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Anti-invasive activity of histone deacetylase inhibitors *via* the induction of Egr-1 and the modulation of tight junction-related proteins in human hepatocarcinoma cells

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The potential anti-metastasis and anti-invasion activities of early growth response gene-1 (Egr-1) and claudin-3, a tight junction (TJ)-related protein, were evaluated using histone deacetylase (HDAC) inhibitors in human hepatocarcinoma cells. The results of wound healing and Transwell assays showed that HDAC inhibitors such as trichostatin A and sodium butyrate inhibited cell migration and invasion. HDAC inhibitors markedly induced Egr-1 expression during the early period, after which expression levels decreased. In addition, the down-regulation of snail and type 1 insulin-like growth factor receptor (IGF-1R) in HDAC inhibitor-treated cells induced the upregulation of thrombospondin-1 (TSP-1), E-cadherin and claudin-3. Cells transfected with Egr-1 and claudin-3 siRNA displayed significant blockage of HDAC inhibitor-induced anti-invasive activity. Collectively, these findings indicate that the up-regulation of Egr-1 and claudin-3 are crucial steps in HDAC inhibitor-induced anti-metastasis and anti-invasion. [BMB reports 2009; 42(10): 655-660]

INTRODUCTION

The propensity of hepatocarcinoma cells to invade surrounding tissues and metastasize to other organ sites is responsible for the majority of liver cancer deaths. Patients whose liver cancer has metastasized have poor prognoses and therefore novel prevention strategies are urgently required in order to save lives (1).

In this study, we present a new paradigm for the prevention of liver cancer metastasis by using histone deacetylase (HDAC) inhibitors to induce early growth response gene-1 (Egr-1), a Cys2-His2-typed zinc-finger transcription factor, and the restoration of tight junctions (TJs) in liver cancer cells. Recent evidence in several animal models has shown that Egr-1 performs a crucial function in the suppression of neovascularization (2). Invasive endothelial cells have become a key target for cancer therapies. However, the effect of Egr-1 induction on the molecular process of invasion by which TJ protein dysfunction associates with cell-cell and cell-matrix adhesion and related invasive proteins remains unknown.

In this study, we assessed the hypothesis that inhibition of HDAC activity induces Egr-1 expression in hepatoma cells, and that metastasis and invasion of hepatoma cells is modulated via the induction of Egr-1 expression and the regulation of claudin-3. In addition, the role of invasion/metastasis associated-proteins such as type 1 insulin-like growth factor receptor (IGF-1R), thrombospondin-1 (TSP-1), E-cadherin and snail was examined. Additionally, in order to confirm the hypothesis that inhibition of HDAC activity inhibits metastasis, motility, invasiveness and TJ protein formation, we down-regulated Egr-1 and claudin-3 expression using siRNA technology and evaluated its effect on HDAC inhibitor-treated cells.

RESULTS AND DISCUSSION

Inhibition of cell proliferation, motility and invasion by HDAC inhibitors

Compared to the untreated control cells, the ability of HDAC inhibitors to inhibit growth in hepatocarcima cells was not significantly influenced by treatment with up to 200 nM trichostatin A (TSA) and 1 mM sodium butyrate (NaB) for 48 h (Fig. 1). However, at concentrations higher than 400 nM TSA and 2 mM NaB, both HDAC inhibitors significantly affected cell viability in a dose-dependent manner. HepG2 cells were more

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sensitive to HDAC inhibitors than Hep3B cells, but the differences were not statistically significant. Wound healing experiments were conducted in order to determine whether the anti-proliferative activity of HDAC inhibitors was associated with the inhibition of the cell motilities in HepG2 and Hep3B cells. The focus was to determine how quickly cells could migrate and fill in empty areas of the tissue culture plate created by scratching of the confluent cell monolayer. As is shown in Fig. 2A, the motilities of cells were inhibited significantly by 24 hours of 500 nM TSA treatment as compared to control cells. Under identical conditions, 2 mM NaB produced a similar effect (data not shown). We then assessed the effects of HDAC inhibitors on invasiveness, which determines the metastatic potential of a cell, using Boyden chamber invasion assays. As is shown in Fig. 2B, the invasiveness of cells was inhibited by approximately 3 fold compared to control cells upon treatment with 500 nM TSA and 2 mM NaB. We noted no significant differences according to cell type or the type of HDAC inhibitor used. Therefore, the results indicated that the HDAC inhibitors TSA and NaB had significant effects on two measures of metastatic potential, motility and invasiveness. This bodes well for the therapeutic use of HDAC inhibitors as an anti-metastatic agent in the treatment of hepatocarcinoma cells.

