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Downregulation of SEMA4C Inhibit Epithelial-Mesenchymal Transition (EMT) and the Invasion and Metastasis of Cervical Cancer Cells via Inhibiting Transforming Growth Factor-beta 1 (TGF-β1)-Induced Hela cells p38 Mitogen-Activated Protein Kinase (MAPK) Activation

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG 1,2 Lilan Yang
ABCF 1 Yayuan Yu
BC 3 Zhenfang Xiong
BC 2 Hongxia Chen
ADG 1 Buzhen Tan
ADG 1 Hui Hu

1 Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, P.R. China
2 Department of Obstetrics and Gynecology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, P.R. China
3 Department of Pathology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, P.R. China

Corresponding Authors: Buzhen Tan, e-mail: tanbuzhen110@126.com, Hui Hu, e-mail: huhui2018@126.com
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Background: Epithelial-mesenchymal transition (EMT) plays a key role in promoting invasion and metastasis of tumor cells. SEMA4C can regulate the generation of transforming growth factor-beta 1 (TGF-β1)-induced EMT in cervical cancer. This study investigated the relationship between the regulation of SEMA4C on TGF-β1-induced p38 mitogen-activated protein kinase (MAPK) activation and invasion and metastasis of cervical cancer.

Material/Methods: Hela-shSEMA4C cell line was established and the success of transfection was confirmed with fluorescence intensity. Cell experiments were divided into 2 groups. Group 1 was Hela, Hela-shNC, and Hela-shSEMA4C; and Group 2 was Hela, Hela-shNC, Hela-shSEMA4C, Hela+TGF-β1, Hela-shNC+TGF-β1, and Hela-shSEMA4C+TGF-β1. Group 1 was detected for SEMA4C mRNA expression by real-time polymerase chain reaction (RT-PCR), cell viability by Cell Counting Kit-8 (CCK-8), F-actin fluorescence intensity by immunofluorescence, cell migration by scratch test, and cell invasion by invasion test. Group 2 was analyzed for E-cadherin fluorescence intensity by immunofluorescence, human fibronectin (FN) content by enzyme-linked immunosorbent assay (ELISA), and SEMA4C, E-cadherin and p-p38 expressions by Western blot.

Results: For Group 1, compared with Hela and Hela-shNC subgroups, the SEMA4C mRNA expression, cell viability, F-actin fluorescence intensity, cell migration and invasion ability in the Hela-shSEMA4C subgroup were significantly decreased ($P<0.05$). For Group 2, compared with Hela and Hela-shNC subgroups, the E-cadherin expression and fluorescence intensity in the Hela-shSEMA4C subgroup were significantly increased ($P<0.01$), while the FN content, SEMA4C, and p-p38 MAPK expressions were significantly decreased ($P<0.01$). Compared with Hela-shNC+TGF-β1 and Hela+TGF-β1 subgroups, the E-cadherin expression and fluorescence intensity in the Hela-shSEMA4C+TGF-β1 subgroup were significantly increased ($P<0.01$), while the FN content, SEMA4C and p-p38 expressions were significantly decreased ($P<0.01$).

Conclusions: Downregulation of SEMA4C can inhibit EMT and the invasion and metastasis of cervical cancer cells via inhibiting TGF-β1-induced Hela cells p38 MAPK activation.

MeSH Keywords: MAP Kinase Signaling System • Neoplasm Invasiveness • Neoplasm Metastasis • Semaphorins • Transforming Growth Factor beta • Uterine Cervical Neoplasms

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Background

Cervical cancer is one of the most common malignant reproductive tumors in women. Despite the improvement in screening and diagnosis techniques, promotion and vaccination, cervical cancer is still the second leading cause of cancer-related death in women worldwide [1,2]. The occurrence and development of cervical cancer are related to many factors. Persistent human papillomavirus (HPV) infection has been recognized as an important cause of cervical cancer [3–5]. HPV16 and HPV18 cause almost 75% of cervical cancer, while HPV31 and HPV45 lead to 10% of cervical cancer [3,4,6]. Studies have shown that HPV proteins can induce epithelial-mesenchymal transition (EMT) in cervical cancer cells. EMT formation is an important cause of primary cervical cancer progression, increase of invasiveness and insensitivity to chemotherapeutics [7–9]. Therefore, inhibiting the formation of EMT can be an important means to reduce the invasion and metastasis of cervical cancer.

SEMA4C gene is a member of the Semaphorin family. It plays an important role in regulating the directional growth of axons and development of myotubes. Previous studies showed that SEMA4C was highly expressed in cervical cancer tissues and correlated with E-cadherin expression. Some studies also found that SEMA4C not only can regulate EMT production, but also affects the generation of transforming growth factor-beta 1 (TGF-β1)-induced EMT via regulation of p38 mitogen-activated protein kinase (MAPK) activity [10–12], suggesting that SEMA4C can regulate the generation of TGF-β1-induced EMT in cervical cancer, which may be related to the regulation of p38 MAPK activity. Therefore, this study is carried out to explore this inference. The aim of this study was to investigate the relationship between the regulation of SEMA4C on TGF-β1-induced p38 MAPK activation, and invasion and metastasis of cervical cancer. The information could be important for the development of new and more effective therapeutics that ameliorate the negative impact of cervical pathogenesis via EMT.

Material and Methods

Ethics approval

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China.

