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Original Article

No tight junctions in tight junction protein-1 expressing HeLa and fibroblast cells

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Abstract: Tight junctions are important structures that form the barrier of cells and tissues, and they play key roles in maintaining homeostasis of our body. The backbone of the tight junction proteins are claudins, which composed more than twenty members. The tight junction protein 1 (TJP1), also called ZO-1 (Zonula Occludens-1), is one of the tight junction related proteins, and it is widely used in literature to label tight junctions. Here we showed that TJP1 (ZO-1) is highly expressed in cancerous HeLa cells, fibroblast cells, HUVEC as well as MDCK cells, while claudin-1 is highly expressed in HUVEC and MDCK cells, but not expressed in HeLa and fibroblast cells. We aimed to investigate whether tight junction is present in HeLa and fibroblast cells. We used transepithelial/transendothelial electrical resistance (TEER) to measure tight junction dynamics in these cells. The results showed that there is no TEERs in HeLa and fibroblast cells, while there is relatively high TEER in HUVEC and MDCK cells. Importantly, the TEER in MDCK cells is dramatically reduced after knockdown of TJP1 (ZO-1). These results suggest that TJP1 (ZO-1) cannot be used as a marker of tight junctions in a variety of cells, while TJP1 (ZO-1) may play an important role in regulation of tight junctions in MDCK cells.

Keywords: Tight junction, tight junction protein-1 (TJP1), transepithelial/transendothelial electrical resistance (TEER), claudin-1

Introduction

Cell-cell communication is important for maintaining the homeostasis of cells and tissues. To date, there are four different cell junctions, which include tight junctions, gap junctions and adherens junctions, as well as desmosome junctions [1-3]. Among these junctions, tight junctions are key players in forming the barrier or fence function in epithelial and endothelial cells, and tight junctions are composed by claudins, occludins, tricellulin and other components [4-6]. Claudins are major backbone of tight junctions [7, 8]. The importance of tight junctions in maintaining the homeostasis of human body is demonstrated by the fact that mutations in tight junctions are associated with a variety of human disease conditions, for example, mutations in claudin16 and claudin19 are associated with hypomagnesaemia, and mutations in claudin14 are associated with hearing loss [9, 10].

Tight junction protein 1 (TJP1), also called zonula occludens-1 (ZO-1), is widely expressed in human epithelial and endothelial cells [11, 12], and there are many literatures using TJP1 (ZO-1) as a marker of tight junctions. TJP1 (ZO-1) is an important integral protein, it contains three protein interacting modules called PDZ domains, and there are a variety of proteins with a PDZ domain binding motif at their extremely C-terminals interacted with different PDZ domains of ZO-1. Our previous work demonstrated that gap junction protein Cx36 contains a PDZ domain interaction motif, consisting of a SAYV amino acid sequences at its carboxyl terminus, that mediates its interaction with the first PDZ domains of PDZ domain containing proteins ZO-1, ZO-2, ZO-3 [13-18]. Importantly, TJP1 (ZO-1) is not only associated with a dozen of different gap junction proteins but is also associated with tight junctions as well as adherens junctions [19, 20]. Further, whole genome sequencing data demonstrated that TJP1 (ZO-1) muta-

tions are associated with arrhythmogenic cardiomyopathy [21].

Here, we investigated TJP1 (ZO-1) and claudin-1 expression in cancerous HeLa cells, fibroblast cells, HUVEC as well as MDCK cells, and we also measured the transepithelial/transendothelial electrical resistance (TEER) to determine whether there is tight junctions in these cells. In addition, we monitored the TEER in MDCK cells after knocking down TJP1 (ZO-1). Our results showed that there is no TEER in HeLa and fibroblast cells even though TJP1 (ZO-1) is highly expressed in these cells. However, the TEER in MDCK cells is significantly reduced after knocking down TJP1 or ZO-1, indicating that TJP1 (ZO-1) may modulate tight junctions in MDCK cells.

Materials and methods

Antibodies

Rabbit polyclonal antibody against claudin-1 (NBP1-77036) was purchased from Novus Biologicals Laboratories (Littleton, CO, USA). Monoclonal anti-ZO-1 antibody (Cat. No. 33-9100) was obtained from Life Technologies (Invitrogen, Carlsbad, CA, USA). The epithelial or endothelial volt/ohm (TEER) meter was purchased from World Precision Instruments.

