Twist1-mediated transcriptional activation of Claudin-4 promotes cervical cancer cell migration and invasion

JIAQI ZHU[1] and QI JIANG[2]

Departments of [1]Gynecology and [2]Obstetrics, Beilun People's Hospital, Beilun Branch of The First Affiliated Hospital, School of Medicine, Zhejiang University, Ningbo, Zhejiang 315826, P.R. China

Received February 18, 2023; Accepted May 25, 2023

DOI: 10.3892/ol.2023.13921

Abstract. Claudin-4, a member of the claudin multigene family, participates in events associated with mesenchymal-like activity of cancerous cells. Claudin-4 expression is upregulated in cervical cancer tissue compared with that in adjoining non-neoplastic tissue. However, the mechanisms that regulate Claudin-4 expression in cervical cancer are poorly understood. Moreover, whether Claudin-4 contributes to the migration and invasion of cervical cancer cells remains unclear. By western blotting, reverse transcription-qPCR, bioinformatics analysis, dual-luciferase reporter assay, chromatin immunoprecipitation assay, wound healing assay and Transwell migration/invasion assay, the present study confirmed that Claudin-4 was a downstream target of Twist1, a helix-loop-helix transcriptional factor, the activity of which has a positive correlation with Claudin-4 expression. Mechanistically, Twist1 directly binds to Claudin-4 promoter, resulting in the transactivation of expression. The depletion of the Twist1-binding E-Box1 domain on Claudin-4 promoter via CRISPR-Cas9 knockout system downregulates Claudin-4 expression and suppresses the ability of cervical cancer cells to migrate and invade by elevating E-cadherin levels and lowering N-cadherin levels. Following activation by transforming growth factor-β, Twist1 induces Claudin-4 expression, thus enhancing migration and invasion of cervical cancer cells. In summary, the present data suggested that Claudin-4 was a direct downstream target of Twist1 and served a critical role in promoting Twist1-mediated cervical cancer cell migration and invasion.

Introduction

Cervical cancer is a key health problem encountered in the female population globally, and it is the fourth most prevalent cancer type in female patients after breast, colorectal and lung cancer (1-3). According to Globocan 2018, cervical cancer constitutes 10% of all gynecological malignancy and 5% of all tumors, with an annual estimate of 570,000 newly diagnosed cases and 311,000 associated fatalities (3). Cervical cancer treatment includes radiotherapy, chemotherapy and surgical procedures including pelvic lymphadenectomy and radical hysterectomy, which are the most commonly used treatment (4,5). Nonetheless, despite treatment, cervical cancer may recur and metastasize to other organs including the liver, lung, bones, and lymph node (6,7). Therefore, treatment failure in cervical cancer is typically attributed to invasion into nearby tissues and/or metastasis of the malignancy to other organs (8). The primary mode by which cervical cancer metastasizes to other organs is via the lymph nodes, which notably worsens patient prognosis (7,9). Compared with 5-year survival rate of >90% in patients with early-stage cervical cancer, the survival rate in those who have lymph node metastasis is <30% (10). Thus far, molecular alterations that promote invasion and metastasis in cervical cancer are not fully known despite evidence for their function in malignancy (11,12). To identify potential treatment targets and enhance patient prognosis, knowledge of the basic molecular pathways of cervical cancer cell migration and invasion is key.

Epithelial-mesenchymal transition (EMT) is a key process that confers invasive and metastatic activity to malignant cells (13,14). During EMT, epithelial cells undergo lose their polarity, become more invasive, disseminate to lymphatic vessels and eventually metastasize to other organs across the body (13,15). Accumulating evidence suggests that specific transcription factors (TFs), known as EMT-TFs, promote EMT, the process by which epithelial cells acquire mesenchymal properties and override senescence, thus enhancing the invasion and metastatic ability of human tumor cells (16,17). Moreover, the contribution of TFs to EMT may vary depending on cell or tissue type and the upstream signaling pathway activated by EMT (18). Among the TFs that participate in EMT induction, Twist-related protein 1 (Twist1) is a basic helix-loop-helix TF known as the master modulator of EMT initiation (19). The Twist1 gene is highly expressed in a wide variety of metastatic tumors and serves a notable role in embryogenesis (20). For example, elevated expression of Twist1 in colorectal cancer tissues is linked to lymph node metastasis and poor prognosis, and specifically with shorter

Correspondence to: Professor Qi Jiang, Department of Obstetrics, Beilun People's Hospital, Beilun Branch of The First Affiliated Hospital, School of Medicine, Zhejiang University, 1288 Lushan East Road, Ningbo, Zhejiang 315826, P.R. China
E-mail: jiangqi2023@yeah.net

Key words: Twist1, claudin-4, epithelial-mesenchymal transition, migration, invasion, cervical cancer

patient survival (21,22). Additionally, Twist1 promotes tumor invasion, metastasis and unfavorable prognosis in breast cancer by triggering EMT induction and downregulating E-cadherin expression (20). Moreover, Twist1 has a key role in the onset and progression of different cancer types in humans through its effects on tumorigenesis, angiogenesis and stemness (23). Like other EMT-TFs, Twist1 induces the onset of EMT by binding to E-Box (5'-CAGATG-3') consensus sites in the promoter of downstream target genes and transcriptional regulation of downstream target genes such as ADP ribosylation factor and P-glycoprotein and performs biological functions (23,24). There is increasing research on the involvement of Twist1 in tumor progression and metastasis (20,23,25). In cervical carcinoma, Twist1 knockdown inhibits cell migration and invasion (26,27). To the best of our knowledge, however, the specific underlying molecular mechanism has not been thoroughly studied and Twist1-mediated transcriptional regulation of downstream target genes in cervical cancer during EMT has not yet been investigated. Knowledge of the events involved in cervical cancer recurrence and metastasis is necessary to gain insight into the interactions and develop targeted cancer treatment.

Claudins primarily act as transmembrane proteins and are essential constituents of tight junctions (TJs) that play a pivotal function in regulating paracellular permeability and maintaining epithelial cells in a polarized state (28). This role, together with the fact that cell-to-cell adhesion interactions are disrupted or rearranged during metastasis, explains why claudin expression is frequently decreased in several cancer types, such as lung adenocarcinomas and colorectal cancer (29). Previous studies have identified the potential role of claudins in signal transduction and may play important roles in tumorigenesis, including tumor cell survival, proliferation, growth, EMT and metastasis (28,29). Nonetheless, increasing evidence suggests that specific claudins may facilitate the metastatic phenotype (30,31). Tissue- and cell-specific expression of the 27 members of the claudin family has been reported, which contributes to the regulation of various biological processes, such as EMT and cancer stem cell (CSC) renewal (28). Claudin-4, a member of the claudin multigene family, is a critical player in TJs by interacting with other claudin-4 proteins expressed at the surface of neighboring cells through extracellular loop interactions and a major research focus (32-34). Claudin-4 is highly expressed in cancerous cells when compared with normal epithelial cells (35). Additionally, Claudin-4 gene deletion can frequently occur in subcellular locations other than TJ structures in normal epithelial cells, particularly along the basolateral membranes (33). Claudin-4 participates in activities linked to the mesenchymal-like behavior of cancer cells beyond its conventional barrier formation function in TJs. Moreover, Claudin-4 is overexpressed in cervical cancer compared with adjacent non-tumor tissue (36). To the best of our knowledge, however, the regulatory and functional role of Claudin-4 in cervical cancer remain unexplored. It is unknown whether Claudin-4 contributes to the spread and invasion of cervical cancer cells.

The aim of the present study was to investigated the regulatory mechanism of Claudin-4 expression in cervical cancer and to clarify the roles of Twist1 and Claudin-4 in cervical cancer cell migration and invasion.

Materials and methods

Bioinformatics analysis. Kmplot software (tnmplot.com/analysis/; updated April 22, 2023) was used to assess the correlation between expression of Twist1 and Claudin-4. It incorporates the survival information and transcriptome data from Gene Expression Omnibus (Genechip from GEO: 3,691 normal, 29,376 tumor and 453 metastasis) and The Cancer Genome Atlas databases (RNA-seq from TCGA: 730 normal, 9,886 tumor and 394 metastasis) (37).

For bioinformatics analysis of the Claudin-4 promoter, 3,000-bp nucleotide sequences upstream of Claudin-4 translation initiation site were downloaded from the National Center for Biotechnology Information (ncbi.nlm.nih.gov/nuccore/NC_000007.14?report=fasta&from=73830996&to=73832690) and imported into the analysis tools (JASPAR 2022: the 9th release of the open-access database of transcription factor binding profiles, jaspar.genereg.net/) to analyze the potential Twist1-binding sites in this region (38).

Cell lines and culture. The American Type Culture Collection supplied the SiHa and HeLa human cervical cancer cell lines. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; cat. no. C11995500BT; Gibco) supplemented with 10% fetal bovine serum (FBS; cat. no. 10099141C; Gibco), 100 g/ml streptomycin, 2 mmol/l L-glutamine and 100 U/ml penicillin (all Thermo Fisher Scientific, Inc.) in accordance with the supplier's recommendations. The cells were maintained at 37˚C and 5% carbon dioxide in a humidified environment (90%).

Reagents and antibodies. TGF-β, a pleiotropic cytokine, was obtained from Sigma-Aldrich (cat. no. T1654; Merck KGaA). Antibodies were as follows: Anti-Claudin-4 (1:1,000; cat. no. ab15104; Abcam), anti-Snail2 (1:1,000; cat. no. 9585T; Cell Signaling Technology, Inc.), anti-Twist1 (1:1,000; cat. no. 90445S; Cell Signaling Technology, Inc.) anti-β-actin (1:10,000; cat. no. ET1701-80; Hangzhou HuaAn Biotechnology Co., Ltd.), anti-Flag (1:1,000; cat. no. 14793S; Cell Signaling Technology, Inc.) anti-E-cadherin (1:1,000; cat. no. 14472S; Cell Signaling Technology, Inc.) and anti-N-cadherin (1:1,000; cat. no. 13116S; Cell Signaling Technology, Inc.).

