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Claudin-10 in the Blood Brain Barrier Function of Cerebral Endothelial Cells and Transendothelial Invasion of Breast Cancer Cells

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Abstract. *Background/Aim: Claudin-10 (CLDN10) is a membrane integral protein. It is one of the widely expressed tight junctional claudins with functions not well defined. In the present study, the expression profile and its role in cerebral endothelial cells and in the interaction between breast cancer and endothelial cells were investigated. Materials and Methods: CLDN10 expression was examined in a wide range of cell types. Brain endothelial cell models with or without CLDN10 expression were generated using the hCMEC/D3 cell line and used to test the barrier and permeability functions. Trans-endothelial drug delivery and invasion were also evaluated. Results: hCMEC/D3 cells express high levels of CLDN10, compared with peripheral endothelial cells, mesothelial cells, fibroblasts, and breast cancer cells, which were either negative or expressed low levels of CLDN10. Knockdown of CLDN10 in hCMEC/D3 cells resulted in impaired tight junctions as seen by reduced transendothelial electric resistance and paracellular permeability. It also accelerated invasion of breast cancer cells through the endothelial cell layer. CLDN10 knockdown in hCMEC/D3 cells led to an increase in transendothelial chemodrug delivery. Furthermore, the SRC kinase inhibitor (AZM475271) was able to decrease the impedance and increase the paracellular permeability in cerebral endothelial cells. Conclusion: Cerebral endothelial cells express high levels of CLDN10, a protein regulating barrier function and thereby drug permeability and cancer invasiveness in brain endothelial cells, suggesting that it is a novel therapeutic target for the treatment of brain metastasis-related diseases.*

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Key Words: Claudin-10, CLDN10, brain endothelial, blood brain barrier, tight junction, breast cancer, metastasis.

The blood-brain barrier (BBB) is composed of brain microvascular endothelial cells (BMECs), astrocytes, pericytes, and many intercellular molecular structures (1, 2). Tight junctions (TJs) between BMECs construct a robust physical barrier preventing the free exchange of substances between the bloodstream and the brain parenchyma and migration of tumor cells (3-7). Patients with brain metastases have poor clinical outcomes. However, there are marked improvement of the clinical course and patient's outcome as a result of improved local and systemic therapies and advanced imaging technologies allowing early diagnosis (8). The blood-brain barrier can also block the entry of drugs into metastatic lesions, especially antibody-based drugs (9). Once breast cancer patients have brain metastases, their quality of life and survival time are seriously affected. The median survival time of breast cancer patients with brain metastases in the United States is only 10 months (10). Therefore, it is essential to understand the molecular mechanisms of the BBB, as it can help us understand the process of breast cancer brain metastasis and develop more targeted therapies. The TJs are formed by the interaction of transmembrane proteins, such as occludin and claudins, and cytoplasmic scaffolding proteins, such as zonula occludens and cingulin.

These proteins work together to regulate the permeability of the BBB (1, 11). However, what makes these highly specialized BBB-TJs extremely restrictive remains unknown. Many studies have demonstrated that CLDNs are the major proteins involved in TJs and are essential for maintaining the BBB structure (12, 13). We have reported that Claudin-5 (CLDN5), a member of this superfamily that plays a key role in TJ formation, is involved in the barrier function of ECs (14). Nevertheless, previous studies have shown that a single claudin subtype, such as claudin-5, is insufficient for completing TJ formation at the BBB (15). Therefore, it is of interest to identify other proteins involved in TJ formation at the BBB.

It has been reported that claudin-10 is highly expressed in mouse brain capillary endothelial cells (BCECs), but not in non-BCECs (16). However, the protein levels and localization of CLDN10 in BCECs have not yet been described. Although CLDN10 has been found in many human epithelial and endothelial cells (17), such as kidney and uterus cells (18), its mRNA and protein expression and localization in human BBB have not been reported. Equally important, it has been reported that CLDN10 is involved in TJ strands (17). Previous studies have shown that claudin-10 not only regulates paracellular ion selectivity, but also plays a role in certain ion channels (18). However, some questions remain unanswered, such as: How is CLDN10 expressed in the human blood-brain barrier? What is the function of CLDN10 in TJs? How does it affect the invasion of breast cancer cells into the brain and trans-endothelial drug delivery?

In the present study, we first explored the expression levels of CLDN10 in brain endothelial cells, common vascular endothelial cells, mesothelial cells, fibroblasts, and breast cancer cells. We found that CLDN10 is highly expressed only in brain endothelial cells and involved in the formation of TJs. We then generated cell models of brain endothelial cells with altered levels of CLDN10 expression. Using these cell models, we studied the roles of CLDN10 in the function of TJs in brain endothelial cells and transendothelial invasion of breast cancer cells, as well as the effect of CLDN10 knockdown in brain endothelial cells on the trans-endothelial drug delivery. Furthermore, we examined the potential role of a SRC kinase in the junctional functions of cerebral endothelial cells, aided by the cell models with differential CLDN10 expression.

Materials and Methods

Cell lines and cell transfection. In this study, three immortalized human brain endothelial cell lines, hCMEC/D3 (kind gift from Dr Guilaume, Flury of Institut COCHIN, Paris, France), TY9, and TY10 (kindly gifted by Dr Yasuteru Sano of Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan) were cultured in endothelial cell (EC) growth medium (EBM-2 medium supplemented with EGM-2 MV, 5% foetal calf serum (FCS) (Sigma-Aldrich, Dorset, UK), and 1% antibiotics (Sigma-Aldrich).

Three vascular endothelial cell lines namely human vascular endothelial cells (HECV) (ICLC, Genova, Italy), primary cultured human umbilical vascular endothelial cells (HuVEC), and human microvascular endothelial cells (HmVEC) (Life Technologies, Paisley, UK) were used. HECV were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Sigma-Aldrich) supplemented with 10% FCS and 1% antibiotics; HmVEC cells were maintained in Medium 131 supplemented with Microvascular Growth Supplement (MVGS) in flasks treated with Attachment Factor Protein (Life Technologies); HuVEC cells were obtained from human umbilical vein and cultured in M199 containing 20%-30% FCS.

The human immortalized mesothelial cells MET5A (ATCC, Manassas, VA, USA) and a human foetal lung fibroblast cell line (MRC5) (European Collection of Animal Cell Cultures, Salisbury, UK) were cultured in DMEM/F12 supplemented with 10% FCS and 1% antibiotics. Five human breast cancer cells lines, MDA-MB-231, MDA-MB-361, BT474, HCC1419, and MCF-7 (European Collection of Animal Cell Cultures) were cultured in DMEM/F12 supplemented with 10% FCS and 1% antibiotics.

hCMEC/D3 cells were transfected with CLDN10 siRNA or control siRNA (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) following the manufacturer's protocol in order to establish CLDN10-manipulated cell models. After incubation for the indicated time, cells were harvested for expression analysis and further experiments.

All the cells were cultured in a humidified incubator at 37˚C, 5% CO_2, and 95% humidity except TY9 and TY10 cells that were cultured at 34˚C.

