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ORIGINAL ARTICLE

Claudin 1 mediates tumor necrosis factor alpha-induced cell migration in human gastric cancer cells

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Abstract

AIM: To investigate the role of claudin 1 in the regulation of genes involved in cell migration and tumor necrosis factor alpha (TNF-α)-induced gene expression in human gastric adenocarcinoma cells.

METHODS: Knockdown experiments were conducted with claudin 1 small interfering RNA (siRNA), and the effects on the cell cycle, apoptosis, migration and invasion were analyzed in human gastric adenocarcinoma MKN28 cells. The gene expression profiles of cells were analyzed by microarray and bioinformatics.

RESULTS: The knockdown of claudin 1 significantly inhibited cell proliferation, migration and invasion, and increased apoptosis. Microarray analysis identified 245 genes whose expression levels were altered by the knockdown of claudin 1. Pathway analysis showed that the top-ranked molecular and cellular function was the cellular movement related pathway, which involved MMP7, TNF-SF10, TGFBR1, and CCL2. Furthermore, TNF- and nuclear frctor-κB were the top-ranked upstream regulators related to claudin 1. TNF-α treatment increased claudin 1 expression and cell migration in MKN28 cells. Microarray analysis indicated that the depletion of claudin 1 inhibited 80% of the TNF-α-induced mRNA expression changes. Further, TNF-α did not enhance cell migration in the claudin 1 siRNA transfected cells.

CONCLUSION: These results suggest that claudin 1 is an important messenger that regulates TNF-α-induced gene expression and migration in gastric cancer cells. A deeper understanding of these cellular processes may be helpful in establishing new therapeutic strategies for gastric cancer.

Key words: Tumor necrosis factor alpha; Claudin 1; Cell migration; Microarray; Gene expression change

Core tip: The objectives of the present research were to investigate the role of claudin 1 in the regulation of genes involved in cell migration and tumor necrosis factor alpha (TNF-α)-induced gene expression in human gastric adenocarcinoma cells. Claudin 1 small interfering RNA transfection significantly inhibited cell migration and invasion in gastric cancer cells. Micro-

array analyses showed that down-regulation of claudin 1changed the expression levels of many genes related to cellular movement and TNF-α signal. We showed that TNF-α treatment induces the expression of claudin 1 in gastric carcinoma cells, and the latter controls gene expression and cell migration as the signal mediator.

Shiozaki A, Shimizu H, Ichikawa D, Konishi H, Komatsu S, Kubota T, Fujiwara H, Okamoto K, Iitaka D, Nakashima S, Nako Y, Liu M, Otsuji E. Claudin 1 mediates tumor necrosis factor alpha-induced cell migration in human gastric cancer cells. *World J Gastroenterol* 2014; 20(47): 17863-17876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17863.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17863

INTRODUCTION

Claudin proteins play an essential role in the function of tight junction (TJ) and the maintenance of the polarity of epithelial cells, and 24 subtypes of the claudin have been identified[1-3]. On the other hand, it has been shown that claudin 1 may influence the biological behavior of tumor progression[4,5]. The expression of claudin 1 increases during tumor development of various types of neoplasm including gastrointestinal cancer[6-10]. Further, expression of claudin 1 has prognostic impact in colon cancer and is related to apoptosis in breast cancer cells[11-13]. In gastric cancer, it has been reported that regulation of claudin 1 is related to transformation in invasive front and metastatic lesion[6,13].

Tumor necrosis factor alpha (TNF-α) is involved in epithelial-mesenchymal transition (EMT)[14] and related to the malignant behavior of epithelial tumors by regulating cell migration and invasion. The progression of EMT usually decreases the expression of TJ and adherens junction proteins, such as claudins, occludins and E-Cadherin[15-20]. However, we recently found that TNF-α treatment increased the expression of claudin 1 in human lung carcinoma A549 cells[21]. Further studies showed that claudin 1 plays an important role in TNF-α-induced gene expression and cellular movement in A549 cells[21].

The objective of the present research was to investigate the role of claudin 1 in the regulation of genes involved in migration and TNF-α-induced gene expression in human gastric adenocarcinoma cells. Our results showed claudin 1 small interfering RNA (siRNA) transfection significantly inhibited cell migration and invasion in gastric cancer cells. Furthermore, microarray analyses showed that down-regulation of claudin 1changed the expression levels of many genes related to cellular movement and TNF-α signal. Our results indicate that claudin 1 plays an important role in TNF-α-induced gene expression and cellular movement in gastric carcinoma cells.

MATERIALS AND METHODS

Cell lines, antibodies, and other reagents

The human gastric adenocarcinoma cell lines MKN28, NUGC4, MKN45 and Kato-Ⅲ were grown in plastic culture flasks (Corning Incorporated, NY, United States) and maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin, as described previously[22]. The flasks were kept in a humidified incubator at 37 ℃ under 5.0% CO_2[22].

The following antibodies were used in the study; claudin 1 antibody (Zymed Laboratories, San Francisco, CA), a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA), and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Human TNF-α was from R&D Systemic Inc. (Minneapolis, MN).

Protein studies

Cells were harvested in M-PER lysis buffer supplemented with protease inhibitors (Pierce, Rockford, IL). The protein concentration was measured with a modified Bradford assay (Bio-Rad, Hercules, CA)[23]. Cell lysates containing equal amounts of total protein were separated by SDS-PAGE and then were transferred onto PVDF membranes (GE Healthcare, Piscataway, NJ)[23]. The membranes were probed with the indicated antibodies, and proteins were detected by the ECL Plus Western Blotting Detection System (GE Healthcare)[23,24].

siRNA transfection

Cells were transfected with 10 nmol/L claudin 1 siRNA (Santa Cruz) using the Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA). The medium containing the siRNA was replaced with fresh medium after 24 h[23,24]. The provided control siRNA (Santa Cruz) was used as a negative control. The siRNA transfected cells were harvested 48 h after transfection for protein studies[23,24]. Further, we used a second independent claudin 1 siRNA, Stealth RNAi siRNA targeting claudin 1 mRNA (Invitrogen) to exclude off target effects.

Real-time quantitative RT-PCR

Total RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA)[24]. Messenger RNA (mRNA) expression was measured by a quantitative real-time PCR (7300 Real-Time PCR System; Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays (Applied Biosystems). The expression level of claudin-1 gene (Hs00221623_m1; Applied Biosystems) was measured, and normalized against the housekeeping gene beta-actin (ACTB, Hs01060665_g1; Applied Biosystems). Each assay was performed in triplicate.