Plasmid construction. Plasmid for Twist1 overexpression (pENTER-CMV-hTwist1-Flag: CH871696) and empty vector control (pENTER-CMV-C-Flag: pAD100004-OE) was purchased from WZ Biosciences, Inc. pGL3 luciferase reporter vectors pGL3-Basic, pGL3-Enhancer, pGL3-Promoter and pGL3-Control were purchased from Promega Corporation. The Claudin-4 promoter (-206 to -2,200 bp) reporter (Claudin-4 Luc WT) was constructed by Laboratory of Obstetrics and Gynecology (Beilun People's Hospital, Ningbo, China). Briefly, cDNAs encoding Claudin-4 promoter region were amplified by PCR from HeLa cells using following primer pair 5'-AGT GCTGGGATTATAGGCATGAGC-3' (forward) and 5'-TCT CTC GGGGACAGGTTGAGC-3' (reverse) and subcloned into pGL3-Basic Vector. The PCR amplification was performed in a Bio-Rad T100 Thermal Cycler using a PrimeSTAR Max DNA Polymerase kit (Takara Bio Inc., R045A) according to the manufacturer's instructions. A total of 30 PCR cycles were

run under the following conditions: Initial DNA denaturation for 5 min, followed by DNA denaturation at 95˚C for 30 sec, primer annealing at 55˚C for 30 sec, and DNA extension at 72˚C for 30 sec. After the final cycle, the reaction was terminated by keeping it at 72˚C for 5 min. The PCR-amplified samples were analyzed by agarose gel electrophoresis by using a horizontal 0.8% (w/v) agarose gel in 1X TBE buffer and with 0.001% (w/v) GelRed® Nucleic Acid Gel Stain (Biotium, 41001) incorporated for DNA staining. The Claudin-4 Luc E-Box1 (-1,500-2,200del) and Claudin-4 Luc E-Box2 (-206-1,500del) promoter reporter were amplified by PCR from Claudin-4 Luc WT plasmid using following primer pairs: 5'-CCCAGT CTCTGGTCAAACTGG-3' (forward) and 5'-TCTCTCGGG GACAGGTTGAGC-3' (reverse) for Claudin-4 Luc E-Box1 and 5'-AGTGCTGGGATTATAGGCATGAGC-3' (forward) and 5'-CTGGGGAGGGAGGGACCAAAG-3' (reverse) for Claudin-4 Luc E-Box2. Mutated Claudin-4 promoter reporter (Claudin-4 Luc ΔE-Box1 and Claudin-4 Luc ΔE-Box2) were constructed by Vigene Biosciences Inc.

Transfection. For transfection of small interfering (si)RNA, Lipofectamine 3000 was used (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions to transfect cells cultivated in 60-mm plates with either 20 nM scrambled control siRNA (siCtrl; Shanghai GenePharma, Shanghai, China) or 20 nM siRNA oligonucleotide targeting Twist1 or Claudin-4 (GenePharma) in a humidified 5% CO2 incubator at 37˚C. Thereafter, the cells were collected for subsequent experiments at 48 h post-transfection. The sequences of siRNA oligonucleotides were as follows: siCtrl, 5'-ATTGTATGCGATCGCAGA C-3'; siTwist1#1, 5'-GGTCA TCGACTTCCTCTA-3'; siTwist1#2, 5'-TTGAGGGTCTGA ATCTTGCTCAGC T-3' and siClaudin-4, 5'-TGCGTGGTG CAGAGCACCGGCCA-3'.

For transient overexpression, Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was applied according to the manufacturer's instructions to transfect cells in 60-mm dishes containing plasmids or negative control. Thereafter, the cells were collected for subsequent experiments at 48 h post-transfection.

Western blotting. In the presence of phosphatase and protease antagonists, whole-cell extracts were obtained and prepared by lysing the cells in NP-40 lysis solution (cat. no. P0013F; Beyotime Institute of Biotech. Inc.). The cell lysate was collected, and total protein was quantified using the bicinchoninic acid protein assay kit. Subsequently, the protein (20-40 μg/lane) was loaded onto 8%-12% SDS-PAGE gel for electrophoretic separation and transferred to nitrocellulose membrane (cat. no. FFN03; Beyotime Biotech. Inc.). After separation, membranes were blocked in TBST buffer (TBS with 0.1% Tween-20) with 5% non-fat skim milk (cat. no. 1706404; Bio-Rad Laboratories, Inc.) for 45 min at room temperature and washed three times with TBST buffer. Following this, membranes were performed using antibodies, as previously described (39).Briefly, membranes were incubated overnight with primary antibodies at 4˚C and with horseradish peroxidase-labeled secondary antibodies (1:5,000; cat. nos. sc-2357 and sc-516102; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Bands were visualized using commercially ECL reagents (cat. no. 32106; Thermo Fisher Scientific, Inc.). ImageLab software (version 4.1; Bio-Rad) was used for densitometry.

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was performed as previously described (40). Briefly, TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was used for extracting total RNA from cell cultures, after which the total RNA was subjected to RT into complementary DNA using PrimeScript™ RT reagent kit (cat. no. RR037A; Takara Biotechnology, Co., Ltd.) according to the manufacturer's protocols. RT-qPCR assay was performed using the SYBR® Premix Ex Taq™ kit (Tli RNaseH Plus; Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions, on an Applied Biosystems StepOne-Plus™ Real-Time PCR instrument. Amplification conditions were set as follows: 95˚C for 30 sec, followed by 95˚C for 10 sec, and 58˚C 30 sec for 40 cycles. The $2^{-\Delta Cq}$ method was used to quantify expression, normalized according to the internal reference gene (GAPDH) (41). The primers used for the assay were as follows: Human Claudin-4 forward, 5'-TGTCACCTCGCAGACCAT CTG-3' and reverse, 5'-CTGCAGGTCCTGCGGCAGTGC-3'; Snail2 forward, 5'-CAGCGAACTGGACACACATAC-3' and reverse, 5'-GAGCAGCGGTAGTCCACACAG-3'; Twist1 forward, 5'-GTCCGCAGTCTTACGAGGAG-3' and reverse, 5'-TGGAGGACCTGGTAGAGGAA-3' and GAPDH forward, 5'-AGGGCATCCTGGGCTACAC-3' and reverse, 5'-GCC AAATTCGTTGTCATACCAG-3'.

Dual-luciferase reporter assay. As per the manufacturer's instructions, Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to co-transfect cells cultured in 24-well plates with siRNA, expression plasmids pGL3 or pGL3 containing the Claudin-4 promoter and a plasmid that expressed *Renilla* luciferase. After 24 h, the cells were collected, lysed and imported into a dual-luciferase reporter assay system (cat. no. E1910; Promega Corporation) to determine the luciferase activity. The results were normalized by comparing the relative firefly luciferase activity with the *Renilla* luciferase activity.

Chromatin immunoprecipitation (ChIP). ChIP assay was performed with the Simple ChIP Enzymatic Chromatin IP kit (cat. no. 9003; Cell Signaling Technology, Inc.), according to the manufacturer's instructions. After purifying the DNA and performing reverse cross-linking, immunoprecipitated DNA was then subjected to PCR amplification using the primers as follows: Claudin-4 E-Box1 forward, 5'-CACGTAACTTTA TCCGGCCAATGC-3' and reverse, 5'-CTGGCAGTTTCA CGACTGTTG-3'; Claudin-4 E-Box2 forward, 5'-CACATT TCTTGAGCATCTGTG A-3' and reverse, 5'-CACATCCGT CTTCCCTGCTAGCCT-3' and GAPDH forward, 5'-TGG CAAAGTGGAGATTGTTGC-3' and reverse, 5'-AAGATG GTGATGGGCTTCCCG-3'.

CRISPR/Cas9-based E-Box1 and E-Box2 knockout. Plasmid pSpCas9(BB)-2 A-Puro (PX459; Addgene, Inc.; cat. no. 48139) was subcloned with a single-guide RNA (sgRNA) targeting the E-Box1 region of the Claudin-4 promoter. For transfection, Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific,

Inc.) was applied according to the manufacturer's instructions to transfect cells in 60-mm dishes containing 3ug sgRNA plasmid or negative control plasmid in a humidified 5% CO_2 incubator at 37˚C. HeLa and SiHa cells were transfected with the construct for 48 h and then maintained in DMEM with 2 ug/ml puromycin at 37˚C for 3 days for selection. By observation under a Nikon inverted light microscope at 100X, single clones were selected after plating cells at 300 cells/plate in 60-mm plates. Sequencing was performed to verify E-Box1 sequence loss. sgRNA with the following sequence: E-Box1-sgRNA, 5'-GATGTATCAAGCCAGATGCT-3'.

Wound healing assay. HeLa and SiHa cells were seeded at a density of $1.5x10^6$ cells/plate in 60-mm plates and, when they reached 80-90% confluence, were used for wound healing assay. After 24 h, the confluent cell monolayers were scraped using a pipette tip to produce a wound. Debris was removed by rinsing the sample using phosphate-buffered saline. The cells that adhered to the culture plate were cultured in a serum-free DMEM (SFM; cat. no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc.) in the presence or absence of 5 ng/ml TGF-β in a humidified 5% CO_2 incubator at 37˚C. Cell migration was photographed using a Nikon inverted microscope at 100X at 0, 24 or 48 h after scratching. Image-Pro Plus 6.0 (Media Cybernetics) was used for assessing the relative width of the wound. Wound healing area was calculated as follows: (Final width/Initial width). Data are expressed as the mean of three separate assays.

Transwell migration and invasion assay. To evaluate migration, cells were maintained in SFM at 37˚C for 18-24 h and seeded at a density of $1.5x10^5$ (HeLa) and $3x10^5$ (SiHa) cells/well in the upper 8.0-μm filter membrane with SFM in a 24-well plate containing DMEM supplemented with 10% FBS (cat. no. 10099141C; Gibco; Thermo Fisher Scientific, Inc.) below the filter membrane. To evaluate invasion, a thin layer of gel was formed using the Matrigel added into the upper cavity insert at 4˚C and incubated for an additional 1 h in a humidified 5% CO_2 incubator at 37˚C. Subsequently, cells were added. After incubating for 12-24 h in a humidified 5% CO_2 incubator at 37˚C, the samples were fixed and stained with 0.1% crystal violet for 30 min at room temperature. Finally, cells on the membrane surface were removed with a cotton swab. Attached cells were photographed and ≥3 randomly selected fields of view per chamber were examined under a Nikon inverted light microscope at 100X to determine the total number of migrating or invading cells.

Statistical analysis. A total of three independent experimental repeats was performed. Data are presented as the mean ± standard error of the mean. GraphPad Prism 6 (GraphPad Software, Inc.) was used to perform statistical analysis using paired two-tailed Student's t test. One-way ANOVA followed by Tukey's post hoc test was used to compare >2 groups. $P<0.05$ was considered to indicate a statistically significant difference. Spearman's correlation analysis was used to analyze the mRNA expression of Twist1 and CLDN-4 1 in human cervical cancer tissue derived from Kmplot database.

Results

Twist1 positively regulates Claudin-4 expression. Twist1, an EMT-inducing TF, activates or inhibits promoters to regulate transcription of genes involved in EMT, thereby upregulating the expression of genes associated with the mesenchymal cell-like phenotype, such as vimentin, and downregulating expression of genes associated with the epithelial phenotype, such as E-cadherin (18,40). To determine whether Twist1 regulates Claudin-4 expression, two siRNA oligonucleotides (siTwist1#1 or siTwist1#2) were used to knock down Twist1 expression and assess its effect on Claudin-4 levels, after which western blot examination of protein levels of Claudin-4, Snail2, and Twist1 was performed. Among all cervical cancer cell lines with wild-type (WT) Twist1, Twist1 silencing in cells led to a notable decrease in protein levels of Claudin-4 and Snail2, which was used as a positive control (Fig. 1A and B). When Twist1 was silenced, both Claudin-4 and Snail2 had a similar reduction in their mRNA and protein levels (Fig. 1C and D). Twist1 overexpression or silencing was established in SiHa and HeLa cells. The results showed that Twist1 overexpression upregulated Claudin-4 protein expression and reversed the reduction in Claudin-4 expression caused by Twist1 knockdown (Fig. 1E and F). Consistently, Twist1 overexpression in HeLa and SiHa cells was associated with enhancement of Claudin-4 and Snail2 mRNA levels (Fig. 1G and H). Furthermore, mRNA expression of Twist1 and Claudin-4 in cervical tumor tissue was assessed (tnmplot.com/analysis/); Spearman's correlation analysis showed no significant correlation between Twist1 mRNA and Claudin-4 expression (Fig. S1). Altogether, these results suggested that Twist1 may cause transcriptional overexpression of Claudin-4.