Main reagents

Hela cells (No. BNCC337633) (BeNa Culture Collection, Beijing, P.R. China; Chinese Academy of Sciences, Beijing, P.R. China); TRIzol reagent (Invitrogen, Calsbad, CA, USA); PrimeScript® RT reagent kit. SYBR® Premix Ex Taq™ II reagent kit (TaKaRa Bio Inc., Shiga, Japan); Dulbecco's Modified Eagle Medium (DMEM) high glucose complete culture medium, Cell Counting Kit-8 (CCK-8) cell proliferation assay kit, ready-to-use 4',6-diamidino-2-phenylindole (DAPI) dye solution (NanJing KeyGen Biotech Co., Ltd., Jiangsu, P.R. China); TGF-β1 (Bioss Antibodies, Beijing, P.R. China); human fibronectin (FN) enzyme-linked immunosorbent assay (ELISA) kit (Meimian, Shanghai, P.R. China); trypsin-ethylenediaminetetraacetic acid (tripsin-EDTA) digestive solution, crystal violet dye solution (Beijing Solarbio Science & Technology Co., Ltd., Beijing, P.R. China); isopropanol (IPA) cell lysis solution, defat dried milk, bovine serum albumin (BSA) (Applygen Technologies Inc., Beijing, P.R. China); bicinchoninic acid (BCA) protein quantification kit (CWBIO, Beijing, P.R. China); polyvinylidene difluoride (PVDF) membrane (Millipore Sigma, Darmstadt, Germany); ultra-sensitive enhanced chemiluminescence (ECL) (Thermo Fisher Scientific Inc., Waltham, MA, USA); mouse monoclonal anti-GAPDH, horseradish-labeled goat anti-mouse IgG (H+L), horseradish-labeled goat anti-rabbit IgG (H+L), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ZSGB-Bio, Beijing, P.R. China); rabbit monoclonal E-cadherin antibody, p38, pp38 (Cell Signaling Technology, Danvers, MA, USA); sheep polyclonal SEMA4C (R&D Systems China, Shanghai, P.R. China); rabbit anti-sheep IgG (H+L) adsorbed antibody horseradish peroxidase (HRP) conjugated (Absin Bioscience Inc., Shanghai, P.R. China).

Main instruments

T100™ PCR Instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA); Tanon 1600 gel imaging system (Tanon, Shanghai, P.R. China); TU-100C dry thermostat (Shanghai Yiheng Scientific Instruments Co., Ltd., Shanghai, P.R. China); Neofuge 13R high speed refrigerated centrifuge, HFI51 CO_2 incubator (Heal Force Bio-Meditech Holdings Ltd., Shanghai, P.R. China); IX51 fluorescence microscope, CKX31 microscope (Olympus Corp., Shinjuku, Tokyo, Japan); HH-2 digital constant temperature water bath pot (Changzhou Ronghua Instrument Manufacturing Co., Ltd., Jiangsu, P.R. China); CFX Connect fluorescent polymerase chain reaction, ChemiDoc XRS+ ultra-high sensitivity chemiluminescence imaging system (Bio-Rad Laboratories,, Shanghai, P.R. China); UV-1600PC ultraviolet visible spectrophotometer (Shanghai Mapada Instrument Co., Ltd., Shanghai, P.R. China); TGL-16G centrifuge (Shanghai Precision Instrument Co., Ltd., Shanghai, P.R. China); 2060R NovoCyte® flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA); RT-6100 microplate reader (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, P.R. China).

Cell transfection and grouping

The Hela cells in logarithmic growth phase were collected. The cell concentration was adjusted to 2×10^7 cells/mL, seeded in a cell culture flask and continued to culture for 24 hours. Cell

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experiments were divided into 2 groups: Group 1 was Hela, Hela-shNC, and Hela-shSEMA4C. Group 2 was Hela, Hela-shNC, Hela-shSEMA4C, Hela+TGF-β1, Hela-shNC+TGF-β1, and Hela-shSEMA4C+TGF-β1. The HeLa cells were transfected with shNC and shSEMA4C respectively, and stably screened.

Hela cells with moderate SEMA4C expression were selected. The cells were spread in a 12-well plate in triplicate until it reached 40% to 50% of each well and then cultured in a 37°C CO_2 incubator. Transfection was performed strictly according to the kit instructions or added with 10 ng/mL TGF-β1.

After transfection, 1% penicillin and streptomycin, and 10% fetal bovine serum were added to the DMEM culture medium and incubated at 37°C, 5% CO_2 for 24 hours. The cells were observed under fluorescence microscope. The cells which were in good condition with high transfection rate and suitable for further growth were analyzed by flow cytometry to select transfected cell line with good purity. The cells of each group were then reserved for further experiments. All experiments were performed in triplicate.

Detection of fluorescence intensity with immunofluorescence

Following transfection of Group 1 and Group 2, the culture dish with crawled cells were rinsed with phosphate buffer saline (PBS) and fixed with 4% polyformaldehyde for 15 minutes. Then, it was rinsed with PBS, added with 0.5% Triton X-100 (prepared with PBS) and permeabilized at room temperature (RT) for 20 minutes. 5% BSA was added dropwise and blocked at 37°C for 30 minutes. This was followed by incubation with diluted primary antibody (1: 100) at 4°C overnight, and with diluted fluorescent secondary antibody (1: 500) at 37°C for 30 minutes. Then, it was rinsed with PBS and incubated with DAPI in dark for 5 minutes. Nuclear staining was performed, then rinsed with PBS, blocked with 50% glycerol in culture dish and observed under fluorescence microscope.

Detection of SEMA4C mRNA expression by real-time polymerase chain reaction (RT-PCR)

Following transfection of Group 1, total RNA was extracted using TRIzol reagent. The purity was determined using ultraviolet spectrophotometer at A_{260}/A_{280}. Total RNA was reverse transcribed into cDNA using PrimeScript® RT reagent kit. SYBR® Premix Ex Taq™ II reagent kit was used for PCR amplification. Primer used were as follows:
SEMA4C: upstream 5'-ACCTTGTGCCGCGTAAGACAG-3',
downstream 5'-CGTCAGCGTCAGTGTCAGGAA-3';
GAPDH: upstream 5'-GACAGTCAGCCGCATCTTCT-3',
downstream: 5'-TTAAAAGCAGCCCTGGTGAC-3'.

GAPDH was used as internal reference. Amplification products were verified by agarose gel electrophoresis, and analysis was performed using $2^{-\Delta\Delta Ct}$ method [13]. Number of cycles was 40.

Detection of cell viability by CCK-8

When the cells observed under the microscope were in good condition and over 90% confluence, the culture medium was discarded, then rinsed with PBS and digested with trypsin for 2 to 3 minutes. Complete medium was added to terminate digestion. The cells were blown down and absorbed into 10 mL centrifuge tubes and centrifuged at 1500 rpm for 3 minutes. The supernatant was discarded, and the cells were resuspended in the added medium. Then, the cells were seeded at a density of 1×10^4 cells/well in the 96-well plate. After 24 hours of adherent cell growth and transfection of Group 1, 10 μL CCK-8 test reagent was added to each well and incubated at 37°C for 2 hours. The microplate reader was set at 450 nm wavelength to measure the optical density (OD) value of each well for calculating the survival rate.

