hPIV2 infection and CLDN1.

2. Materials and methods

2.1. Cells, antibodies and virus

Vero cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FCS. A549 and MDCK cells were cultured in Dulbecco's modified Eagle's MEM (DMEM) supplemented with 10% FCS. All cells were maintained in a humidified incubator at 37 °C with 5% CO_2. Anti-CLDN1 rabbit polyclonal antibody and anti-actin mAb were purchased from Abcam (Cambridge, United Kingdom) and Santa Cruz (Dallas, TX), respectively. Anti-hPIV2 P/V protein (315-1) mAb was as described previously (Nishio et al., 1997). hPIV2 (Toshiba strain) and recombinant hPIV2 (rPIV2) $V_{C193/197A}$ that possesses point mutations in V gene were as previously described (Nishio et al., 2005).

2.2. Quantitative real-time reverse transcription-PCR

CLDN1, Zonula occludens-1 (ZO-1), ZO-2 and Occludin mRNA expression was measured by quantitative real-time reverse transcription-PCR (qRT-PCR). Total RNAs were isolated from virus-infected cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA synthesis was carried out by using a PrimeScript RT reagent kit (Takara, Shiga, Japan) with oligo-dT primer. qRT-PCR was performed by using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The primers were as follows; CLDN1 (F: 5′-AAAGTCTTTGACT CCTTGCTGAATC-3′, R: 5′-GGTGTTGGGTAAGAGGTTGTTTTTC-3′), ZO-1 (F: 5′-GGGACAACAGCATCCTTCCA-3′, R: 5′-ATCACAGTGTGGTAAG CGCA-3′), ZO-2 (F: 5′-TGAAGACACGGACGGTGAAG-3′, R: 5′-GGGCTG GGTTTCCTTATGCT-3′), Occludin (F: 5′-CAGGCCTCTTGAAAGTCC ACC-3′, R: 5′-AGGCTGGCTGAGAGAGCATT-3′), GAPDH (F: 5′-GAAGG TCGGAGTCAACGGATTT-3′, R: 5′-ATCTTGAGGCTGTTGTCATACT TCT-3′).

2.3. Western blot analyses

Cells were lysed in a lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.6% NP-40), and they were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoretic transfer from gels onto nitrocellulose membrane was carried out. The membranes were blocked with 5% skim milk in PBS, treated with primary antibody, and washed with PBS including 0.2% Tween20 (PBS-T). The membranes treated with peroxidase-labeled horse anti-mouse IgG or peroxidase-labeled goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), and washed with PBS-T. Signals were visualizes by using ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz) according to the manufacturer's instructions.

2.4. Plasmid construction

Human CLDN1 expressing plasmid (pcDNA-CLDN1) was generated by inserting a cDNA clone of the claudin-1 gene into plasmid pcDNA 3.1(+) (Invitrogen, Carlsbad, CA). To establish CLDN1 knockout (KO) MDCK cell line, GeneArt CRISPR Nuclease Vector (Thermo Fisher Scientific, Waltham, MA) was used according to the manufacturer's instruction with some modifications. To allow the antibiotic selection, a hygromycin-resistant gene was inserted into the vector. The target sequence of canine CLDN1 is 5′-caccgcaacgcggggctgcagctcctgg-3′. hPIV2 V was cloned into the mammalian expression vector pEBS-PL, to generate pEBS-V, as described previously (Nishio et al., 2005).

2.5. Establishment of cell lines constitutively over-express CLDN1 and V proteins

A549 cells were transfected with pcDNA-CLDN1 using XtremeGENE HP (Roche, Basel, Switzerland) according to the manufacturer's protocols. At two days post transfection, cells were transferred to 12-well plates, and cultured in DMEM containing 10% FCS and 100 μg/ml hygromycin B (Invitrogen). Cells were cultured for 2–4 weeks and hygromycin-resistant colonies were isolated. A549 cells were transfected with pEBS-V plasmids using XtremeGENE HP. At two days post transfection, the cells were transferred to 12-well plates and cultured in DMEM containing 10% FCS and 100 μg/ml hygromycin B. Cells were cultured for 2–4 weeks and hygromycin-resistant colonies were isolated. Overexpression of CLDN1 was confirmed by western blot analysis using specific antibody against CLDN1.