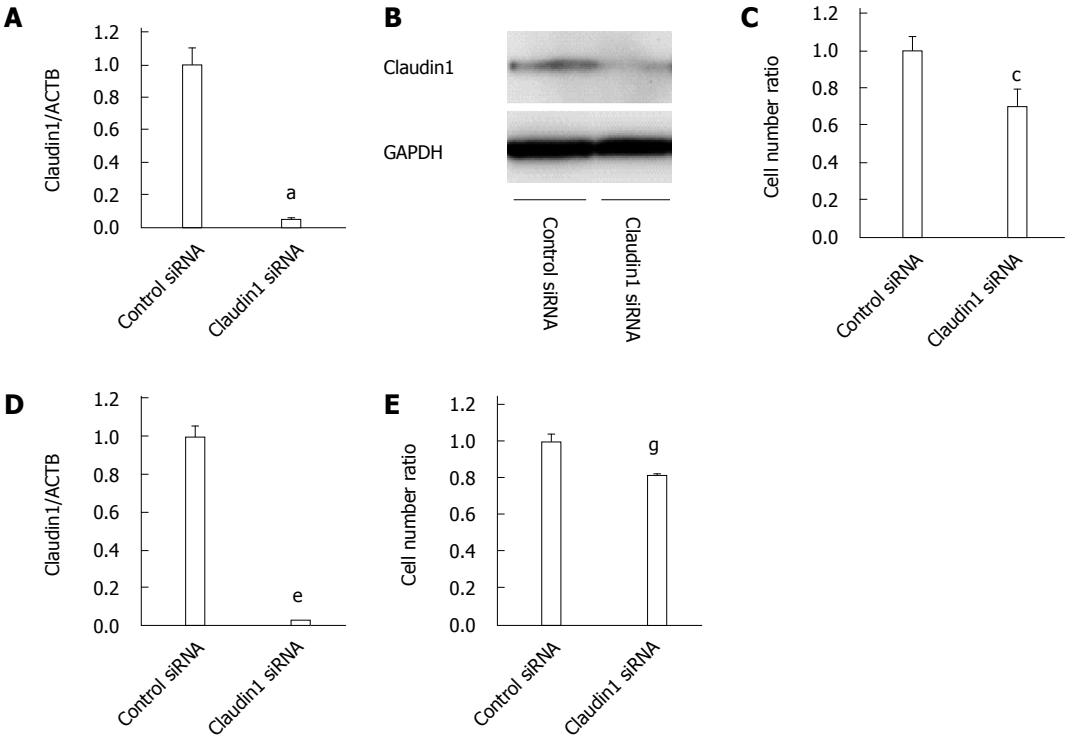


Figure 1 Claudin 1 controls cell proliferation in MKN28 cells. A: Claudin 1 siRNA effectively reduced the mRNA levels of claudin 1 in MKN28 cells. Mean ± SE. $n = 3$. [a]$P < 0.05$ *vs* control siRNA; B: Western blotting revealed that claudin 1 siRNA reduced the protein levels of claudin 1 in MKN28 cells; C: Downregulation of claudin 1 inhibited the proliferation of MKN28 cells. The number of cells was counted 48 h after siRNA transfection. Mean ± SE. $n = 3$. [c]$P < 0.05$ *vs* control siRNA; D: A second independent claudin 1 siRNA also reduced the mRNA levels of claudin 1 in the MKN28 cells. Mean ± SE. $n = 3$. [e]$P < 0.05$ *vs* control siRNA; E: A second independent claudin 1 siRNA also inhibited the proliferation of MKN28 cells. The number of cells was counted 48 h after siRNA transfection. Mean ± SE. $n = 3$. [g]$P < 0.05$ *vs* control siRNA.

Cell proliferation

Cells were seeded onto 6 well plastic plates at a density of 1.0×10^5 cells per well and incubated at 37 ℃ with 5% CO_2. At 24 h after the cell seeding, siRNA transfection was performed. At 48 h after siRNA transfection, the cells were detached from the plates using a trypsin-EDTA and were counted using Countess® Automated Cell Counter (Invitrogen). Each sample was independently counted for three times, and each assay was performed in triplicate.

Analysis of apoptotic cells

As control, non-transfected cells were treated with staurosporine for 24 h. At 48 h after transfection, the siRNA transfected cells were harvested and stained with fluorescein isothiocyanate (FITC) conjugated annexin V and phosphatidylinositol using the annexin V Kit (Beckman Coulter, Brea, CA)[23]. A Becton Dickinson Accuri™ C6 Flow Cytometer was used to analyze the proportion of apoptotic cells.

Analysis of cell migration and invasion

The migration assay was conducted using a cell culture insert with 8 μm pore size (BD Biosciences, Bedford, MA). Biocoat Matrigel (BD Biosciences) was used to evaluate cell invasion potential. Briefly, at 24 h after siRNA transfection, cells (2.0×10^4 cells per well) were seeded in the upper chamber in serum free medium[25]. The lower chamber contained medium with 10% FBS. The chambers were incubated for 48 h for migration assay and 60 h for invasion assay at 37 ℃ in 5% CO_2, and then, non-migrated or non-invaded cells were removed from the upper side of the membrane by scrubbing with cotton swabs[25]. Migrated or invaded cells were fixed on the membrane and stained with Diff-Quick staining reagents (Sysmex, Kobe, Japan)[25]. The migrated or invaded cells on the lower side of the membrane were counted in four independent fields of view at magnification × 100 of each insert[25]. Each assay was performed in triplicate.

Microarray sample preparation and hybridization

MKN28 cells were transfected with control siRNA and claudin 1 siRNA. At 48 h after siRNA transfection, total RNA was extracted using an RNeasy kit (Qiagen). Quality of RNA was monitored using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Cyanine-3 (Cy3)-labeled cRNA was prepared from 0.1 μg total RNA using the Low Input Quick Amp Labeling Kit (Agilent). Samples were purified using RNAeasy columns (Qiagen). A total of 0.60 μg of Cy3-labelled cRNA was fragmented and hybridized to an Agilent SurePrint G3 Human Gene Expression 8 × 60K Microarray for 17 h. After washing, slides were scanned on the Agilent DNA Microarray Scanner (G2565CA) using the one color scan setting for 8 K × 60 K array slides.

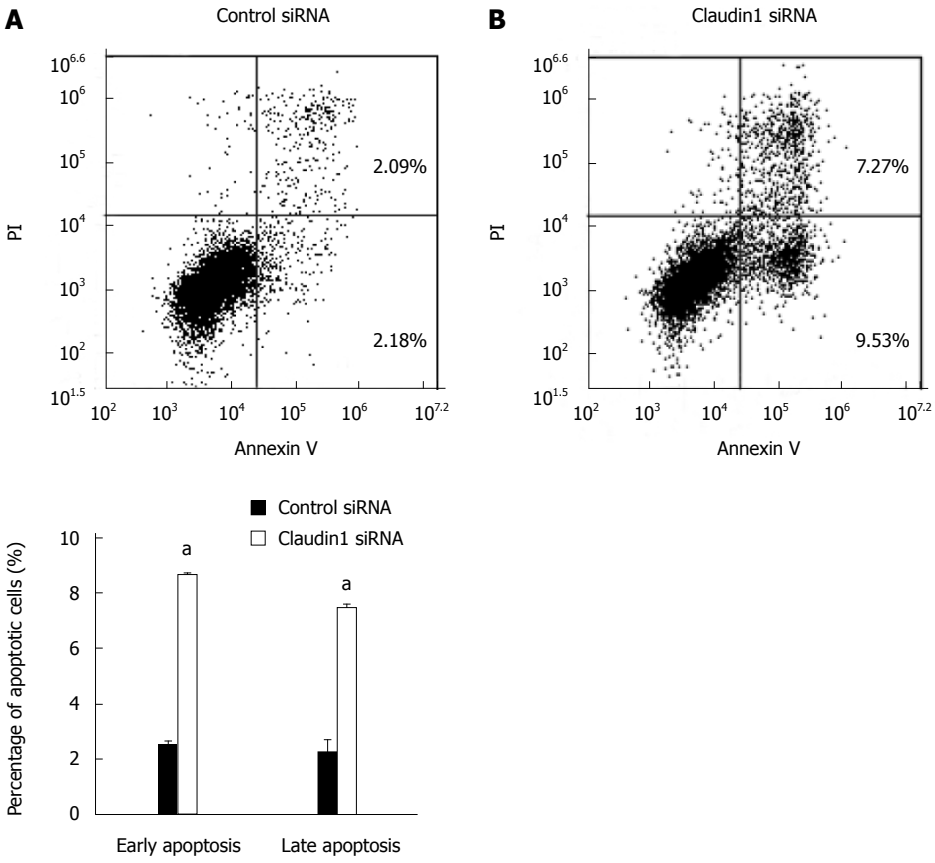


Figure 2 Claudin 1 controls apoptosis in MKN28 cells. Down-regulation of claudin 1 induced both early (annexin V positive/PI negative) and late apoptosis (annexin V/PI double positive) in MKN28 cells 48 h after siRNA transfection. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA.