Detection of cell migration by scratch test

Following transfection of Group 1, the cells cultured in good condition were prepared for scratch migration. Two parallel lines were drawn at the bottom of the 12-well plate with a ruler. After trypsinization, the cells were centrifuged, and the supernatant was discarded. The pellet was resuspended in medium, seeded in wells and cultured in the incubator. After reaching 100% density, each well was scratched with a pipette tip. The primary medium was discarded, washed with PBS and added with serum-free medium. Position where the scratches were straighter were selected to take photographs. Each well was photographed at fixed points for 0 hours. Then, it was placed in the incubator and cultured. After 24 hours, the scratches of each well were rephotographed and location of the scratches should coincide with that of 0 hours (fixed point). That is, scratches in the same position were taken at 2 time points. The corresponding scratch width was calculated using the 24 hours and 0 hours scratch data, and the cell migration rate was calculated according to the formula: migration rate=migration distance (μm)/migration time (hour).

Detection of cell invasion by invasion test

Following transfection of Group 1, the cells cultured in good condition were prepared for invasion test. The medium was discarded and washed with PBS for 5 minutes. Then, it was added with 0.1% crystal violet stain for 1 hour. The non-invading cells on the upper surface were removed with a cotton swab, and the Transwell chamber was inverted on a glass slide and photographed. The staining solution in the well was removed, and 2 mL 33% acetic acid solution was added

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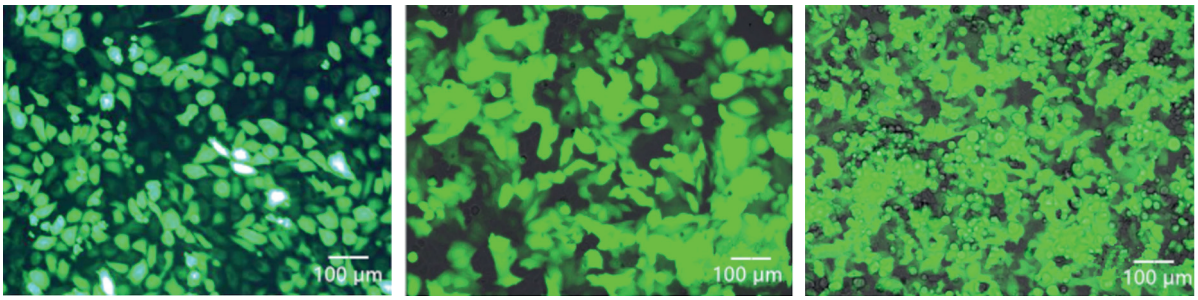

Figure 1. Construction of Hela-shSEMA4C cell line (200×). The experimental tests were performed 3 times.

to dissolve the staining solution in the cells. After thoroughly mixed and let stand, measurement was done with ultraviolet spectrophotometer at 570 nm wavelength. Zero adjustment was performed with 33% acetic acid solution and the absorbance value in each well was measured 3 times. Images of cells in 5 microscopic field vision were randomly selected and captured and the number of cells was counted. The average number of cells was used to assess the invasive capacity.

Detection of FN content by ELISA

Following transfection of Group 2, all the kit components and reagents were taken out from the refrigerator and restored to RT. The standard wells were prepared, each was added with different concentrations of 50 μL standard solution. The blank and sample wells were also set up. Then 40 μL of sample diluent was added to each well of enzyme-labeled coated plate followed by 10 μL sample, and each well was added 100 μL of enzyme-labeled reagent, except for the blank wells. The plate was sealed with sealing film and incubated at 37°C for 60 minutes. Then, the film was carefully removed, and the liquid was discarded. The plate was washed, and pat dry. 50 μL chromogenic agent A was added to each well followed by 50 μL chromogenic agent B, then gently shaken and mixed. The color was developed at 37°C in dark for 15 minutes. 50 μL of stop solution was added to each well to terminate reaction, and the OD value of each well was measured at 450 nm wavelength.

Detection of SEMA4C expression, E-cadherin and phosphorylated p38 (p-p38) expression with Western blot

Following transfection of Group 2, SDS-PAGE separating gel and spacer gel were prepared and added slowly to the casting chamber until 2/3 filled up. Absolute ethanol was added to the gel. After the separation gel was solidified, the spacer gel was added, and the tooth comb was inserted. After the spacer gel was completely solidified, the electrophoretic solution was diluted 10×. The frame was placed in the electrophoretic solution, the tooth comb was then removed, and the protein sample and marker were added. Electrophoresis was performed, with 60 V and 80 V, respectively to compress and isolate the protein. When the protein band was halfway through the gel, transfer buffer was prepared and precooled. The PVDF membrane was cut according to the expected band position and activated for 15 seconds in methanol. The appropriate size of gel containing the target band was cut. A "sandwich" (sponge – filter paper – gel – membrane – filter paper – sponge) was assembled, followed by membrane transfer and blocking with BSA blocking solution overnight. The PVDF membrane was incubated with primary antibody for 3 hours (1: 100), followed by secondary antibody the next day for 2 hours (1: 500), and exposed under the gel imaging system.

Statistical analysis

The data were plotted using GraphPad Prism software. All data were analyzed using SPSS 19 software (SPSS Inc., Chicago, IL, USA). Comparison between subgroups was performed with ANOVA test. The experimental results were expressed as mean±standard deviation ($\bar{\chi}\pm s$). $P<0.05$ was considered as significant difference.

Results

Construction of Hela-shSEMA4C cell line

The fluorescence intensity of Hela-shSEMA4C subgroup was higher than that of Hela-shNC and Hela subgroups, indicated that transfection was successful (Figure 1).

SEMA4C mRNA expression

Compared with Hela and Hela-shNC subgroups, the SEMA4C mRNA expression in the Hela-shSEMA4C subgroup was significantly decreased ($P<0.01$) (Figure 2).