Cell culture

HeLa, NIH3T3 fibroblast, HUVEC and MDCK cells were obtained from ATCC. HeLa cells and HUVEC cells have been used in our previous studies [22-25]. All the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, Utah) and 1% penicillin-streptomycin-fungizone (Sigma-Aldrich, St. Louis, MO).

TEER measurement

We measured the transepithelial or transendothelial resistance (TTER) of MDCK cells, HeLa cells, fibroblast cells as well as HUVEC cells under confluence condition cultured in serum free medium in 6 well dishes. The EVOM reading in the above cells after subtracting the readings in 6 wells cell free dishes containing serum free cell culture medium was the real EVOM value of these cells under confluence condition.

Western immunoblotting

The procedure for western blotting was adopted from our previously used protocols [26-28]. Briefly, cells were washed in a cold PBS buffer (50 mM sodium phosphate buffer, pH 7.4, 0.9% saline) and were lysed in an IP buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin). Homogenates were collected and centrifuged at 20,000×g for 20 min at 4°C, then the supernatants were taken for protein determination utilizing Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Proteins were boiled for 3-5 minutes and were separated by SDS-PAGE (10 μg of protein per lane) using 7.5% gel for ZO-1 antibody, 12.5% gel for claudin-1 antibody. The gels were then transblotting to polyvinylidene difluoride membranes (Bio-Rad Laboratories) in a Tris-glycine transfer buffer, pH 8.3, containing 0.5% SDS. Membranes were blocked for 2 h at room temperature using 5% non-fat milk powder blocking buffer, rinsed 4 times in TBSTw (TBS +0.05% tween 20), and incubated overnight at 4°C with polyclonal or monoclonal primary antibodies diluted at 1: 1000 in PBS buffer containing 0.05% Tween. Membranes were washed 4 times in TBSTw for 40 min, incubated with specific secondary antibodies (Li-Cor Bio-sciences, Lincoln, NE). Membranes were then scanned on an imaging system (Odyssey Infrared; Li-Cor).

Immunohistochemistry

The procedures for Immunofluorescence labeling has been described previously [29-32]. Briefly, HeLa, fibroblast, HUVEC and MDCK cells were grown in glass slides separately. When they reached confluence, the cells were fixed with 1% cold paraformaldehyde (PA) for 15 min, and washed with PBS. Then slides were incubated in 50 mM Tris-HCl, pH 7.4, containing 1.5% sodium chloride (TBS), 0.3% Triton X-100 (TBSTr) and 5% normal goat serum (NGS) overnight at 4°C simultaneously with two primary antibodies. Slides were then washed for 1 h in TBSTr and incubated for 1 h at room temperature simultaneously with appropriate combinations of secondary antibodies, which included:

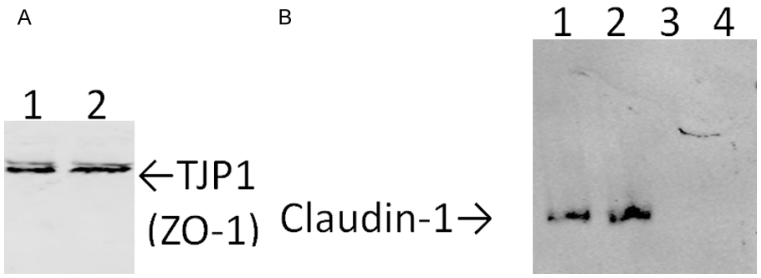

Figure 1. Characterization of TJP1 (ZO-1) and claudin-1 antibodies using WB. As shown in (A), a doublet bands migrating at approximately 220-225 kDa corresponding to the different isoforms of TJP1 (ZO-1) in homogenates of HeLa (lane 1) and HUVEC cells (lane 2). For claudin-1 detection, as shown in (B), a single band migrating at approximately 20 kDa corresponding to the monomer form of claudin-1 was detected in homogenates of HUVEC (lane 1) and MDCK cells (lane 2), but no detection in homogenates of HeLa (lane 3) and fibroblast cells (lane 4).