Processing of microarray data

The scanned images were analyzed with Feature Extraction Software 10.10 (Agilent) using default parameters to obtain background subtracted and spatially detrended Processed Signal intensities[26]. The fold change of each molecule was calculated by using raw signal data of two samples, and a fold change cutoff of 5 was set to identify molecules whose expression was significantly differentially regulated. The networks and functional analyses were generated using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc., Redwood City, CA).

Immunofluorescent Staining and Confocal Microscopy

MKN28 cells were cultured on glass coverslips and stained as previously described[21]. After different stimulation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA, stained with designated antibodies and rhodamine phalloidin. Slides were mounted with VECTASHIELD mounting medium and 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, United States), and distribution of Claudin 1 was examined by confocal fluorescence microscopy (LSM510; Carl Zeiss Co. Ltd, Germany).

Statistical analysis

Student's *t*-test was used to evaluate continuous variables. Differences were considered significant when the relevant *P* value was < 0.05. These analyses were performed using JMP version 10 (SAS Institute Inc., Cary, NC).

RESULTS

Claudin 1 controlled cell proliferation, apoptosis, migration and invasion in MKN28 cells

We conducted knockdown experiments with claudin 1 siRNA in MKN28 cells, and analyzed the effects of claudin 1 knockdown on cell proliferation, apoptosis, migration and invasion. Claudin 1 siRNA effectively reduced claudin 1 mRNA levels (Figure 1A) and claudin 1 protein levels (Figure 1B). The cell counts of claudin 1 siRNA transfected cells were significantly lower than those of control siRNA transfected cells 48 h after siRNA transfection (Figure 1C). Similar results were obtained by using another independent claudin 1 siRNA (Figure 1D, E). Down-regulation of claudin 1 increased both early (annexin V; positive and PI; negative) and late apoptosis (annexin V; positive and PI; positive) 48 h after siRNA transfection (Figure 2). Furthermore, down-regulation of claudin 1 significantly inhibited cell migration and invasion (Figure 3). These results suggest that claudin 1 plays a crucial role in regulating cell proliferation, apoptosis, migration and invasion in MKN28 cells.

Gene expression profile of claudin 1 siRNA transfected cells

We analyzed the gene expression profiles of claudin 1

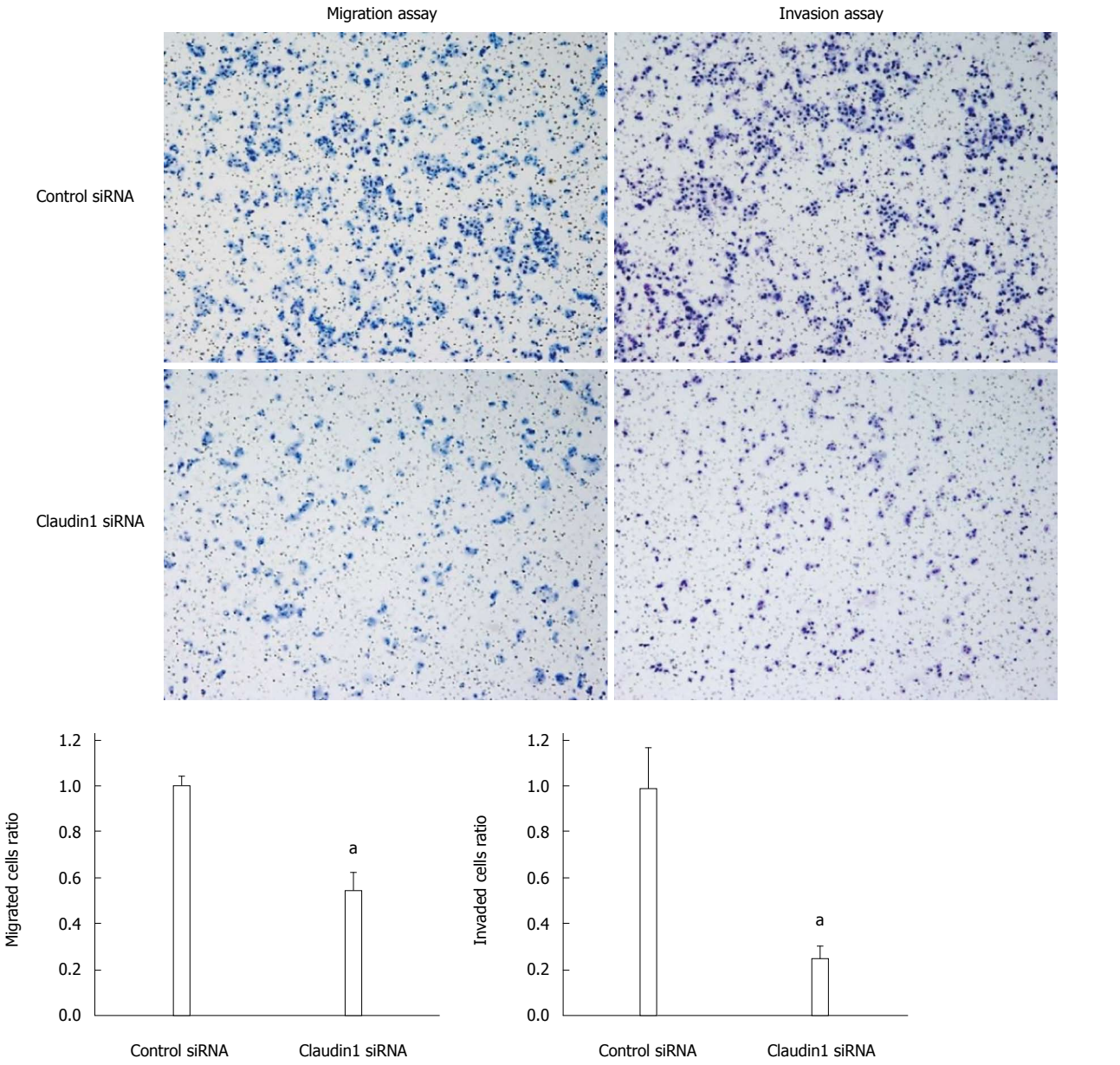


Figure 3 Claudin 1 controlls cell migration and invasion in MKN28 cells. Down-regulation of claudin 1 significantly inhibited cell migration and invasion in MKN28 cells. Cell migration and invasion were determined by Boyden chamber assay. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA.

siRNA transfected MKN28 cells with microarray and bioinformatics. Microarray analysis showed that the expression levels of 245 genes displayed fold changes of > 5.0 in MKN28 cells subjected to claudin 1 knockdown. Of these genes, 76 were upregulated, and 169 were downregulated in claudin 1 siRNA transfected cells. The 20 genes whose expression levels were the most strongly up- or down-regulated in claudin 1 siRNA transfected cells are shown in Table 1. Claudin 1 expression was down-regulated in claudin 1 siRNA transfected cells (fold change: -26.87; Table 1). Ingenuity Pathway Analysis showed that "Cellular movement" was the top-ranked molecular and cellular functions (Table 2(. Furthermore, TNF- and nuclear factor (NF)-κB were the top-ranked upstream regulators related to claudin 1 (Table 2). We then examined the signal transduction networks induced by the knockdown of claudin 1 expression (Table 2). One of the top-ranked signal networks was related to the cellular movement (Table 2, Figure 4A). These results indicate that the expression level of claudin 1 influences genes related to cellular movement, and that TNF-α signal may be its important upstream regulator.