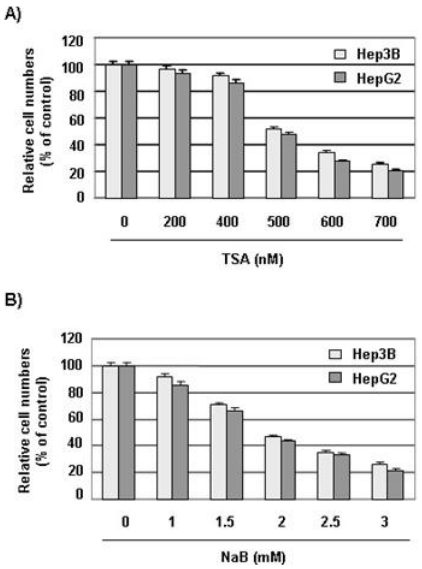

Fig. 1. Inhibition of cell proliferation by HDAC inhibitor treatment in HepG2 and Hep3B cells. Approximately 2 x 10^5 HepG2 cells and 1 x 10^5 Hep3B cells were seeded on 6-well plates and treated with 500 nM of TSA (A) and 2 mM NaB (B), respectively, for 48 h during the MTT assay. The data are shown as the means of triplicate samples (error bars, ± S.D.) and represent the viability of the cells as compared to controls.

Modulation of Egr-1 expression as well as metastasis-involved and TJ-related proteins by HDAC inhibitors

Western blot analysis was performed in order to determine how Egr-1 expression was associated with the anti-invasive activity of HDAC inhibitors. As is shown in Fig. 3, Egr-1 expression in both cell lines was induced to a maximum level within 2 or 3 h of treatment with TSA and NaB, followed by a gradual decrease to basal levels. This finding is very consistent with results reported by Maehara *et al*. (3). In a previous study, we reported that TSA inhibited cyclooxygenase-2 (COX-2) ac-

