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EFEMP2 increases the invasion ability of cervical cancer cells by promoting EMT via the Raf/MEK/ERK signaling pathway

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EFEMP2 has been reported as a candidate oncogene. To investigate the role of EFEMP2 in cervical cancer cell proliferation and invasion, the mRNA and protein expressions of EFEMP2 in 5 different cervical cancer cell lines were detected. And then the effects of up- or down-regulation of EFEMP2 expression on the biological behavior of cervical cancer cells were further investigated by transfection experiments and cell function assays *in vitro* and *in vivo*. The results revealed that EFEMP2 was highly expressed in highly invasive Ca Ski cells and lowly expressed in less invasive HT-3 cells. When EFEMP2 was knocked down, the proliferation and invasion ability of cervical cancer cells were also reduced, accompanied by the decreased expression of MMP-1, MMP-13, MMP-3, and MMP-10, meanwhile, the EMT process was blocked and the Raf/MEK/ERK signaling pathway was inhibited. On the contrary, the upregulation of EFEMP2 could promote the proliferation and invasion of cervical cancer cells by inducing EMT and activating the Raf/MEK/ERK pathway. In conclusion, EFEMP2 could increase the invasion ability of cervical cancer cells by upregulating the expression of MMP-1, MMP-13, MMP-3, and MMP-10 and inducing the EMT process through the Raf/MEK/ERK pathway. EFEMP2 played a promoting role in the development of cervical cancer and provided a potential therapeutic target for inhibiting the invasion and metastasis of cancer cells and improving the prognosis of cervical cancer patients.

Key words: EFEMP2, cervical cancer, invasion, EMT, MMPs, Raf/MEK/ERK signaling pathway

In 2018, over 570,000 women worldwide suffered from cervical cancer, which is the fourth most common malignancy in women, causing 311,365 deaths annually, with about 85% occurring in underdeveloped or developing countries [1, 2]. Although, extensive research on cervical cancer vaccines, screening tests, diagnostic equipment, and treatment played a very important role in reducing the incidence of cervical cancer, however, the invasion and metastasis of cervical cancer is still a major challenge in the treatment process, and a large number of patients die from metastasis and recurrence [3]. Therefore, on the basis of conventional surgery, radiotherapy, and chemotherapy, new treatment methods are needed to improve the prognosis of patients with cervical cancer. In this regard, targeted gene delivery therapy is presented as a promising approach, but the clinical effect is not significant in the treatment of cervical cancer patients [4]. So, it is of great significance to elucidate the mechanism of invasion and metastasis of cervical cancer and to find effective targeted drugs to improve the prognosis.

The fibulin family is the main component of elastic fibers in the extracellular matrix (ECM) and includes 8 extracellular glycoproteins, fibulin 1–8 [5]. Based on length and domain, fibulins-3, -4, -5, and -7 have been recognized as short fibulins, while long fibulins include fibulins-1, -2, -6, and -8 [6, 7]. They participate in the regulation of cell morphology, growth, adhesion, and invasion, and are closely related to the progression of a variety of tumors [8, 9]. EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2), also known as mutant p53 binding protein 1 (MBP1) or fibulin-4, is a 443-amino acid secreted protein and has been reported as a candidate oncogene [10, 11]. This gene is often found in tissues and organs rich in elastic fibers, closely related to the formation of elastic fibers and the development of connective tissue [12, 13]. Influenced by the tumor microenvironment, the role of EFEMP2 in tumorigenesis and development may have tissue specificity [14, 15]. So far, different studies have shown different conclusions, including both tumor inhibition and carcinogenic activity. For instance, in ovarian

cancer [16], gliomas [17], and colon cancer [18], EFEMP2 acted as an oncogene to promote the progression of cancer, however, in bladder cancer [19], breast cancer [20, 21], lung cancer [22], and endometrial carcinoma [23], this gene had tumor suppressive functions. In cervical carcinoma [24], EFEMP2 was found highly expressed in cancer tissues and highly invasive subclones, and related to poor prognostic clinicopathologic features. However, how EFEMP2 promotes the progression of cervical cancer is unclear.