Key reagents. The fluorescence dye, DiI (1,10-Dioctadecyl-3,3,30,30Tetramethylindocarbocyanine Perchlorate), was purchased from Sigma-Aldrich. A small molecule inhibitor to human SRC, namely AZM475271 was from Tocris (Bristol, UK). Cell Counting Kit-8 was from NBS Biologicals (Huntingdon, UK). Cisplatin was from Tocris Cookson Ltd.; Neratinib (Puma Biotechnologies, Los Angeles, CA, USA). Antibodies to CLDN10 (sc-373700) and a housekeeping protein anti-GAPDH antibodies were from Santa Cruz Biotechnologies Inc.

Extraction of RNA from cells and PCR analysis of gene transcripts. Total RNA was extracted using the TRI Reagent (Sigma-Aldrich). Reverse transcription of the RNA was then performed using a reverse transcription kit from Promega (Southampton, UK). For conventional PCR, GoTaq Green Master Mix (Promega) was applied using primers for CLDN10 and GAPDH. Quantitative analysis of gene transcripts was conducted using a StepOne Plus thermocycler from Fisher Scientific (Loughborough, Leicestershire, UK). The primers used in the study are listed in Table I and a FAM tagged Uniprimer™ and reverse primer with a unique z-sequence were used. GAPDH was used as a housekeeping control to normalize the data.

Western blot analysis. Cells were harvested, lysed in a RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide, pH of 7.4), and proteins were separated using SDS-PAGE. The proteins were then transferred to an Immobilon-P PVDF membrane (Merck Millipore, Hertfordshire, UK) for further analysis. After blocking with a skimmed milk mixture, the membrane was incubated overnight with the appropriate primary

Table I. *Primers used in the study.*

Target	Forward primer	Reverse primer*
GAPDH	AAGGTCATCCATGACAACTT	ACTGAACCTGACCGTACAGCCATCCACAGTCTTCTG
GAPDH	GGCTGCTTTTAACTCTGGTA	GACTGTGGTCATGAGTCCTT
SRC	TGTGGCCCTCTATGACTATG	ACTGAACCTGACCGTACAAAACTCCCCTTGCTCATGTA
ZO-1	TGGTGATGACACACATGGTA	ACTGAACCTGACCGTACAGGTGGTACTTGCTCGTAA
CLDN10	CTGGACGGTTATATACAGGC	ACTGAACCTGACCGTACACATCATTCCAAAGAGCGCAA
CLDN10	CTGGACGGTTATATACAGGC	CGTTGTATGTGTATCTGGGT

*The underlined are the Z-sequence.

antibody, washed, and incubated with the secondary antibody conjugated with HRP. The proteins were visualized using the EZ-ECL solution (Geneflow Ltd., Litchfield, UK), and images were captured using a G-BOX detection system (Syngene, Cambridge, UK).

Dynamic monitoring of TJ of human brain endothelial cell (hCMEC/D3) layer and tumour- human brain endothelial cell (hCMEC/D3) layer interactions by ECIS. Electric cell-substrate impedance sensing (ECIS) can be used to investigate the formation and dynamics of epithelial TJs. ECIS can provide a continuous measurement of cell growth and changes in cell behaviour, including the formation and disassembly of TJs, by monitoring the impedance of cells growing on a substrate with electrodes (Applied Biophysics Inc., Troy, NJ, USA).

The assay was performed as previously described with modifications (19). Briefly, ECIS arrays (96W1E) (Applied Biophysics Inc.) were first activated, stabilized, and cleaned; 7×10^4 hCMEC/D3 cells were seeded in ECIS arrays and incubated for 24 h to reach confluence before the ECIS arrays were placed in the incubated array station. The resistance/impedance were measured over the course of the experiment. In selected wells, an appropriate concentration of SRCi (AZM475271) was added into medium, and changes in impedance were measured and analysed to assess the dynamics of TJ disassembly.

ECIS was also used to monitor interactions of breast cancer and endothelial cells. 7×10^4 hCMEC/D3 control cells, or hCMEC/D3$^{CLDN10\text{-}KD}$ were plated in the ECIS array and incubated for 24 h to reach confluence. Breast cancer cells were then added to the endothelium and migration of breast cancer cells was automatically tracked and recorded.

Trans-endothelial resistance (TER). TER was measured using an EVOM voltohmmeter (EVOL, World Precision Instruments, Aston, Herts, UK) and a pair of STX-2 chopstick electrodes (WPI, Sarasota, FL, USA). The resistance was measured with a voltohmmeter after electrodes were placed in the upper and lower chambers. hCMEC/D3 cells were seeded into the 0.4 mm pore size insert (upper chamber) and allowed to reach full confluence for further experiments. The TER of inserts with hCMEC/D3^{Control} cells, or hCMEC/D3$^{CLDN10\text{-}KD}$ cells was then examined.

Paracellular permeability (PCP). PCP was determined using fluorescently labeled dextran FITC-Dextran-40, molecular weight being 40 kDa, as we previously reported (20). hCMEC/D3 cells were seeded into the 0.4 μm pore size insert (upper chamber) and allowed to reach full confluence for further experiments. With or without SRCi (AZM475271), Dextran-40 alone or in combination with breast cells was added to the upper chamber. Then the medium from the lower chamber was collected at the indicated time. The fluorescence from these collections was read on a multichannel fluorescence reader (Promega).

Transendothelial invasion of breast cancer cells assay. Trans-well chambers equipped with 6.5 mm diameter polycarbonate filter (pore size 8 μm) (Becton Dickinson Labware, Oxford, UK) were pre-coated with Collagen (Type I solution from rat tail) from Sigma Aldrich (St. Louis, MO, USA). Then, hCMEC/D3 cells were seeded and left to reach confluency. Following this, 30,000 breast cancer cells previously labeled with DiI (Tetramethylindocarbocyanine Perchlorate) were aliquoted into each insert. After 96 h co-culture, non-invasive cells are removed from the inner chamber with a cotton swab. The breast cancer cells passing through the insert were fixed (4% formalin) and counted using fluorescent microscopy.

Trans-endothelial drug delivery. hCMEC/D3 cells with or without CLDN10 knockdown were seeded into the 0.4 μm pore size inserts (upper chamber) and allowed to reach full confluence. Meanwhile, 1×10^5 breast cancer cells were seeded to each well of a 24-well plate and incubated overnight. Then inserts were transferred to the 24-well plate and indicated concentrations of antitumor agents were added. After 96 h co-culture, the breast cancer cells were fixed with 4% formalin and stained with crystal violet solution. The cells were then counted using microscopy.

Immunofluorescence (IFC). Cells were seeded in a chamber slide to reach confluence. They were then fixed with ice cold pure ethanol for overnight. After rehydration with PBC, cells were first permeabilized with 0.5% Triton X100 for 5 min, followed by washing and blocking with 10% horse serum. Anti-CLDN10 antibody (1:200 dilution) was added and the slide kept in dark on a rocking platform for 2 h. Following washing, FITC-conjugated secondary antibody (1:1,000 dilution) together with DAPI (4',6-diamidino-2-phenylindole) (1:1,000, as a nucleus counter stain) was added to the cells for a further 2 h. The slides were mounted with FluoSave for photographing.

Statistical analysis. Statistical Product and Service Solutions (SPSS) 27.0 (IBM Corp., Armonk, NY, USA), as well as GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) were used to conduct the statistical analyses. Statistical significance was determined using Student's *t*-test or Anova test, where appropriate. $p<0.05$ was considered statistically significant.

