Original Article
CLDN6-mediates SB431542 action through MMPs to regulate the invasion, migration, and EMT of breast cancer cells

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Abstract: Our previous research confirmed the repression of SMADs signaling pathway inhibits the invasion, migration, and EMT in breast cancer MCF-7 and SKBR-3 cell lines by DNMT1 up-regulating CLDN6, but the mechanism is unclear. Western blot was performed to detect the expression of SMAD2, SMAD3, P-SMAD2, and P-SMAD3. Then RT-PCR was carried out to examine the expression of tight junctions and cell adhesion molecule E-cadherin. According to the gene sequence of Claudin6, shRNA was linked with the green fluorescent protein-expressing eukaryotic expression vector pGC silencer TMM6/Neo/GFP, and it was transfected into breast cancer MCF-7 cells and SKBR-3 cells. RT-PCR and western blot were applied to verify the Claudin6 gene-silencing effect. We observed cellular morphology with inverted microscope, analyzed the capacity for clone formation, and detected transepithelial electrical resistance. The level of MMP2, and MMP9 in the cells treated with or without SB431542 and MCF-7-shGFP, MCF-7-shClaudin-6, SKBR-3-shGFP, and SKBR-3-shClaudin-6 cells pretreated with SB431542 were examined by RT-PCR and western blot. The expressions of Claudin-6, occludin, and cell adhesion molecule E-cadherin were enhanced by SB431542. SB431542 transformed mesenchymal cell morphology into epithelial cell morphology, inhibited capacity for clone formation, increased transepithelial electrical resistance, and downregulated the expression of MMP2 and MMP9. Knock down of Claudin6 can abolish SB431542 effects. We conclude that Claudin6 mediates the effects of SB431542 on the biologic phenotypes of the breast cancer cells we studied. We speculate Claudin6-mediated the SB431542 inhibition of invasion, migration, and EMT in breast cancer cells via MMP2/9.

Keywords: SMADs, Claudin6, TEERs, clone formation, morphology, MMPs

Introduction

Cancer cells escape from the primary sites to distant sites by local infiltration or intravasation into the blood or lymphatic vessels. The transportation is required during the formation of metastases [1, 2]. This process facilitates changes in the morphology and movement of cancer cells and leads to changes in cell-cell adhesion molecules [1]. In epithelial tissues, the linker complex including adhesive junctions (AJs), tight junctions (TJs), desmosomes, and gap junctions is located between cells [3]. Epithelial cells are connected each other by adhesion molecules at the AJs that mediate stable cohesion between cells [4, 5]. These junctional complexes include E-Cadherin (CDH1), which binds with β-catenin and α-catenin in the junctional complex [4-6]. The loss of cell-cell adhesion and cell junctions mediated by CDH1 homophilic binding promotes cells to dissociate from the primary tumor, invade neighboring tissues, and migrate to remote sites [7].

Occludin, Claudins (CLDNs) and junctional adhesion molecules (JAMs) constitute TJs [8]. CLDNs are a pivotal component of TJs [9]. CLDNs family consists 27 members [10]. As major transmembrane proteins, CLDNs play key roles in the formation and maintenance of tight junctions [11]. It is generally acknowledged that the disruption of tight junctions causes the loss of intercellular cohesion. It promotes the aggressiveness and lack of differentiation of cancer cells, which leads to metastasis. Previous studies have demonstrated

that mRNA or membrane protein expression levels of CLDNs were strongly correlated with carcinogenesis in breast cancer [12-14].

Unlimited proliferation is a characteristic of cancer cell activity [12]. It is accompanied by morphologic manifestations both in vitro in cell culture and in vivo during tumor proliferation, invasion, and metastasis. During *in vitro* cell culture, the proliferation results in the formation of cell clones. The biologic activity of cancer cells can be reflected by the clone formation ability and morphologic features [16-18].

Cancer cells acquire invasive and migratory abilities by epithelial-to-mesenchymal transition (EMT). The capability facilitates tumor dissemination and metastasis [19]. Matrix metalloproteinases (MMPs) play a role in regulating EMT, invasion, and migration. Various MMPs play an important role in cancer progression and metastasis [20].

CLDNs can regulate a variety of cancers' proliferation, invasion, and metastasis. The CLDNs possess different functions in different kinds of tumors. They play a key role during tumorigenesis [21]. Studies have shown that CLDN6 can induce the apoptosis, and inhibit the invasion, and migration of breast cancer cells MCF-7 [22]. Our previous studies showed that Smads signaling pathway can regulate the Claudin6 expression by DNA methyltransferase1 (DNMT1), and can regulate the invasion, migration, and EMT in breast cancer cells, but the mechanism is not clear [23]. Here, we show that CLDN6 mediates the Smads signaling pathway inducing morphologic change, enhances transepithelial resistance and inhibits clone formation in breast cancer. Knock down of CLDN6 can abolish downregulation of MMP2 and MMP9 expression by SB431542.

Materials and methods

Cell culture and reagents

We acquired the human breast cancer cell lines MCF-7 and SKBR-3 from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured the cells in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT,

USA) and 100 units/ml penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). All the cell lines were cultured in a humidified incubator (5% CO_2, 37°C). SB431542 was obtained from Sigma (St. Louis, MO, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. One microgram of total RNA was used for reverse transcription to synthesize cDNA using the MMuLVreverse transcriptase (TaKaRa, Japan) for 1 hour at 42°C, and 0.5 µg cDNA was used for PCR. ZO-2, ZO-1, Occludin, CLDN7, CLDN6, CDH1, MMP2 and MMP9 were amplified along with GAPDH as an endogenous control following the instructions of Premix LA Taq Kit (TaKaRa, Japan). The primer sequences and the PCR reaction conditions are listed in **Table 1**. After electrophoresis, the gel was imaged and analyzed by an imaging system (Syngene, Cambridge, UK).