Verification of gene expression by real-time quantitative RT-PCR

Four genes (*MMP7*, *TNF-SF10*, *TGFBR1*, and *CCL2*) were examined further using quantitative RT-PCR. All these genes were chosen from Figure 4A. The expression levels of *MMP7*, *TNF-SF10*, and *TGFBR1* mRNA were significantly lower in claudin 1 siRNA transfected cells than in control siRNA transfected ones (Figure 4B). The expression levels of *CCL2* tended to be increased

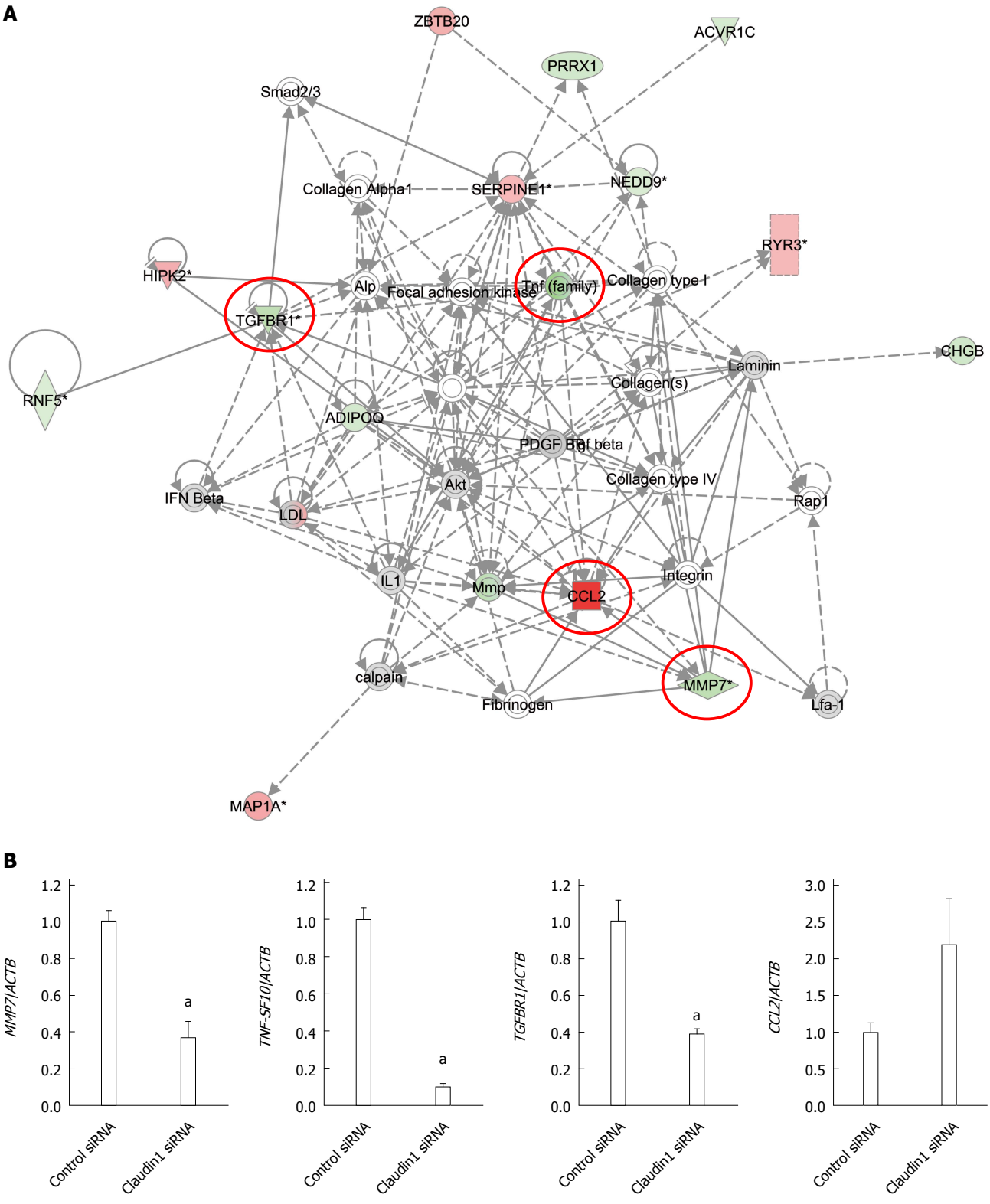


Figure 4 Analysis of gene expression change in claudin 1 siRNA transfected MKN28 cells. A: One of the top-ranked signaling networks related to claudin 1 down-regulation according to ingenuity pathway analysis. Red and green indicate genes whose expression levels were higher or lower, respectively, than reference RNA levels. Genes analyzed for verification were highlighted by red circles; B: Verification of gene expression by real-time quantitative RT-PCR. The expression levels of for selected genes *MMP7*, *TNF-SF10*, *TGFBR1*, and *CCL2* in claudin 1 siRNA transfected MKN28 cells were compared with those in control siRNA transfected cells. Gene expression levels were normalized to the level of *ACTB*. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA.