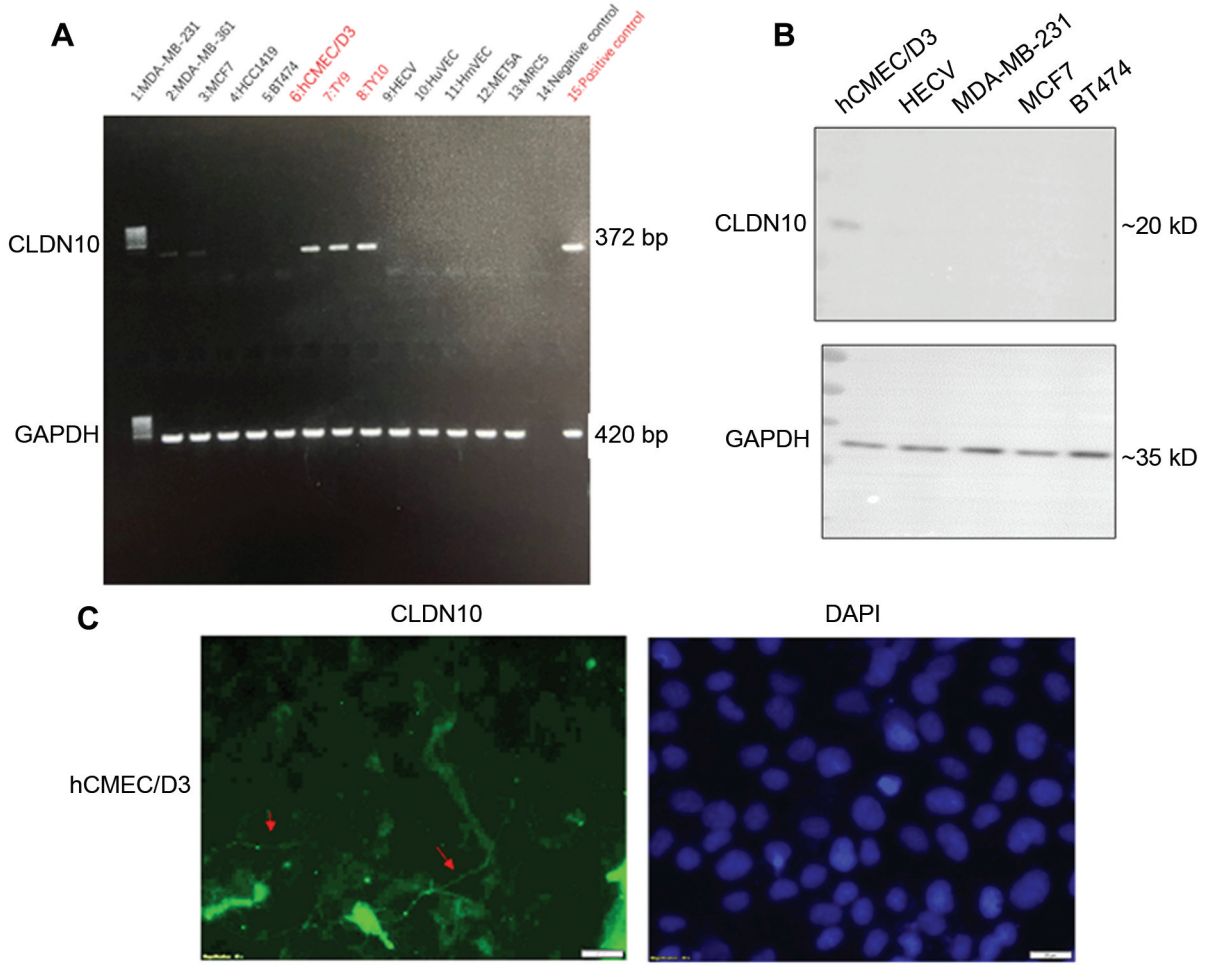

Figure 1. *The expression and distribution of CLDN10 in brain endothelial cells, vascular endothelial cells, mesothelial cells, fibroblasts, and breast cancer cells. (A): The CLDN10 transcript expression in human cerebrovascular endothelial cells, vascular endothelial cells, mesothelial cells, fibroblasts, and breast cancer cells assayed using RT-PCR. (B): CLDN10 protein in the representational cell lines assayed using protein blotting. (C) The ultrastructure of TJ strands reconstituted by stable expression of CLDN10 is shown using immunofluorescent staining between hCMEC/D3 cells. Data are representative of three independent experiments (n=3).*

Results

The expression of CLDN10 is relatively high in brain endothelial cells. Claudins (Cldns) are the pivotal transmembrane proteins that establish paracellular tightness between neighboring endothelial and epithelial cells (21). Members of this family have been described to play a vital role in BBB to resist the brain metastasis of tumour cells (5, 7, 15). However, few studies discuss whether CLDN10 (claudin-10), as a member of the claudins family, plays a role in breast cancer brain metastasis.

To investigate whether CLDN10 is involved in the process of breast cancer cell invasion through the blood-brain barrier, we first examined the expression of CLDN10 in brain endothelial cells and breast cancer cells using PCR; MET5A non-cancerous mesothelial cells and MRC5 fibroblasts were used for comparison (Figure 1A). We found that the CLDN10 transcript was relatively highly expressed in brain endothelial cells, while its expression was very low in breast cancer cells, and undetectable in other types of cells (Figure 1A). On the protein level, we found relatively high expression levels of CLDN10 in brain endothelial cells hCMEC/D3 cells, but it was not detected in other cells (Figure 1B). This indicates that CLDN10 expression is relatively high in brain endothelial cells, but very low in breast cancer cells. We then used immunofluorescent staining (IFC) to determine the cellular localization of CLDN10 in hCMEC/D3 cells and found that CLDN10 staining was