Western blotting analysis

Western blotting experiments were carried out as described previously [24]. Anti-SMAD2, SMAD3, P-SMAD2, and P-SMAD3 antibodies were from Cell Signaling Technology (Beverly, MA, USA); Anti-MMP2, MMP9, CLDN6 antibodies were purchased from Abcam (Cambridge, UK). The anti-β-actin antibody was obtained from Santa Cruz (Santa Cruz, CA, USA). Primary and secondary antibodies were both diluted at 1:1000. The blots were imaged and analyzed applying an ECL western blotting system (GE, Fairfield, CT, USA).

Cell clone formation assay

Tumor cells were digested using 0.25% trypsin/0.02% EDTA solution at the logarithmic phase to make a single-cell suspension in culture medium. The cells were plated into six-well culture plates at 1,000 cells each well. The medium was changed every 3 days until cell clones could be observed by the unaided eye [25].

Transient transfection

Cells were transfected with short hairpin RNA (shRNA) by applying Lipofectamine 2000 (Invitrogen), according to the specification. ShRNA

Table 1. Primers and information for RT-PCR

Primer Name	Primer Sequence	Length (bp)	Annealing Temp (°C)	Cycles
GAPDH	5'-TGTTGCCATCAATGACCCCTT-3'	178	56	25
	5'-CTCCACGACGTACTCAGCG-3'			
CLDN6	5'-TTCATCGGCAACAGCATCGT-3'	345	58	35
	5'-GGTTATAGAAGTCCCGGATGA-3'			
CLDN4	5'-TGCACTCTGCGAACGTTAAG-3'	141	56	35
	5'-GCGATGGCCATTACCTGTAG-3'			
CLDN7	5'-GGAGATCCCAGGTCACACAT-3'	140	50	30
	5'-CAGGGTCTGCCCTAGTCATC-3'			
Occludin	5'-AAAATGTGTCTGCAGGCACACAGGACG-3'	101	55	35
	5'-AGGCTGCCTGAAGTCATCCACAGGC-3'			
ZO-2	5'-GCCAAAACCCAGAACAAAGA-3'	203	55	30
	5'-AACACTGGCAAATATCACAGC-3'			
ZO-1	5'-CACGATGCTCAGAGACGAAGG-3'	156	55	30
	5'-TTCTACATATGGAAGTTGGGGATC-3'			
CDH1	5'-ATTCTGGGGGATTCTTGGAGG-3'	337	56	30
	5'-GGTCAGTATCAGCCGCTTTC-3'			
MMP2	5'-TCTCCGACATTGACCTTGGC-3'	302	60	30
	5'-CAGGGTGCTGGCTGAGTAGATC-3'			
MMP9	5'-TTGACAGCGACAAGAAGTGG-3'	180	58	30
	5'-GCCATTCACGTTCGTCCTTAT-3'			

targeting of CLDN6 (50-GTGCAAGGTGTACGA-CTCA-30) and a negative control shRNA were both obtained from GeneChem Co. Ltd.

Transepithelial electrical resistance (TEER) measurement

The cells were cultured in Millicell chambers with 6-well culture plates at 5.0×10^5 cells per well; TEERs of cells were detected according to the Millicell-ERS instructions when cells were cultured until a monolayer of cell fused. The resistance of Millicell chambers' unseeded cells was detected as blank control (R_{blank}). The resistance of each group of cells is the difference between the average of the resistance of each group of cells and the R_{blank} multiplied by the diameter of the chamber.

Statistical analysis

All analysis was performed using the SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). Unpaired t-tests were performed to assess data for target mRNA and protein. The data are presented as means ± standard error from at least three independent experiments. P < 0.05 was considered significant.
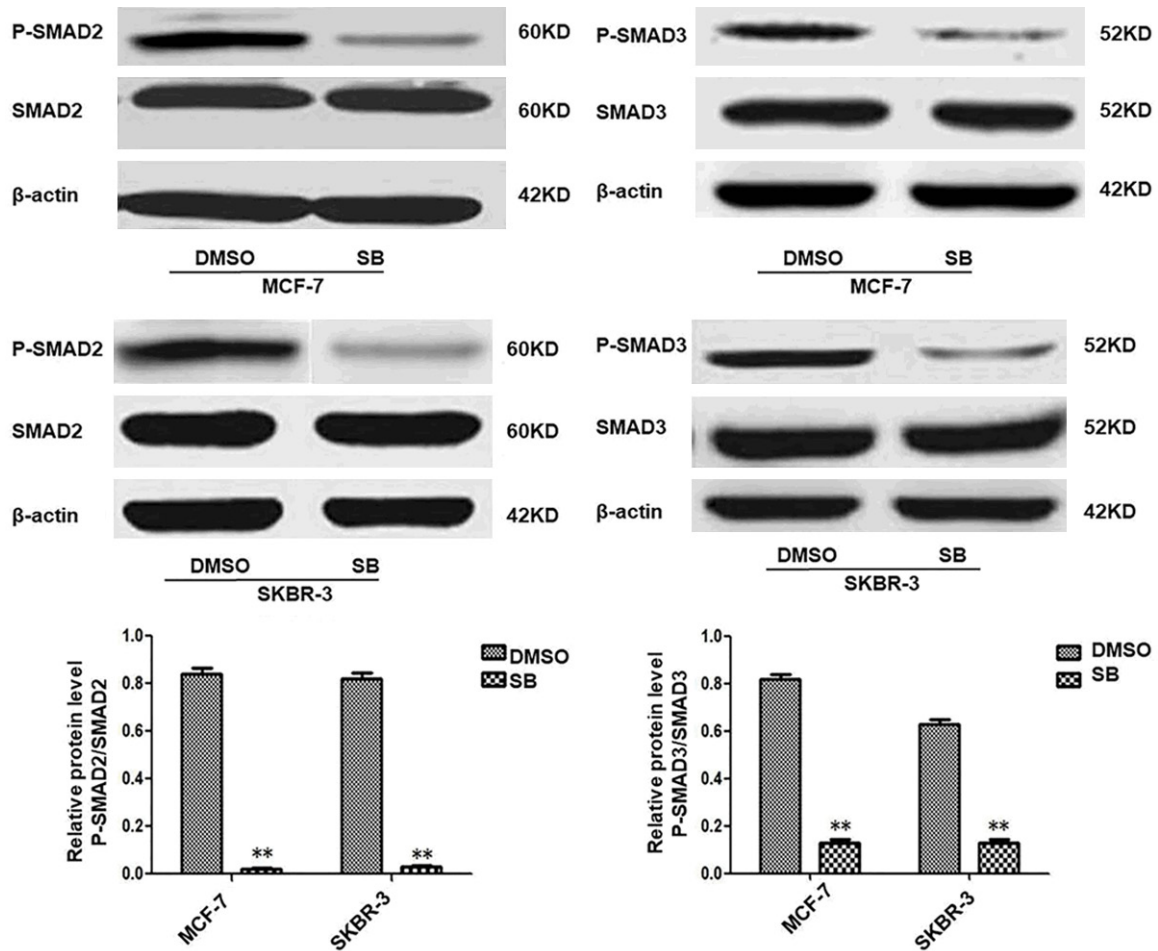
Results