In our study, lentivirus transfection technology was used to knock down the expression of EFEMP2 in highly invasive cervical cancer cells and increase the expression of EFEMP2 in lowly invasive cervical cancer cells, further illustrating the effects of altered expression of EFEMP2 on cell proliferation and invasion.

Materials and methods

Cell culture. Human cervical cancer cell lines Ca Ski, HeLa, SiHa, C33A, and HT-3 were obtained from the National Collection of Authenticated Cell Cultures, Chinese Academy of Sciences. Ca Ski cells containing an integrated human papillomavirus type 16 genome (HPV-16) and HPV-18 related sequences, were cultured in medium RPMI-1640 (Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin solution. HeLa cells containing HPV-18 sequences and SiHa cells containing an integrated HPV-16 genome were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) with 10% FBS. And C33A and HT-3 cells containing no virus particles of HPV-16 and HPV-18 were cultured with McCoy's 5A medium (Gibco) supplemented with 10% FBS.

Table 1. The sequence of primer in real time RT-qPCR.

Primer name	Sequences
EFEMP2	F: 5'-GCTGCTACTGTTGCTCTTGGG-3' R: 5'-GGGATGGTCAGACACTCGTTG-3'
CDH1	F: 5'-GGATTGCAAATTCCTGCCATTC-3' R: 5'-AACGTTGTCCCGGGTGTCA-3'
CDH2	F: 5'-CGAATGGATGAAAGACCCATCC-3' R: 5'-GCCACTGCCTTCATAGTCAAACACT-3'
VIM	F: 5'-AACCTGGCCGAGGACATCA-3' R: 5'-TCAAGGTCAAGACGTGCCAGA-3'
SNAIL	F: 5'-GCTCCCTCTTCCTCTCCATACC-3' R: 5'-AAGTCCTGTGGGGCTGATGT-3'
SLUG	F: 5'-GAAGCATTTCAACGCCTCCAA-3' R: 5'-GTTGTGGTATGACAGGCATGGAGTA-3'
TWIST	F: 5'-CAGCTACGCCTTCTCGGTCT-3' R: 5'-CTGTCCATTTTCTCCTTCTCTGG-3'
ZEB2	F: 5'-AAATGCACAGAGTGTGGCAAGG-3' R: 5'-CTGCTGATGTGCGAACTGTAGGA-3'
MMP-1	F: 5'-CACAAACCCCAAAAGCGTGT-3' R: 5'-TCGGCAAATTCGTAAGCAGC-3'
MMP-8	F: 5'-TGCTATCACCACACTCCGTG-3' R: 5'-ATACCAGTTGGAAGGGATGGC-3'
MMP-13	F: 5'-TCCTGGGCCAAATTATGGAG-3' R: 5'-GGGTCCTTGGAGTGGTCAAGA-3'
MMP-2	F: 5'-CTCATCGCAGATGCCTGGAA-3' R: 5'-TTCAGGTAATAGGCACCCTTGAAGA-3'
MMP-9	F: 5'-ACGCACGACGTCTTCCAGTA-3' R: 5'-CCACCTGGTTCAACTCACTCC-3'
MMP-3	F: 5'-CTGGGCCAGGGATTAATGGAG-3' R: 5'-CAATTTCATGAGCAGCAACGAGA-3'
MMP-10	F: 5'-TGCTTTGTCCTTCGATGCCA-3' R: 5'-GGTTCCAGTGGGATCTTCGC-3'
MMP-11	F: 5'-CCCAAGACTCACCGAGAAGG-3' R: 5'-AGCGAAAGGTGTAGAAGGCG-3'
ACTB	F: 5'-TGGCACCCAGCACAATGAA-3' R: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'