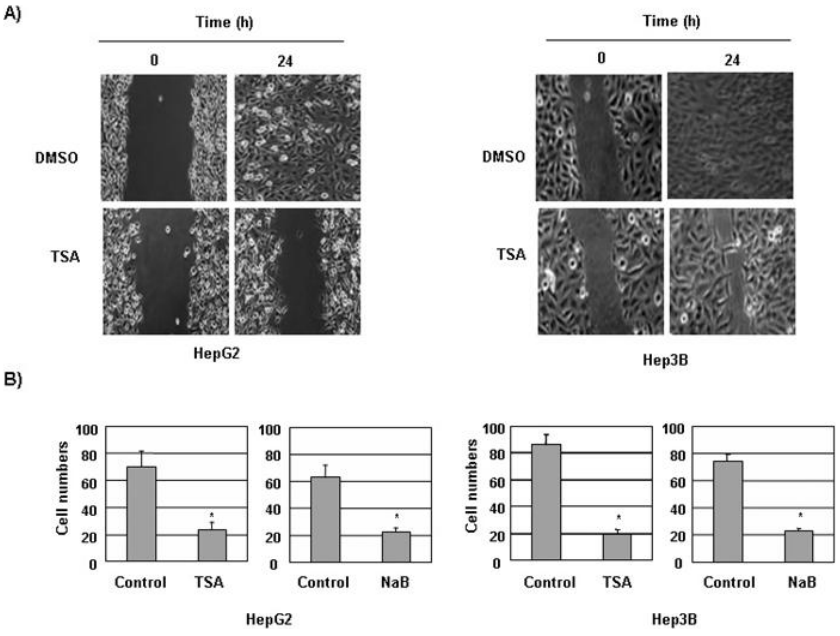

Fig. 2. Inhibition of motility and invasion by HDAC inhibitors in HepG2 and Hep3B cells. (A) Cells were grown to confluence on 30-mm cell culture dishes coated with rat tail collagen and then treated with vehicle or 500 nM TSA for 24 h. A scratch was made through the cell layer using a pipette tip. Photographs of the wounded area were taken immediately after the scratch was made (0 h) and 24 h later in order to monitor cell movement into the wounded area. (B) Cells were plated onto the apical side of Matrigel-coated filters in 0.5 ml of serum-free medium. The cells were then exposed to 500 nM of TSA and 2 mM NaB, respectively, for 24 h. The cells on the bottom of the filter were stained using hematoxylin and counted (three fields of each triplicate filters) using an inverted microscope. *P < 0.05, significantly different from the corresponding control cells.

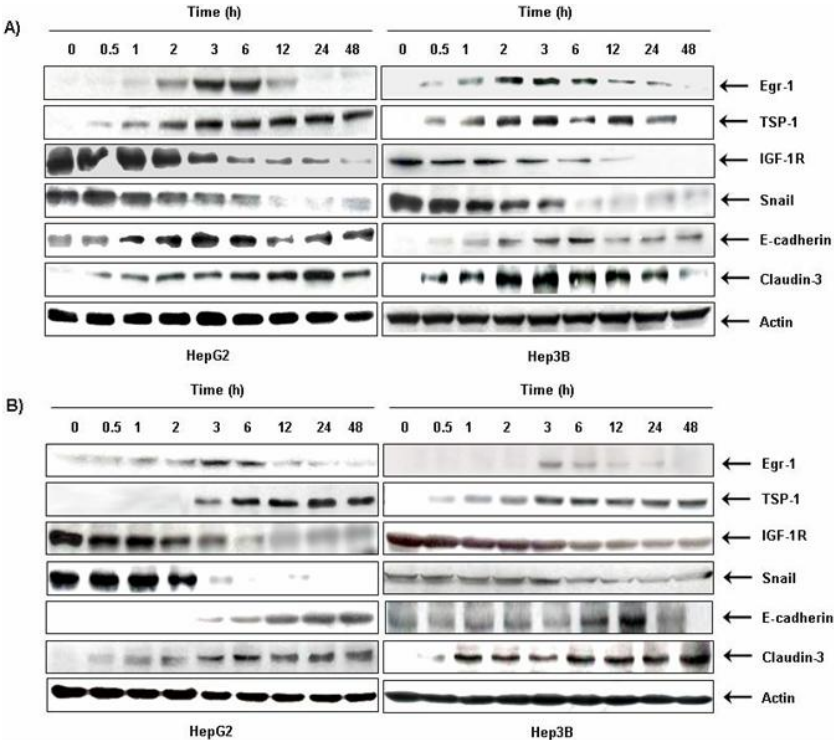

Fig. 3. Modulation of Egr-1 and TJ-related protein expression via HDAC inhibitor treatment in HepG2 and Hep3B cells. Western blotting was conducted in order to quantitate several listed proteins. The cells were treated with 500 nM TSA (A) and 2 mM NaB (B), respectively, for 48 h, after which the proteins were extracted. Aliquots containing 40 μg of total protein were fractionated on 8-12% SDS-PAGE, followed by transfer of protein onto PVDF membranes. Specific complexes were detected by chemiluminescence in accordance with enhanced Western blotting detection protocols. The β-actin protein was utilized as a protein loading control.

tivity in A549 human lung carcinoma cells (4). Moreover, it has been shown that several COX inhibitors possess anti-tumorigenic activity and increase Egr-1 expression during early stages (5, 6). This phenomenon also regulates the expression of numerous genes, some of which may possibly exhibit anti-invasion activities. One possible candidate gene proposed to mediate this invasion is TSP-1. Human TSP-1 contains one Egr-1 binding site in its promoter region and its transcription can be modulated by Egr-1 (7). Recently, Moon *et al*. showed that COX-2 inhibitors can suppress tumor cell invasion via the activity of TSP-1, which occurs downstream of Egr-1 (8). In the current study, we determined that TSP-1 expression was induced by TSA as well as by NaB in a time-dependent manner in both HepG2 and Hep3B cells (Fig. 3). Compared with Egr-1 induction, TSP-1 expression increased over a 2-3 h treatment period, and maximal levels were observed after 12-24 h. This supports the notion that HDAC inhibitor-induced Egr-1 expression results in TSP-1 expression, and that TSP-1 may function as an anti-invasive mediator in hepatocarcinoma cells.