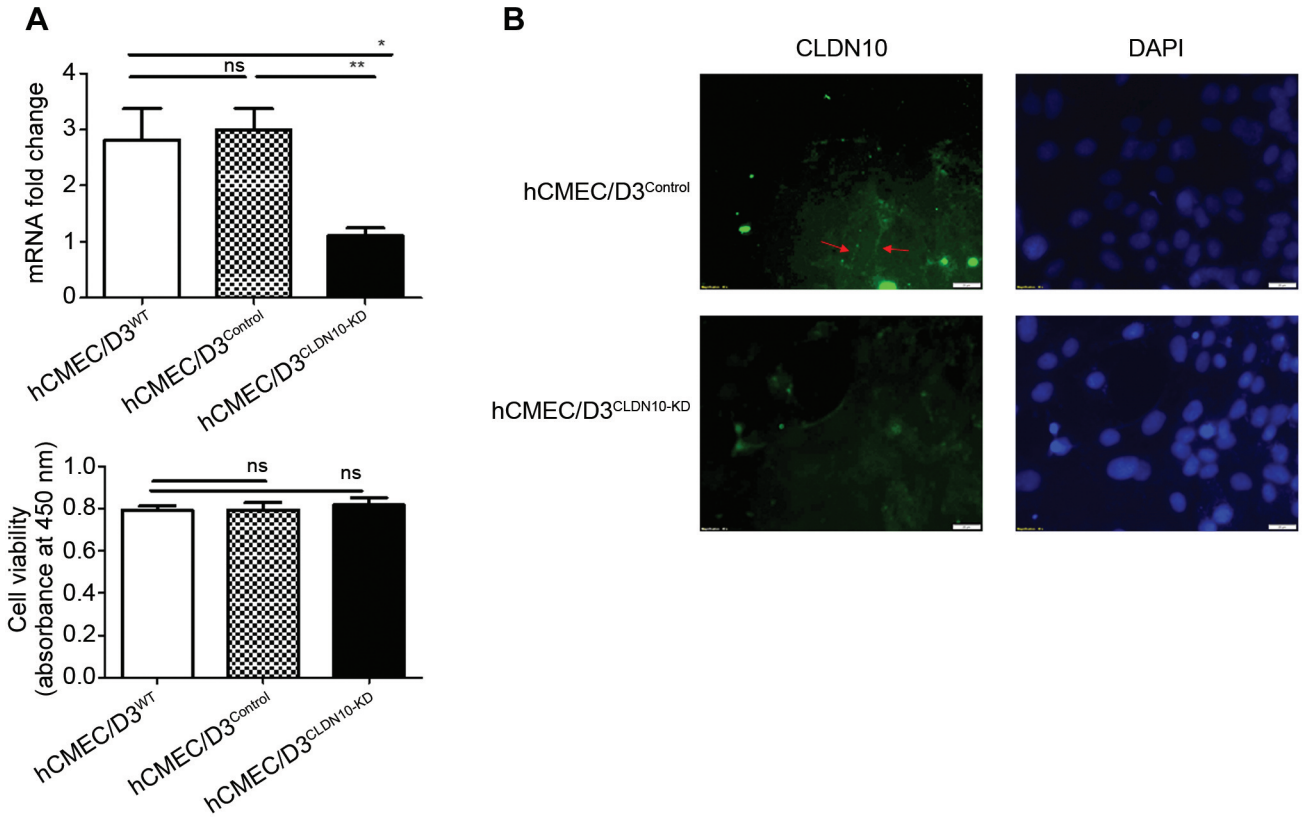

Figure 2. *Generation of cell models with differential expression of CLDN10 as confirmed using quantitative PCR and immunofluorescent staining. (A): The CLDN10 transcript expression in hCMEC/D3 wild type cells (hCMEC/D3WT), hCMEC/D3 control cells (hCMEC/D3^{Control}) and CLDN10 knockdown cells (hCMEC/D3$^{CLDN10-KD}$). (B): TJ strands reconstituted by CLDN10 in the respective hCMEC/D3 cells as shown using IFC. (C) Viability hCMEC/D3 cells assayed using Cell Counting Kit-8 a after 36 h of incubation. Data are representative of three independent experiments (n=3). Data are given as the mean± SEM, n=3. *p<0.05; **p<0.01; Student's t-test.*

indeed located at the intercellular junction are of the hCMEC/D3 cells (Figure 1C), in line with the location of tight junction in these cells.

The generation of cell models with altered levels of CLDN10. Brain endothelial cells are an important components of the blood-brain barrier, and the claudin protein family in brain endothelial cells plays an important role in the strength of the blood-brain barrier, which can seriously affect substance diffusion (e.g. drug delivery) and brain metastasis of tumours including breast cancer (4, 22, 23). However, the role of CLDN10 in TJs of the blood brain barrier and brain metastasis of breast cancer has not been reported. To further explore the role of CLDN10 in TJs of brain endothelial cells, a key component of the blood brain barrier (BBB), and the potential impact of CLDN10 on brain metastases in breast cancer, we generated cell models by manipulating CLDN10 expression levels in brain endothelial cells to explore tumour-brain endothelial cell interactions *in vitro*. Brain endothelial hCMEC/D3 cells were used to generate CLDN10 knockdown cell models because of their relatively higher CLDN10 expression. As demonstrated in Figure 2A, CLDN10 expression of these cells showed a clear decrease following transfection, as detected by both quantitative PCR and IFC (Figure 2B). Additionally, in order to verify whether CLDN10 gene expression level affects the proliferation of hCMEC/D3 cells, we performed a CCK-8 proliferation assay and found that reduction in the levels of CLDN10 did not affect hCMEC/D3 cell proliferation (Figure 2C).

Effect of reduction in CLDN10 expression on transendothelial resistance (TER) of brain endothelial cells. The BBB is a diffusion barrier that selectively excludes most blood-derived substances and cells from entering the brain. TER measurement is a reliable parameter characterizing the integrity of BBB (24). In order to examine the effect of CLDN10 changes on the barrier integrity between brain endothelial cells, we established the brain endothelial cell