Twist1 upregulates Claudin-4 transcription via E-Boxes in the Claudin-4 promoter. It was next determined whether Twist1 functions as a TF by directly activating the transcription of Claudin-4 to elucidate the processes involved in the upregulation of Claudin-4 transcription by Twist1. First, 3,000-bp nucleotide sequences from the upstream of Claudin-4 translation initiation site as the promoter region of Claudin-4 were downloaded and then analysis tools (jaspar.genereg.net/) were used to analyze the potential Twist1-binding sites in the region. There were two probable Twist1 consensus-binding E-Box domains at -722 to -727 (E-Box1) and -2,123 to -2,128 (E-Box 2) upstream of the Claudin-4 start codon (Fig. 2A). Additionally, dual-luciferase reporter assay revealed that compared with pGL3 control, there was a fourfold increase in luciferase reporter activity mediated by the Claudin-4 promoter (Claudin-4 Luc), which contained both potential Twist1 consensus-binding E-Box domains in HeLa and SiHa cells, whereas after Twist1 was knocked down, Claudin-4 Luc activity was significantly attenuated (Fig. 2B and C), demonstrating that the transcriptional activation of Claudin-4 was dependent on Twist1. To investigate how Twist1 regulates Claudin-4 transcription and the location of Twist1-binding domains in the Claudin-4 promoter, deletion mutants of the Claudin-4 promoter were established (-1,500-2,200del and -206-1,500del), followed by dual-luciferase reporter assay analysis (Fig. 2A). Promoter activity of the -206-1,500del mutant without E-Box1 (Claudin-4 Luc E-Box2) was

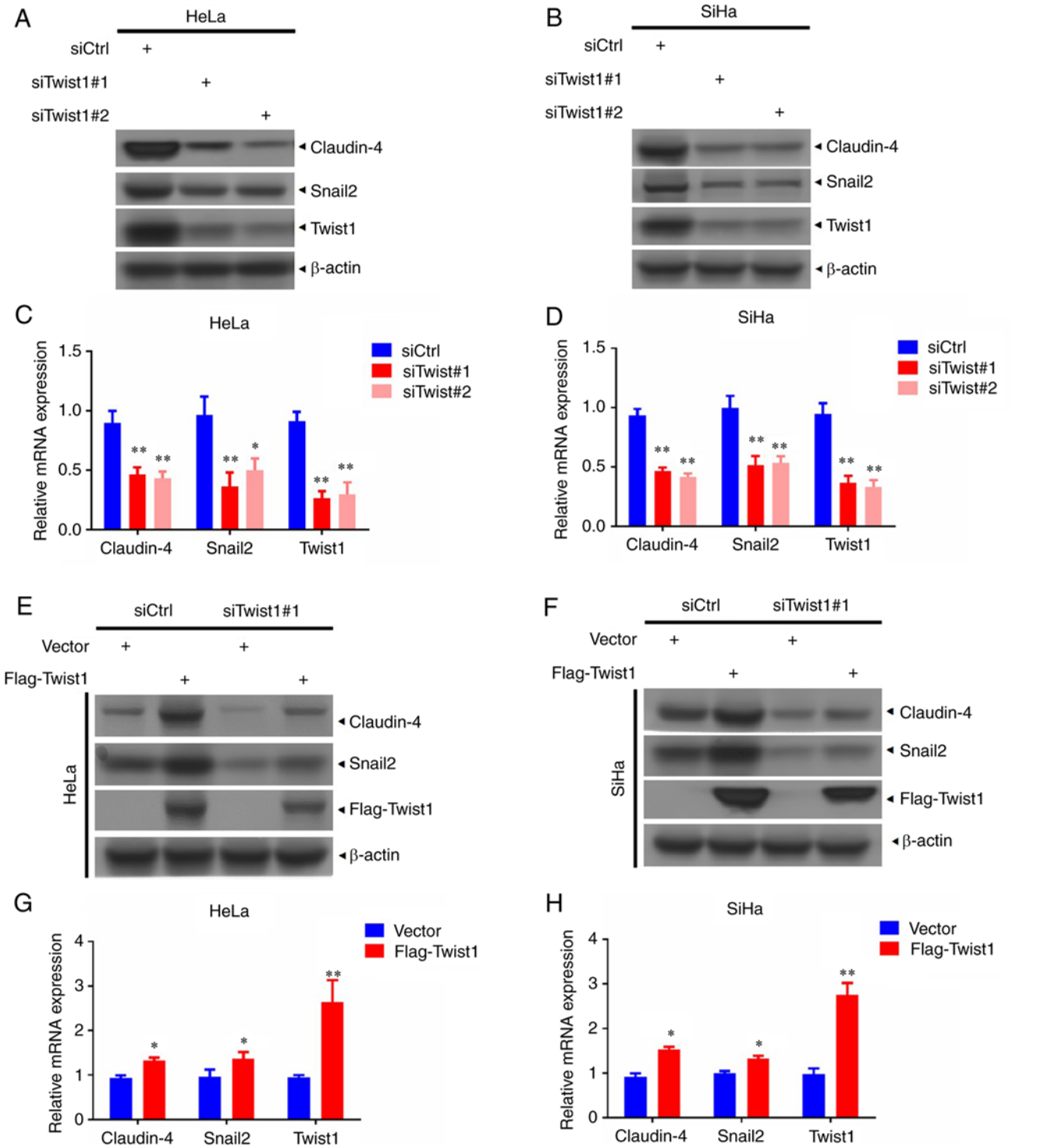

Figure 1. Twist1 positively regulates Claudin-4 expression. Transfection of HeLa and SiHa cells with 20 nM siRNA targeting Twist1 or scrambled control siRNA was performed. Briefly, 48 h after transfection, HeLa (A) and SiHa (B) cells were subjected to western blotting. Transfection of HeLa and SiHa cells with 20 nM siRNA targeting Twist1 or scrambled control siRNA was performed. 48 h after transfection, HeLa (C) and SiHa (D) cells were subjected to RT-qPCR analysis. *P<0.05, **P<0.01 vs. siCtrl. Transfection of 20 nM siRNA targeting Twist1 or scrambled control siRNA into (E) HeLa and (F) SiHa cells. Co-transfection with carrier or an overexpression plasmid for Twist1 was performed 24 h after transfection. Cells were collected 48 h after transfection and examined by western blotting using specific antibodies. After treatment, (G) HeLa and (H) SiHa cells were collected 48 h after transfection and analyzed by RT-qPCR. *P<0.05, **P<0.01 vs. vector. RT-q, reverse transcription-quantitative PCR; siRNA, small interfering RNA; Ctrl, control.

significantly decreased compared with that of the WT promoter (Claudin-4 Luc WT), whereas the -1,500-2,200del mutant (Claudin-4 Luc E-Boxl) promoter activity was comparable to that of Claudin-4 Luc WT (Fig. 2D and E). Twist1 overexpression enhanced the promoter activity of Claudin-4 Luc WT and Claudin-4 Luc E-Boxl to a comparable degree but with no effect on the promoter activity of Claudin-4 Luc E-Box2 (Fig. 2F), suggesting that the -206-1,500-bp region and E-Box1 within the Claudin-4 promoter is necessary for the transcriptional activation of Claudin-4 by Twist1.