Alexa Fluor 488 and Alexa Fluor 594-conjugated IgG diluted at 1:1000 (Molecular Probes, Eugene, Oregon). Following incubation with secondary antibodies, slides were sequentially washed in TBSTr for 30 min, in 50 mM Tris-HCl buffer, pH 7.4, for 30 min, and then coverslipped using antifade medium. In addition, in order to exclude inappropriate cross-reactions between primary and secondary antibodies or between different secondary antibodies, control procedures included omission of one of the primary antibodies with inclusion of the relevant secondary antibody. The confocal immunofluorescence images were gathered on a Fluoview confocal microscope using Fluoview software with z-stacks of ten scans at z scanning intervals of 0.5 μm.

Gene transfection

The protocol for ZO-1 shRNA transfection into MDCK cells using lipofectamine 3000 transfection reagents has been described previously [24, 33, 34]. The ZO-1 shRNA 3253 that specifically target canine TJP1/ZO-1 was obtained from Addgene with Plasmid #37206, and the shRNA sequence is 5'-GCGCATTCCCACATATGAA-3'. This shRNA has been used to knockdown TJP1 (ZO-1) in MDCK cells by others [35].

Statistics analyses

Graphpad Prism software and ANOVA was conducted to determine the differences in protein expression level and TEER value in different cells or groups. Data were shown as the average ± standard error of the mean, and P value < 0.05 was considered to be statistically significant.

Results

Characterization of ZO-1 and claudin-1 antibodies

The specificity of anti-TJP1 (ZO-1) and claudin-1 antibodies was validated using WB and lysates of HeLa, fibroblast, HUVEC or MDCK cells. For TJP1 (ZO-1) antibody, as shown in **Figure 1A**, a doublet bands migrating at approximately 220-225 kDa corresponding to the different isoforms of TJP1 (ZO-1) were detected in homogenates of HeLa (lane 1) and HUVEC cells (lane 2). Similarly, for claudin-1 antibody, as shown in **Figure 1B**, a single band migrating at approximately 20 kDa corresponding to the monomer form of claudin-1 was detected in homogenates of HUVEC (lane 1) and MDCK cells (lane 2); however, there is no claudin-1 detection in homogenates of HeLa (lane 3) and fibroblast cells (lane 4). These results indicated that the antibodies we used for detecting TJP1 (ZO-1) and claudin-1 were specific.

Double Immunofluorescence labeling showing HeLa and fibroblast cells express TJP1 (ZO-1) but not claudin-1

Double Immunofluorescence labeling of TJP1 (ZO-1) and claudin-1 was carried out in HeLa and fibroblast cells and the results were investigated using a confocal microscope with multiple scanning. As shown in **Figure 2**, the punctate and cell surface labeling of TJP1 (ZO-1) is clearly seen in cell-cell contacts of HeLa cells (**Figure 2A**); however, no claudin-1 labeling is seen in the same field with DPAI labeling nucleus (**Figure 2B**). For fibroblast cells, the punctate, strands of labeling for TJP1 (ZO-1) at cell-cell contacts or intracellularly were clearly seen (**Figure 2C**), however, there is no claudin-1 labeling in the same field with DPAI labeling nucleus (**Figure 2D**). These results indicated that HeLa and fibroblast cells express TJP1 (ZO-1) but no claudin-1.

HUVEC and MDCK cells express both TJP1 (ZO-1) and claudin-1

Double immunofluorescence labeling of TJP1 (ZO-1) and claudin-1 was further performed in HUVEC and MDCK cells. The results were deter-