TJs are apical intercellular junctions whose general function is the maintenance of epithelial polarity as well as functioning as selective barriers to molecules. TJs are dynamic structures subject to modulation during wound repair, inflammation and tumor progression. Claudins, key components of TJ, are transmembrane proteins whose extracellular domains interact with claudin proteins of adjacent cells, thereby regulating paracellular permeability (9). Emerging evidence has shown that disruption of TJs caused by the dysregulation of TJ proteins is an early event in cancer cell invasion as well as in metastasis, causing the levels of claudin proteins tend to be irregular according to the specific tissues involved. For example, the expression of claudin-1, -3 and -4 is associated with significant disorganization of the TJ strand in addition to increased paracellular permeability of the dye ruthenium red in cancer tissues. Claudin-3 and claudin-4 have also been shown to be overexpressed in breast, gastric, ovarian and pancreatic cancers (10-12). In contrast, Higashi *et al*. (1) reported reduced levels of claudin-1 in malignant hepatocellular carcinoma, and another researcher demonstrated that claudin down-regulation is associated with higher invasiveness (13). The results described above confirm not only that claudins are dysregulated in many types of cancer, but also that the nature of this dysregulation is highly specific according to cancer type. Our proposed experiments are expected to increase our current understanding of the function of individual claudins in TJ proteins in the context of HDAC inhibitor treatment. In the present study, we determined that HDAC inhibitors dramatically increased the levels of both claudin-3 and E-cadherin in a time-dependent manner (Fig. 3). However, the levels of IGF-1R and snail, an E-cadherin repressor, upon HDAC inhibitor treatment were negatively correlated with those of TSP-1, claudin-3 and E-cadherin. As a regulator of invasion and metastasis, snail represses E-cadherin transcription and results in the disruption of adherent junctions (14). It also appears to contribute to the de-

velopment of malignant carcinomas, in which the loss of E-cadherin expression is frequently observed (15). On the other hand, IGF-1R is highly expressed in many human cancer types and promotes tumor invasion and metastasis (16). Thus, these results support the concept that TSA and NaB, as HDAC inhibitors, can restore the levels of claudin protein. Moreover, the function of TJs is expected to inhibit metastasis via multiple mechanisms, including the inhibition of both cell motility and invasiveness.

Effect of siRNA-mediated Egr-1 and claudin-3 inhibition on the expression of TJ and invasion-related proteins as well as invasiveness

The induction of Egr-1 was examined for its role in the repression of metastasis by HDAC inhibitors. Therefore, we successfully down-regulated Egr-1 gene expression in cells using siRNA technology and evaluated its effect on invasion- and TJ-related gene expression as well as on tumor cell invasion. As is shown in Fig. 4A, inhibition of Egr-1 expression effectively mitigated the TSA-induced up-regulation of TSP-1, E-cadherin and claudin-3. In addition, it prevented the down-regulation of IGF-1R and snail as the result of TSA treatment. This result validates our conclusion that induction of TSP-1 and TJ proteins, as well as the inhibition of IGF-1R and snail by TSA, occurs in an Egr-1 dependent manner. Furthermore, in cases in which cell invasion was examined by the transfection of Egr-1 siRNA with TSA it was found that invasiveness was significantly higher than in the irrelevant siRNA cells. These numbers were decreased to a substantially greater degree with TSA than without TSA in both the HepG2 and Hep3B cells (Fig. 4B). Therefore, these results support the idea seen in other cancer cells that induction of Egr-1 by HDAC inhibition mediates the suppression of cell motility and invasiveness by modulating TJ related-proteins (8, 17, 18). In this study, the anti-metastatic mechanism through which HDAC inhibitors induce Egr-1 expression has yet to be fully elucidated in relation