2.6. Generation of CLDN1 knockout MDCK cell line

MDCK cells were transfected with GeneArt CRISPR Nuclease Vector targeting canine CLDN1 using Lipofectamin 3000 (Invitrogen) according to the manufacturer's protocols. At two days post transfection, the cells were transferred to 12-well plate and cultured DMEM containing 10% FCS and 100 μg/ml hygromycin B. The cells were cultured for 2–4 weeks and hygromycin-resistant colonies were isolated. Knockout of CLDN1 was confirmed by western blot analysis using a specific antibody against CLDN1.

2.7. Virus titration

Virus titers were measured by a plaque assay using Vero cells as described previously, with a little modification (Nishio et al., 2005, 2007). Briefly, 300 μl of serial 10-fold dilution of the virus solutions in MEM without FCS was added to each well of a 12-well plate with Vero cells. After 1 h of adsorption, virus solutions were removed and MEM containing 2% FCS and 1% SeaKem ME agarose (FMC BioProducts, Philadelphia, PA) was added. At 4–5 days post infection, the cells were stained with 0.1% neutral red and the plaques were counted.

2.8. Immunofluorescence analysis (IFA) to examine cell-to-cell spread of hPIV2

Cells cultured at UV sterilized cover glass in 6-well plates were inoculated with hPIV2 at a multiplicity of infection (MOI) of 0.01. After 1 h of adsorption, virus solutions were removed and MEM containing 2% FCS and 1% SeaKem ME agarose was added. At 24 and 36 h post infection, the cells were fixed with 4% paraformaldehyde at room temperature and rinsed with PBS. The cells were permeabilized with PBS containing 0.2% (w/v) TritonX-100 and washed with PBS. The cells were incubated with anti-hPIV2 P/V mAb. The secondary antibody reaction was performed with AlexaFluor 488 goat anti-mouse IgG (Invitrogen). After washing the cells with PBS, cells were observed under a fluorescence microscope.

3. Results

3.1. Induction of CLDN1 expression in hPIV2 infected cells

To gain insight into the involvement of tight junction molecules in viral infection, we examined the mRNA expression of CLDN1, ZO-1, ZO-

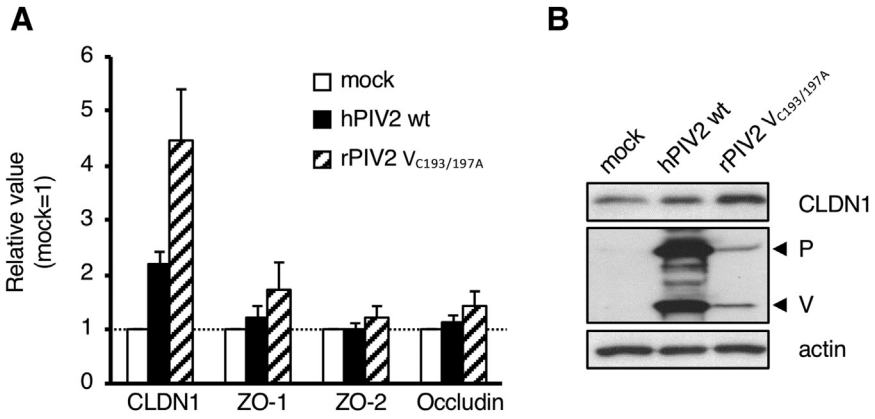

Fig. 1. Induction of CLDN1 in hPIV2-infected A549 cells. (A) A549 cells were infected with hPIV2 wt or rPIV2 $V_{C193/197A}$ at an MOI of 0.01 for 48 h. Expressions of CLDN1, ZO-1, ZO-2 and Occludin mRNA were measured by qRT-PCR, and relative values are shown (mock = 1). Data represent means and standard deviations of triplicate experiments. **(B)** A549 cells were infected with hPIV2 wt or rPIV2 $V_{C193/197A}$ at an MOI of 0.01 for 48 h. The cell lysates were subjected to western blot analysis using the indicated antibodies. Expression of actin in cell lysates was used as a loading control.