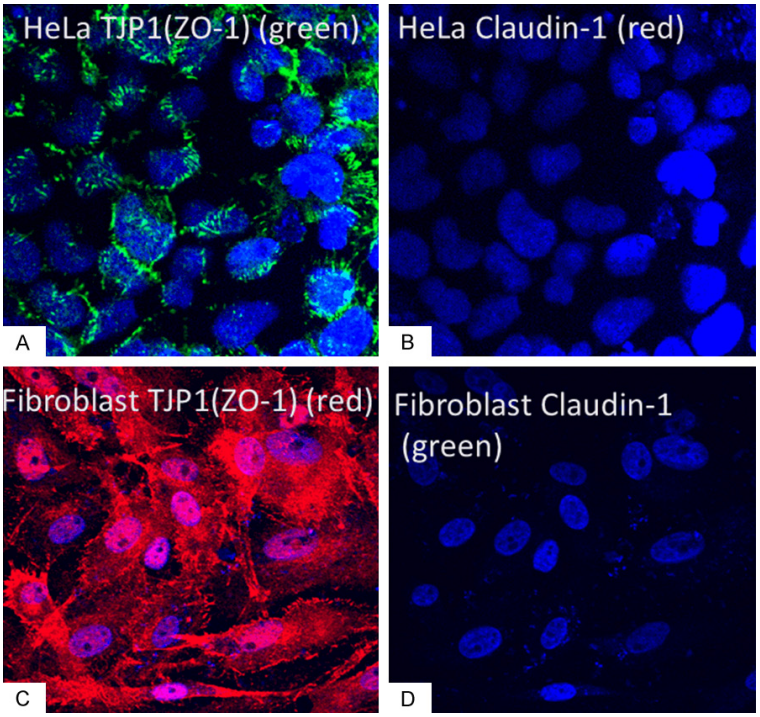

Figure 2. Immunofluorescence labeling of TJP1 (ZO-1) and claudin-1 in HeLa and fibroblast cells. The punctate and cell surface labeling of TJP1 (ZO-1, green) is clearly seen in cell-cell contacts of HeLa cells (A), however, no claudin-1 labeling (red) is seen in the same field with DAPI labeling nucleus in blue color (B). Similarly, punctate and strands of labeling for TJP1 (ZO-1, red) at cell-cell contacts or intracellularly were clearly seen in fibroblast cells (C), however, there is no claudin-1 labeling (green) in the same field with DAPI labeling nucleus in blue color (D).

mined using a confocal microscope with multiple scanning. Punctate immunolabeling for TJP1 (ZO-1) was detected at appositions between HUVEC cells, and contained some strong intracellular TJP1 (ZO-1) immunoreactivity (**Figure 3A**). Immunolabeling for claudin-1 (**Figure 3B**) in these cells exhibited a similar distribution to TJP1 (ZO-1) around the periphery of these cells, but intracellular labeling for claudin-1 was also present. The overlay results revealed a relatively high degree of TJP1 (ZO-1)/claudin-1 colocalization with yellow color at sites of cell-cell contact (**Figure 3C**). For MDCK cells, strong long strands of immunolabeling for TJP1 (ZO-1) at sites of cell-cell contacts between MDCK cells, and in contract to HUVEC cells, there is barely intracellular TJP1 (ZO-1) immunolabeling (**Figure 3D**). Immunolabeling for claudin-1 (**Figure 3E**) in these cells exhibited a similar distribution to TJP1 (ZO-1) at cell-cell contacts of these cells, but intracellular labeling for claudin-1 was very weak. The overlay results revealed a high degree of TJP1 (ZO-1)/claudin-1 colocalization with yellow color at sites of cell-cell contact (**Figure 3F**).

TEER measurement in HUVEC and MDCK cells while no TEER in HeLa and fibroblast cells

The TEER measurement was conducted in confluent 6-well HeLa, fibroblast as well as HUVEC and MDCK cells, and the 6 well cell culture dishes with medium only were used as a reading control. As shown in **Figure 4**, after subtraction of the reading value obtained from the cell free 6 well cell culture dishes, it showed that there is no TEER in HeLa and fibroblast cells, while there is strong TEER reading in both HUVEC and MDCK cells. Because HeLa cells and fibroblast cells showed strong TJP1 (ZO-1) but not TEER labeling, we conclude that TJP1 (ZO-1) is not correlated with tight junction TEER reading in these cells. However, there is strong TEER reading in both HUVEC and MDCK cells, and these cells also showed strong claudin-1 immunolabeling. These results indicated that claudin-1 is related to TEER reading in these cells.