SMADs signaling pathway was inhibited by SB431542 in breast cancer cells

The breast cancer cells were incubated with SB431542, an inhibitor of SMADs signaling pathway, RT-PCR and western blot analysis showed that P-SMAD2 and P-SMAD3 expression was decreased in MCF-7 and SKBR-3 cells treated with SB431542 (**Figure 1**).

SMADs signaling pathway regulates the expression of tight junction protein and E-cadherin in breast cancer cells

We have already reported that inhibition of the SMADs signal pathway can reverse the EMT process which is one of the key steps in the tumor metastasis and inhibits the migration and invasion of breast cancer cells. In order to study further the effects of CLDN6-mediated inhibition of the SMADs signaling pathway on the occurrence and development of breast cancer, MCF-7 and SKBR-3 cells were treated with 10 μM SB431542 for 24 h. RT-PCR demonstrated that inhibition of SMADs signaling pathway dysregulated the expression of tight junction protein CLDN6,

Figure 1. SB431542 inhibits SMADs signaling in MCF-7 and SKBR-3 cells. SB431542 downregulated P-SMAD2 and P-SMAD3 expression. MCF-7 and SKBR-3 cells were incubated with SB431542 for 24 h at 10 μM. Results of densitometry analysis of relative expression levels of P-SMAD2 and P-SMAD3 after normalization to SMAD2 and SMAD3 are presented (*P < 0.05 and **P < 0.01 are considered significant and highly significant, respectively). Bars represent mean ± SE (n = 3).

occludin, and E-cadherin in MCF-7 and SKBR-3 cells (**Figure 2**). TEER is a generally acknowledged quantitative technique to measure the intactness of tight junction dynamics in cell culture models of endothelial and epithelial monolayers [21]. The TEER was detected. The result showed that SB431542 increased the TEER in MCF-7 and SKBR-3 cells (**Table 2**; **Figure 3A**) Thus, our results suggested that SB431542 enhanced TEER by regulating the expression of tight junction protein and E-cadherin in breast cancer cells. The changes in cell-cell connections are closely related to tumor formation and development. In order to confirm whether CLDN6 mediates the effect of SB431542 on TEER, CLDN6 in MCF-7 and SKBR-3 cells treated with SB431542 as knocked down by shRNA. The result showed that the

TEER was decreased (**Table 3**; **Figure 3B**). RT-PCR and western blot analysis demonstrated that CLDN6 was significantly downregulated in the cells that had knocked down CLDN6, compared to the control group (**Figure 3C**). Therefore, we conclude that CLDN6 mediates the SB431542 effect on TEER in MCF-7 and SKBR-3 cells.

CLDN6 effects on breast cancer cellular morphology

MCF-7 and SKBR-3 cells were treated with 10 μM SB431542 for 24 h, and the cell morphology was observed. SB431542 caused a profound effect on the morphology of MCF-7 and SKBR-3 cells. MCF-7 cell morphology changed from long spindle shape to short spindle sha-
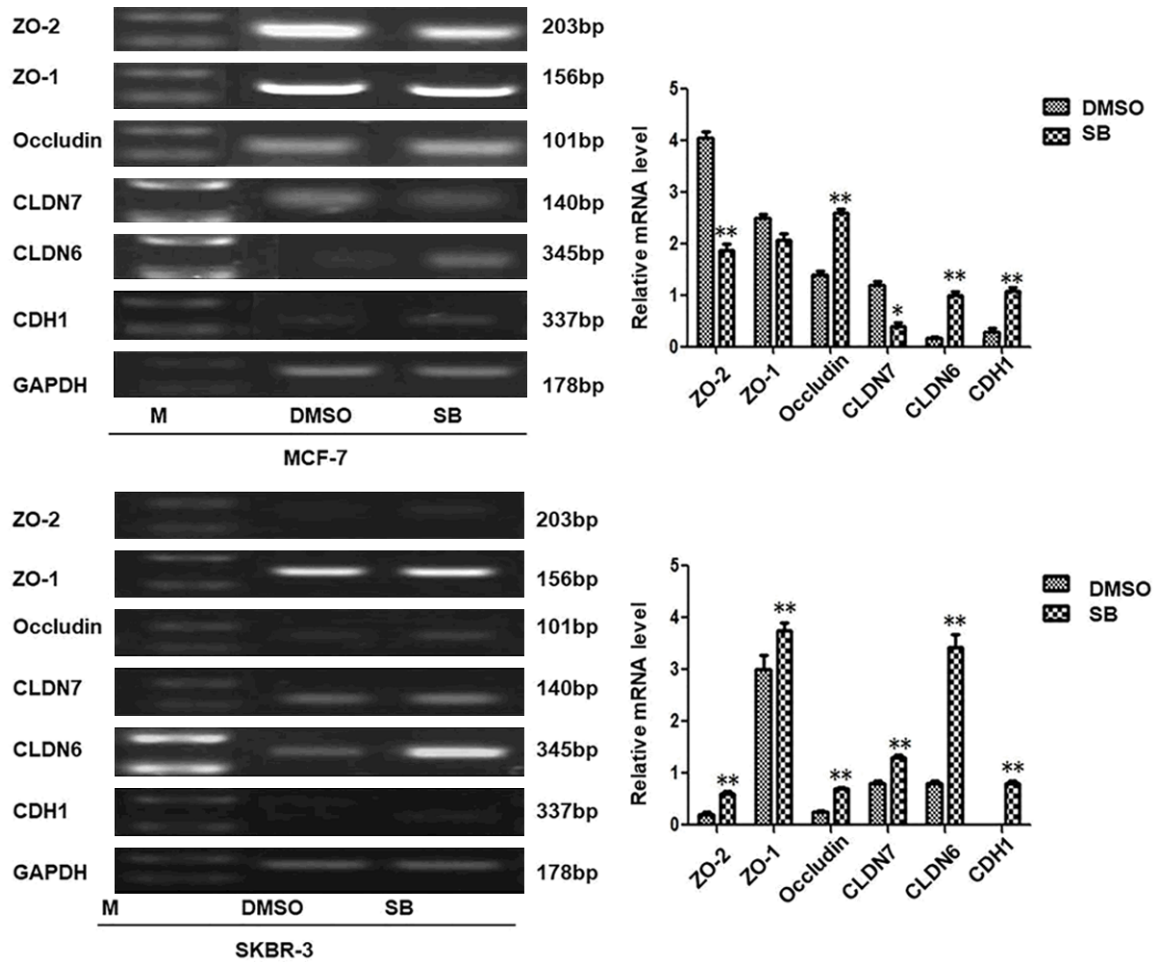
Figure 2. SMADs signaling regulates expression of tight junction protein and E-cadherin. Real-time polymerase chain reaction (RT-PCR) analysis of tight junction protein (ZO-2, ZO-1, occludin, CLDN7, and CLDN6) and E-cadherin; and densitometric analysis of relative gene expression levels after normalization to loading control GAPDH are presented. The lane "M" stands for marker; (*P < 0.05 and **P < 0.01 are considered significant and highly significant, respectively). Bars represent mean ± SE (n = 3).