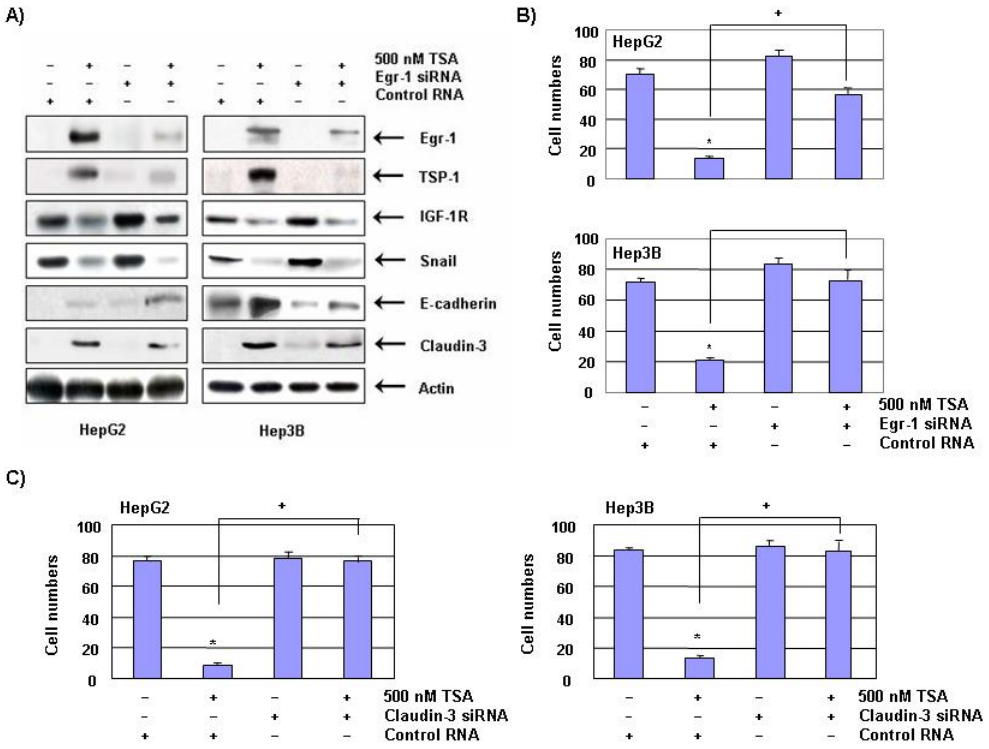

Fig. 4. Effects of siRNA-mediated Egr-1 or claudin-3 inhibition on the expression of TJ and invasion-related proteins as well as on invasiveness in TSA-treated HepG2 and Hep3B cells. (A) Cells were transfected with Egr-1 siRNA and control cells were treated with an equal amount of non-specific irrelevant RNA. After siRNA transfection, the cells were incubated for 4 h for Egr-1 induction and transfected until 24 h for the expression of other proteins. (B, C) cells were transfected for 24 hours with Egr-1 or claudin-3 siRNA, respectively. Approximately 50,000 transfected cells were plated onto the apical sides of Matrigel-coated filters in serum-free medium containing either DMSO or TSA. Medium containing 20% FBS was placed in the basolateral chamber to function as a chemoattractant. The cells on the bottom of the filter were stained using hematoxylin and counted (three fields of each triplicate filters) using an inverted microscope. The data are shown as the means of triplicate samples (error bars, ± S.D.) and represent the invasive cell numbers as compared with those of control cells. $^{\dagger}P < 0.05$, significantly different compared to control cells with Egr-1 siRNA and TSA; $*P < 0.05$, significantly different compared to control cells with non-targeting irrelevant siRNA and TSA via the Student's t-test.

with TJ proteins, particularly claudin-3. In order to evaluate the hypothesis that inhibition of claudin-3 expression exerts anti-metastatic effects, we induced the knockdown of claudin-3 expression in HepG2 and Hep3B cells using siRNA transfection. The results of the Matrigel invasion assay in Fig. 4C show that claudin-3 siRNA significantly increased the invasiveness of these cell lines when compared to cells treated with irrelevant siRNA. Therefore, we propose that Egr-1 induction and/or claudin-3 expression levels may be closely related with metastatic potential in HepG2 and Hep3B hepatoma cells.