Reduced TEER in MDCK cells after knocking down TJP1 (ZO-1)

Because TJP1 (ZO-1) was detected and co-localized with claudin-1 in HUVEC as well as MDCK cells, and these cells also showed strong TEER reading, we asked whether TJP1 (ZO-1) has a regulatory role in TEER in these cells. So we transfected ZO-1 shRNA into MDCK cells using lipofectamine 3000 high efficiency gene transfection reagent as we previously described [24, 33]. After 3 days of shRNA transfection, MDCK cells were harvested for WB detection, and the result showed TJP1 (ZO-1) expression was significantly reduced ($P < 0.01$, N=4) (**Figure 5A** and **5B**). Meanwhile, the TEER reading in these cells showed a dramatic reduction compared with MDCK cells transfected utilizing siRNA controls ($P < 0.001$, N=5) (**Figure 5C**). Therefore, we conclude that TJP1 (ZO-1) can

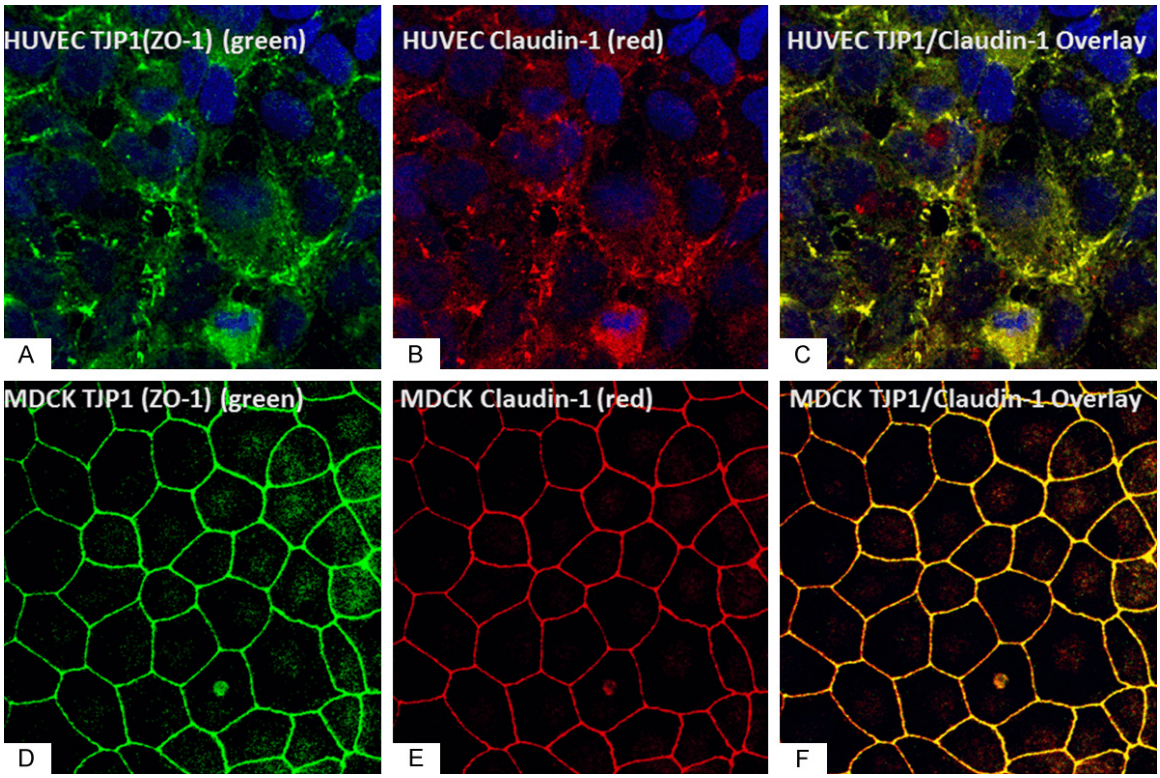

Figure 3. Immunofluorescence labeling of TJP1 (ZO-1) and claudin-1 in HUVEC and MDCK cells. Punctate immunolabeling for TJP1 (ZO-1) at appositions between HUVEC cells, and contained some strong intracellular TJP1 (ZO-1) immunoreactivity (A). Immunolabeling for claudin-1 in these cells exhibited a similar distribution to TJP1 (ZO-1) around the periphery of these cells, but intracellular labeling for claudin-1 was also present (B). The overlay results revealed a relatively high degree of TJP1 (ZO-1)/claudin-1 colocalization with yellow color at sites of cell-cell contact (C). For MDCK cells, strong long strands of immunolabeling for TJP1 (ZO-1) at sites of cell-cell contacts between MDCK cells (D). Immunolabeling for claudin-1 (E) in these cells exhibited a similar distribution to TJP1 (ZO-1) around the periphery of these cells, the overlay results revealed a high degree of TJP1 (ZO-1)/claudin-1 colocalization with yellow color at sites of cell-cell contacts (F).