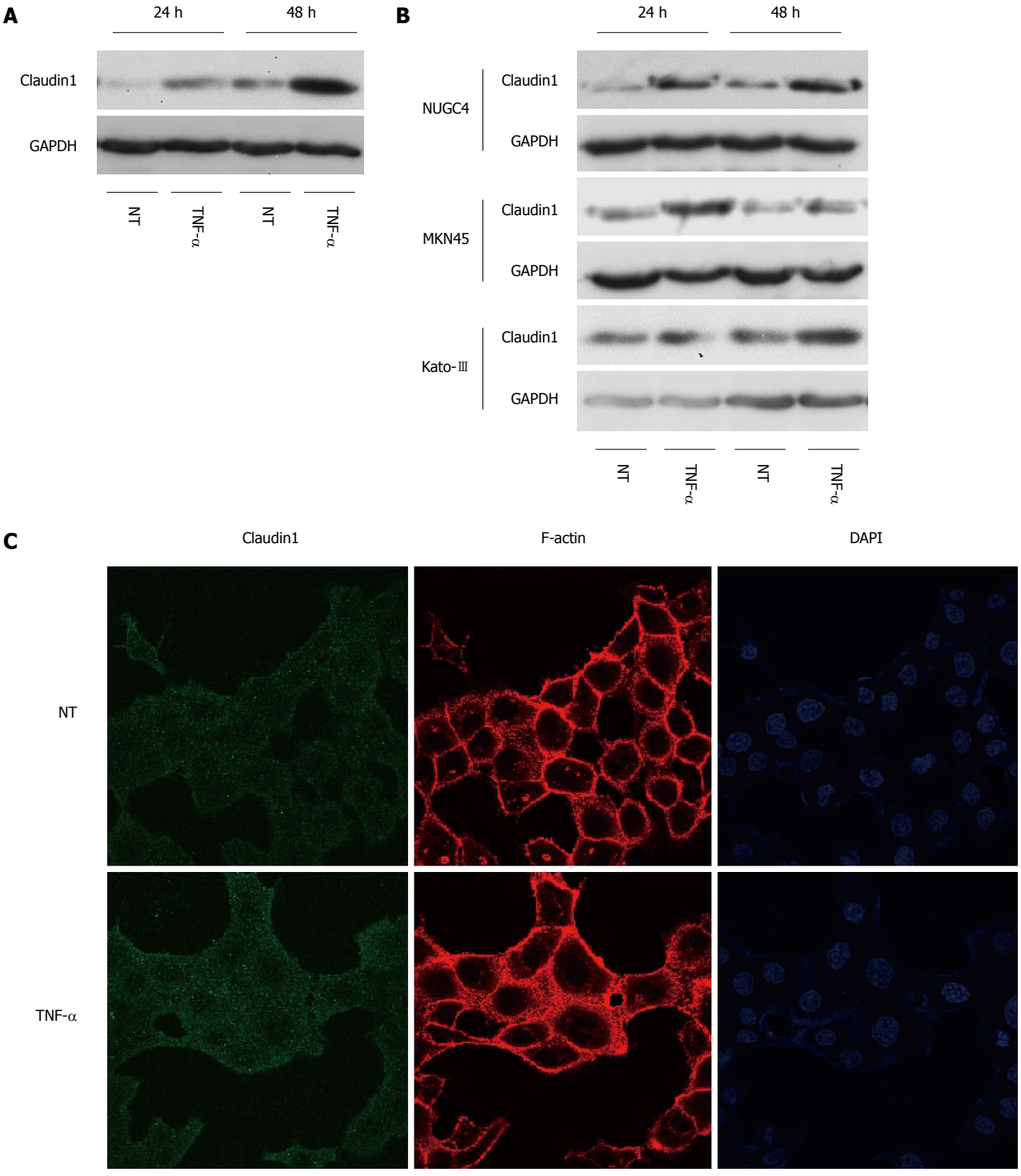
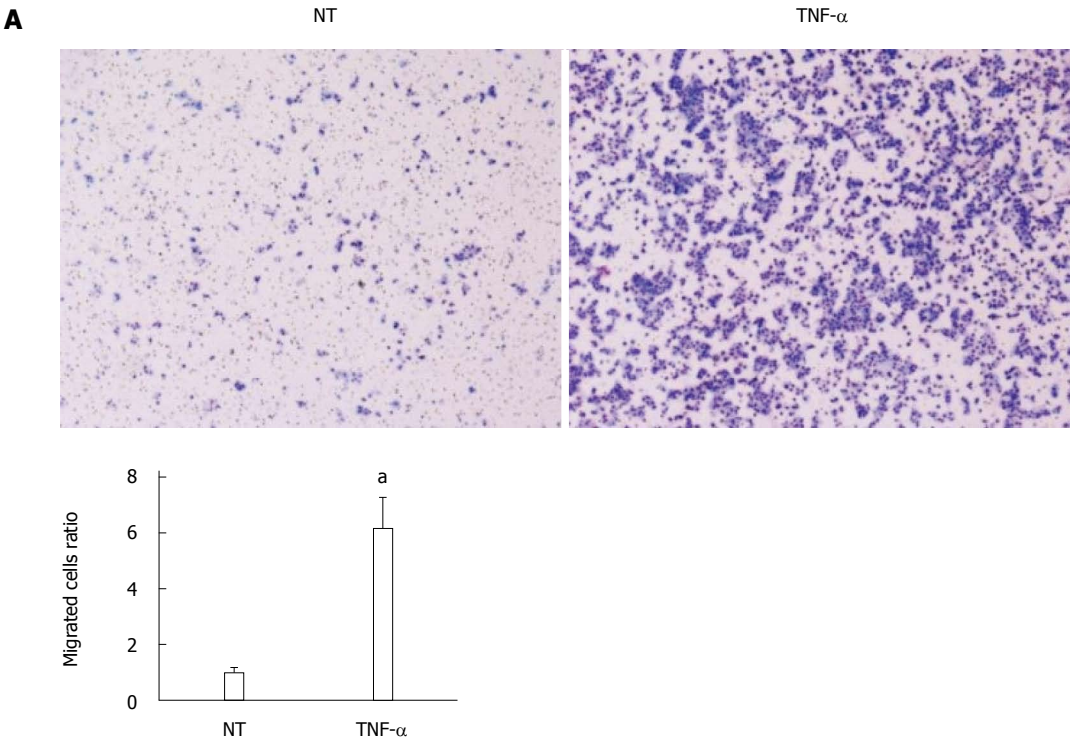


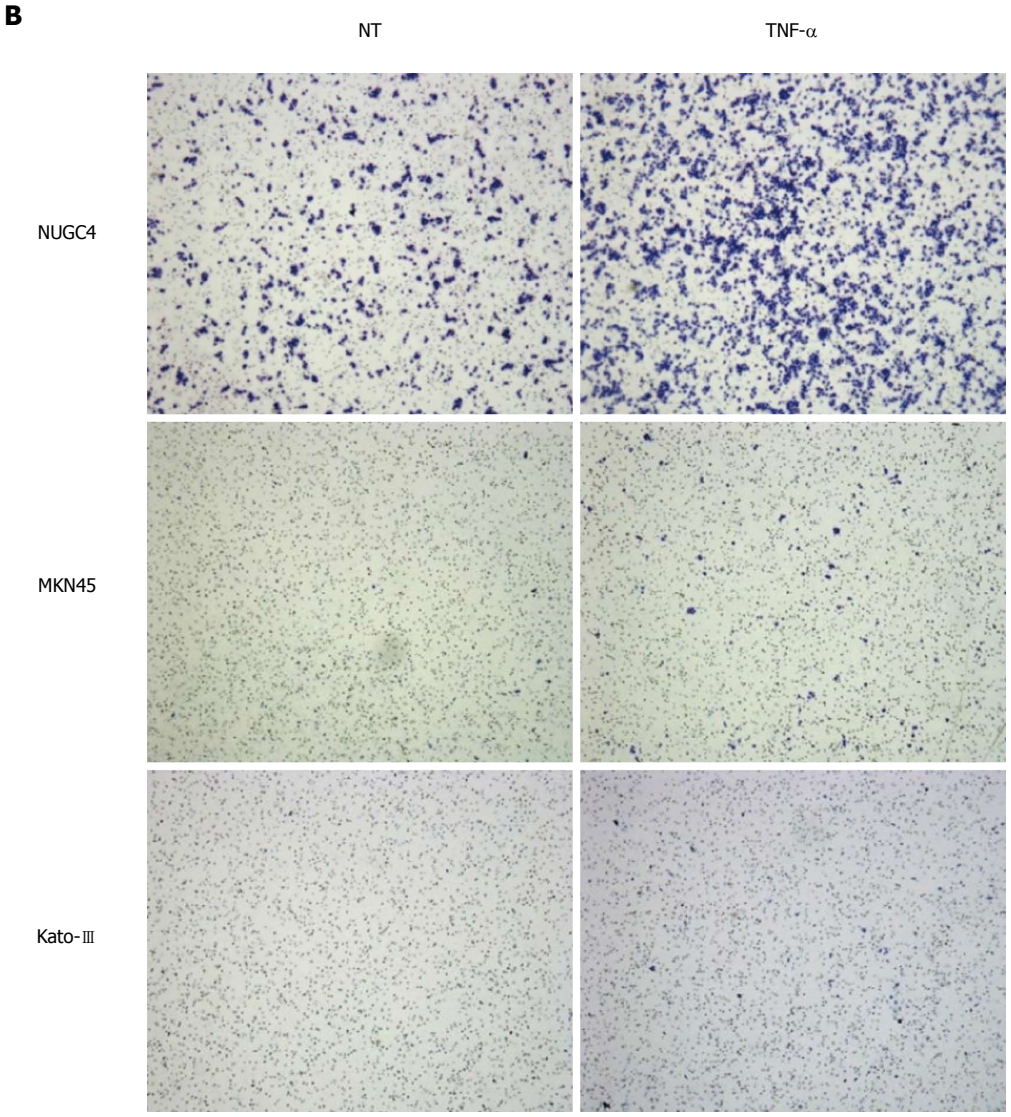
Figure 5 Tumor necrosis factor α induces claudin 1 expression in gastric cancer cells. A: Western blotting revealed that the basal expression level of claudin 1 was increased in a time-dependent manner at 24 and 48 h, which was further increased by tumor necrosis factor α (TNF-α) (20 ng/mL) stimulation in MKN28 cells; B: Western blotting revealed that the expression level of claudin 1 was increased by TNF-α (20 ng/mL) stimulation in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ; C: At 24 h after treating the cells with TNF-α, expression of claudin 1 protein was increased mainly in cytoplasm. MKN28 cells were immunostained with an anti-claudin 1 antibody and counterstained F-actin and nuclei with rhodamine phalloidin and DAPI, respectively.