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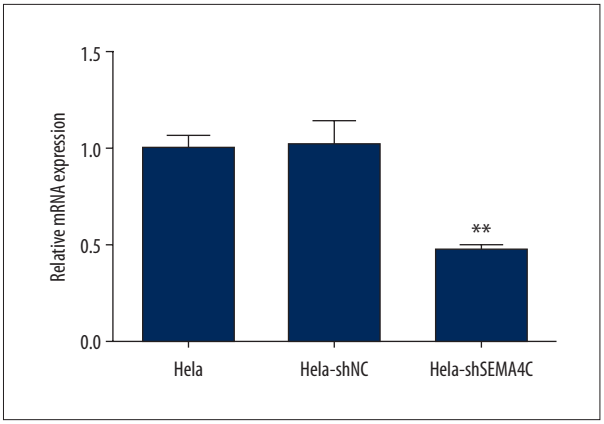

Figure 2. SEMA4C mRNA expression. ** *P*<0.01 versus Hela and Hela-shNC subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

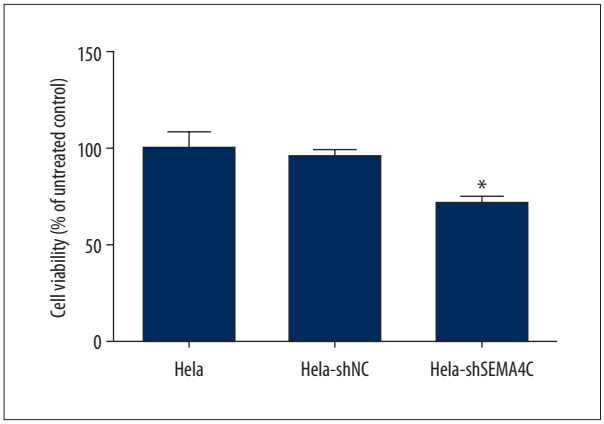

Figure 3. Effects of shSEMA4C on Hela cell viability. * *P*<0.05 versus Hela and Hela-shNC subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

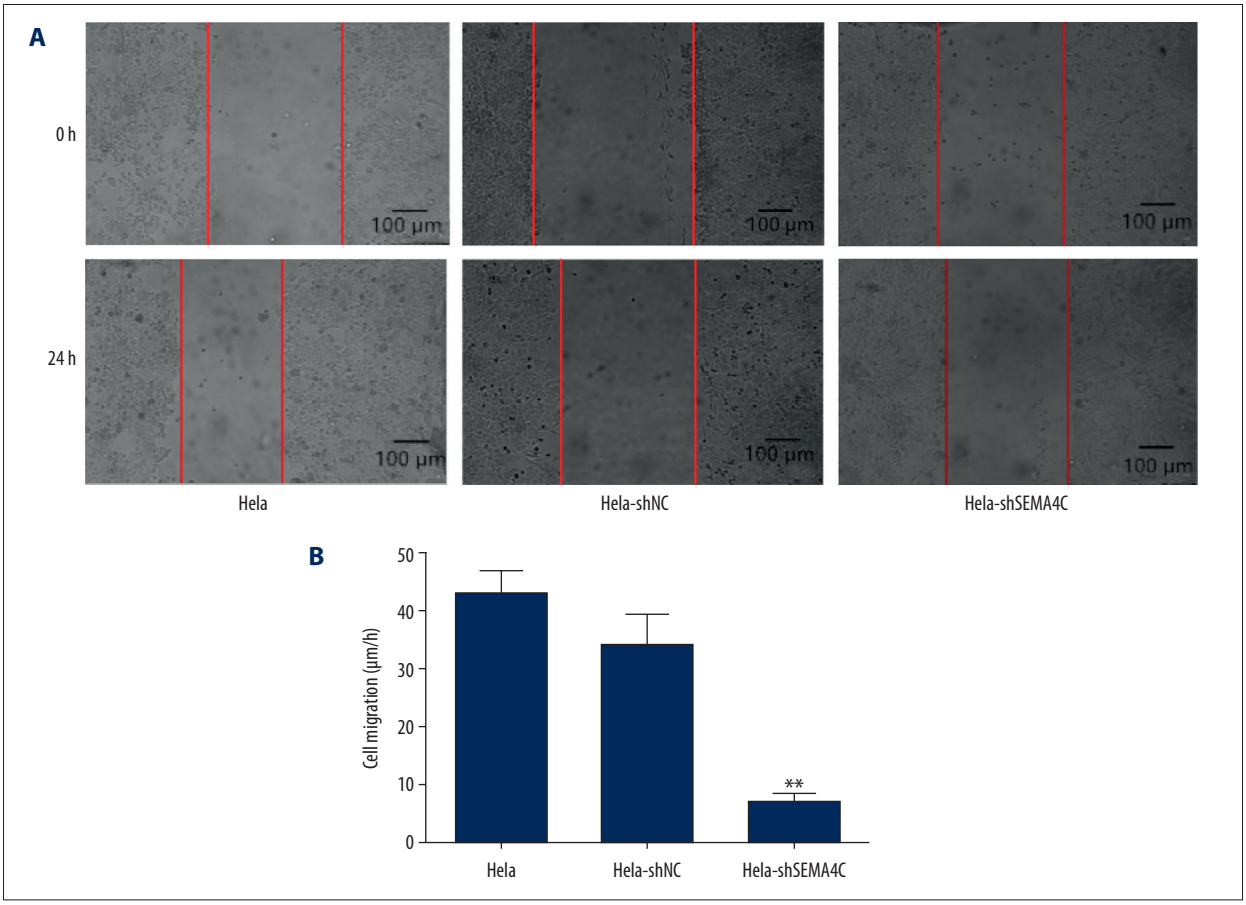

Figure 4. Effect of shSEMA4C on the migration ability of Hela cells. (**A**) photographs of scratch wound-induced migration assay (200×). (**B**) Histogram of cell migration rate. ** *P*<0.01 versus Hela and Hela-shNC subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