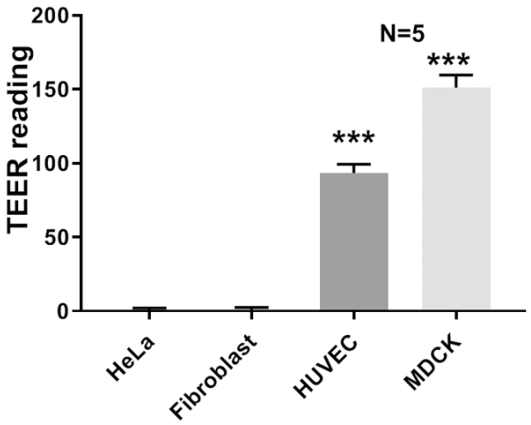

Figure 4. TEER measurement in HUVEC, MDCK, HeLa and fibroblast cells. The results showed that there is no TEER in HeLa and fibroblast cells, while there is strong TEER reading in both HUVEC and MDCK cells.

regulate TEER in MDCK cells which express TJP1 (ZO-1) and claudin-1.

Discussion

The present results demonstrate that (1) TJP1 (ZO-1) are wildly expressed in HeLa, fibroblast, HUVEC and MDCK cells, while only HUVEC and MDCK cells express claudin-1; (2) TEER measurement showed the higher TEER value in HUVEC and MDCK cells, but undetectable in HeLa and fibroblast cells; while knockdown TJP1 (ZO-1) in MDCK cells also can reduce TEER value. These findings indicate that TJP1 (ZO-1) may not be used as a marker of tight junctions, but TJP1 (ZO-1) can regulate tight junctions (containing claudin-1 protein) when tight junctions are present in these cells. Therefore, claudin-1 or other claudins can be used as a marker of tight junctions.

Tight junction protein 1 (TJP1), also called Zonula Occludens-1 (ZO-1), was first cloned in the 1980s [11], and it was originally considered as a tight junction protein. The widespread

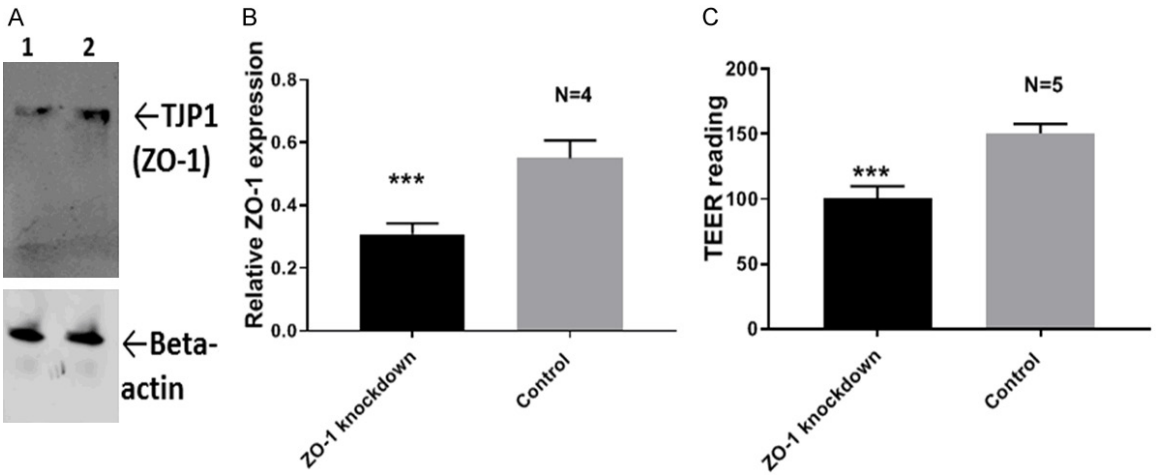

Figure 5. Reduced TEER in MDCK cells after Knocking down TJP1 (ZO-1). (A) after 3 days of TJP1 (ZO-1) shRNA transfection, MDCK cells were harvested for WB detection, the result showed that TJP1 (ZO-1) expression was significantly reduced ($P < 0.01$, N=4) (B), meanwhile, the TEER reading in these cells showed a dramatic reduction compared with MDCK cells transfected with shRNA controls ($P < 0.001$, N=5) (C).