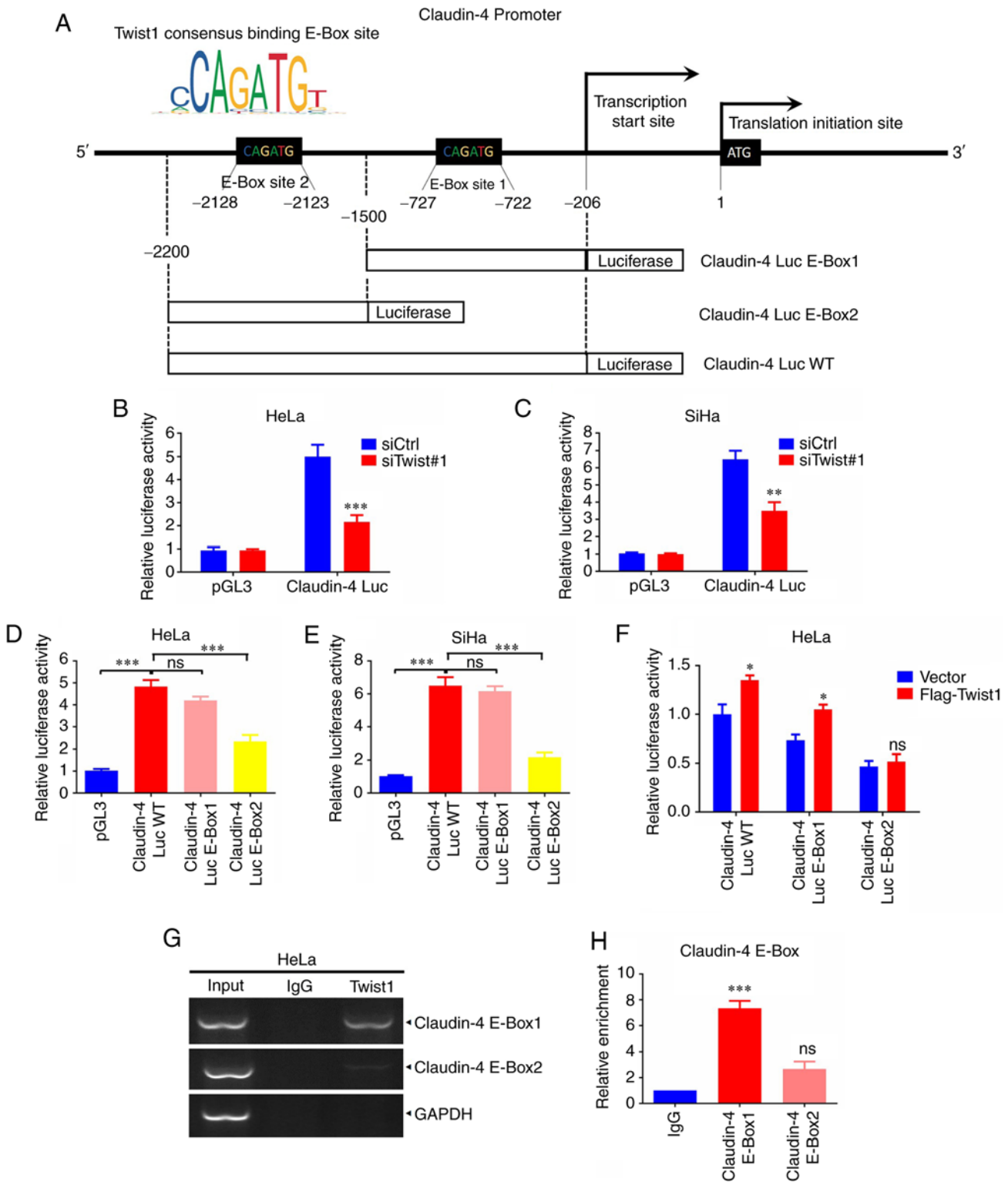

Figure 2. Twist1 upregulates Claudin-4 transcription via E-Boxes in the Claudin-4 promoter. (A) Potential Twist1 consensus binding E-Boxes sites in the Claudin-4 promoter region. E-Box1 and E-Box2 sites upstream of the start codon of Claudin-4 are shown. The constructed deletion mutants of the Claudin-4 promoter (-1,500-2,200del and -206-1,500del) and Claudin-4 full-length promoter reporter constructs are presented. Transfection of (B) HeLa and (C) SiHa cells with 20 nM siRNA targeting Twist1 or scrambled control siRNA was performed. Plasmids expressing pGL3 or pGL3 carrying the Claudin-4 promoter (Claudin-4-Luc) with two potential Twist1 binding E-Box domains and Renilla luciferase were co-transfected. Luciferase activity was measured after 24 h using the dual-luciferase reporter assay system and normalized to *Renilla* luciferase activity. **P<0.01, ***P<0.001 vs siCtrl. (D) HeLa and (E) SiHa cells containing co-expression of plasmids that express *Renilla* luciferase and pGL3 or pGL3 bearing the Claudin-4 promoter with two potential Twist1-binding E-Box sites (WT), with the ablation of E-Box2 (Claudin-4 Luc E-Box1) or with the ablation of E-Box1 (Claudin-4 Luc E-Box2), followed by dual-luciferase reporter assay. ***P<0.001 vs. Claudin-4 Luc WT. (F) Control or an overexpression plasmid of Twist1 was transfected into HeLa cells. Co-transfection with Claudin-4 Luc WT, Claudin-4 Luc E-Box1, or Claudin-4 Luc E-Box2 plasmids was performed 24 h after transfection. Luciferase activity was measured after another 24 h. Fresh HeLa cells were harvested and lysed for chromatin immunoprecipitation assay with normal IgG or anti-Twist1 antibody. *P<0.05 vs. vector. (G) Promoter fragments were amplified by PCR utilizing primers specific for putative E-Box on the Claudin-4 promoter region or GAPDH (negative control). (H) Reverse transcription-quantitative PCR was performed to quantify the relative enrichment of Twist1 at potential Twist1-binding E-Box domains. ***P<0.001 vs. IgG. siRNA, small interfering RNA; siCtrl, scrambled control siRNA; WT, wild-type; del, deletion; ns, not significant.

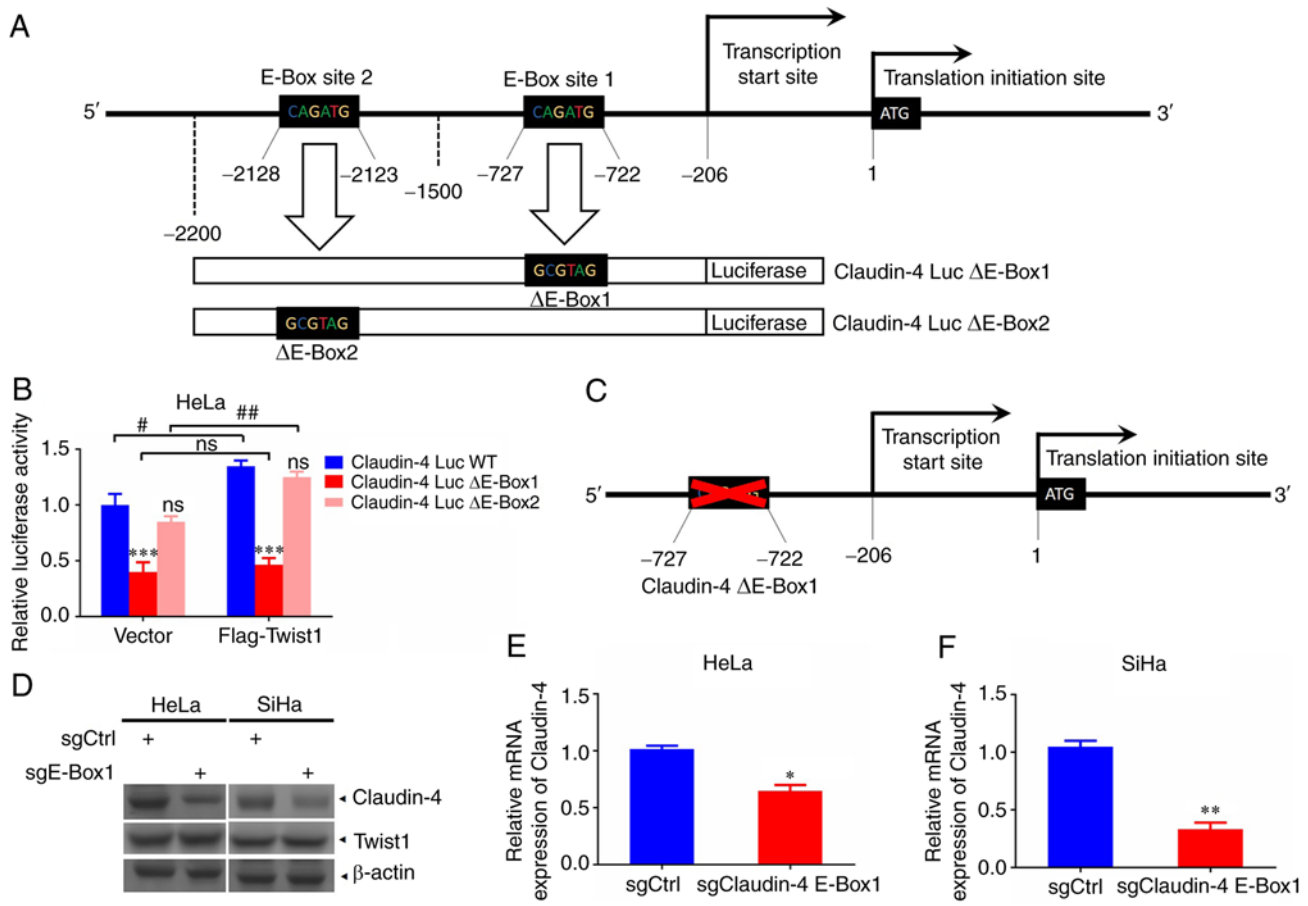

Figure 3. E-Box1, but not E-Box2, is necessary for Claudin-4 promoter activity. (A) E-Box1 and E-Box2 mutations in Claudin-4 promoter-luciferase constructs. (B) Twist1-overexpressing or a control plasmid was transfected in HeLa cells. The cells were transfected with Claudin-4 Luc plasmids 24 h after transfection, and then luciferase activity was measured 24 h later. ***P<0.001 vs. Claudin-4 Luc WT; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. vector. (C) *In situ* deletion of the E-Box1 site in the Claudin-4 promoter region. (D) Transfection of the construct carrying the specific single-guide RNA into HeLa and SiHa cells was followed by puromycin selection. Western blotting on cells that had been cultured from single clones with E-Box1 deletion. Reverse transcription-quantitative PCR on (E) HeLa and (F) SiHa cells that had been cultured from single clones with E-Box1 deletion. *P<0.05, **P<0.01 vs. sgCtrl. WT, wild-type; ns, not significant; sg, single-guide.

ChIP assay revealed that the Twist1 protein was preferentially recruited to the Claudin-4 E-Box1 region of the Claudin-4 promoter but not to the Claudin-4 E-Box2 region (Fig. 2G). RT-qPCR analysis confirmed that the Twist1-binding E-Box1 region on the Claudin-4 promoter was enriched sevenfold compared with the control group (Fig. 2H). Overall, in addition to direct binding action, Twist1 transactivated the Claudin-4 promoter, confirming that Twist1 binds to Claudin-4 as one of its downstream targets. Moreover, Claudin-4 promoter activity and Twist1-mediated Claudin-4 transcription were both dependent on the E-Box1 region, which was located primarily near the transcription initiation site.

E-Box1, but not E-Box2, is necessary for Claudin-4 promoter activity. To confirm that the E-Box1 domain in the Claudin-4 promoter was the Twist1-binding site, potential Twist1-binding domains were deleted while constructing two luciferase reporters (Claudin-4 ΔE-Box1 and ΔE-Box2; Fig. 3A). Following E-Box1 deletion, the activity of the luciferase reporter was significantly decreased (Fig. 3B). Twist1 overexpression induced promoter activity of Claudin-4 Luc WT and Claudin-4 ΔE-Box2 to a similar degree but there was no evident effect on Claudin-4 ΔE-Box1 (Fig. 3B), indicating that the Claudin-4 promoter relied on the potential Twist1-binding E-Box1 domain, but not the E-Box2 domain.

Furthermore, to ascertain whether Claudin-4 expression was regulated by the putative Twist1-binding E-Box1 domain under physiological conditions. CRISPR-Cas9 knockout was performed to remove E-Box1 from the chromosome 7 Claudin-4 promoter without altering the transcriptional start codon (Fig. 3C). Notably, following E-Box1 knockout, both Claudin-4 mRNA and protein expression levels were significantly reduced in HeLa and SiHa cells (sgClaudin-4 E-Box1; Fig. 3D-F). Collectively, these findings provide proof of concept that Twist 1 specifically bound to the E-Box1 domain of the Claudin-4 promoter and stimulated its transcription.

TGF-β induces Claudin-4 expression by upregulating Twist1. TGF-β is a pleiotropic cytokine that impedes cell proliferation, stimulates cell migration, invasion and differentiation and causes Twist1 overexpression (42). Next, it was examined whether TGF-β promoted Claudin-4 expression at the gene and protein expression levels. TGF-β treatment of HeLa and SiHa cells dose-dependently increased Twist1, Snail2 and Claudin-4 protein levels (Fig. 4A), indicating that TGF-β can stimulate the expression of Twist1 and thus increase expression of Snail2