2 and Occludin in hPIV2 infected cells. A polarized cell line, A549 cells (Bose et al., 2001) were infected with hPIV2 wt at an MOI of 0.01. Relative mRNA expressions were measured by qRT-PCR using specific primers. Among four genes, CLDN1 mRNA expression was specifically up-regulated by approximately two-fold in hPIV2 wt infection (Fig. 1A).

We next examined the contribution of V protein for the up-regulation of cellular gene expression. Paramyxovirus V proteins are composed of the N-terminal half of the P protein and a unique C-terminal region. hPIV2 V protein influences viral growth efficiency, and the C terminus of the V protein-specific region contains functionally important groups of Cys (zinc finger) and Trp residues (Nishio et al., 2001, 2007). We previously generated rPIV2 $V_{C193/197A,}$ which expresses a mutant V protein that no longer antagonize multiple antiviral host proteins (Nishio et al., 2005). We hypothesized that the functional V affects the induction of tight junction molecules during viral infection to obtain an ideal condition for virus propagation. The relative mRNA expressions of tight junction molecules in A549 cells infected with rPIV2 $V_{C193/197A}$ were compared with those with hPIV2 wt. Although expressions of mRNA coding for ZO-1, ZO-2 and Occludin did not exhibit clear changes, the up-regulation of CLDN1 mRNA expression was enhanced in the case of rPIV2 $V_{C193/197A}$ infection by approximately four-fold higher than that of mock (Fig. 1A). Remarkable induction of CLDN1 protein expression was observed in rPIV2 $V_{C193/197A}$ infected A549 cells by western blot analysis. (Fig. 1B).

We further examined the mRNA expressions of CLDN 3, 4 and 7, all of which express in human respiratory tract (Coyne et al., 2003) in A549 cells infected with hPIV2 wt, rPIV2 $V_{C193/197A}$ and mock by qRT-PCR, and found that CLDN1 is the only factor that was specifically induced by viral infection (data not shown).

3.2. CLDN1 inhibits hPIV2 growth

To investigate the effects of CLDN1 on hPIV2 growth, A549 cell lines stably over-expressing CLDN1 (A549/CLDN1), as well as their control cell lines (A549/ctrl), were generated (Fig. 2A). hPIV2 wt was inoculated into these cells at an MOI of 0.1 for 48 h. Virus titers and viral protein expression were measured by a plaque assay and western blot analysis, respectively. We observed that hPIV2 wt growth and protein expression were decreased to approximately one third in the presence of excessive amount of CLDN1 (Fig. 2A). The similar result was observed at an MOI of 2 for 48 h (data not shown).

MDCK cells endogenously express CLDN1 protein abundantly (Fig. 2B). We generated a CLDN1 knockout MDCK cell line (MDCK/CLDN1-KO) and its control (MDCK/ctrl) by using a clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (CRISPR/Cas9) system. These cells were infected with hPIV2 wt at an MOI of 0.1 for 48 h, and virus titers and viral protein expression were measured. Depletion of CLDN1 elicited an approximately ten-fold enhancement of hPIV2 growth and protein expression (Fig. 2B). We next attempted to confirm these results using MDCK cells cultured on filters that exhibit both apical and basolateral surfaces. Release of hPIV2 from the apical surface was approximately ten-fold higher in MDCK/CLDN1-KO cells than that in MDCK/ctrl cells at an MOI of 0.1 (Fig. 2C). The similar result was observed at an MOI of 2 (data not shown). Thus, we confirmed that hPIV2 growth is inhibited in the presence of CLDN1.