in claudin 1 siRNA transfected cells although the difference was not statistically significant (Figure 4B). These changes were in agreement with the microarray results.

Claudin 1 plays important roles in TNF-α-induced cell migration in gastric cancer cells

We previously showed that claudin 1 mediates TNF-α-


A
NT
TNF-α
Migrated cells ratio
8
6
4
2
0
a
NT
TNF-α


B
NT
TNF-α
NUGC4
MKN45
Kato-Ⅲ

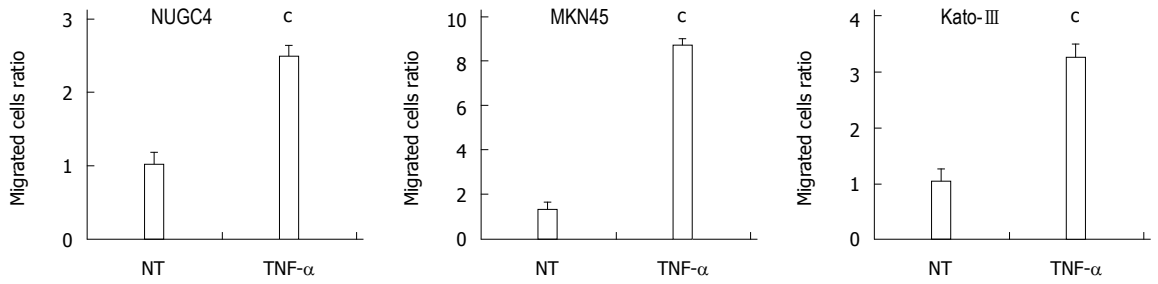


Figure 6 Tumor necrosis factor α induces cell migration in gastric cancer cells. A: Tumor necrosis factor α (TNF-α) stimulation (20 ng/mL, 24 h) significantly increased cell migration in MKN28 cells. Cell migration was determined by Boyden chamber assay. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA, NT; B: TNF-α stimulation (20 ng/mL, 48 h) significantly increased cell migration in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ. Cell migration was determined by Boyden chamber assay. Mean ± SE. $n = 3$. $^{c}P < 0.05$ *vs* control siRNA. NT: No treatment.

Table 1 Genes that displayed the greatest changes in their expressions in the claudin 1 small interfering RNA transfected MKN28 cells

Gene symbol	Gene ID	Gene name	Fold change
Up-regulated genes			
FGF6	NM_020996	Fibroblast growth factor 6	151.34
TM4SF20	NM_024795	Transmembrane 4 L six family member 20	72.03
B4GALNT2	NM_153446	Beta-1,4-N-acetyl-galactosaminyl transferase 2	47.30
SLC2A13	BC047507	Solute carrier family 2 (facilitated glucose transporter), member 13	43.02
HS3ST2	NM_006043	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	28.39
ZNF596	NM_001042416	Zinc finger protein 596	26.36
IL2RA	NM_000417	Interleukin 2 receptor, alpha	22.00
CCL2	NM_002982	Chemokine (C-C motif) ligand 2	18.05
SGOL2	BC048349	Shugoshin-like 2	17.64
SYT4	NM_020783	Synaptotagmin Ⅳ	17.29
FGF19	NM_005117	Fibroblast growth factor 19	16.44
ARID5B	NM_032199	AT rich interactive domain 5B	14.47
DEFB105B	NM_001040703	Defensin, beta 105B	14.34
DSG3	NM_001944	Desmoglein 3	12.99
RSPH1	NM_080860	Radial spoke head 1 homolog (Chlamydomonas)	12.82
COL5A2	NM_000393	Collagen, type Ⅴ, alpha 2	12.72
NRG2	NM_013982	Neuregulin 2	12.63
TMEM26	NM_178505	Transmembrane protein 26	12.22
CCL3L3	NM_001001437	Chemokine (C-C motif) ligand 3-like 3	11.54
OR51E1	NM_152430	Olfactory receptor, family 51, subfamily E, member 1	9.43
Down-regulated genes			
LY6G6F	NM_001003693	Lymphocyte antigen 6 complex, locus G6F	-332.04
WT1	NM_024426	Wilms tumor 1	-68.28
MADCAM1	NM_130760	Mucosal vascular addressin cell adhesion molecule 1	-60.84
SLC2A9	NM_001001290	Solute carrier family 2 (facilitated glucose transporter), member 9	-48.05
SULT4A1	NM_014351	Sulfotransferase family 4A, member 1	-39.30
SYNPR	NM_144642	Synaptoporin	-36.73
C10orf47	NM_153256	Chromosome 10 open reading frame 47	-29.96
MAS1L	NM_052967	MAS1 oncogene-like	-29.67
CLDN1	NM_021101	Claudin 1	-26.87
CBX1	NM_006807	Chromobox homolog 1	-26.37
MPPED2	NM_001145399	Metallophosphoesterase domain containing 2	-25.00
SCUBE2	NM_020974	Signal peptide, CUB domain, EGF-like 2	-22.13
CHI3L2	NM_001025199	Chitinase 3-like 2	-21.67
SLIT2	NM_004787	Slit homolog 2	-21.60
HFM1	NM_001017975	HFM1, ATP-dependent DNA helicase homolog	-20.56
CR1	NM_000651	Complement component (3b/4b) receptor 1	-18.04
PLA2G10	NM_003561	Phospholipase A2, group X	-16.70
GRIP2	NM_001080423	Glutamate receptor interacting protein 2	-16.45
XIST	NR_001564	X (inactive)-specific transcript	-15.79
TNF-SF10	NM_003810	Tumor necrosis factor (ligand) superfamily, member 10	-15.26