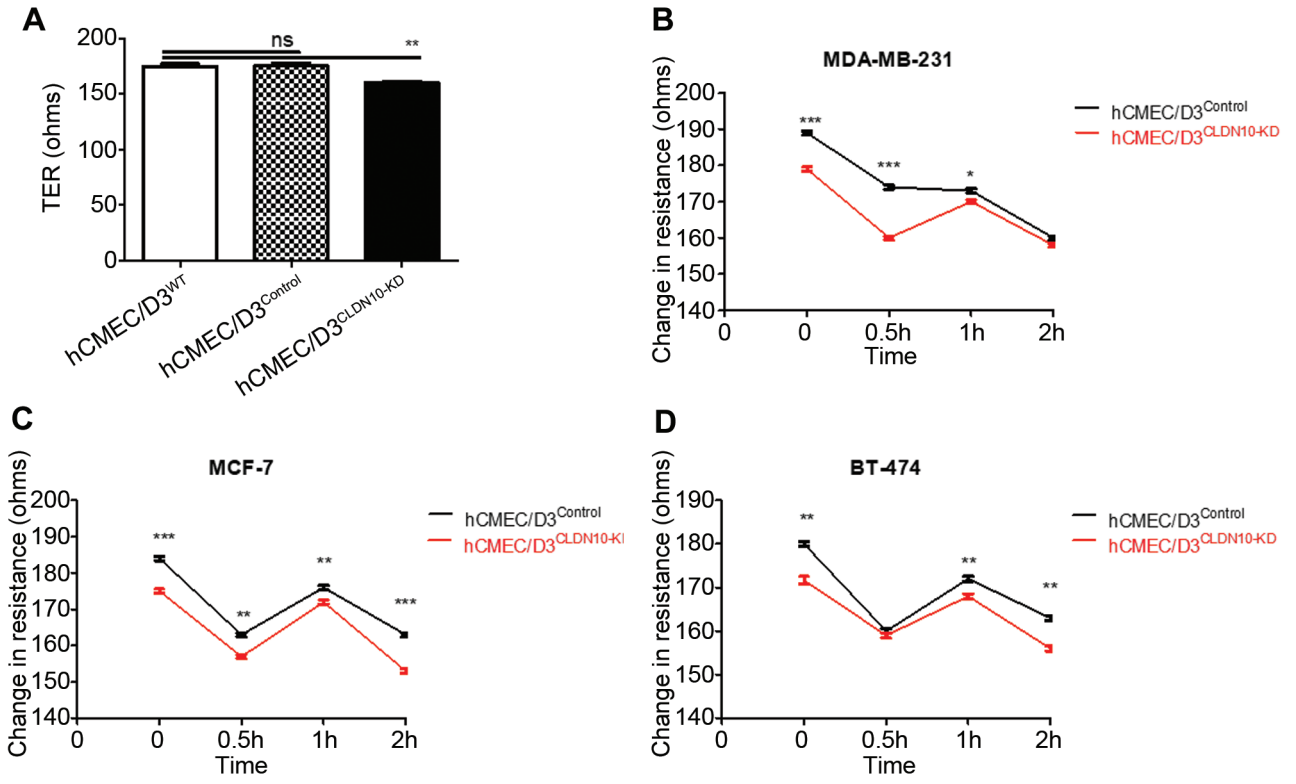

Figure 3. *Effect of CLDN10 down-regulation on TER of brain endothelial cells. (A) Trans-epithelial resistance (TER) (ohms) of hCMEC/D3 cells simulating the blood-brain barrier; (B-D) Change in trans-epithelial resistance (TER) (ohms) of hCMEC/D3 cells simulating the blood-brain barrier treated with human breast cancer cells. Data are given as the mean±SEM, n=3. *p<0.05; **p<0.01; Student's t-test.*

barrier models *in vitro* using CLDN10 knockdown hCMEC/D3 and the parental hCMEC/D3 cell line that expresses high levels of CLDN10. The TER of CLDN10KD hCMEC/D3 brain endothelial cell barrier was decreased (Figure 3A). We further examined TER changes following coculture of brain endothelial cells with three different breast cancer cell lines. As shown in Figure 3B–D, after the addition of tumour cells TER showed a downward trend, which may be related to the destruction of BBB by tumour cells.

Effect of CLDN10 down-regulation on PCP between brain endothelial cells. Paracellular permeability (PCP) of BBB is governed by TJs between brain endothelial cells (20); as another assessment method of TJ function, its aberration of which is linked to substance transport from the blood to the brain and is also explored as means to enhance brain drug delivery (1, 3). In order to determine whether paracellular permeability of the brain endothelial cell barrier is altered in the presence of reduced CLDN10 levels the PCP was measured. The result showed that permeability in hCMEC/D3$^{CLDN10\text{-}KD}$ cell is higher than that of hCMEC/D3^{Control} cells over a 2 h period, indicating changes in PCP between brain endothelial cells (Figure 4A). Addition of three breast cancer cell lines had differential effects on permeability over a 4 h period: addition of MCF7 cells reduced the permeability over 2 h; whereas BT-474 cells had no effect. It is interesting to note that addition of MDA-MB-231 cells led to a contrasting trend to that seen with MCF-7 cells (Figure 4B-D). This could imply that adding different breast cancer cells into the brain endothelial cell model has differential effects on PCP.

Effects of CLDN10 down-regulation on barrier functions of brain endothelial cells assessed by ECIS. ECIS was further employed to evaluate TJ function of brain endothelial cells in order to evaluate if barrier functions simulating the BBB using the hCMEC/D3 cell model. hCMEC/D3 cells were first allowed to grow to confluence on the gold-plated electrodes. Upon the formation of cell monolayer in the well overnight, the resistance (or impedance) of the system was measured. The rate of resistance represents the sealing and permeability properties of the brain endothelial cell barrier. Impedance in hCMEC/D3$^{CLDN10\text{-}KD}$ cells was lower than that in hCMEC/D3^{Control} cells (Figure 5A). The addition of three different types of breast cancer cells to the brain endothelial barrier model in ECIS caused a slight decrease in resistance

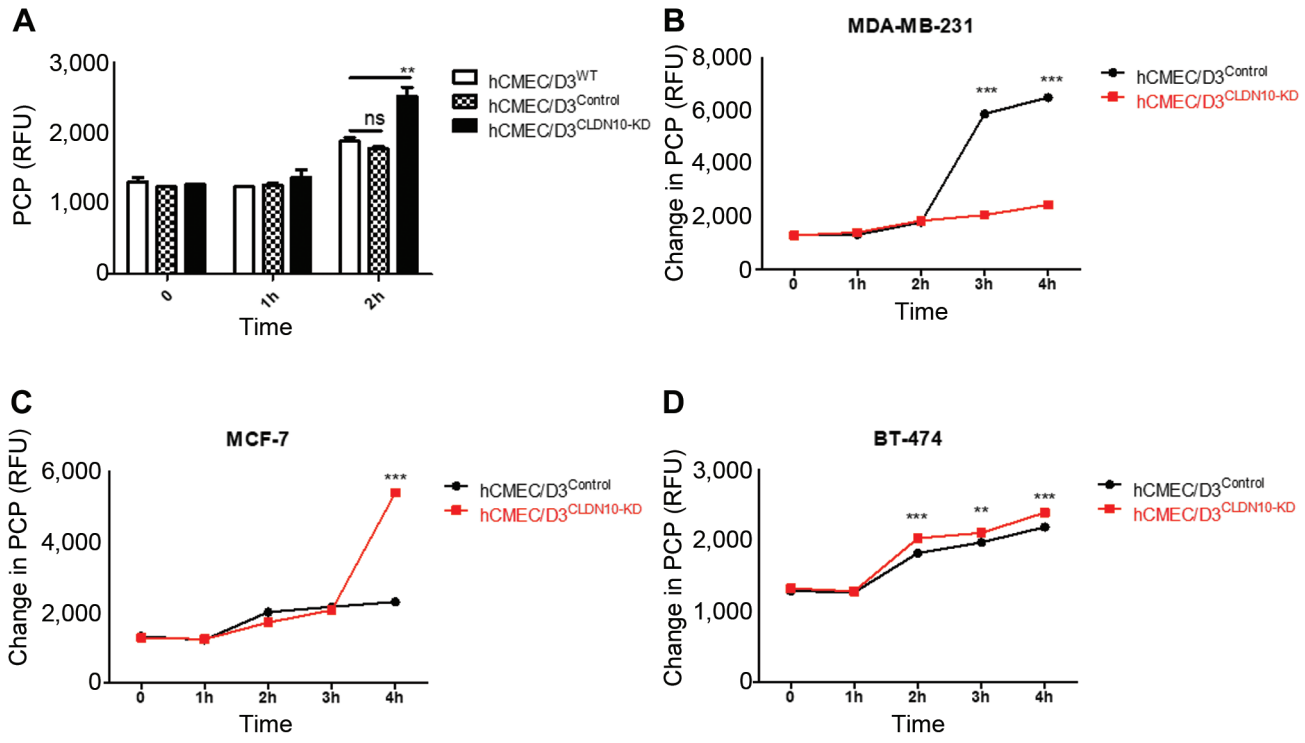

Figure 4. *Effect of CLDN10 down-regulation on PCP between human brain capillary endothelial cells. (A): Change in paracellular permeability (PCP) (RFU) of hCMEC/D3 cells simulating the blood-brain barrier over 2 h. (B-D): Change in paracellular permeability (PCP) (RFU) of hCMEC/D3 cells simulating the blood-brain barrier treated with human breast cancer cells. Data are given as the mean±SEM, n=3. *p<0.05; **p<0.01; ***p<0.001; Student's t-test.*

at first, but resistance increased over time, and the resistance level of the hCMEC/D3^{Control} cells remained higher than that of the hCMEC/D3$^{CLDN10\text{-}KD}$ cells (Figure 5B-D).