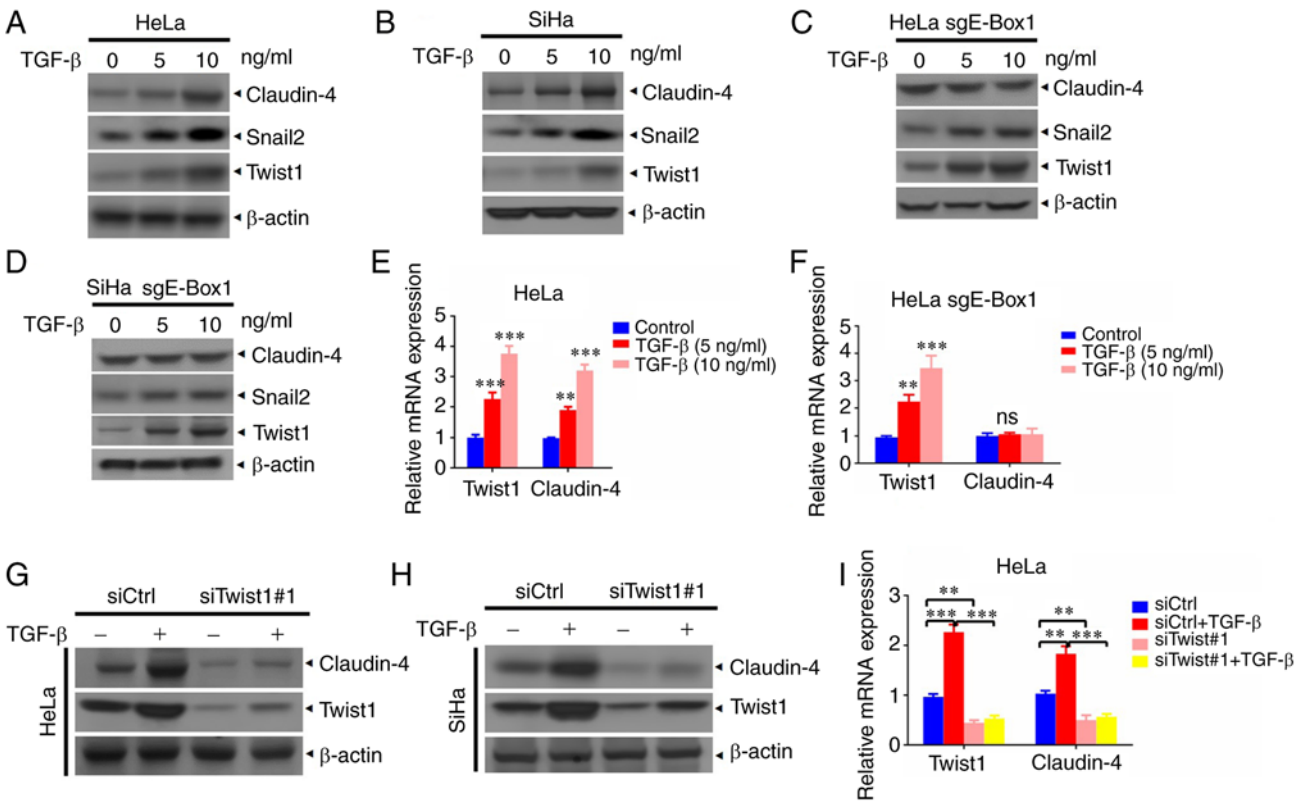

Figure 4. TGF-β induces Claudin-4 expression by upregulating Twist1. Treatment of (A) HeLa and (B) SiHa cells with TGF-β for 48 h, and whole-cell extracts were harvested for western blotting using specific antibodies. (C) HeLa and (D) SiHa cells with E-Box1 of Claudin-4 deletion using the CRISPR-Cas9 system were treated with TGF-β for 48 h, and whole-cell extracts were harvested for western blotting using specific antibodies. (E) Wild-type HeLa and (F) E-Box1-deleted HeLa cells were exposed to TGF-β for 48 h before RT-qPCR analysis of Twist1 and Claudin-4. **P<0.01, ***P<0.001 vs. control. A total of 20 nM siRNA against Twist1 or scrambled control siRNA was transfected into HeLa and SiHa cells. Cells were treated with TGF-β. (G) HeLa and (H) SiHa cells were subsequently harvested for immunoblotting with specific antibodies. (I) Following treatment, HeLa cells were subsequently harvested for RT-qPCR analysis of Twist1 and Claudin-4. **P<0.01, ***P<0.001 vs. siCtrl or siCtrl + TGF- β. RT-q, reverse transcription-quantitative; siRNA, small interfering RNA; Ctrl, control; ns, not significant.

and Claudin-4 (Fig. 4B). Twist1 and Claudin-4 expression was transcriptionally stimulated by TGF-β (Fig. 4E). Additionally, Twist1 knockdown prevented TGF-β from inducing Claudin-4 expression at the gene and protein expression levels in SiHa and HeLa cells (Fig. 4G-I), suggesting that TGF-β promoted Claudin-4 expression by upregulating Twist1 expression.

To clarify the regulation mechanisms of TGF-β on Claudin-4, sgClaudin-4 E-Box1 HeLa and SiHa cells were treated with a gradient concentration of TGF-β. TGF-β treatment caused dose-dependent upregulation of Twist1 and Snail2 expression but had no effect on Claudin-4 protein levels (Fig. 4C and D). Similar effects were observed at the transcriptional level (Fig. 4F). Collectively, these data confirmed that TGF-β mediated Claudin-4 expression through the TGF-β/Twist1/E-Box1 axis.

Claudin-4 knockdown inhibits TGF-β-induced cell migration and invasion. TGF-β causes cancer cells to migrate and invade their surroundings (43,44). Whether Claudin-4 contributes to migration and invasion abilities of cervical cancer cells is not yet known. After treating HeLa cells with TGF-β and transfecting them with Twist1 and Claudin-4 siRNA, wound healing assay was performed to ascertain whether Claudin-4 was involved in the TGF-β-triggered migration and invasion of cervical cancer cells. At 24 and 48 h after wound establishment, the wound area in the control group was considerably enlarged compared with that in the TGF-β group, demonstrating that TGF-β stimulated HeLa cell migration (Fig. 5A and B). Consistently, as demonstrated by Transwell migration experiment, TGF-β significantly boosted HeLa cell migration (Fig. 5C and D). However, the knockdown of Twist1 or Claudin-4 reduced cell migration and diminished the ability of TGF-β to promote cell migration (Fig. 5A-D). Similarly, TGF-β improved cell invasion, as evidenced by the results of the Transwell invasion assay (Fig. 5E and F). Moreover, TGF-β promoted cell invasion, whereas silencing of Twist1 or Claudin-4 reversed this effect (Fig. 5E and F). Western blotting was performed to evaluate the efficiency of knocking down Twist1 and Claudin-4 (Fig. 5G). In summary, Claudin-4, similar to Twist1, mediated TGF-β-stimulated migration and invasion of cervical cancer cells.

Claudin-4 knockdown suppresses cell migration and invasion caused by Twist1. As the aforementioned data showed that the oncogenic transcriptional regulator Twist1 targets Claudin-4, it was analyzed whether Claudin-4 functioned downstream of Twist1 to facilitate cancer cell migration and invasion. Transwell migration/invasion experiment showed that upregulated expression of Twist1 in HeLa cells facilitated the migration and invasion of cervical cancer cells (Fig. 6A-D).

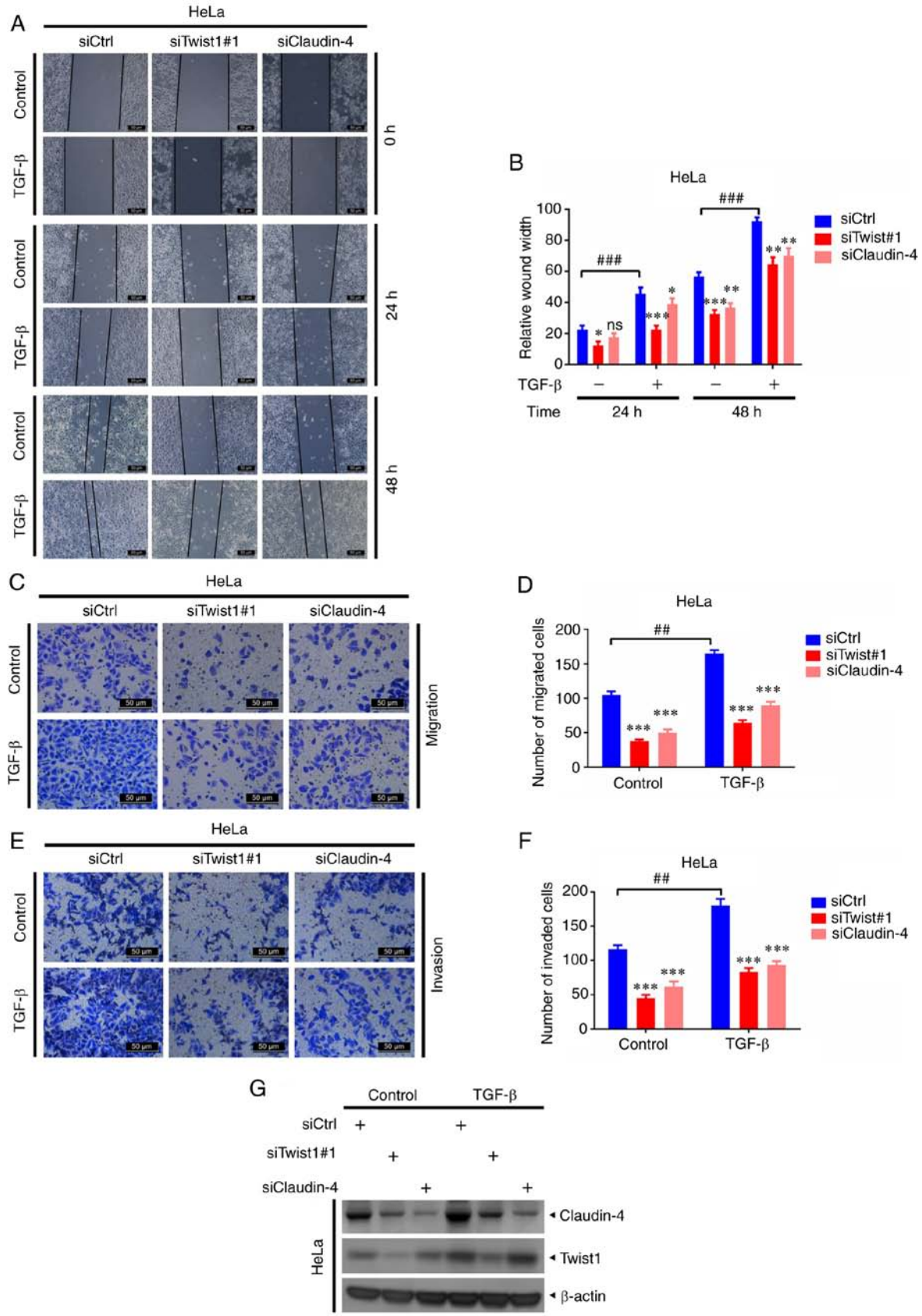

Figure 5. Claudin-4 knockdown inhibits TGF-β-induced cell migration and invasion. (A) Wound healing experiments were performed after transfecting HeLa cells with 20 nM siRNA and TGF-β treatment. (B) Cell migratory ability was measured as relative healing wound area compared with that at 0 h. (C) Transfection of HeLa cells with 20 nM siRNA followed by treatment with or without TGF-β before the Transwell migration assay. Images captured at 12-24 h demonstrate cells that migrated. (D) Cells that could migrate were counted in three randomly selected fields of view/chamber insert. (E) Transfection of HeLa cells with 20 nM siRNA followed by treatment with or without TGF-β before the Transwell invasion assay. Images captured at 12-24 h demonstrate cells that invaded. (F) Cells that could invade were counted in three randomly selected fields of view/chamber insert. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. TGF-β untreated. *P<0.05, **P<0.01, ***P<0.001 vs. siCtrl. (G) Western blotting assay was performed to evaluate efficiency of knocking down Twist1 and Claudin-4. siRNA, small interfering RNA; siCtrl, scrambled control siRNA; ns, not significant.