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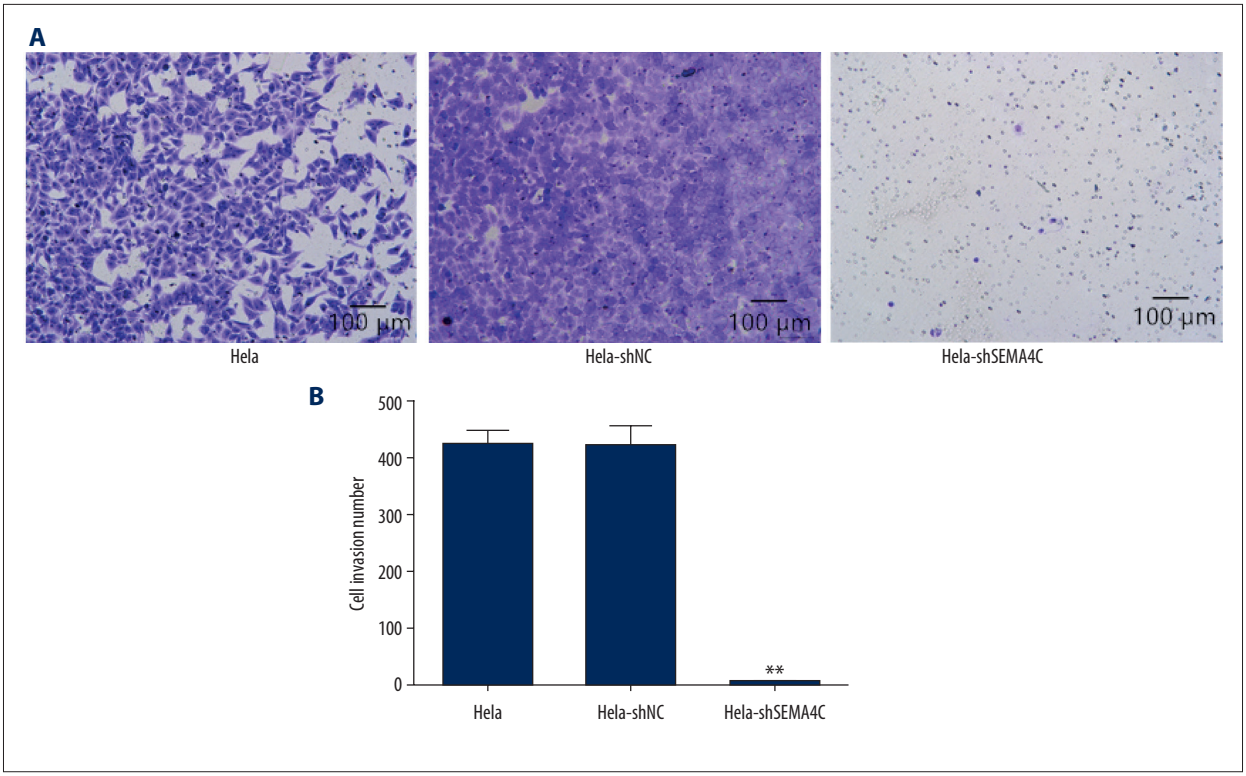

Figure 5. The effect of shSEMA4C on the invasive ability of Hela cells. (**A**) Hela cells under crystal violet stain (200×). (**B**) Histogram of cell invasion number. ** *P*<0.01 versus Hela and Hela-shNC subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

Effect of shSEMA4C on Hela cell viability

CCK-8 test results showed that compared with Hela and Hela-shNC subgroups, cell viability of Hela-shSEMA4C subgroup was significantly decreased (*P*<0.05) (Figure 3).

Effect of shSEMA4C on migration ability of Hela cells

The results of cell migration test showed that compared with Hela and Hela-shNC subgroups, the cell migration rate of Hela-shSEMA4C subgroup was significantly decreased (*P*<0.01) (Figure 4).

Effect of shSEMA4C on invasive ability of Hela cells

The results of cell invasion test showed that compared with Hela and Hela-shNC subgroups, the number of cell invasion in Hela-shSEMA4C subgroup was significantly decreased (*P*<0.01) (Figure 5).

Effect of shSEMA4C on the fluorescence intensity of F-actin in Hela cells

The results of immunofluorescence test showed that compared with Hela and Hela-shNC subgroups, the fluorescence intensity of F-actin in the Hela-shSEMA4C subgroup was significantly decreased (*P*<0.01) (Figure 6).

Effect of shSEMA4C on E-cadherin fluorescence intensity in TGF-β1-induced Hela cells

The results of immunofluorescence test showed that compared with Hela and Hela-shNC subgroups, the fluorescence intensity of E-cadherin in the Hela-shSEMA4C subgroup was significantly increased (*P*<0.01). Compared with Hela-shNC+TGF-β1 and Hela+TGF-β1 subgroups, the fluorescence intensity of E-cadherin in the Hela-shSEMA4C+TGF-β1 subgroup was significantly increased (*P*<0.01) (Figure 7).

Effect of shSEMA4C on FN content in TGF-β1-induced Hela cells

The results of ELISA test showed that compared with Hela and Hela-shNC subgroups, the FN content in the Hela-shSEMA4C subgroup was significantly decreased (*P*<0.01). Compared with Hela+TGF-β1 and Hela-shNC+TGF-β1 subgroups, the FN content in the Hela-shSEMA4C+TGF-β1 subgroup was significantly decreased (*P*<0.01) (Figure 8).