Effect of CLDN10 knockdown in hCMEC/D3 cells on the invasion of breast cancer cells through a human brain endothelial cell (hCMEC/D3) monolayer. The blood–brain barrier (BBB) protects the brain from pathogens and cancer cells. The effect of CLDN10 knockdown in hCMEC/D3 cells on the invasive potential of breast cancer cells was assessed using an *in vitro* invasion model composed of a semi-permeable membrane layered with basement membrane and brain endothelial cells with or without CLDN10 knockdown. As seen in Figure 6, invasion of all three breast cancer cell lines was significantly increased due to CLDN10 knockdown in the brain endothelial cells hCMEC/D3 (Figure 6).

Effect of CLDN10 alteration in hCMEC/D3 cells on drug trans-endothelial permeability. To investigate the effects of CLDN10 knock-down in the BBB on the delivery of chemotherapy and targeted drugs across the brain endothelial cell barrier, we evaluated a number of chemotherapeutics. We modified the transwell assay to assess differences in killing cancer cells after chemotherapy and targeted drugs passed through the brain endothelial cell barrier to determine the effect of CLDN10 knockdown on drug trans-endothelial barrier delivery. Cisplatin and the targeted drug Neratinib that passes through the hCMEC/D3$^{CLDN10\text{-}KD}$ cell layer were found to increase the death rate of breast cancer cells, as it was evident by decreased cell number after drug treatment, implying that an increased amounts of the therapeutics were able to cross the blood-brain barrier (Figure 7A-D).

CLDN10 is closely related to ZO-1 in forming TJs, which is regulated by the SRC Pathway in hCMEC/D3. A functional TJ requires the interactions between the membrane integral TJ proteins (claudins and occluding), the subcoat scaffolding proteins (Zonula Occludens, ZOs) and the cytoskeleton. Claudin association with actin dependents on ZO-1, but colocalization demonstrates intermittent rather than continuous association between claudin, ZO-1, and actin (25). To determine whether inhibiting CLDN10 had an impact on ZO-1, we measured the transcription levels of ZO-1 in the hCMEC/D3 cells. As shown in Figure 8A, the transcription level of ZO-1 decreased. Additionally, we found a slight decline in the expression of SRC, a known key signaling

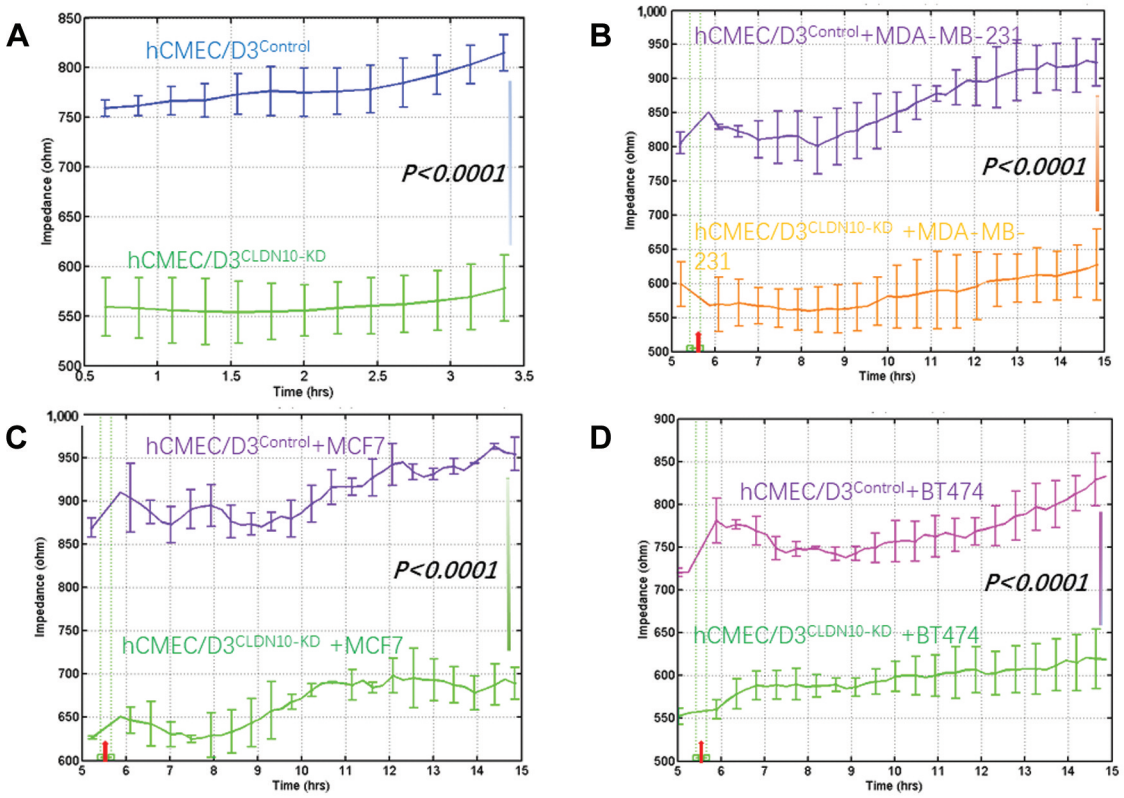

Figure 5. *Effects of CLDN10 down-regulation on TJ of hCMEC/D3 cells simulating BBB using ECIS with or without breast cancer cells. (A) The impedance of hCMEC/D3 cells simulating BBB. (B-D) The impedance of hCMEC/D3 cell simulating BBB cocultured with breast cancer cells. The red arrow indicates the addition of breast cancer cells. Data are given as the mean±SEM, n=3. One way ANOVA was used for statistical analysis.*

regulator for CLDN10. To investigate whether SRC influences TJ function *via* CLDN10, we performed ECIS and PCP experiments to assess the effect of the SRC inhibitor (SRCi) AZM475271 on TJ function in hCMEC/D3 cells with or without CLDN10 knockdown. After adding the SRCi, ECIS detected resistance of hCMEC/D3$^{CLDN10\text{-}KD}$ cells and hCMEC/D3^{Control} cells was differentially affected; ECIS detected resistance was significantly reduced in hCMEC/D3$^{CLDN10\text{-}KD}$ cells when SRC were inhibited, while PCP was increased in the hCMEC/D3$^{CLDN10\text{-}KD}$ cells more than in the hCMEC/D3^{Control} cells (Figure 8B and C).