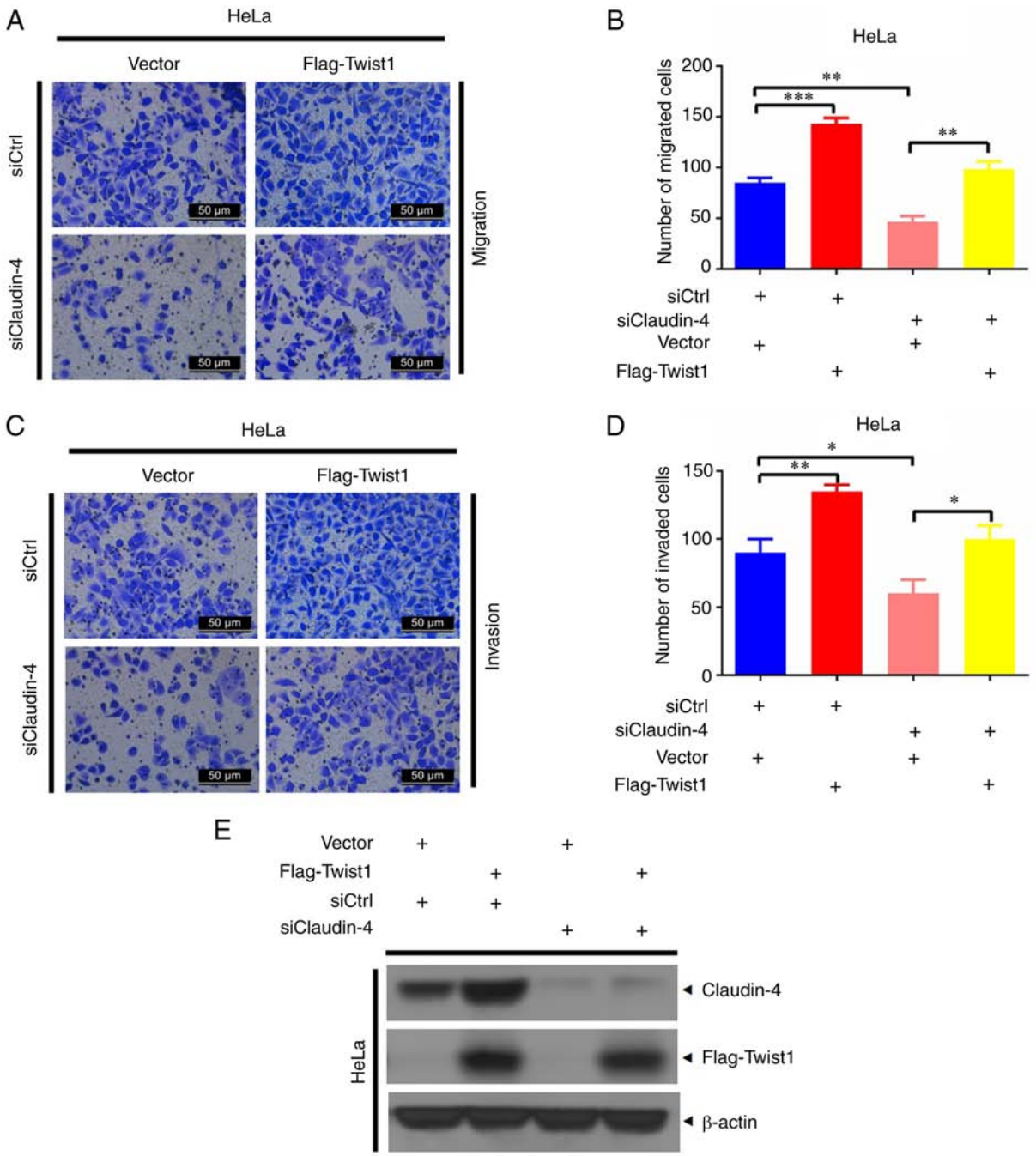

Figure 6. Claudin-4 knockdown suppresses cell migration and invasion caused by Twist1. (A) Specific siRNA (20 nM) was transfected into HeLa cells. Co-transfection with vector or a Twist1-overexpressed plasmid was performed on cells 24 h after transfection, and 12-24 h later, cells were assessed by Transwell migration assay. (B) Cells that could migrate were counted in three randomly selected fields of view/chamber insert. (C) After treatment, HeLa cells were assessed by Transwell invasion assay. (D) Cells that could invade were counted in three randomly selected fields of view/chamber insert. *P<0.05, **P<0.01, ***P<0.001 vs. siCtrl + vector or siClaudin-4 + vector. (E) Cell lysates were collected, and efficiency of Claudin-4 knockdown or Twist1 overexpression was assessed by western blot analysis. siRNA, small interfering RNA; siCtrl, scrambled control siRNA.

However, Claudin-4 silencing halted cell migration and counteracted Twist1 pro-invasive effects (Fig. 6A-D). Additionally, the western blot assay verified the efficacy of Twist1 overexpression and Claudin-4 knockdown (Fig. 6E). Collectively, these results suggested that Claudin-4 served as a downstream effector of Twist1 to increase cell migration and invasion.

Deletion of E-box 1 on the Claudin-4 promoter inhibits cervical cancer cell migration and invasion. Given that E-Box1 was critical for Twist1-induced Claudin-4 transcription and Claudin-4 promoted cell migration and invasion downstream of Twist1. Migration and invasion abilities of Claudin-4 WT and sgClaudin-4 E-Box1 HeLa and SiHa cells were assessed to determine the role and potential mechanism of Claudin-4 in cervical cancer cells. Downregulation of Claudin-4 by sgClaudin-4 E-Box1 significantly suppressed cervical cancer cell migration, as demonstrated by decreased number of migrating cells in the Transwell migration assay (Fig. 7A-C) and slower wound healing (Fig. 7G-J). Consistently, sgClaudin-4 E-Box1 significantly decreased SiHa and HeLa cell invasion in

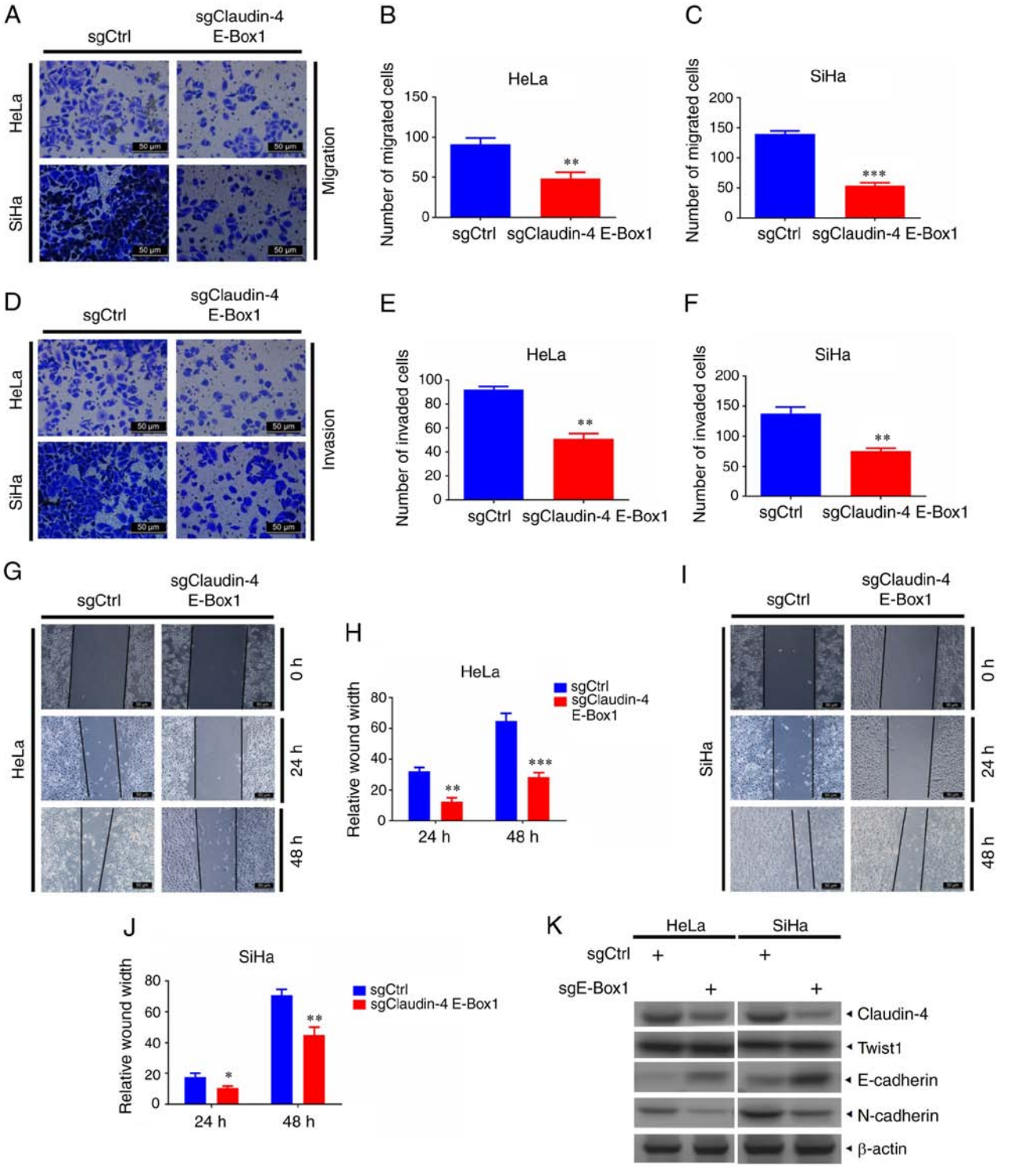

Figure 7. Deletion of E-box 1 on Claudin-4 promoter inhibits cervical cancer cell migration and invasion. (A) HeLa and SiHa cells were transfected with sgRNA and subjected to the Transwell migration assays. Representative images of migratory cells at 12-24 h are presented. The number of migratory (B) HeLa and (C) SiHa cells were counted in three random fields/chamber. (D) HeLa and SiHa cells were transfected with sgRNA and then seeded into a 24-well plate in a serum-free medium with Matrigel and cultured for 12-24 h, followed by staining. Representative images of invasive cells at 12-24 h. The number of invaded (E) HeLa and (F) SiHa cells were counted in three random fields/chamber insert. (G) HeLa cells were transfected with sgRNA and then assessed using the wound healing assay. (H) The cell migration was determined as the relative healing wound area compared with that observed at 0 h. (I) SiHa cells were transfected with sgRNA and assessed using the wound healing assay. (J) Cell migration was determined as the relative healing wound area compared with that observed at 0 h. *P<0.05, **P<0.01, ***P<0.001 vs. sg Ctrl). (K) Cell lysates were collected and assessed using western blotting. Ctrl, control; sgRNA, single-guide RNA.

Transwell invasion assay (Fig. 7D-F). Furthermore, deletion of E-Box1 led to upregulated expression of the epithelial marker E-cadherin and downregulated expression of the mesenchymal marker N-cadherin in HeLa and SiHa cells (Fig. 7K), indicating that E-Box1 deletion suppressed EMT induction, one of the hallmarks of tumor progression that is associated with

cellular migration and invasion (45). Collectively, these results suggested that deletion of E-Box1 on the Claudin-4 promoter inhibited the ability of cervical cancer cells to migrate and invade via suppressing EMT by promoting E-cadherin expression and lowering N-cadherin levels.