3.3. CLDN1 inhibits cell-to-cell spread of hPIV2

We hypothesized that CLDN1 is involved in the spread of hPIV2 via cell-to-cell contact. A549/ctrl and /CLDN1 were inoculated with hPIV2 wt at a low MOI (0.01), and 1% agarose was added to the culture medium to prevent the spread of released virions, but allowing cell-to-cell spread. Virus dissemination was examined by IFA using P/V mAb. hPIV2-infected A549/ctrl cells were observed as foci consisting of multiple cells at 24 h, they subsequently widely expanded at 36 h (Fig. 3A). In contrast, viral foci were rarely observed in A549/CLDN1 at 24 h. Although they expanded at 36 h, the size was smaller than those observed in A549/ctrl. Similar experiments were performed using MDCK/ctrl and /CLDN1-KO cells (Fig. 3B). The viral foci observed in MDCK/ctrl cells, which abundantly express endogenous CLDN1, were very small at 24 and 36 h. However, those in MDCK/CLDN1-KO cells were larger, especially at 36 h (Fig. 3B). Next, the cell numbers within the viral foci observed in these cell lines were counted, when the most remarkable difference was seen (24 h for A549 cells, and 36 h for MDCK cells). Cell numbers within the viral foci in A549/CLDN1 cells (1.95 on average) were clearly fewer than those in A549/ctrl cells (6.35 on average) (Fig. 3C). Moreover, those in MDCK/ctrl (1.98 on average) were fewer than those in MDCK/CLDN1-KO (6.98 on average) (Fig. 3D). These results demonstrated that the cell-to-cell spread of hPIV2 is inhibited in the presence of CLDN1.

3.4. Effect of V protein for induction of CLDN1 expression in infected cells

hPIV2 wt infection induced CLDN1 expression, and rPIV2 V mutant infection facilitated the induction, indicating that V protein acts to repress CLDN1 expression. To evaluate this, we created A549 cells that stably express hPIV2 V protein (A549/V), and their control (A549/ctrl) (Fig. 4A, mock). CLDN1 expression was not affected by the stable expression of hPIV2 V. hPIV2 wt and rPIV2 $V_{C193/197A}$ were used to infect A549/ctrl and A549/V. CLDN1 mRNA and protein expressions were measured by qRT-PCR and western blot analysis, respectively. hPIV2 wt infection induced CLDN1 mRNA expression both in A549/ctrl and /V, whereas rPIV2 $V_{C193/197A}$ infection did not induce CLDN1 mRNA expression in A549/V as strongly compared to A549/ctrl (Fig. 4B). CLDN1 protein expression was remarkable in rPIV2 $V_{C193/197A}$ infected A549/ctrl cells, but not in that infected A549/V cells (Fig. 4A). These data suggest that V protein reduces CLDN1 expression during viral infection.