Discussion

This study reports for the first time the differential expression of CLDN-10 among human brain endothelial cells, vascular endothelial cells, mesothelial cells, fibroblasts, and breast cancer cells. We observed that CLDN-10 is highly expressed in brain endothelial cells and is involved in the formation of TJ strands between brain endothelial cells. Its expression level affects the function of the brain endothelial

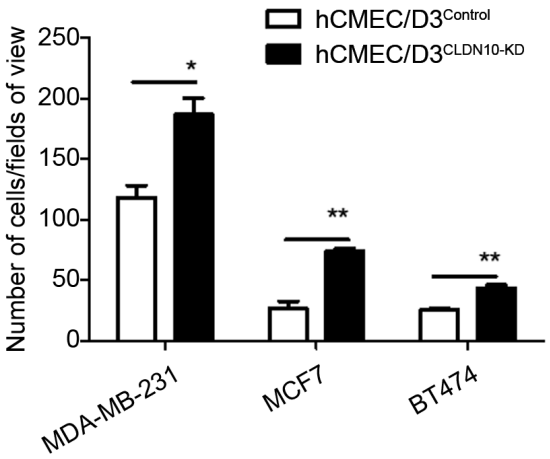

Figure 6. *CLDN10 knockdown in hCMEC/D3 cells boosts breast cancer cell invasion through a human brain endothelial cell (hCMEC/D3) layer. The three breast cancer cells have different abilities to invade the endothelial cell layer, but they are more likely to cross the hCMEC/D3$^{CLDN10\text{-}KD}$ cell layer. The images were taken with a Nikon microscope (original magnification, ×100). Data are given as the mean±SEM, n=3. *p<0.05; **p<0.01; Student's t-test.*

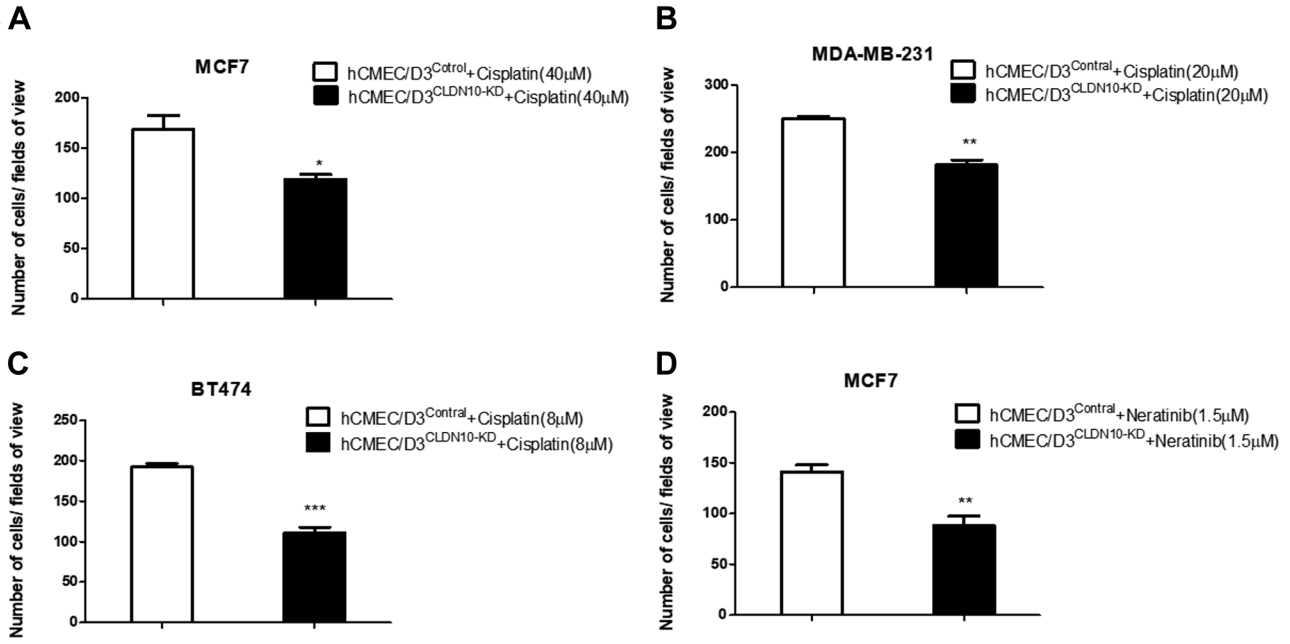

Figure 7. *Effect of CLDN10 down-regulation in hCMEC/D3 cells on drug trans-endothelial barrier delivery. (A-C) The corresponding concentration of the chemotherapy drug cisplatin passed through the endothelial cell layer and killed the three breast cancer cells. (D) The corresponding concentration of the targeted drug neratinib killed the three breast cancer cells after crossing the endothelial cell layer. Data are given as the mean±SEM, n=3. *p<0.05; **p<0.01; ***p<0.001; Student's t-test.*

barrier-TJ and correspondingly, the transendothelial invasion of breast cancer cells and drug delivery. This study also revealed for the first time that ZO-1 gene expression is decreased upon CLDN10 knock down in brain endothelial cells; meanwhile, the TJ function of the brain endothelial cell barrier was more vulnerable to being altered by a SRC kinase inhibitor.

Claudins are the main proteins forming the backbone of TJ and important determinants of paracellular "tightness" between adjacent brain endothelial cells (6, 26). Previous research suggests that claudin-5 (CLDN-5) is the dominant TJ protein at the BBB, with minor contributions from CLDN3 and -12, as well as occluding (27). However, the BBB appears ultrastructurally normal in CLDN5 knock-out mice, which suggests that additional claudins and/or TJ-associated marvel proteins (TAMPs) may be involved (27). In this study, we report that CLDN10 was highly expressed in three immortalized human brain capillary cell lines, which is consistent with Ohtsuki's report that claudin-10 is highly expressed in mouse brain capillary endothelial cells (16). It has been recognized in recent years that 27 members of the claudin family exhibit tissue-specific expression, resulting in different barrier properties in different tissues (5, 6, 28, 29). Previous studies have also reported that CLDN10 is expressed in epithelial cells in organs such as the kidney and uterus and is associated with TJs between epithelia (18, 30, 31). However, we did not observe its expression in vascular endothelial cells, mesothelial cells, and fibroblasts. Furthermore, we detected the very low levels of CLDN10 in breast cancer cells that were prone to brain metastasis. In summary, CLDN10 is differentially expressed in different vascular endothelial cells and different tumor cells.

To investigate the role of CLDN-10 in BBB TJs, we used immunohistochemistry to determine its distribution and discovered that it is involved in TJ strands, which is consistent with other reports (18, 30). To further study the role of CLDN10 in brain endothelial cell barrier-TJ, we generated cell models that express decreased levels of CLDN10 expression compared to controls. To monitor the impact of CLDN10 down-regulation on the sealing and permeability properties of brain endothelial cell barrier -TJ, we performed TER, ECIS (measuring the barrier function between brain endothelial cells), and PCP assays. Knocking down CLDN10 in brain endothelial cells dramatically reduced transendothelial barrier resistance. The changes in TER and ECIS indicated that CLDN10 plays an important role in the integrity and sealing properties of brain endothelial cell barrier. The changes in PCP indicated that CLDN10 plays a regulatory role in the permeability of the brain endothelial cell barrier. Furthermore, it is critical that the TJs of the blood-brain barrier strictly limit the entry of tumor cells and drugs into the brain. In order to evaluate the impact of