Discussion

Metastasis is a process wherein cancerous cells move from the original site to various parts of the body (46). A cancer cell must pass through several stages before it metastasizes. EMT is an essential stage in cancer progression (13). In this stage, epithelial cells become more detached and motile due to the downregulation of cell-to-cell adhesion structures, alteration in polarity, and reorganization of their cytoskeleton (13). Sealing off spaces between polarized epithelium or endothelium with large intercellular adhesion complexes called TJs is essential to maintain tissue barrier integrity (30). TJs in cancerous cells become 'loosened' or disassembled throughout the metastatic phase, which facilitates migration and dissemination. Claudins serve a key role in TJs, wherein they create heteromeric and homomeric connections between adjoining cells and are key regulators of cancer development and metastasis (47). Claudin serves as a tumor suppressor and is often downregulated in cancers. Accumulating evidence suggests that claudin-1 downregulation in gastric cancer cells improves tumorigenicity *in vivo* and the metastatic, migratory and invasive potential of lung cancer cells is inhibited by claudin-1 overexpression (48,49). Claudin-6 expression is decreased in invasive ductal carcinoma of the breast and this decline is negatively associated with lymph node metastases (50). A correlation exists between downregulated claudin-7 expression and higher tumor grade as well as locoregional and distant metastasis, particularly locoregional tumor recurrence (51).

Certain claudin proteins exhibit upregulated expression in cancers and serve as a cancer-promoting factor, which may potentially promote the metastatic phenotype. For example, melanoma cells have greater cell migration and invasion abilities due to claudin-1 expression, which activates matrix metalloproteinase (MMP)-2 (52). Claudin-3 overexpression in ovarian epithelial cells enhances invasion via MMP stimulation and upregulation of claudin-3 expression is associated with ovarian cancer progression (53,54). Claudin-6 overexpression in gastric cancer cell lines increases ability to invade, migrate and proliferate (55). In esophageal squamous cell carcinoma, claudin-7 overexpression promotes both cell proliferation and metastatic activity (56). Decreased claudin expression in various cancers together with upregulated claudin in normal tissues predicts poor survival. However, unfavorable prognosis may also be predicted by the ectopic expression of claudin in a tissue that, under normal circumstances, does not express this claudin (28,57). Thus, claudins function in a cell- or a tissue-specific manner to either promote or suppress cancer formation.

Similar to other claudin proteins, Claudin-4 may either inhibit or promote tumor growth. Notably, Claudin-4 exerts a tumor-suppressing effect in diverse malignancies. Pancreatic cancer cells that express Claudin-4 have decreased invasive and metastatic ability (58). Kwon *et al* (48) confirmed that Claudin-4 overexpression suppresses gastric cancer cell migration and invasion without slowing cellular proliferation. Moreover, decreased Claudin-4 expression is associated with poor prognosis in individuals with pancreatic, colon, esophageal and breast cancer (30). Conversely, Claudin-4 overexpression occurs in esophageal, gastric, biliary and ovarian cancer (34,54). Moreover, tumor invasion and MMP-2 activity and expression are correlated with Claudin-4 expression in ovarian and gastric cancer (53,59). Claudin-4 overexpression occurs in cervical cancer tissue compared with that in the adjacent non-tumor tissue (36). To the best of our knowledge, however, functional implications of Claudin-4 in cervical cancer are unknown. It is unknown whether Claudin-4 contributes to spread and invasion of cervical cancer cells. The present study demonstrated that Claudin-4 promoted cervical cancer cell migration and invasion by decreasing E-cadherin and increasing N-cadherin levels, which clarifies its role in these processes.

Numerous mechanisms have been implicated in claudin expression regulation. Claudin expression may be regulated transcriptionally by TFs. Snail is a transcription suppressor that serves a key role in EMT. In mouse epithelium, Snail suppresses the gene expression of claudin-1, -3, -4, and -7 and E-cadherin by binding specifically to promoter regions of these genes (60). Epigenetic mechanisms serve a key role in transcriptional regulation of claudin expression. For example, DNA hypermethylation is associated with claudin-7 expression suppression in breast cancer cells (61). Conversely, DNA hypomethylation is associated with Claudin-4 overexpression in ovarian cancer (62). In gastric cancer, Claudin-4 overexpression is linked to DNA hypomethylation and depletion of suppressive histone methylations such as H4K20me3 (63). Additionally, the regulation of claudin expression by microRNAs (miRs) is a second epigenetic process that has been uncovered (64,65). For example, miR-155 inhibits claudin-1 protein and mRNA expression in ovarian cancer precursor cells (64). To the best of our knowledge, the present study identified a novel mechanism for regulating Claudin-4. Specifically, there was a positive correlation between Twist1 activity and Claudin-4 expression as Claudin-4 is a downstream target of this helix-loop-helix transcriptional regulator. Mechanistically, Twist1 transactivated production of Claudin-4 by binding specifically to the gene promoter. CRISPR-Cas9 was used to delete Twist1-binding E-Box1 domain from the Claudin-4 promoter, which resulted in low expression of Claudin-4 and suppression of cervical cancer cell migration and invasion by increasing E-cadherin and decreasing N-cadherin expression. Furthermore, Twist1, following activation by TGF-β, induced Claudin-4 expression, leading to cervical cancer cell migration and invasion. While mRNA expression of Twist1 and Claudin-4 in cervical tumor tissue showed no significant correlation by Spearman's correlation analysis. The reason for this negative result may be due to the small number of cervical cancer samples in the database which leads to the bias of the results, or the GC preference of RNA-sequencing data may affect the final results.

Because Claudin-4 serves a key function in the metastasis of cervical cancer, antibody-based treatments targeting this protein hold promise. Antibodies that can identify human Claudin-4 at its extracellular loops have been successfully produced and anti-Claudin-4 antibody has demonstrated anticancer efficacy in both cell culture and animal models (66,67).

Additionally, a dual-targeting anti-Claudin-4 monoclonal antibody with anticancer activity has been developed and validated both *in vivo* and *in vitro* (68). To the best of our knowledge, the processes that drive Claudin-4-facilitated tumor progression and metastasis are not known. Claudin-4-targeted therapies for cervical cancer treatment require further exploration.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ collected and analyzed data and wrote and edited the manuscript. QJ conceived and designed the study and edited the manuscript. Both authors have read and approved the final manuscript. JZ and QJ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Stelzle D, Tanaka LF, Lee KK, Ibrahim Khalil A, Baussano I, Shah ASV, McAllister DA, Gottlieb SL, Klug SJ, Winkler AS, *et al*: Estimates of the global burden of cervical cancer associated with HIV. Lancet Glob Health 9: e161-e169, 2021.
2. Zhang S, Xu H, Zhang L and Qiao Y: Cervical cancer: Epidemiology, risk factors and screening. Chin J Cancer Res 32: 720-728, 2020.
3. Arbyn M, Weiderpass E, Bruni L, de Sanjosé S, Saraiya M, Ferlay J and Bray F: Estimates of incidence and mortality of cervical cancer in 2018: A worldwide analysis. Lancet Glob Health 8: e191-e203, 2020.
4. Park SH, Kim M, Lee S, Jung W and Kim B: Therapeutic potential of natural products in treatment of cervical cancer: A Review. Nutrients 13: 154, 2021.
5. Vu M, Yu J, Awolude OA and Chuang L: Cervical cancer worldwide. Curr Probl Cancer 42: 457-465, 2018.
6. Wang D, Li Q, Li K, Xiao P and Yin R: Twist-related protein 1-mediated regulation of mesenchymal change contributes to the migration and invasion of cervical cancer cells. Oncol Lett 10: 3107-3112, 2015.
7. Liao Y, Huang J, Liu P, Zhang C, Liu J, Xia M, Shang C, Ooi S, Chen Y, Qin S, *et al*: Downregulation of LNMAS orchestrates partial EMT and immune escape from macrophage phagocytosis to promote lymph node metastasis of cervical cancer. Oncogene 41: 1931-1943, 2022.
8. Cohen PA, Jhingran A, Oaknin A and Denny L: Cervical cancer. Lancet 393: 169-182, 2019.
9. Liu Y, Fan H, Dong D, Liu P, He B, Meng L, Chen J, Chen C, Lang J and Tian J: Computed tomography-based radiomic model at node level for the prediction of normal-sized lymph node metastasis in cervical cancer. Transl Oncol 14: 101113, 2021.
10. Chen C, Shen N, Chen Y, Jiang P, Sun W, Wang Q, Wang Z, Jiang Y, Cheng W, Fu S and Wang S: LncCCLM inhibits lymphatic metastasis of cervical cancer by promoting STAU1-mediated IGF-1 mRNA degradation. Cancer Lett 518: 169-179, 2021.
11. Roszik J, Ring KL, Wani KM, Lazar AJ, Yemelyanova AV, Soliman PT, Frumovitz M and Jazaeri AA: Gene expression analysis identifies novel targets for cervical cancer therapy. Front Immunol 9: 2102, 2018.
12. Balasubramaniam SD, Balakrishnan V, Oon CE and Kaur G: Key molecular events in cervical cancer development. Medicina (Kaunas) 55: 384, 2019.
13. Ribatti D, Tamma R and Annese T: Epithelial-Mesenchymal transition in cancer: A historical overview. Transl Oncol 13: 100773, 2020.
14. Sheng L and Zhuang S: New insights into the role and mechanism of partial epithelial-mesenchymal transition in kidney fibrosis. Front Physiol 11: 569322, 2020.
15. Ramesh V, Brabletz T and Ceppi P: Targeting EMT in Cancer with repurposed metabolic inhibitors. Trends Cancer 6: 942-950, 2020.
16. Navas T, Kinders RJ, Lawrence SM, Ferry-Galow KV, Borgel S, Hollingshead MG, Srivastava AK, Alcoser SY, Makhlouf HR, Chuaqui R, *et al*: Clinical evolution of Epithelial-Mesenchymal transition in human carcinomas. Cancer Res 80: 304-318, 2020.
17. Singh M, Yelle N, Venugopal C and Singh SK: EMT: Mechanisms and therapeutic implications. Pharmacol Ther 182: 80-94, 2018.
18. Lamouille S, Xu J and Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 15: 178-196, 2014.
19. Xiong H, Nie X, Zou Y, Gong C, Li Y, Wu H, Qiu H, Yang L, Zhuang L, Zhang P, *et al*: Twist1 Enhances hypoxia induced radioresistance in cervical cancer cells by promoting nuclear EGFR localization. J Cancer 8: 345-353, 2017.
20. Yeeravalli R, Kaushik K and Das A: TWIST1-mediated transcriptional activation of PDGFRbeta in breast cancer stem cells promotes tumorigenesis and metastasis. Biochim Biophys Acta Mol Basis Dis 1867: 166141, 2021.
21. Fattahi F, Saeednejad Zanjani L, Vafaei S, Habibi Shams Z, Kiani J, Naseri M, Gheytanchi E and Madjd Z: Expressions of TWIST1 and CD105 markers in colorectal cancer patients and their association with metastatic potential and prognosis. Diagn Pathol 16: 26, 2021.
22. Valdes-Mora F, Gomez del Pulgar T, Bandrés E, Cejas P, Ramírez de Molina A, Pérez-Palacios R, Gallego-Ortega D, García-Cabezas MA, Casado E, Larrauri J, *et al*: TWIST1 overexpression is associated with nodal invasion and male sex in primary colorectal cancer. Ann Surg Oncol 16: 78-87, 2009.
23. Wang XX, Yin GQ, Zhang ZH, Rong ZH, Wang ZY, Du DD, Wang YD, Gao RX and Xian GZ: TWIST1 transcriptionally regulates glycolytic genes to promote the Warburg metabolism in pancreatic cancer. Exp Cell Res 386: 111713, 2020.
24. Qin Q, Xu Y, He T, Qin C and Xu J: Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. Cell Res 22: 90-106, 2012.
25. Zheng Y, Dai M, Dong Y, Yu H, Liu T, Feng X, Yu B, Zhang H, Wu J, Kong W, *et al*: ZEB2/TWIST1/PRMT5/NuRD multicomplex contributes to the epigenetic regulation of EMT and metastasis in colorectal carcinoma. Cancers (Basel) 14: 3426, 2022.
26. Li J, Khan MA, Wei C, Cheng J, Chen H, Yang L, Ijaz I and Fu J: Thymoquinone Inhibits the Migration and Invasive Characteristics of Cervical Cancer Cells SiHa and CaSki In Vitro by Targeting Epithelial to Mesenchymal Transition Associated Transcription Factors Twist1 and Zeb1. Molecules 22: 2105, 2017.
27. Wei X, Wei Z, Li Y, Tan Z and Lin C: AKR1C1 contributes to cervical cancer progression via regulating TWIST1 expression. Biochem Genet 59: 516-530, 2021.
28. Li J: Targeting claudins in cancer: Diagnosis, prognosis and therapy. Am J Cancer Res 11: 3406-3424, 2021.
29. Tabaries S and Siegel PM: The role of claudins in cancer metastasis. Oncogene 36: 1176-1190, 2017.
30. Kwon MJ: Emerging roles of claudins in human cancer. Int J Mol Sci 14: 18148-18180, 2013.
31. Yoon CH, Kim MJ, Park MJ, Park IC, Hwang SG, An S, Choi YH, Yoon G and Lee SJ: Claudin-1 acts through c-Abl-protein kinase Cdelta (PKCdelta) signaling and has a causal role in the acquisition of invasive capacity in human liver cells. J Biol Chem 285: 226-233, 2010.