Table 2. Effect of SB431542 on TEER in MCF-7 and SKBR-3 cells

Experimental Group	TEER (ohm·cm^2)	
	MCF-7	SKBR-3
DMSO	105 ± 7	114.3 ± 11.5
SB431542	155.3 ± 12.5**	180.3 ± 17.5**

**p<0.01 is considered highly significant.

pe. SKBR-3 cells acquired a cobble-shaped epithelial phenotype as opposed to a polygon. MCF-7 and SKBR-3 cells treated with SB43-1542 were used to knock down CLDN6 through shRNA. The change in morphology caused by SB431542 was reversed (**Figure 3D**, **3E**). Therefore, we conclude that knocking down CLDN6 blocks the effect of SB431542 on the morphology of MCF-7 and SKBR-3 cells.

CLDN6 effects on breast cancer cell clone formation

MCF-7 and SKBR-3 cells were treated with 10 μM SB431542 for one week. We observed the cell clone formation. SB431542 inhibited the cell clone formation (P < 0.05) (**Figure 3F**). Knocking down CLDN6 showed that the cell clone formation was increased significantly (**Figure 3G**).

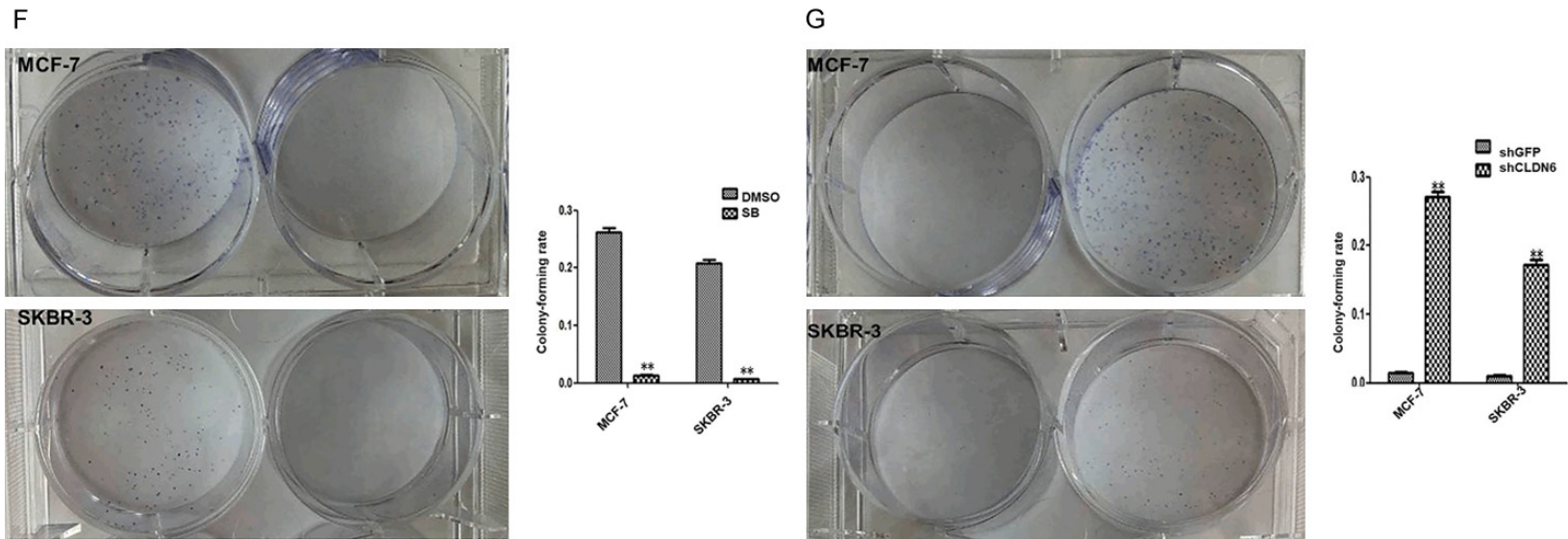*CLDN6 effects on the expression of MMP2 and MMP9*