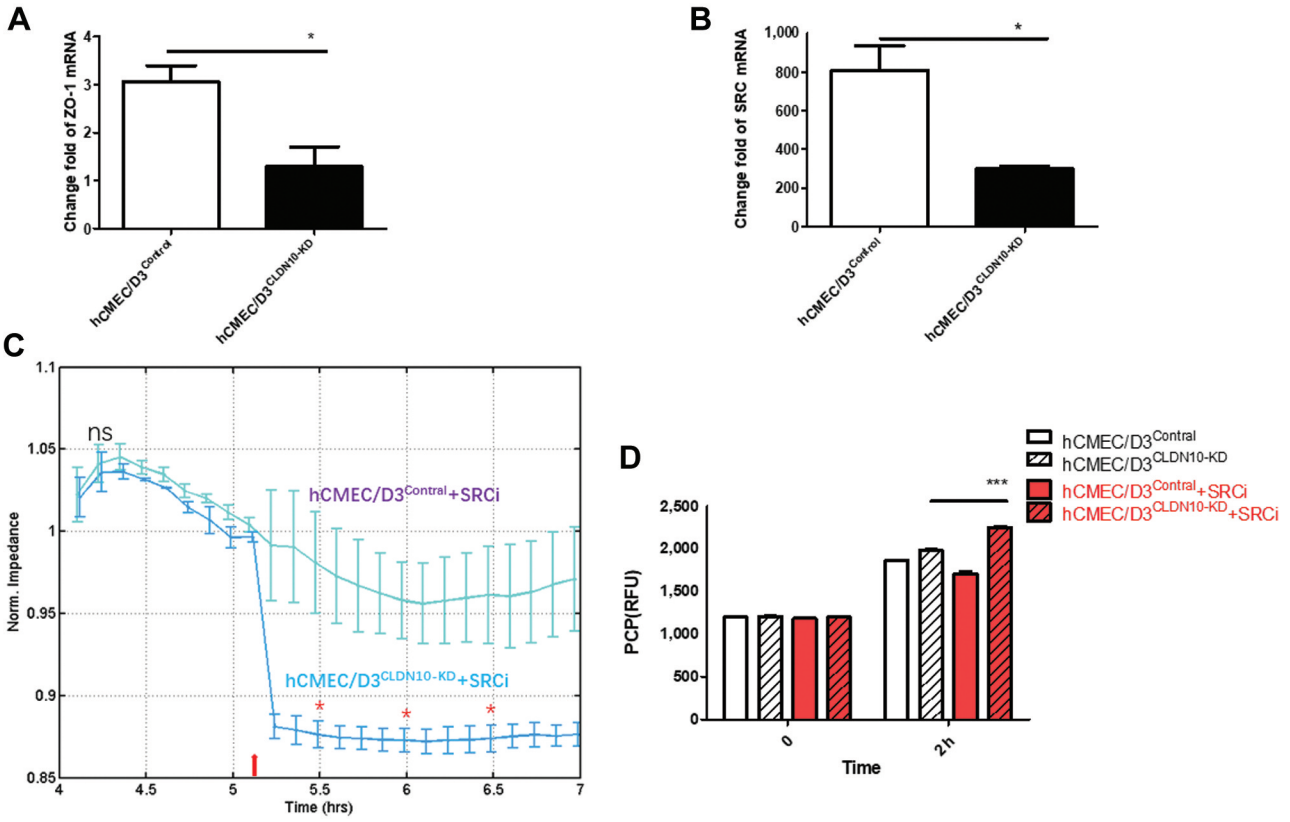

Figure 8. *The relation between CLDN10 and ZO-1 and the role of SRC kinase. (A) Expression of potential CLDN10 interacting partners and regulatory factor in hCMEC/D3 cells. (B) The effects of the SRC inhibitor (SRCi) AZM475271 on the tight junction function of hCMEC/D3^{Control} cells and hCMEC/D3$^{CLDN10\text{-}KD}$ cells were assessed using the ECIS assay. (C) The effects of the SRC inhibitor (SRCi) AZM475271 on paracellular permeability in hCMEC/D3^{Control} cells and hCMEC/D3$^{CLDN10\text{-}KD}$ cells were measured using the PCP assay. Data are given as the mean±SEM, n=3. Comparisons were made between control hCMEC/D3 and hCMEC/D3$^{CLDN10\text{-}KD}$ after adding SRCi (indicated by red arrow). (D). Paracellular permeability (PCP) following CLDN10 knockdown and cells response to SRCi. *p<0.05; **p<0.01; ***p<0.001; Student's t-test.*

CLDN10 down-regulation on trans-endothelial drug delivery and breast cancer cell invasion, we designed experiments in which chemotherapy and targeted drugs penetrate through the endothelial barrier and kill breast cancer cells. It was discovered that CLDN10 knockdown improved the ability of chemotherapy and targeted drugs to kill breast cancer cells after crossing the endothelial barrier. In addition, breast cancer cell transendothelial invasion assays showed that knockdown of CLDN10 in hCMEC/D3 cells also facilitated transendothelial invasion by breast cancer cells.

The organization and integrity of epithelial TJs depend on interactions between claudins, ZO scaffolding proteins, and the cytoskeleton (25). ZO-1 is a TJ protein that interacts with both occludin and claudins (32, 33). Additionally, ZO-1 is necessary for claudins to associate with actin. Prot-Bertoye *et al*. reported that CLDN10 was colocalized with ZO-1 in the human kidney cortex (34). However, Anderson *et al*. reported that although colocalized, the association between claudin, ZO-1, and actin is intermittent rather than continuous, and breaking and reannealing of claudin strands are independent of ZO-1 or actin interactions in fibroblasts (25).

A small number of previous studies have also investigated the association and role of CLDN10, ZO-1 and SRC in brain endothelial cells. In this study, we found that ZO-1 transcript levels decreased in CLDN10 knockdown brain endothelial cells. Junyan *et al*. report that decreased expression of p-SRC is associated with increased levels of claudin-5, occludin and ZO-1 TJ proteins (35). SRC kinase activity appears to be involved in both TJ assembly and disassembly. However, the role of SRC kinase in TJ regulation in CLDN10 knockdown brain endothelial cells is unknown. In this study, we also examined SRC mRNA levels, and our result showed that they decreased along with CLDN10 knockdown.

We further assessed the effect of SRC kinases on TJ function of brain endothelial cells using ECIS detected resistance and PCP by adding a SRCi. The result showed that ECIS detected resistance in the hCMEC/D3$^{CLDN10\text{-}KD}$ cells was significantly reduced following inhibition of SRC kinase

inhibited, whereas ECIS detected resistance was moderately decreased in the hCMEC/D3^{Control} cells. In contrast, in the hCMEC/D3$^{CLDN10\text{-}KD}$ cells, there was a significant elevation in PCP with the addition of the SRCi, while there was no significant change in in hCMEC/ D3^{Control} cells. These results demonstrate that CLDN10 knockdown in hCMEC/D3 cells suppresses SRC transcript levels, and in return, that TJ function in hCMEC/D3$^{CLDN10\text{-}KD}$ cells becomes more drug sensitive.

Conclusion

In conclusion. claudin-10 has a unique pattern of expression in cerebral endothelial cells. Its high expression in brain endothelial cells is important in the formation of TJs and the function of brain endothelial cell barrier. Moreover, CLDN10 expression affects the function of endothelial TJs, which in turn affects the entry of tumor cells and drugs across the blood brain barrier. Collectively, CLDN10 is an important factor in the pathophysiology of the cerebral endothelium and cancer invasion, and thereby is a favorable therapeutic target.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

WJ, XZ, and TAM conceived the study. XZ, ZF, YY, QPD FR, and WJ carried out the experimental work. WJ, TAM, and XZ conducted the statistical analyses. All participated in manuscript preparation.

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