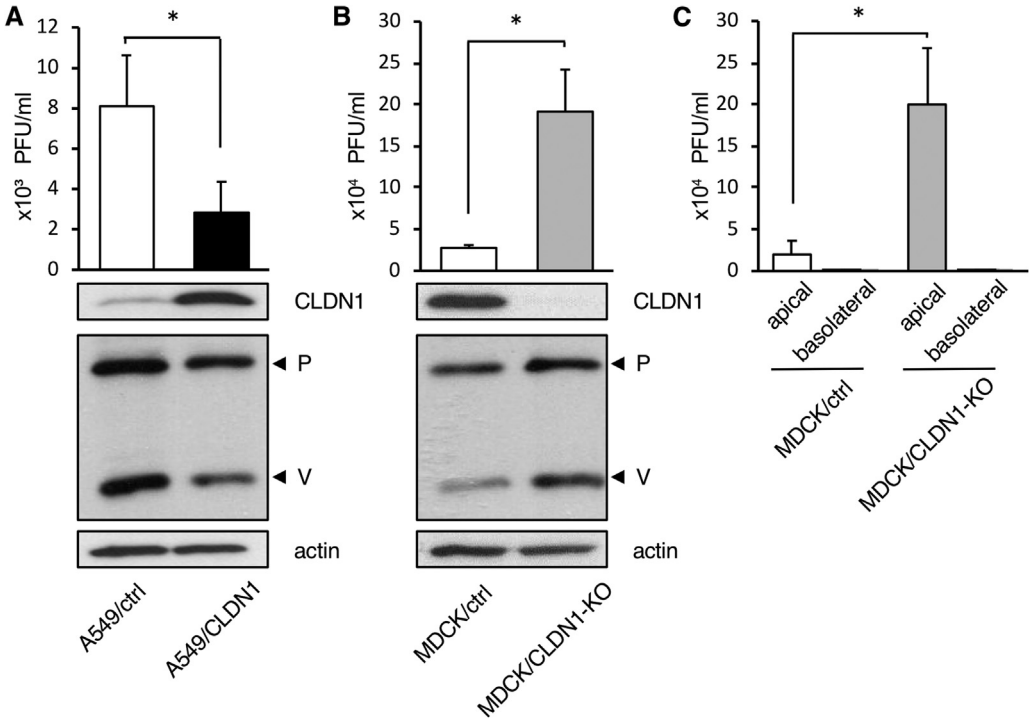

Fig. 2. Effect of CLDN1 over expression and knockout on hPIV2 growth. **(A)** A549/CLDN1 and its control cells were infected with hPIV2 wt at an MOI of 0.1 for 48 h. **(B)** MDCK/CLDN1-KO and the control cells (MDCK/ctrl) were infected with hPIV2 at an MOI of 0.1 for 48 h. **(C)** MDCK cells were grown on the filter, and infected with hPIV2 via the apical surface at an MOI of 0.1 for 48 h. Samples were collected from both apical and basolateral sides. Virus titers were measured by a plaque assay. Data represent means and standard deviations of triplicate experiments. Significance is indicated as $p < 0.05$ (*). The cell lysates were subjected to western blot analysis.

4. Discussion

Tight junctions enable epithelial cells to form physical barriers that help to defend against infection. In the present study, we observed an increase of CLDN1 mRNA and protein expression levels during infection of hPIV2 wt, and infection with hPIV2 expressing dysfunctional V proteins caused further enhanced induction of CLDN1 (Fig. 1). We focused on this induction of CLDN1, and examined its role in hPIV2 propagation. Although tight junctions were thought of as a host defense, their implication for respiratory viral infection has not been examined in depth, and direct evidence that tight junction molecules affect viral growth was insufficient (Colpitts and Baumert, 2016). We showed that hPIV2 growth was reduced in a A549 cell line over-expressing CLDN1 (Fig. 2A). Moreover, by using a CRISPR/Cas9 based strategy to knockout the CLDN1, we demonstrated that hPIV2 release from apical surface was enhanced in the absence of CLDN1 (Fig. 2B, C). These results support the importance of CLDN1 in constructing a barrier against viral growth. CLDN1 expresses in epithelial cells of human respiratory tract (Coyne et al., 2003), which could be effective for hPIV2 that infects airway epithelial cells and causes respiratory illness (Hall, 2001; Henrickson, 2003).