In our previous experiment, it was confirmed CLDN6-mediated repression of SMADs signaling pathway inhibited the invasion, migration and EMT. CLDN6-mediated inhibition of Smad signaling pathway changed the breast cancer
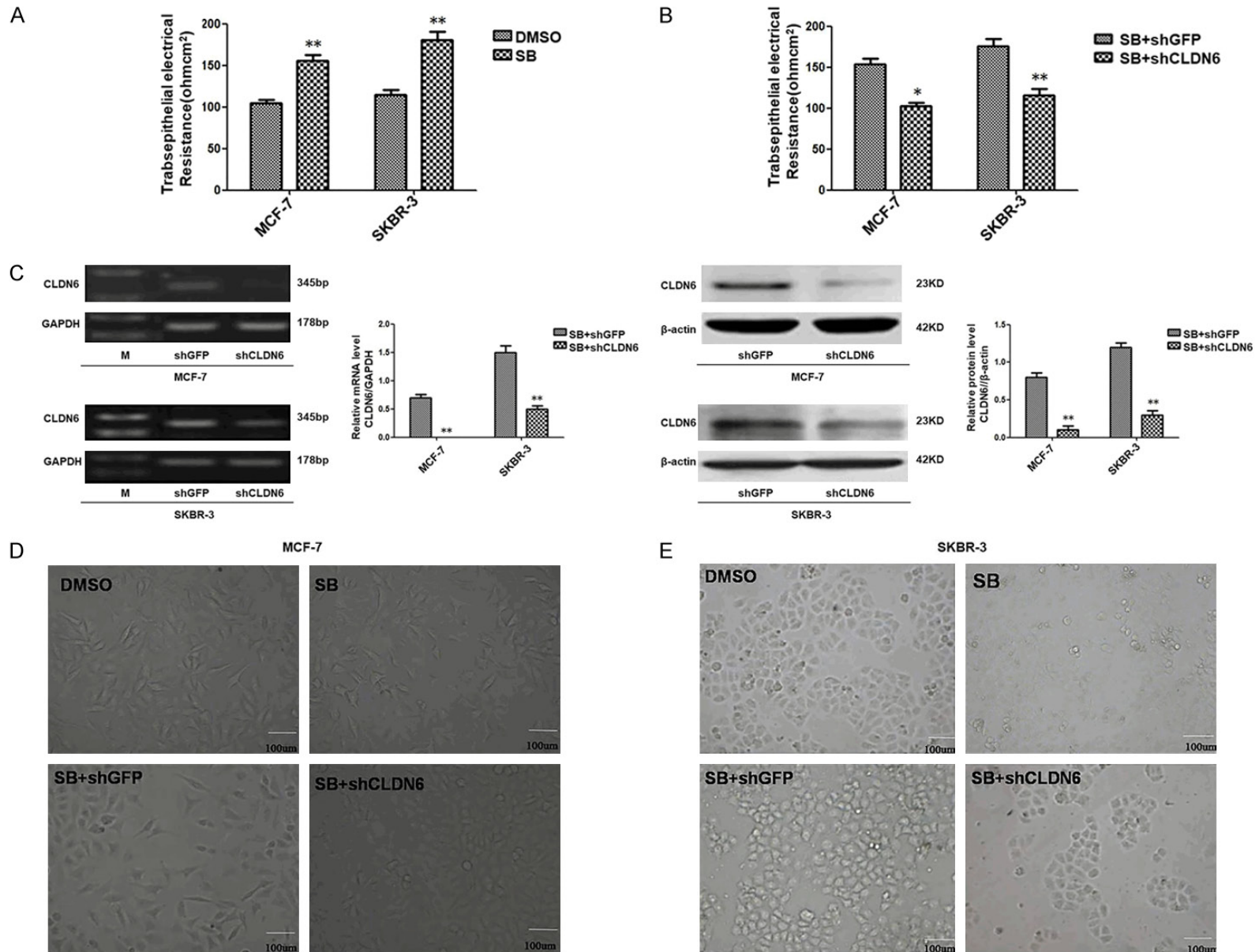
Figure 3. CLDN6 is required for the breast cancer cell TEER, morphology, and clone formation. A, B. CLDN6-mediated inactivation of SMADs signaling pathway regulated TEER (*$P < 0.05$ and **$P < 0.01$ are considered significant and highly significant, respectively). bars represent mean ± SE (n = 3). C. RT-PCR and immunoblot expression analyses of CLDN6 in MCF-7-shGFP, MCF-7-shCLDN6, SKBR-3-shGFP, and SKBR-3-shCLDN6 cells pretreated by SB431542, and densitometric analysis of relative expression levels after normalization to loading control GAPDH or β-actin are presented. The lane "M" stands for marker (*$P < 0.05$ and **$P < 0.01$ are significant and highly significant, respectively). Bars represent mean ± SE (n = 3). D, E. CLDN6-mediated inactivation of SMADs signaling pathway affected cell morphology (Bar, 100 μm). F, G. CLDN6 was required to inhibit cell clone formation (*$P < 0.05$ and **$P < 0.01$ are significant and highly significant, respectively). Bars represent mean ± SE (n = 3).

Table 3. CLDN6 mediates the effect of SB431542 on TEER in MCF-7 and SKBR-3 cells

Experimental Group	TEER (ohm·cm^2)	
	MCF-7	SKBR-3
SB431542 + shGFP	153.3 ± 12.5	176 ± 15
SB431542 + shCLDN6	102.3 ± 7*	116 ± 13**

*p<0.05, **p<0.01 are considered significant and highly significant, respectively.

cell morphology, increased transepithelial resistance, and reduced clone formation which played a crucial role during the EMT, invasion, and migration progression in our current study. It is unclear that the molecular mechanism of CLDN6-mediated inhibition of SMADs signal pathway represses the invasion, migration, and EMT. To address this problem, MMP2 and MMP9 were detected by RT-PCR and Western blot. MMP2 and MMP9 were down-regulated following SB431542 treatment. CLDN6 was knocked down after the cells were treated with SB431542. The down-regulation of MMP2 and MMP9 expression by SB431542 was blocked in MCF-7 cells (**Figure 4A**, **4B**). We got similar results in SKBR-3 cells (results not shown).