Immunocytochemistry (ICC). The streptavidin-biotin-peroxidase (SP) kits were used for ICC experiments. Logarithmic growing cells were inoculated in a 6-well plate on the cover slide, fixed with 4% paraformaldehyde, and then stained by the SP method; the specific steps referred to the instructions of the SP staining kit (ZSGB-BIO, China). EFEMP2 (ab125073, Abcam) positive staining showed brown granules in the cell membrane and cytoplasm. The results of cell staining were evaluated by a semi-quantitative scoring method [25], and the total expression score was calculated according to the sum of the staining intensity score and cell positive number score. The two pathologists scored the cell coverslips separately, and if the results were inconsistent, the consensus was reached through a discussion.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). Logarithmic growing cells were completely lysed by RNAiso Plus (TaKaRa) to get the total RNA, of which 2 μl were used to carry out reverse transcription to obtain complementary DNA (cDNA) by the RT reagent Kit (TaKaRa). PCR amplification was performed using the TB Green™ Premix Ex Taq™ II kit (TaKaRa) by LightCycler®480 System (Roche Diagnostics), and a total reaction volume was 20 μl. After the PCR reaction, the CT values were relatively quantified by the $2^{-\Delta\Delta CT}$ method [26]. Primers of internal reference β-actin and target genes were designed and synthesized by TaKaRa Bioengineering Co., Ltd. The primer sequences are shown in Table 1. The experiments were repeated 3 times.

Western blotting. Log growth period cells were collected into an EP tube, in which was added a pre-mixed lysate (RIPA:PMSF = 100:1) for cell lysis. After preparing the 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel, 40 μg/lane sample protein was added to corresponding lanes and underwent phased electrophoresis. After transferring in ice box, the polyvinylidene difluoride (PVDF) membranes with electrophoretic-separated proteins were taken out and blocked with 5% bovine serum albumin (BSA) for 1h, and then incubated with primary antibodies (E-cadherin #3195, N-cadherin #13116, Vimentin #5741, Snail #3879, Twist #69366, Slug #9585, Zeb2 #97885, P-Raf-1 #9431, Raf-1 #9422, P-MEK1/2 #9154, MEK1/2 #9126, P-ERK1/2 #4370, ERK1/2 #4695, Cell Signaling Technology; MMP-1 ab134184, MMP-2 ab86607, MMP-3 ab52915, MMP-8 ab81286, MMP-9 ab76003, MMP-10 ab261733, MMP-11 ab119284, MMP-13

ab219620, EFEMP2 ab125073, Abcam) with the dilution of 1:1000 at 4 °C overnight. The next day, the membranes were incubated with corresponding secondary antibodies (Boster Biology) at 25 °C for 1 h. Protein bands were developed by the enhanced chemiluminescence (ECL) method (Bio-Rad ECL kit, Solarbio), while the gray value was processed with the ImageJ software. The experiment was repeated 3 times.

Lentiviral transfection for upregulation or downregulation of EFEMP2. The lentiviral overexpression or knockdown vectors for EFEMP2 were constructed by Genechem Co. (Shanghai, China), and all vectors were labeled with GFP fluorescence and puromycin. Log growth period cells were digested, centrifuged, inoculated into a 24-well plate, and then cultured for 24 h at 37 °C and 5% CO_2. Lentiviral transfection was performed with the multiplicity of infection value (MOI, number of virus particles/number of living cells) of 50 for RNAi and 100 for overexpression. After routine culture for 72 h, under the fluorescence microscope, the proportion of cells with high fluorescence to the total number of cells was more than 80%, which was considered to be ideal transfection efficiency. The mRNA and proteins of overexpression or knockdown groups were extracted and qRT-PCR, western blotting, and ICC were used to verify the transfection effect.

Cell growth curve. Log growth period cells were digested, centrifuged, inoculated into a 24-well plate (10,000 cells/well), and then cultured at 37 °C and 5% CO_2. From the next day, three wells were taken every day to count and calculate the mean value, and the observation and counting lasted for 1 week. According to the observation results, the growth curve was drawn with time as the horizontal axis and the average daily cell number as the longitudinal axis.

Cell cycle assay. Cell cycle analysis was performed by flow cytometry (CytoFLEX, Beckman), firstly, log growth period cells were digested with trypsin and washed with ice PBS, the final concentration of which was adjusted to 5×10^5 cells/ml. After that the cells were fixed with 5 ml pre-cooled 70% ethanol and placed at –20 °C overnight. And then the fixed cells were treated with RNase at 37 °C for 30 min and stained with propidium iodide (PI) at 25 °C in darkness for 10 min, finally, the proportion of cells at each stage of the cell cycle was analyzed by the ModFit LT 4.1 (Verity Software House, USA).