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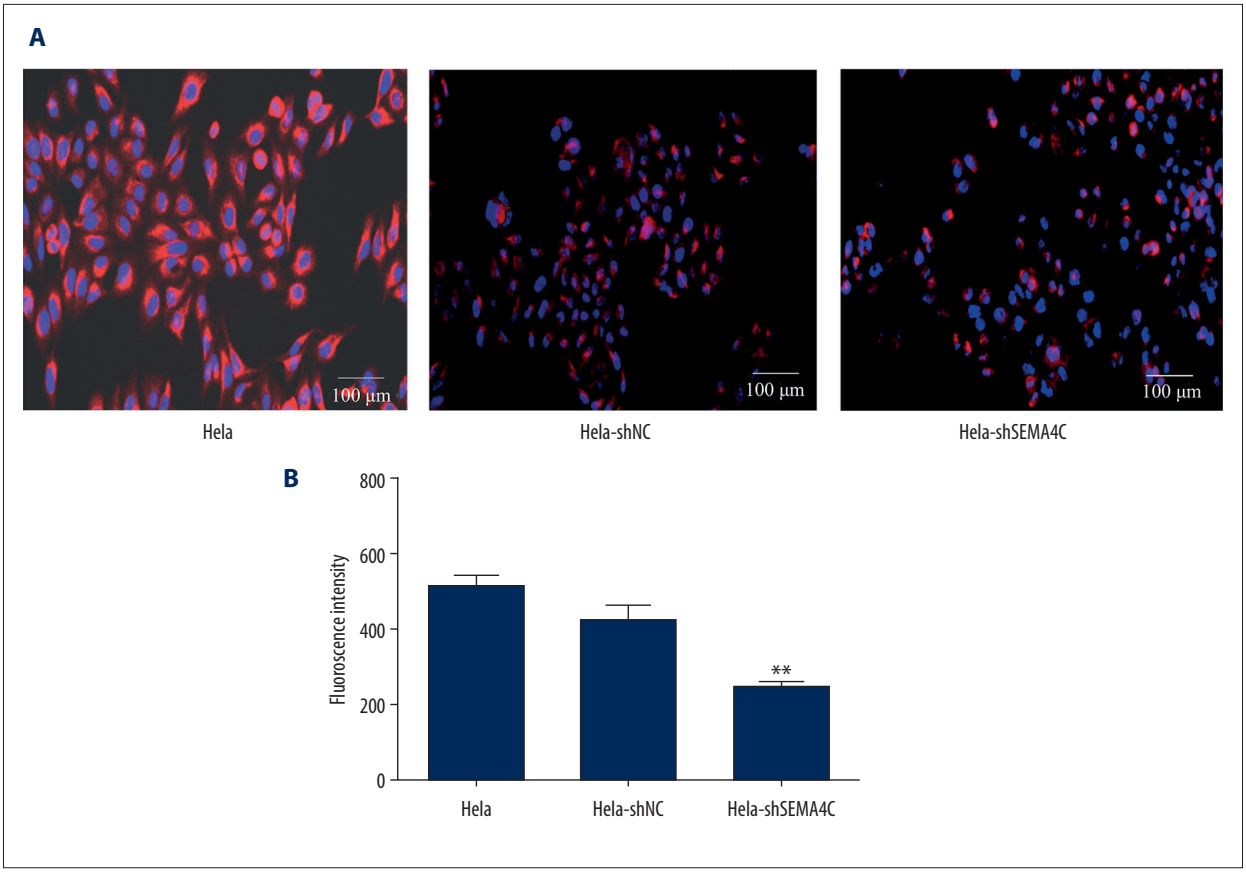

Figure 6. Effects of shSEMA4C on F-actin fluorescence intensity in Hela cells. (**A**) Hela cells under immunofluorescence (200×). (**B**) Histogram of fluorescence intensity. ** *P*<0.01 versus Hela and Hela-shNC subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

Effects of shSEMA4C on the expression of SEMA4C, E-cadherin and p-p38 in TGF-β1-induced Hela cells

The results of western blot showed that compared with Hela and Hela-shNC subgroups, the expression of E-cadherin was significantly increased (*P*<0.01) in the Hela-shSEMA4C subgroup, while the expressions of SEMA4C and p-p38 were significantly decreased (*P*<0.01). Compared with Hela+TGF-β1 and Hela-shNC+TGF-β1 subgroups, the expression of E-cadherin was significantly increased (*P*<0.01) in the Hela-shSEMA4C+TGF-β1 subgroup, while the expressions of SEMA4C and p-p38 were significantly decreased (*P*<0.01) (Figure 9).

Discussion

EMT is a phenomenon proposed by Greenburg and Hay in 1982. It is a process whereby the epithelial cells are transformed into mesenchymal cells under specific physiological and pathological conditions, and is closely related to embryonic development, wound healing, tissue and organ fibrosis, and formation of tumor stem cells [14]. EMT plays a key role in promoting the metastasis and invasion of tumor cells, resulting in loss of polarity of epithelial cells, acquiring mesenchymal characteristics, and manifesting as downregulation of epithelial cell markers such as E-cadherin, α-catenin and β-catenin, upregulation of mesenchymal phenotype markers such as vimentin and N-cadherin, and cytokine-induced EMT formation such as Snail, Slug, Twist, Rho, TGF-β, and fibroblast growth factor (FGF) [15]. Studies found that these phenotypic changes are determined by a series of gene expression changes in cells, and among these, the downregulation of E-cadherin expression is an important molecular event in EMT [16,17]. The TGF-β family-induced EMT plays an important role in growth, development and tumor formation [18,19]. In advanced cervical cancer, the level of TGF-β1 in extracellular increases. Treatment of cervical cancer cell lines with TGF-β1 not only leads to morphological changes of EMT, but also down-regulation of E-cadherin, up-regulation of fibronectin and rearrangement of cytoskeleton fibers [20].

MAPK is a highly conserved serine/threonine protein kinase in cell. This signaling pathway includes 3 subfamilies, ERK, JNK, and p38 MAPK. After being activated by upstream cytokines,