Discussion

Breast cancer is one of the most common tumors. It is derived from the ductal epithelium of the breast [27-29]. The metastasis and recurrence of breast cancer is the main cause of death [30]. The metastases require the cancer cells to break away from the original site. They infiltrate the surrounding tissue, invade into the vasculature, extravasate to distal sites, and grow [1, 2]. The process accompanies morphologic change, movement of carcinoma cells, and repression of cell-cell adhesion molecule expression [1]. In an earlier study, we reported that CLDN6 can reverse EMT which is a step in tumor metastasis, and inhibit breast cancer cell migration and invasion by mediating the inhibition of the SMADs signaling pathway [23], In the current research, we aimed to further explore the effects of CLDN6 mediating the inhibition of SMADs signaling pathway on the development of the MCF-7 and SKBR-3 breast cancer cells.

RT-PCR was performed. The result manifested that inhibition of SMADs signaling pathway up-regulated some of the expression of occludin and CLDN6 tight junction proteins and E-cad-

herin in MCF-7 and SKBR-3 cells. TEER is a quantitative technique to detect the integrity of tight junction dynamics in cell culture models of endothelial and epithelial monolayers [26]. The TEER was detected. The results showed that SB431542 increased the TEER in MCF-7 and SKBR-3 cells. Thus, our results suggested that SB431542 enhanced TEER by regulating the expression of tight junction protein and E-cadherin in breast cancer cells. Similar to our results, Krizbai [31] also found that SB-431542 enhanced TEER, indicating disruption in rat brain endothelial cell monolayer integrity. The changes in cell-cell connections were closely related to tumor formation and development. CLDNs play crucial roles in the formation and maintenance of tight junctions [11]. It is generally accepted that the disruption of tight junctions leads to the loss of intercellular cohesion, which causes the invasiveness and de-differentiation of cancer cells and facilitates metastasis. Earlier studies have suggested that mRNA or membrane protein expression levels of CLDNs were strongly correlated with carcinogenesis in BC [12-14].

In order to confirm that CLDN6 mediates the SB431542 regulation of the TEER in MCF-7 and SKBR-3 cells, the cells were treated with SB431542. CLDN6 in the cells was knocked down. The result showed that the TEER was decreased. Therefore, we can conclude that CLDN6 mediates the SB431542 effect on TEER in MCF-7 and SKBR-3 cells.

Uncontrolled proliferation is a hallmark of cancer cells [15]. It accompanies morphologic manifestations in cells cultured in vitro and during tumor proliferation, invasion and metastasis in vivo. In cells cultured in vitro, cell proliferation results in the formation of cell clones. The biologic behavior of cancer cells can be reflected by the clone formation rate and morphologic characteristics [16-18]. CLDNs can regulate proliferation, invasion, and metastasis in a variety of cancer types [32-35]. In the current study, SB431542 inhibited the cell clone formation. Knocking down CLDN6 obstructed the inhibitory effects of SB431542 on clone formation.

In the present research, SB431542 resulted in a profound effect on the morphology of MCF-7 and SKBR-3 cells. MCF-7 cell morphology changed from a long spindle shape to short spindle shape. SKBR-3 cells acquired a cobble-
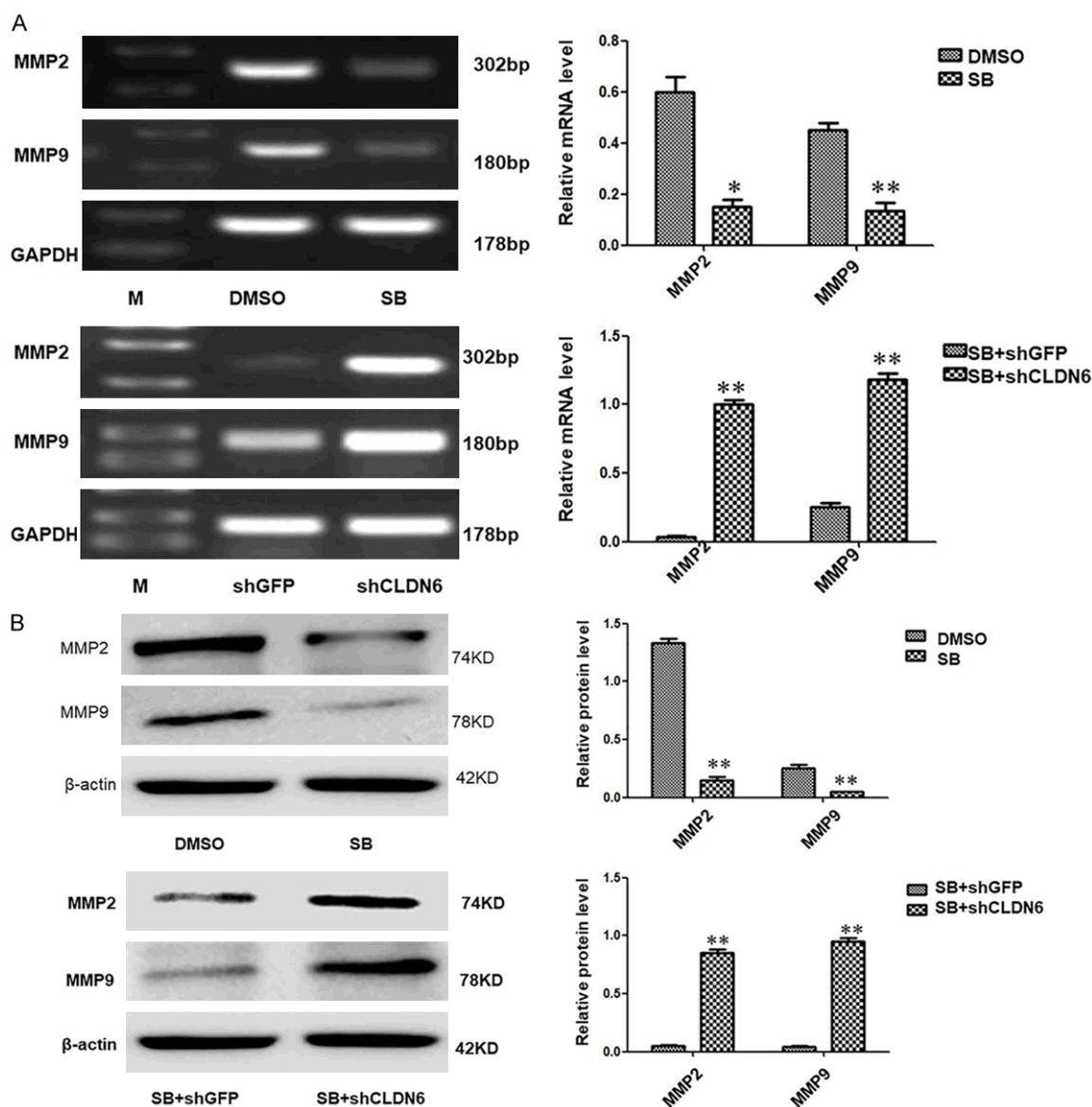
Figure 4. CLDN6-mediated Smads signaling pathway regulates the expression of MMP2 and MMP9. (A) RT-PCR and (B) immunoblot expression analyses of MMP2 and MMP9 in MCF-7 cells treated with or without SB431542, MCF-7-shGFP, and MCF-7-shCLDN6 pretreated with SB431542, and densitometric analysis of relative expression levels after normalization to loading control GAPDH or β-actin are presented (*P < 0.05 and **P < 0.01 are significant and highly significant, respectively). Bars represent mean ± SE (n = 3).