In conclusion, the data presented herein show that the HDAC inhibitors TSA and NaB can enhance anti-metastatic activity by inducing Egr-1 expression and by regulating TJ protein expression in cancer cells. To the best of our knowledge, these conclusions are the result of novel findings and are therefore unprecedented. Although further studies will clearly be necessary to confirm these findings in an *in vivo* model of hepatoma cancer metastasis, our findings are timely considering the importance of Egr-1 and TJs and their composite proteins in cancer progression and metastasis. Not only are our findings novel, but the ensuing paradigm of metastasis inhibition through the strengthening of TJs is extremely innovative.

MATERIALS AND METHODS

Cell culture and HDAC inhibitors

The human hepatocarcinoma cell lines, HepG2 and Hep3B, were cultured in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 1% penicillin-streptomycin in an incubator maintained at 37°C with 5% CO_2. TSA and NaB (Sigma-Aldrich, St. Louis, MO) were dissolved in DMSO (Sigma-Aldrich) and distilled water, respectively, at concentrations of 1 mM TSA and 2 M NaB.

MTT assay

Cell viability was determined by methylthiazoletetrazolium (MTT) assay. In brief, cells were seeded at 2×10^5 cells for HepG2 and 1×10^5 cells for Hep3B in 6-well plates. After 24 h of culturing, the cells were treated for 48 hours with TSA or NaB, followed by incubation with 0.5 mg/ml of MTT (Chemicon, Temecula, CA) for 4 h. DMSO was then added in order to solubilize the newly-formed, colored formazan product. The optical density of the solution at 540 nm (absorbed by formazan dye) was determined using an ELISA plate reader (Molecular Devices Co., Sunnyvale, CA) (19).

Protein extraction and Western blot analysis

Cells were harvested and lysed, and the protein concentrations were determined via Bio-Rad protein assay (BioRad Lab., Hercules, CA) in accordance with the manufacturer's instructions. The total proteins in Laemmli buffer were resolved on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with the desired antibodies, incubated with diluted enzyme-linked secondary antibody and visualized by enhanced chemiluminescence (ECL) in accordance with the recommended procedure (Amersham Corp., Arlington Heights, IL). Antibodies against Egr-1, E-cadherin, IGF-1Rβ and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-claudin-3 antibody was acquired from Zymed (San Francisco, CA). Antibodies against snail and TSP-1 were purchased from Abgent (San Diego, CA) and Labvision Co. (Fremont, CA), respectively. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were obtained from Amersham (Oakville, ON).

Wound healing experiment

To assess the effect of HDAC inhibitors on hepatocarcinoma cell motility, we conducted wound healing experiments. In brief, cells were grown to confluence on 30-mm cell culture dishes coated with rat tail collagen (20 μg/ml, BD Biosciences, Bedford, MA), then treated for 6 hours with vehicle or TSA. A scratch was made in the cell layer using a pipette tip. After washing with PBS, serum-free media (to prevent cell proliferation) containing either vehicle (DMSO) or TSA was added. In order to monitor cell movement into the wounded area, photographs of the wounded area were taken immediately after the scratch was made and 24 h later.

Matrigel invasion assay

In order to determine the effects of HDAC inhibitors on cell invasiveness, the cells were exposed for 6 hours to TSA and NaB, which induced no cytotoxic effects as shown by the results of the MTT assay, and were evaluated by the Boyden chamber (BD Biosciences) invasion assay. Treated cells (50,000) were plated onto the apical side of Matrigel-coated filters in serum-free medium containing either DMSO or HDAC inhibitors. Medium containing 20% FBS was placed in the basolateral chamber as a chemoattractant. After 24 h, the cells on the apical side were wiped off with a Q-tip. Cells on the bottom of the filter were stained with hematoxylin (Sigma-Aldrich) and counted (three fields of each triplicate filter) with an inverted microscope (20).

siRNA treatment

The cells were transfected with Egr-1 and claudin-3 siRNA (Dharmacon, Lafayette, CO). Control cells were treated with an equal quantity of non-specific irrelevant RNA. Transfection of synthetic RNA was conducted with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. After transfection with siRNA, the cells were incubated for 24 h under the indicated conditions. The expression of related TJ proteins as well as invasiveness was assessed in transfected cells by Western blotting and Matrigel invasion.

Statistical analysis

All experiments were conducted in duplicate or triplicate at least

three times. Values in the figures are expressed as the means ± S.D. Statistical analyses were conducted using Student's t-test for the comparison of two data groups.

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