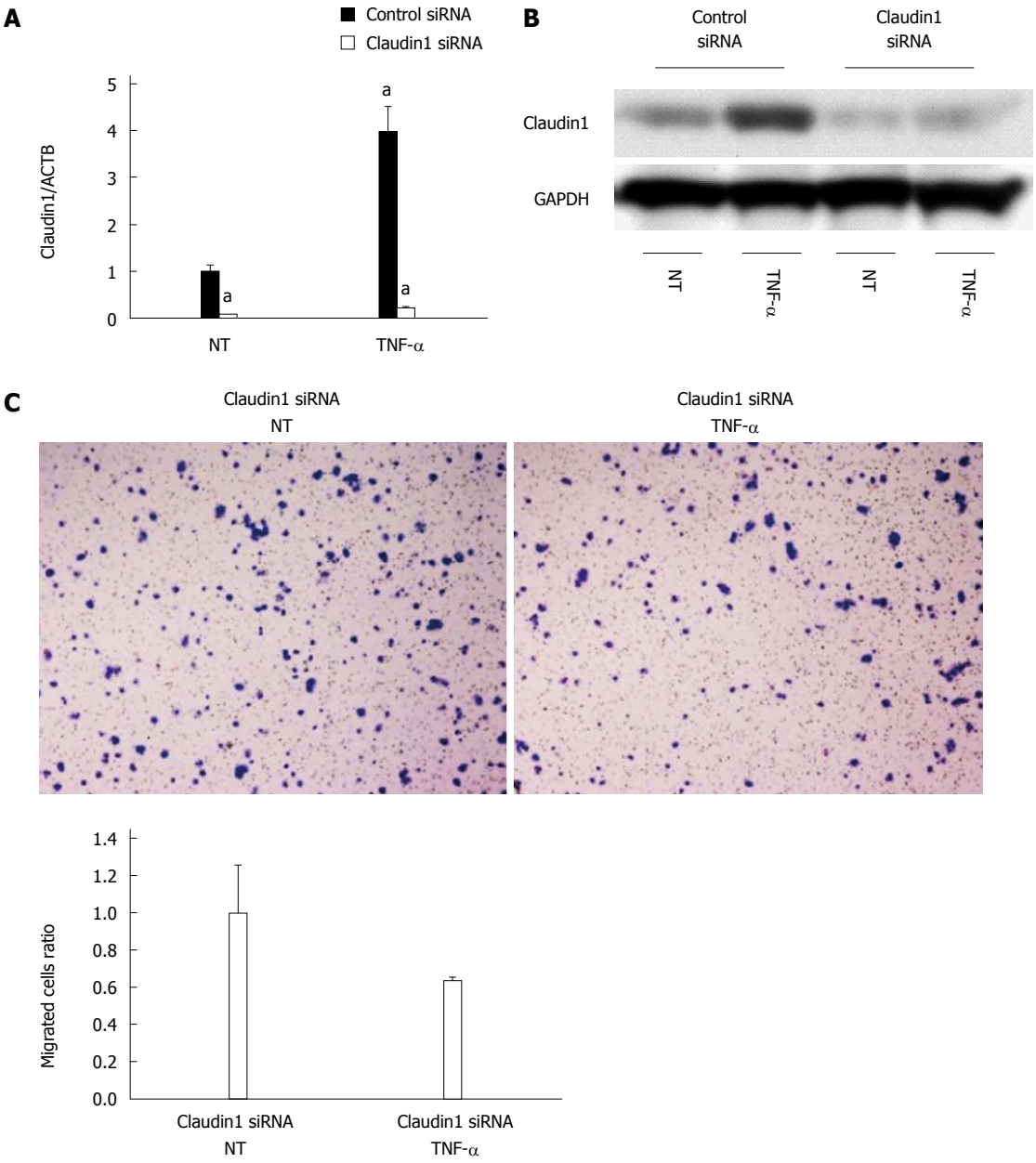


Figure 7 Claudin 1 is important for tumor necrosis factor α-induced cell migration in MKN28 cells. A: Claudin 1 siRNA effectively reduced both the basal and tumor necrosis factor α (TNF-α)-induced expression of claudin 1 mRNA. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA, NT; B: Western blotting revealed that transfection of claudin 1 siRNA effectively reduced both the basal and TNF-α-induced expression of claudin 1 protein; C: TNF-α stimulation (20 ng/mL, 48 h) did not enhance cell migration in the claudin 1 siRNA transfected cells. Cell migration was determined by Boyden chamber assay. Mean ± SE. $n = 3$. NT: No treatment.

induced gene expression and cellular movement in human lung carcinoma A549 cells[21]. In the present study, our microarray data showed TNF- and NF-κB were the top-ranked upstream regulators related to claudin 1 in MKN28 cells. To determine the effect of TNF-α on claudin 1 expression in MKN28 cells, we analyzed protein levels of claudin 1, using western blotting. The basal expression level of claudin 1 was increased in a time-dependent manner at 24 and 48 h (Figure 5A). And, it was further increased by TNF-α treatment in MKN28 cells (Figure 5A). Similar trends that the expression level of claudin 1 was increased by TNF-α stimulation were found in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ (Figure 5B). Furthermore, results of immunofluorescent staining showed that TNF-α-induced claudin 1 expression was mainly detected in the cytoplasm of MKN28 cells (Figure 5C). TNF-α treatment increased cell migration in MKN28 cells (Figure 6A). Similar trends were found in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ (Figure 6B).We then transfected MKN28 cells with claudin 1 siRNA and found that both the basal and TNF-α-induced expression of claudin 1 mRNA and protein were effectively reduced (Figure 7A, B). To examine TNF-α-induced gene expression, we performed microarray to determine gene expression profiles in cells treated with or without TNF-α and in the presence of claudin 1 siRNA or control siRNA. In control siRNA transfected MKN28 cells, TNF-α

Table 2 Top molecular and cellular functions, upstream regulators, and networks of claudin1 according to ingenuity pathway analysis

Name	*P* value	Molecules	Score
Top molecular and cellular functions			
Cellular movement	2.77×10^{-9}-1.01×10^{-2}	51	
Cell-to-cell signaling and interaction	7.24×10^{-7}-1.02×10^{-2}	55	
Molecular transport	1.23×10^{-6}-9.98×10^{-3}	51	
Small molecule biochemistry	1.23×10^{-6}-9.98×10^{-3}	55	
Cellular development	3.80×10^{-6}-9.50×10^{-3}	59	
Top upstream regulators			
TNF-RSF1A	1.80×10^{-7}		
TNF-	1.70×10^{-6}		
NF-κB (complex)	2.01×10^{-6}		
Escherichia coli B5 lipopolysaccharide	2.09×10^{-6}		
NF-κB1	3.23×10^{-6}		
Top networks			
Associated network functions			
Cellular assembly and organization, hair and skin development and function, cellular function and maintenance			33
Cell-to-cell signaling and interaction, cellular growth and proliferation, tissue morphology			32
Cellular movement, hematological system development and function, immune cell trafficking			31
Cardiovascular system development and function, organismal development, cellular movement			22
Hereditary disorder, respiratory disease, tissue development			22

TNF-: Tumor necrosis factor; NF: Nuclear factor.

stimulation changed expression of 169 genes, of which changes of 135 genes were not seen in claudin 1 siRNA transfected cells. For example, of the top 10 genes up-regulated by TNF-α, the fold of change was reduced in 7 of them, and all the top 10 genes down-regulated by TNF-α, the fold of changes was dramatically reduced in claudin 1 siRNA transfected MKN28 cells (Table 3). Moreover, TNF-α did not enhance cell migration in the claudin 1 siRNA transfected MKN28 cells (Figure 7C). These results show that claudin 1 has crucial role in mediating TNF-α-induced gene expression and migration in gastric cancer cells.