shaped epithelial phenotype as opposed to a polygon. After knocking down CLDN6, the changes in morphology caused by SB431542 were abrogated. Therefore, we conclude that CLDN6 mediates the function of SB431542 on the clone formation and morphology of MCF-7 and SKBR-3 cells.

The CLDNs have different functions in different tumors. They play a key role during tumorigenesis [19]. Studies have shown that claudin6 can inhibit the apoptosis, invasion, and migra-

tion of breast cancer cells MCF-7 [22]. Our previous studies showed that Smads signaling pathway can regulate the CLDN6 expression by DNMT1, and can regulate the invasion, migration and EMT in breast cancer cells, but the mechanism is not clear [23]. Here, we show that Cloudin 6 mediates the inhibition of Smads signaling pathway regulating the breast cancer cell morphology, increasing transepithelial resistance, and lessening clone formation, which are closely involved in EMT [36, 37]. The levels of MMP2 and MMP9 in cells were down-

regulated following SB431542 treatment. CL-DN6 was knocked down after the cells treated with SB431542, and the down-regulation of MMP2 and MMP9 was blocked. We speculate that Claudin6-mediated effects of SB431542 inhibit the invasion, migration, and EMT in breast cancer cells through MMP2 and MMP9. We will further investigate the molecular mechanisms by which SB431542/CLDN6 regulate the expression of MMPs to inhibit EMT, migration, and invasion in breast cancer.

Despite recent prevention, diagnosis, and therapy advances, lomany patients still die due to metastasis and recurrence. Evidence reveals that the EMT process plays an essential role in metastasis and recurrence of cancer [38]. It reveals the mechanism responsible for EMT. Genes, especially CLDN6 and signaling associated with EMT should be given high priority in order to decrease cancer morbidity and mortality.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-74.

[2] Lambert AW, Pattabiraman DR and Weinberg RA. Emerging biological principles of metastasis. Cell 2017; 168: 670-691.

[3] Franke WW. Discovering the molecular components of intercellular junctions--a historical view. Cold Spring Harb Perspect Biol 2009; 1: a003061.

[4] Wirtz-Peitz F and Zallen JA. Junctional trafficking and epithelial morphogenesis. Curr Opin Genet Dev 2009; 19: 350-6.

[5] Ragkousi K and Gibson MC. Cell division and the maintenance of epithelial order. J Cell Biol 2014; 207: 181-8.

[6] Valenta T, Hausmann G and Basler K. The many faces and functions of beta-catenin. EMBO J 2012; 31: 2714-36.

[7] Petrova YI, Schecterson L and Gumbiner BM. Roles for E-cadherin cell surface regulation in cancer. Mol Biol Cell 2016; 27: 3233-3244.

[8] Brennan K, Offiah G, McSherry EA and Hopkins AM. Tight junctions: a barrier to the initiation and progression of breast cancer? J Biomed Biotechnol 2010; 2010: 460607.

[9] Nakagawa S, Miyoshi N, Ishii H, Mimori K, Tanaka F, Sekimoto M, Doki Y and Mori M. Expression of CLDN1 in colorectal cancer: a novel marker for prognosis. Int J Oncol 2011; 39: 791-6.

[10] Mineta K, Yamamoto Y, Yamazaki Y, Tanaka H, Tada Y, Saito K, Tamura A, Igarashi M, Endo T, Takeuchi K and Tsukita S. Predicted expansion of the claudin multigene family. FEBS Lett 2011; 585: 606-12.

[11] Hewitt KJ, Agarwal R and Morin PJ. The claudin gene family: expression in normal and neoplastic tissues. BMC Cancer 2006; 6: 186.

[12] Tokés AM, Kulka J, Paku S, Szik A, Páska C, Novák PK, Szilák L, Kiss A, Bögi K and Schaff Z. Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. Breast Cancer Res 2005; 7: R296-305.

[13] Kim TH, Huh JH, Lee S, Kang H, Kim GI and An HJ. Down-regulation of claudin-2 in breast carcinomas is associated with advanced disease. Histopathology 2008; 53: 48-55.