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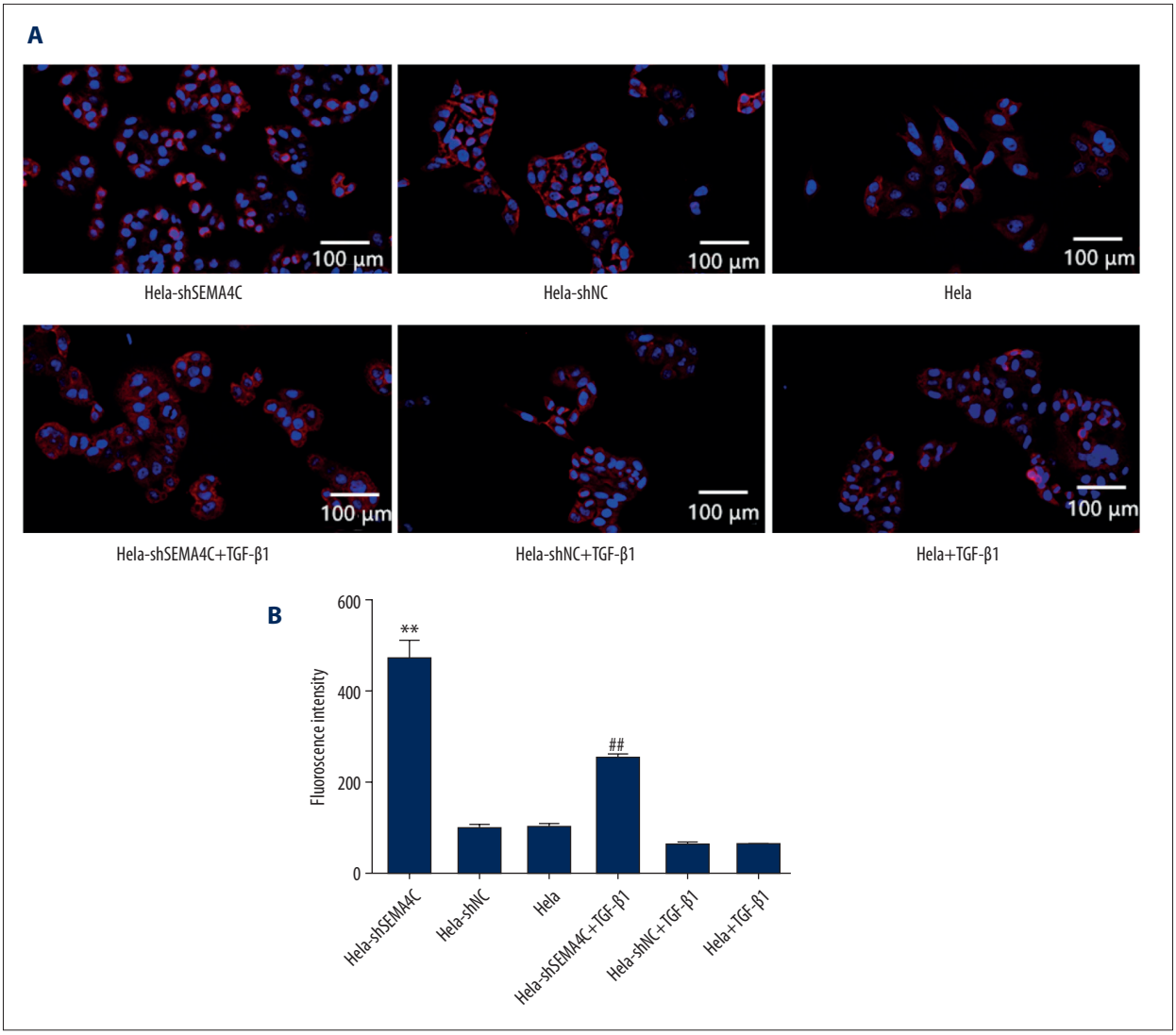

Figure 7. Effect of shSEMA4C on E-cadherin fluorescence intensity in TGF-β1 induced Hela cells. (**A**) Hela cells under immunofluorescence (200×). (**B**) Histogram of fluorescence intensity. ** $P<0.01$ versus Hela and Hela-shNC subgroups; ## $P<0.01$ versus Hela+TGF-β1 and Hela-shNC+TGF-β1 subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

hormones, and neurotransmitters, this signaling pathway can amplify the signal cascade. Take p38 MAPK signaling pathway as an example, activation of MKKK can further activate MKK, which then phosphorylates p38 MAPK. Phosphorylated p38 MAPK translocates into the nucleus and regulates the transcription factor expression, and then participates in the cellular inflammatory reaction, stress responses, and fibrosis process. Studies have shown that TGF-β1 can promote the formation of EMT by activating p38 MAPK, thereby promoting tumor invasion and metastasis [21–23]. This suggested that formation of TGF-β1-induced EMT in cervical cancer cells can be inhibited by blocking the p38 MAPK signaling pathway. The Semaphorin family is a secretory or transmembrane protein family. Years ago, it was discovered as a factor in the nervous system that exerts axon-directing function. Semaphorins can be divided into 8 classes according to their molecular structure. The family members have secretory functions and a conservative Sema domain. Members of this family regulate biological processes such as cell migration, angiogenesis, immune response and tumor formation by binding to their receptor, Plexins family. Some members of Semaphorin family involved in the isolation of epithelial cell barrier, which was related to the formation of EMT in cells, such as SEMA3E and SEMA7A [24,25]. SEMA4C, as a class IV subfamily is mainly found in developing neural tissue, and it plays an important role in regulating the directional growth of axons and the development of myotubules. Subsequent studies have also found that SEMA4C is abnormally expressed in various tissues such as gastric cancer,

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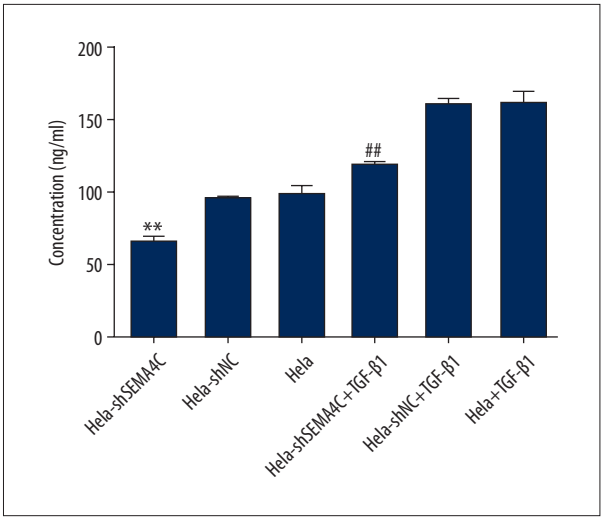

Figure 8. Effects of shSEMA4C on FN content in TGF-β1-induced Hela cells. ** $P<0.01$ versus Hela and Hela-shNC subgroups; ## $P<0.01$ versus Hela+TGF-β1 and Hela-shNC+TGF-β1 subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

colon cancer, lung cancer, and breast cancer, and is closely related to the degree of malignancy, suggesting that SEMA4C plays a certain role in the occurrence and development of malignant tumors [26–29]. Secretory SEMA4C is highly expressed in breast cancer and cervical cancer serums, and the expression in serum of patients with metastasis is higher. High expression of secretory SEMA4C promotes proliferation and invasion of cancer cells by activating PlexinB2-MET signaling pathway, thereby promoting lymph node metastasis [27]. The expression of miR-125 was downregulated in paclitaxel-induced MCF-7 and SKBR3 breast cancer cells EMT changes [30]. Upregulation of miR-25 expression can downregulate its target SEMA4C expression in cisplatin-resistance cervical cancer cells and reverse EMT [31], indicating that SEMA4C has a significant regulatory effect on EMT. In addition, SEMA4C is an important activator of P38MAPK signaling pathway during differentiation of myocytes [32]. The expression of TGF-β1-induced SEMA4C in human proximal tubular epithelial cells is upregulated, and Sema4C knockout expression can inhibit p38 MAPK activation, thereby reverse the generation of TGF-β1-induced EMT. Overexpression of SEMA4C can activate p38 MAPK signaling pathway, which promotes generation of TGF-β1-induced EMT. Moreover, the regulation function of EMT by SEMA4C can be blocked by p38 specific inhibitor [10]. This suggests that SEMA4C can affect the formation of EMT by regulating the p38 MAPK signaling pathway.