Tight junctions are thought to act as a physical barrier between apical and basolateral membrane domains of polarized epithelial cells, which restrict pathogens to a cellular compartment. An additional property that inhibits cell-to-cell spread of viruses has been suggested. Human immunodeficiency virus type 1 (HIV-1) protein expression disrupts oral epithelial tight junctions, leading to the facilitation of cell-to-cell spread of herpes simplex virus (Sufiawati and Tugizov, 2014). We hypothesized that the involvement of tight junctions in inhibiting viral cell-to-cell spread broadly applies to many viruses, and examined this for hPIV2. Using CLDN1 over-expressing and -KO cell lines, we found that hPIV2 cell-to-cell spread is inhibited in the presence of CLDN1, and improved in its absence (Fig. 3). Our results suggest that a property of tight junctions is to restrict infection of various viruses via cell-to-cell contact at least, and CLDN1 alone could be sufficient for this inhibition. It is currently unclear how the tight junction proteins are involved in the inhibition of cell-to-cell spread of hPIV2 and other paramyxoviruses. It is broadly known that hPIV2 spread is facilitated by formation of cell fusion caused by the cooperation of viral F and HN proteins expressed on the infected cell surface (Ah-Tye et al., 1999; Tsurudome et al., 2008), which may contribute to a direct transmission of virus particle or replication machinery to neighboring cells. PIV5, Sendai virus and MeV can infect polarized epithelial cells and form cell fusions or intracellular membrane pores that contribute to the rapid spread of these viruses (Villenave et al., 2010; Zhang et al., 2011; Singh et al., 2015). The presence or absence of CLDN1 (and other tight junction-related proteins) would alter the environment of intercellular connection, and may influence the efficiency of the cell-to-cell spread of paramyxoviruses. In addition to the cell-to-cell spread via direct contact, these viruses are disseminated from a cell to adjacent cells as released viral particles. The involvement of tight junction molecules in this type of virus dissemination is also an important issue in complete elucidation of the mechanism of cell-to-cell spread of these viruses.

Viruses have evolved to counter the antiviral effect of tight junctions. As mentioned above, HIV-1 protein degrades tight junction molecules (Sufiawati and Tugizov, 2014). West Nile virus (WNV) and Japanese encephalitis virus (JEV) are mosquito-borne viruses. Following mosquito transmission, these viruses cross several polarized cell layers. The WNV capsid protein induced the degradation of CLDN1, CLDN2, CLDN3 and CLDN4 in various cultured cells (Medigeshi et al., 2009). JEV infection also caused degradation of CLDN1 in endothelial and epithelial cells (Agrawal et al., 2013). These observations suggest that mosquito-borne viruses establish their infection in their vertebrate hosts by degrading tight junction molecules to disrupt the epithelial barriers. Additionally, the destruction of tight junctions is also implicated in the enhancement of virus-mediated nervous pathogenesis. In the present study, we propose that this destruction of tight junctions during viral infection contributes not only to the virus entry and tissue damage, but also to the enhanced dissemination of viruses whose cell-to-cell spread should be intrinsically restricted by tight junctions.

When compared with HIV-1, WNV and DENV, hPIV2 infection does not employ an active strategy to degrade tight junction molecules. V protein appears to counter the tight junction-mediated antiviral response. Interestingly, rPIV2 $V_{C193/197A}$ infection caused further enhanced CLDN1 expression compared with hPIV2 wt infection (Fig. 1). In V-expressing A549 cells, intrinsic induction of CLDN1 caused by