expression of TJP1 (ZO-1) protein promoted the scientists to argue that ZO-1 could be a "ubiquitous components of all mammalian tight junctions" [11, 36, 37]. However, ZO-1 is a peripheral membrane protein, and it was not the integral tight junction-forming proteins. With the dedicated discoveries by Dr. Tsukita's group in 1990's, they demonstrated that claudins are the major tight junctions, because mouse epithelial cells lacking occludin still can form tight junctions. Just as Tsukita said "the identification of ZO-1, occludin, and claudins opened a new way to understand the barrier and fence properties of tight junctions in molecular terms" [8, 38-42]. There are more than twenty different claudin genes, which are distributed in different cells and tissues, and mutations in claudins are associated with a variety of human disease conditions [10, 43, 54]. Most importantly, claudin-1 knockout mice showed the lethal phenotype due to the loss of water by skin, indicating the pivotal role of claudins in maintaining the barrier and fence functions of cells [44, 45].

TJP1 (ZO-1) is one of important proteins in mammalian cells. It contains three PDZ domains, one SH3 domain, a kinase domain and other protein interacting sequences, through these domains or related structures, it associates with gap junctions, tight junctions, adherens junction proteins, actin cytoskeleton, actinins, and transcription factor or signaling molecules [46]. For interaction of TJP1 (ZO-1) with gap junctions, it has been reported that at least a dozen different gap junction proteins (called connexin, Cx) are associated with TJP1 (ZO-1), which includes Cx36 binds to the first PDZ domain of TJP1 (ZO-1) [13-16], while other connexins (Cx30, Cx31.9, Cx43, Cx46, Cx47, and Cx50) bind to the second PDZ domain of TJP1 (ZO-1) [47-49]. Elimination of TJP1 (ZO-1) in mouse is lethal, indicating the functional roles of ZO-1 in maintaining homeostasis, and other zonula occluden proteins such as TJP2 (ZO-2) and TJP3 (ZO-3) cannot rescue the loss of TJP1 (ZO-1) [50-52]. Further, it has been reported that mutation in TJP1 (ZO-1) is associated with arrhythmogenic cardiomyopathy [21].

Our current data showed that TJP1 (ZO-1) is widely distributed in all four cell lines we investigated, while claudin-1 is only present in HUVEC and MDCK cells. TJP1 (ZO-1) expression in HeLa cells is consistent with our previous reports [13, 15, 17]. The TEER measurement is correlated with the expression of claudin-1 in HUVEC and MDCK cells, but not correlated with TJP1 (ZO-1) expression in HeLa and fibroblast cells. Therefore, we conclude that TJP1 (ZO-1) cannot predict the presence of tight junctions in cells and tissues, and it may not be used as a marker to label tight junctions. However, in literature and international conferences, to date, there are still many studies that use TJP1 (ZO-1) to label tight junctions in cells or tissues.

There are some cases that cells may avoid tight junction expression, such as HeLa cells, fibro-

blast cells. In the trabecular meshwork cells of eye, strong TJP1 (ZO-1) expression was detected [53], but there is no tight junction structure in these trabecular meshwork cells, which is demonstrated by studies using freeze fracture electron microscopy [55]. Our previous studies also demonstrated that weak fixation condition may enhance the detection of TJP1 (ZO-1) in rodent brain tissues [29]. In our current study, we have tried different fixation condition, and the results showed that TJP1 (ZO-) can be detected using all these different fixation conditions, while claudin-1 can only be detected in HUVEC and MDCK cells. Our present data also showed that knockdown of TJP1 (ZO-1) by shRNA in MDCK cells can significantly reduce TEER reading. It is consistent with the previous report which demonstrated that TJP1 (ZO-1) can regulate or stabilize claudin function [35, 55].

In summary, it may be worth the attention to select the appropriate protein markers for labeling tight junctions, and it appears that claudin-1 or other claudins can be used as good candidates to accurately label tight junctions, while tight junction protein-1 (TJP1) (also called ZO-1), even this protein contains the word of tight junction, may not be used as a marker to label tight junctions because there are some cell types or tissues that only express TJP1 (ZO-1), but no claudins formed tight junctions.

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Disclosure of conflict of interest

None.

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