Previous studies found that SEMA4C could be expressed in a variety of cancer cells [33–35], and it was generally expressed in cervical cancer cell lines [35]. Among these, the expression in Hela cell line was higher than that in SiHa cell line [35]. Downregulation of E-cadherin was a critical step in EMT process [36,37]. Our previous study found correlation between SEMA4C and E-cadherin expression in cervical cancer cells. On this basis, in this study, we further explored the effect of SEMA4C on TGF-β1-induced EMT in cervical cancer cells and the effect on p38 MAPK activation. The Hela-shSEMA4C and Hela-shNC cell lines were established, and the success of transfection was confirmed by immunofluorescence. RT-PCR was used to determine the SEMA4C mRNA expression. The results showed that the SEMA4C mRNA expression was significantly

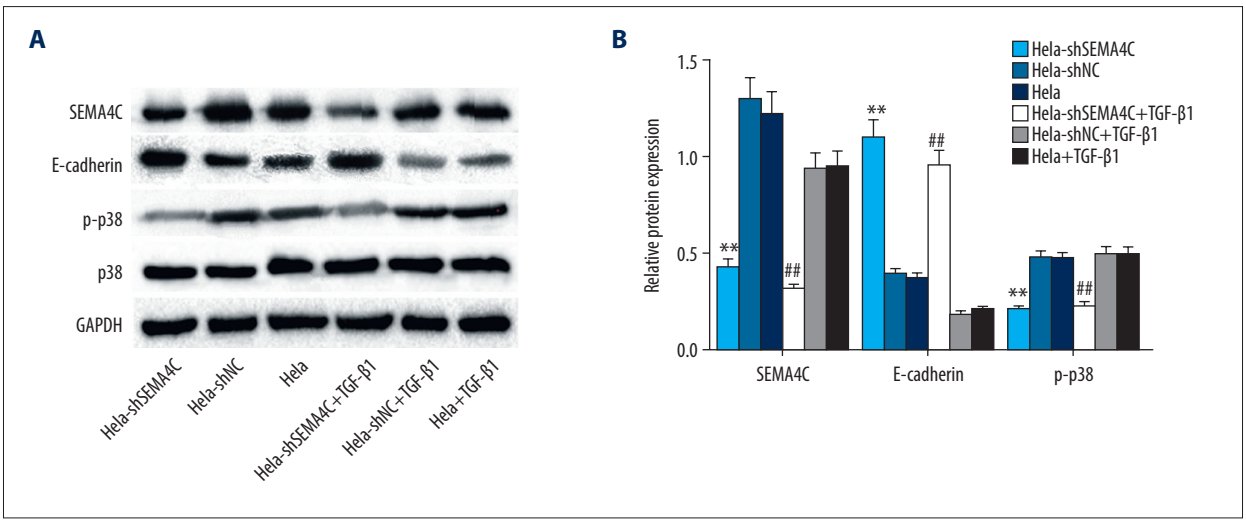

Figure 9. Effects of shSEMA4C on the expression of SEMA4C, E-cadherin and p-p38 in TGF-β1-induced Hela cells. (**A**) Western blot. (**B**) Histogram of relative protein expression. ** $P<0.01$ versus Hela and Hela-shNC subgroups; ## $P<0.01$ versus Hela+TGF-β1 and Hela-shNC+TGF-β1 subgroups. Comparison between subgroups was performed with ANOVA test. The experimental tests were performed 3 times.

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decreased in the transfected SEMA4C Hela cells. CCK-8 test, scratch test and invasion test were used to detect the effect of SEMA4C on the viability, migration and invasion of Hela cells, respectively. The results showed that downregulation of SEMA4C expression could significantly inhibit the viability, migration, and invasion ability of Hela cells. The ability to migrate and invade is important for metastasis to occur. In addition, the results of immunofluorescence test showed that downregulation of SEMA4C expression could attenuate the intensity of F-actin, suggesting that it could lead to rearrangement of cytoskeletal fibers in Hela cells. This may also contribute to the decrease in cell migration [38]. Next, the effects of SEMA4C on FN content, E-cadherin expression, and fluorescence intensity in TGF-β1-induced Hela cells were investigated. FN was found to be upregulated in cervical cancer tissues in previous studies [39], while TGF-β1 promotes EMT and cell invasion in Hela cells [40]. The results of this study showed that downregulation of SEMAC4 expression could significantly reduce FN content, upregulate E-cadherin expression and increase fluorescence intensity in TGF-β1-induced Hela cells, suggesting that it could significantly inhibit EMT in TGF-β1-induced Hela cells. Finally, western blot was used to investigate the effect of SEMA4C on p38 MAPK activation in TGF-β1-induced Hela cells. The results showed that downregulation of SEMA4C expression could significantly reduce the phosphorylation level of p38 MAPK in TGF-β1-induced Hela cells, which would then upregulate the expression of E-cadherin and inhibit EMT in Hela cells. This finding signifies the importance of the Sema4C-MAPK signaling pathway which can be a potential therapeutic target in the treatment of cervical cancer.

This is our preliminary report. We are currently establishing other cervical cancer cell lines with C33A and SiHa, the results and comparisons will be presented in the subsequent studies.

Conclusions

Our study demonstrated that downregulation of SEMA4C can inhibit EMT and the invasion and metastasis of cervical cancer cells via inhibiting TGF-β1-induced Hela cells p38 MAPK activation.

Conflict of interests

None.

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