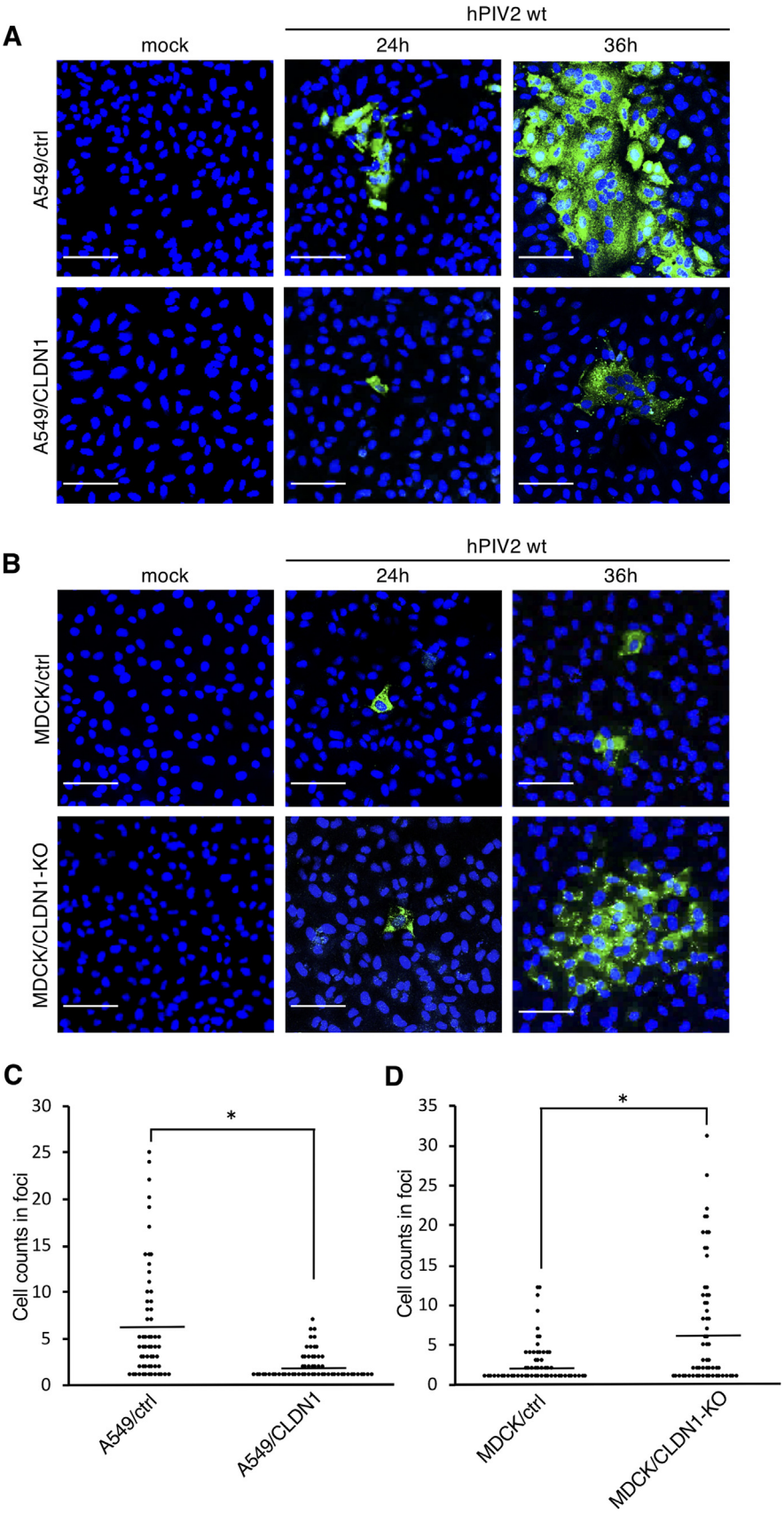

Fig. 3. Effect of CLDN1 for the spread of hPIV2 via cell-to-cell contact. (A and B) A549/CLDN1 and MDCK/CLDN1-KO or their control cells were infected with hPIV2 wt at an MOI of 0.01 for 24 and 36 h, and stained with anti-P/V mAb (green). Nuclei were stained with 4′, 6′-diamidino-2-phenylindole (DAPI; blue). Scale bars correspond to 100 μm. **(C and D)** The cell numbers of hPIV2 infected foci were counted, and those of total sixty foci per each cell line are shown. Results of A549 cells at 24 h **(C)**, and those of MDCK cells at 36 h **(D)** are shown. Significance is indicated as $p < 0.05$ (*).