DISCUSSION

Recent reports have indicated that claudin proteins were up-regulated and mis-located in cancer cells[2]. Although there are several reports on the expression of claudin 1 in gastric cancer, no consensus has been reached about the relationship between claudin 1 expression and clinicopathological parameters[5,6,13,27]. Some reports have indicated that claudin 1 was highly expressed in intestinal subtype of adenocarcinomas[5,27], while others found that it was highly expressed in diffuse subtype of adenocarcinomas[6,13]. Several studies reported claudin 1 expression at invasive front of gastric carcinomas, which suggests that over expression of claudin 1 is related to carcinogenesis in invasive and metastatic gastric cancer[6,13,27]. The up-regulation of claudin 1 in colon cancer cells increased tumor growth and metastasis *in vivo*, whereas depletion of claudin 1 in metastatic colon cancer cells with siRNA inhibited cellular invasion[7]. Furthermore, over expression of claudin 1 increased cellular movement in melanoma[8], oral squamous cell carcinoma[9], hepatocellular carcinoma[10] and lung carcinoma cells[21]. In the present study, our results also showed that knocking down of claudin 1 decreased cell proliferation, cell migration and invasion, and increased apoptosis in gastric cancer cells, which suggests the importance of claudin 1 in the progression of gastric carcinomas.

Our data showed that "Cellular Movement" was the top-ranked molecular and cellular functions related to claudin 1 down-regulation according to Ingenuity Pathway Analysis in gastric cancer cells. MMP7 was found in the signal network related to cellular movement, strongly suggesting that the expression of claudin 1 may involve in migration, invasion and metastasis of gastric cancer. Furthermore, TNF- and NF-κB were the top-ranked upstream regulators related to claudin 1. Tumor necrosis factor superfamily member 10 (TNF-SF10), was significantly down-regulated by claudin 1 siRNA in MKN28 cells. These data show the important role of claudin 1 in TNF-α-induced cell migration.

It has been shown that TNF-α stimulation induced EMT in colonic organoids[14], renal carcinoma[28-30] and skin cells[31]. TJ and adherens junction proteins are usually down-regulated during the progression of EMT[15-20]. In this manner, claudin 1 expression is generally decreased by TNF-α stimulation, and the decreased protein expression leads to the increase in the paracellular permeability of epithelial cells[32,33]. On the other hand, TNF-α was reported to increase claudin 1 protein expression in pancreatic cancer cells[34] and airway smooth muscle cells[35]. Our recent report showed that TNF-α stimulation remarkably increased claudin 1 protein expression in the cytoplasm in A549 cells, and that depletion of claudin

Table 3 Top 10 up or down-regulated genes induced by tumor necrosis factor α in control small interfering RNA or claudin 1 small interfering RNA transfected MKN28 cells

Gene symbol	Gene ID	Gene name	Control siRNA	Claudin1 siRNA
			Fold change	Fold change
Up-regulated genes				
IL6	NM_000600	Interleukin 6	11.16	7.16
MMP9	NM_004994	Matrix metallopeptidase 9	10.49	18.13
BMP2	NM_001200	Bone morphogenetic protein 2	10.12	5.62
VSTM1	NM_198481	V-set and transmembrane domain containing 1	9.83	2.53
CLEC1A	NM_016511	C-type lectin domain family 1, member A	9.71	-1.22
CXCL10	NM_001565	Chemokine (C-X-C motif) ligand 10	9.33	1.82
IL2RG	NM_000206	Interleukin 2 receptor, gamma	9.14	15.56
TFPI	NM_001032281	Tissue factor pathway inhibitor	8.98	11.79
KLHDC7B	NM_138433	Kelch domain containing 7B	8.90	1.79
CCL5	NM_002985	Chemokine (C-C motif) ligand 5	8.85	7.84
Down-regulated genes				
LY6G6F	NM_001003693	Lymphocyte antigen 6 complex, locus G6F	-311.81	1.05
RFTN2	NM_144629	Raftlin family member 2	-291.41	1.39
WT1	NM_024426	Wilms tumor 1	-75.96	1.05
MADCAM1	NM_130760	Mucosal vascular addressin cell adhesion molecule 1	-67.39	1.06
SLC2A9	NM_001001290	Solute carrier family 2, member 9	-49.12	-1.21
C10orf47	NM_153256	Chromosome 10 open reading frame 47	-44.11	1.85
SYNPR	NM_144642	Synaptoporin	-40.64	1.08
GGT7	NM_178026	Gamma-glutamyltransferase 7	-40.33	1.24
LRRC36	NM_018296	Leucine rich repeat containing 36	-38.25	1.06
MAS1L	NM_052967	MAS1 oncogene-like	-32.62	1.07

1 inhibited the TNF-α-induced gene expression and cell migration[21]. Similarly, in the present study, TNF-α induced over expression of claudin 1 in the cytoplasm in gastric carcinoma MKN28 cells, and knocking down of claudin 1 blocked the TNF-α-induced gene expression and cellular movement in human gastric cancer cells. Generally, claudin 1 participates in cell-to-cell adhesion as TJ proteins, and its down-regulation may promote cell migration. However, our findings showed claudin 1 induced by TNF-α is mainly in cytoplasm, and regulates TNF-α-induced gene expression[21]. In addition, many of these claudin 1 dependent genes are related to cellular movement, suggesting that claudin 1 mediates TNF-α-initiated cell migration with various mechanisms[21].

In conclusion, we found that claudin 1 played a role in the proliferation, apoptosis, cell migration and invasion in gastric carcinoma cells, suggesting the importance of claudin 1 expression in the progression of gastric carcinomas. Our microarray results also suggest that claudin 1 has marked effects on the expression of genes related to cellular movement. Furthermore, we showed that TNF-α induces the gene expression of claudin 1 in gastric carcinoma cells, and the latter acts as the signal mediator to regulate gene expression and cellular movement. A deeper understanding of this pathway may serve as a mean to establish a new therapeutic target for gastric carcinoma.

COMMENTS

Background

Recent reports have indicated that claudin proteins were up-regulated and mislocated in cancer cells, and influenced the biological behavior of tumor progression. Although there are several reports on the expression of claudin 1 in gastric cancer, no consensus has been reached about the relationship between claudin 1 expression and clinicopathological features.

Research frontiers

The authors recently found that the expression of claudin 1 was increased in response to tumor necrosis factor alpha (TNF-α) stimulation, and that claudin 1 played an important role in TNF-α-induced gene expression and cellular movement in human lung carcinoma A549 cells. The objectives of the present research were to investigate the role of claudin 1 in the control of genes involved in cell migration and TNF-α-induced gene expression in human gastric adenocarcinoma cells.

Innovations and breakthroughs

The authors showed that the knockdown of claudin 1 significantly inhibited cell migration and invasion in gastric cancer cells. Microarray analyses showed that down-regulation of claudin 1changed the expression levels of many genes related to cellular movement and TNF-α signal. They found that TNF-α stimulation induced the gene expression of claudin 1 in gastric carcinoma cells, and the latter acted as the signal mediator to regulate gene expression and migration.

Applications

The results of the present study suggest that claudin 1 acts as a crucial signal mediator in TNF-α induced gene expression and cell migration in gastric carcinoma cells. A deeper understanding of these cellular processes may be helpful in establishing new therapeutic strategies for gastric cancer.

Terminology

Claudin proteins play an essential role in the function of TJ, and 24 subtypes of the claudin have been identified. They interact with each other through homo- and heterophilic interactions and are crucial for the maintenance of cellular polarity of epithelial cells.

Peer review

This is a good descriptive study in which the role of claudin 1 in the regulation of genes involved cell migration and TNF-α-induced gene expression in human gastric cancer cells. They reported that claudin 1 knock down significantly inhibited cell migration and invasion in gastric carcinoma cells. And the depletion of claudin 1 changed the expression level of TNF-α signal. The results are interesting and meaningful for further understand the role of claudin 1 on cancer development.

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