32. Liu W and Li M: The role of claudin-4 in the development of gastric cancer. Scand J Gastroenterol 55: 1072-1078, 2020.
33. Hicks DA, Galimanis CE, Webb PG, Spillman MA, Behbakht K, Neville MC and Baumgartner HK: Claudin-4 activity in ovarian tumor cell apoptosis resistance and migration. BMC Cancer 16: 788, 2016.
34. Neesse A, Griesmann H, Gress TM and Michl P: Claudin-4 as therapeutic target in cancer. Arch Biochem Biophys 524: 64-70, 2012.
35. Luo Y, Kishi S, Sasaki T, Ohmori H, Fujiwara-Tani R, Mori S, Goto K, Nishiguchi Y, Mori T, Kawahara I, *et al*: Targeting claudin-4 enhances chemosensitivity in breast cancer. Cancer Sci 111: 1840-1850, 2020.
36. Chenhong Di and Jin F: Value of combined detection of claudin 4 and high-risk human papilloma virus in high-grade squamous intraepithelial lesion and cervix squamous cell carcinoma. Zhejiang Da Xue Xue Bao Yi Xue Ban 47: 344-350, 2018 (In Chinese).
37. Lanczky A and Gyorffy B: Web-Based survival analysis tool tailored for medical research (KMplot): Development and implementation. J Med Internet Res 23: e27633, 2021.
38. Castro-Mondragon JA, Riudavets-Puig R, Rauluseviciute I, Lemma RB, Turchi L, Blanc-Mathieu R, Lucas J, Boddie P, Khan A, Manosalva Pérez N, *et al*: JASPAR 2022: The 9th release of the open-access database of transcription factor binding profiles. Nucleic Acids Res 50: D165-D173, 2022.
39. Shu J, Wang X, Yang X and Zhao G: ATM inhibitor KU60019 synergistically sensitizes lung cancer cells to topoisomerase II poisons by multiple mechanisms. Sci Rep 13: 882, 2023.
40. Meng J, Chen S, Han JX, Qian B, Wang XR, Zhong WL, Qin Y, Zhang H, Gao WF, Lei YY, *et al*: Twist1 Regulates Vimentin through Cul2 Circular RNA to Promote EMT in Hepatocellular Carcinoma. Cancer Res 78: 4150-4162, 2018.
41. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
42. Shao T, Song P, Hua H, Zhang H, Sun X, Kong Q, Wang J, Luo T and Jiang Y: Gamma synuclein is a novel Twist1 target that promotes TGF-β-induced cancer cell migration and invasion. Cell Death Dis 9: 625, 2018.
43. Zhang Y, Alexander PB and Wang XF: TGF-β family signaling in the control of cell proliferation and survival. Cold Spring Harb Perspect Biol 9: a022145, 2017.
44. Wrighton KH, Lin X and Feng XH: Phospho-control of TGF-beta superfamily signaling. Cell Res 19: 8-20, 2009.
45. Cho ES, Kang HE, Kim NH and Yook JI: Therapeutic implications of cancer epithelial-mesenchymal transition (EMT). Arch Pharm Res 42: 14-24, 2019.
46. Chambers AF, Groom AC and MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2: 563-572, 2002.
47. Hewitt KJ, Agarwal R and Morin PJ: The claudin gene family: Expression in normal and neoplastic tissues. BMC Cancer 6: 186, 2006.
48. Chang TL, Ito K, Ko TK, Liu Q, Salto-Tellez M, Yeoh KG, Fukamachi H and Ito Y: Claudin-1 has tumor suppressive activity and is a direct target of RUNX3 in gastric epithelial cells. Gastroenterology 138: 255-265.e1-e3, 2010.
49. Chao YC, Pan SH, Yang SC, Yu SL, Che TF, Lin CW, Tsai MS, Chang GC, Wu CH, Wu YY, *et al*: Claudin-1 is a metastasis suppressor and correlates with clinical outcome in lung adenocarcinoma. Am J Respir Crit Care Med 179: 123-133, 2009.
50. Osanai M, Murata M, Chiba H, Kojima T and Sawada N: Epigenetic silencing of claudin-6 promotes anchorage-independent growth of breast carcinoma cells. Cancer Sci 98: 1557-1562, 2007.
51. Sauer T, Pedersen MK, Ebeltoft K and Naess O: Reduced expression of Claudin-7 in fine needle aspirates from breast carcinomas correlate with grading and metastatic disease. Cytopathology 16: 193-198, 2005.
52. Leotlela PD, Wade MS, Duray PH, Rhode MJ, Brown HF, Rosenthal DT, Dissanayake SK, Earley R, Indig FE, Nickoloff BJ, *et al*: Claudin-1 overexpression in melanoma is regulated by PKC and contributes to melanoma cell motility. Oncogene 26: 3846-3856, 2007.
53. Agarwal R, D'Souza T and Morin PJ: Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. Cancer Res 65: 7378-7385, 2005.
54. Uthayanan L and El-Bahrawy M: Potential roles of claudin-3 and claudin-4 in ovarian cancer management. J Egypt Natl Canc Inst 34: 24, 2022.
55. Zavala-Zendejas VE, Torres-Martinez AC, Salas-Morales B, Fortoul TI, Montano LF and Rendon-Huerta EP: Claudin-6, 7, or 9 overexpression in the human gastric adenocarcinoma cell line AGS increases its invasiveness, migration, and proliferation rate. Cancer Invest 29: 1-11, 2011.
56. Lioni M, Brafford P, Andl C, Rustgi A, El-Deiry W, Herlyn M and Smalley KS: Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells. Am J Pathol 170: 709-721, 2007.
57. Kohmoto T, Masuda K, Shoda K, Takahashi R, Ujiro S, Tange S, Ichikawa D, Otsuji E and Imoto I: Claudin-6 is a single prognostic marker and functions as a tumor-promoting gene in a subgroup of intestinal type gastric cancer. Gastric Cancer 23: 403-417, 2020.
58. Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, Menke A, Fensterer H, Giehl K, Löhr M, *et al*: Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. Cancer Res 63: 6265-6271, 2003.
59. Hwang TL, Lee LY, Wang CC, Liang Y, Huang SF and Wu CM: Claudin-4 expression is associated with tumor invasion, MMP-2 and MMP-9 expression in gastric cancer. Exp Ther Med 1: 789-797, 2010.
60. Ikenouchi J, Matsuda M, Furuse M and Tsukita S: Regulation of tight junctions during the epithelium-mesenchyme transition: Direct repression of the gene expression of claudins/occludin by Snail. J Cell Sci 116: 1959-1967, 2003.
61. 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 22: 2021-2033, 2003.
62. Litkouhi B, Kwong J, Lo CM, Smedley JG III, McClane BA, Aponte M, Gao Z, Sarno JL, Hinners J, Welch WR, *et al*: Claudin-4 overexpression in epithelial ovarian cancer is associated with hypomethylation and is a potential target for modulation of tight junction barrier function using a C-terminal fragment of Clostridium perfringens enterotoxin. Neoplasia 9: 304-314, 2007.
63. Kwon MJ, Kim SH, Jeong HM, Jung HS, Kim SS, Lee JE, Gye MC, Erkin OC, Koh SS, Choi YL, *et al*: Claudin-4 overexpression is associated with epigenetic derepression in gastric carcinoma. Lab Invest 91: 1652-1667, 2011.
64. Qin W, Ren Q, Liu T, Huang Y and Wang J: MicroRNA-155 is a novel suppressor of ovarian cancer-initiating cells that targets CLDN1. FEBS Lett 587: 1434-1439, 2013.
65. Sonoki H, Sato T, Endo S, Matsunaga T, Yamaguchi M, Yamazaki Y, Sugatani J and Ikari A: Quercetin Decreases Claudin-2 Expression Mediated by Up-Regulation of microRNA miR-16 in Lung Adenocarcinoma A549 Cells. Nutrients 7: 4578-4592, 2015.
66. Suzuki M, Kato-Nakano M, Kawamoto S, Furuya A, Abe Y, Misaka H, Kimoto N, Nakamura K, Ohta S and Ando H: Therapeutic antitumor efficacy of monoclonal antibody against Claudin-4 for pancreatic and ovarian cancers. Cancer Sci 100: 1623-1630, 2009.
67. Fujiwara-Tani R, Mori S, Ogata R, Sasaki R, Ikemoto A, Kishi S, Kondoh M and Kuniyasu H: Claudin-4: A new molecular target for epithelial cancer therapy. Int J Mol Sci 24: 5494, 2023.
68. Kato-Nakano M, Suzuki M, Kawamoto S, Furuya A, Ohta S, Nakamura K and Ando H: Characterization and evaluation of the antitumour activity of a dual-targeting monoclonal antibody against claudin-3 and claudin-4. Anticancer Res 30: 4555-4562, 2010.