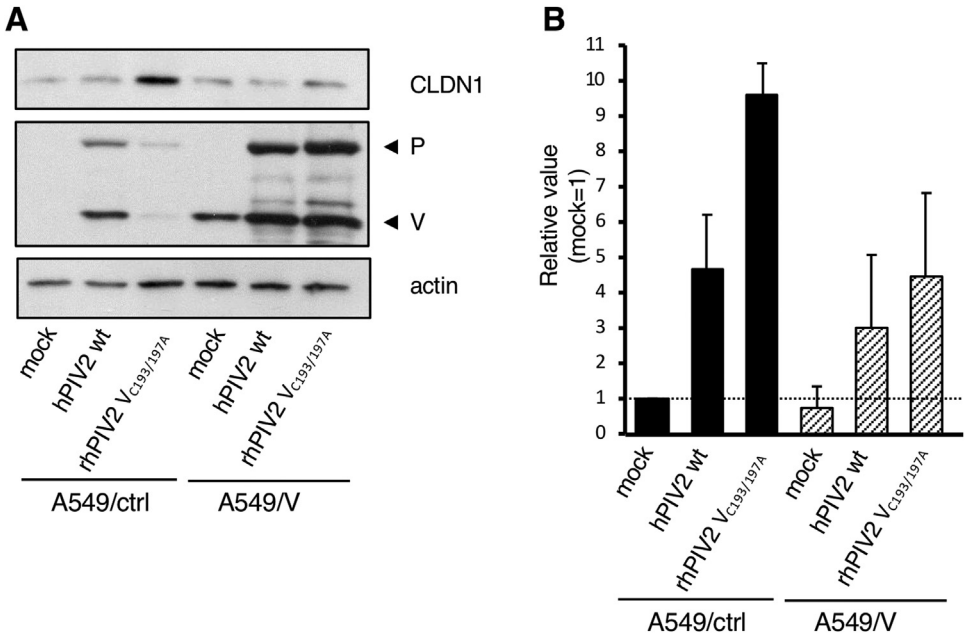

Fig. 4. Induction of CLDN1 in the hPIV2 infected A549 cells expressing V protein. **(A)** V protein over expressing A549 (A549/V) or control (A549/ctrl) cells were infected with hPIV2 wt or rPIV2 $V_{C193/197A}$ at an MOI of 0.01 for 48 h. CLDN1, hPIV2 P, V and actin were detected by western blot analysis. **(B)** Expression of CLDN1 mRNA were measured by qRT-PCR and relative values are shown (A549/ctrl mock = 1). Data represent means and standard deviations of triplicate experiments.

rPIV2 $V_{C193/197A}$ infection was not observed (Fig. 4A, B). However, it is shown that V expression alone does not affect CLDN1 expression (compare protein expressions between mock-A549/ctrl and mock-A549/V; Fig. 4A). We also confirmed that V does not interact with CLDN1 by an immunoprecipitation assay (data not shown), indicating that other molecules may contribute to the regulatory mechanism of CLDN1 expression during hPIV2 infection. V protein acts to restrict multiple antiviral cellular responses. In the infection of hPIV2 expressing dysfunctional V, cellular responses that are normally limited by V function could be activated, some of which may be responsible for CLDN1 induction. Because V protein acts to prevent IFN-mediated antiviral responses, we have focused on the relationship between IFN and CLDN1 induction. However, we could not find that the treatment of IFN-α to A549 cells induce the CLDN1 mRNA expression (data not shown). Thus, we would focus on the other possibility. CLDN1 mRNA and protein are induced by several pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (Kondo et al., 2008; Fujita et al., 2011; Al-Sadi and Ma, 2007). We found that TNFα mRNA expression was induced by hPIV2 wt infection, and this was stronger in hPIV2 infections that express dysfunctional V (data not shown). Expression pattern of CLDN1 during hPIV2 infection may be affected by this increase of TNFα. Moreover, we have recently reported that normal V interacts with caspase-1 and suppresses the production of activated IL-1β (Ohta et al., 2018). Inhibition of IL-1β triggered by functional V is also considered to be a strategy that indirectly inhibits CLDN1 expression.

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