[14] Kominsky SL, Argani P, Korz D, Evron E, Raman V, Garrett E, Rein A, Sauter G, Kallioniemi OP and Sukumar S. Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. Oncogene 2003; 22: 2021-33.

[15] Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, Ellis M, Henry NL, Hugh JC, Lively T, McShane L, Paik S, Penault-Llorca F, Prudkin L, Regan M, Salter J, Sotiriou C, Smith IE, Viale G, Zujewski JA and Hayes DF; International Ki-67 in Breast Cancer Working Group. Assessment of Ki67 in breast cancer: recommendations from the international Ki67 in breast cancer working group. J Natl Cancer Inst 2011; 103: 1656-64.

[16] Soltysova A, Altanerova V and Altaner C. Cancer stem cells. Neoplasma 2005; 52: 435-40.

[17] Franken NA, Rodermond HM, Stap J, Haveman J and van Bree C. Clonogenic assay of cells in vitro. Nat Protoc 2006; 1: 2315-9.

[18] Munshi A, Hobbs M and Meyn RE. Clonogenic cell survival assay. Methods Mol Med 2005; 110: 21-8.

[19] Nieto MA, Huang RY, Jackson RA and Thiery JP. Emt: 2016. Cell 2016; 166: 21-45.

[20] Vos MC, Hollemans E, Ezendam N, Feijen H, Boll D, Pijlman B, van der Putten H, Klinkhamer P, van Kuppevelt TH, van der Wurff AA and Massuger LF. MMP-14 and CD44 in epithelial-to-mesenchymal transition (EMT) in ovarian cancer. J Ovarian Res 2016; 9: 53.

[21] Kwon MJ. Emerging roles of claudins in human cancer. Int J Mol Sci 2013; 14: 18148-80.

[22] Wu Q, Liu X, Liu YF, Lu Y, Wang LP, Zhang XW, Li YL and Quan CS. Inhibition of p38 activity reverses claudin-6 induced cell apoptosis, invasion, and migration. Chin Med J (Engl) 2013; 126: 3539-44.

[23] Lu Y, Wang L, Li H, Li Y, Ruan Y, Lin D, Yang M, Jin X, Guo Y, Zhang X and Quan C. SMAD2 inactivation inhibits CLDN6 methylation to suppress migration and invasion of breast cancer cells. Int J Mol Sci 2017; 18: 1863.

[24] Liu Y, Jin X, Li Y, Ruan Y, Lu Y, Yang M, Lin D, Song P, Guo Y, Zhao S, Dong B, Xie Y, Dang Q and Quan C. DNA methylation of claudin-6 promotes breast cancer cell migration and invasion by recruiting MeCP2 and deacetylating H3Ac and H4Ac. J Exp Clin Cancer Res 2016; 35: 120.

[25] Geng XF, Fang M, Liu SP and Li Y. Quantum dot-based molecular imaging of cancer cell growth using a clone formation assay. Mol Med Rep 2016; 14: 3007-12.

[26] Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML and Hickman JJ. TEER measurement techniques for in vitro barrier model systems. J Lab Autom 2015; 20: 107-26.

[27] Coleman MP, Quaresma M, Berrino F, Lutz JM, De Angelis R, Capocaccia R, Baili P, Rachet B, Gatta G, Hakulinen T, Micheli A, Sant M, Weir HK, Elwood JM, Tsukuma H, Koifman S, E Silva GA, Francisci S, Santaquilani M, Verdecchia A, Storm HH and Young JL; CONCORD Working Group. Cancer survival in five continents: a worldwide population-based study (CONCORD). Lancet Oncol 2008; 9: 730-56.

[28] Siegel R, Naishadham D and Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.

[29] Akram M, Iqbal M, Daniyal M and Khan AU. Awareness and current knowledge of breast cancer. Biol Res 2017; 50: 33.

[30] Wolfe AR, Debeb BG, Lacerda L, Larson R, Bambhroliya A, Huang X, Bertucci F, Finetti P, Birnbaum D, Van Laere S, Diagaradjan P, Ruffell B, Trenton NJ, Chu K, Hittelman W, Diehl M, Levental I, Ueno NT and Woodward WA. Simvastatin prevents triple-negative breast cancer metastasis in pre-clinical models through regulation of FOXO3a. Breast Cancer Res Treat 2015; 154: 495-508.

[31] Krizbai IA, Gasparics Á, Nagyőszi P, Fazakas C, Molnár J, Wilhelm I, Bencs R, Rosivall L and Sebe A. Endothelial-mesenchymal transition of brain endothelial cells: possible role during metastatic extravasation. PLoS One 2015; 10: e0123845.

[32] Lu Z, Ding L, Hong H, Hoggard J, Lu Q and Chen YH. Claudin-7 inhibits human lung cancer cell migration and invasion through ERK/MAPK signaling pathway. Exp Cell Res 2011; 317: 1935-46.

[33] Dahiya N, Becker KG, Wood WH 3rd, Zhang Y and Morin PJ. Claudin-7 is frequently overexpressed in ovarian cancer and promotes invasion. PLoS One 2011; 6: e22119.

[34] Tabaries S, Dupuy F, Dong Z, Monast A, Annis MG, Spicer J, Ferri LE, Omeroglu A, Basik M, Amir E, Clemons M and Siegel PM. Claudin-2 promotes breast cancer liver metastasis by facilitating tumor cell interactions with hepatocytes. Mol Cell Biol 2012; 32: 2979-91.

[35] Ashikari D, Takayama KI, Obinata D, Takahashi S and Inoue S. CLDN8, an androgen-regulated gene, promotes prostate cancer cell proliferation and migration. Cancer Sci 2017; 108: 1386-1393.

[36] Thiery JP and Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 2006; 7: 131-42.

[37] Tomaskovic-Crook E, Thompson EW and Thiery JP. Epithelial to mesenchymal transition and breast cancer. Breast Cancer Res 2009; 11: 213.

[38] Gupta GP and Massague J. Cancer metastasis: building a framework. Cell 2006; 127: 679-95.