Plate cloning formation test. Log growth period cells were digested and suspended, 1,000 cells/well were added to the 6-well plate, mixed evenly, and then cultured at 37 °C and 5% CO_2. After 10 days of routine culture, the culture medium from the well was sucked out, and the cell clones were stained with crystal violet for 10 min. The six-well plate was placed on a white background with sufficient light, each well was photographed, and the number of clones in each well was counted. The experiment was repeated 3 times.

Cell invasion and migration assay *in vitro* with Boyden chamber. For cell migration assay *in vitro*, 2×10^5 cells suspended in 200 µl culture medium were added in the upper compartment, and 600 µl NIH3T3 cell serum-free conditioned medium was added to the lower compartment as a chemokine. After 12 h of routine culture, the excess cells without penetrating the membrane on the upper layer of the filter were removed, and the penetrated cells on the lower layer of the membrane were stained and counted under an inverted microscope. For cell invasion assay *in vitro*, Matrigel (BD BioCoat) was mixed with a serum-free medium at 1:7 ratio, 50 µl of which was added to the upper layer of the membrane to simulate the structure and function of extracellular matrix (ECM). The remaining steps were the same as the cell migration assay *in vitro*, except that the culture time allowing cells to penetrate the microporous membrane was 24 h.

Establishment of models with nude mice orthotopic transplantation tumor. Twenty-five 3 or 4-week-old female BALB/C/nu/nu mice were randomly divided into 5 groups: Ca Ski, Ca Ski-shRNA1, Ca Ski-shRNA2, HT-3, and HT-3-exEFEMP2. Each nude mouse was injected with about 0.2 ml cell suspension (1.0×10^7 cells/ml) subcutaneously in the neck and back. The state of the nude mice was observed regularly, and the size of the tumor was measured weekly with vernier calipers. According to the formula Volume = Length × Width^2 × 0.5, the tumor volume of nude mice was calculated and the growth curve of the tumor was drawn. After 8 weeks of inoculation, all nude mice were euthanized with carbon dioxide (CO_2), and the tumors of each group were completely stripped and measured. All animal experiments were approved by the Institutional Medical Ethics Committee of Shandong University. All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Statistical analysis. Data storage and management were processed and statistically analyzed by Excel 2016 and SPSS 24.0. For quantitative data, mean ± standard deviation was used to describe it. A t-test and F-test were used for intergroup comparison, and ANOVA test was used for pair comparison. A p-value <0.05 (two-sided) was considered statistically significant.

Results

Protein and mRNA expressions of EFEMP2 in 5 different cervical cancer cell lines. The invasive abilities of 5 cervical cancer cell lines Ca Ski, HeLa, SiHa, C-33A, and HT-3 were detected by the Boyden chamber invasion assay, the results showed that Ca Ski cells had the highest invasive ability with the most cells penetrating the Matrigel and membrane, at the same time C33A and HT-3 cells had the lower invasion capability with the less penetrating cells, and the invasive ability of HeLa and SiHa were weaker than Ca Ski but stronger than C33A and HT-3 (Figure 1A). The protein and mRNA expressions of EFEMP2 in these cervical

cancer cell lines were detected by ICC (Figure 1B), western blotting (Figure 1C), and real-time RT-qPCR (Figure 1D), respectively. The high-invasive cell line Ca Ski had the highest expression of EFEMP2, and the low-invasive cell lines C-33A and HT-3 had the lowest expression of EFEMP2. The expression of EFEMP2 increased with the increase of cell invasion ability. In the subsequent experiments, RNA interference was used to reduce the expression of EFEMP2 in Ca Ski cell line, and lentivirus transfection was performed to increase the expression of EFEMP2 in HT-3 cells, so as to investigate the effects of altered expression of EFEMP2 on proliferation and invasion ability of cervical cancer cells.

Determination of the transfection efficiency of EFEMP2 up- or down-regulation. As shown in Figures 1E and F, the fluorescent cell number >80% indicated that the transfection efficiency was satisfied. EFEMP2-RNAi-lentivirus transfected Ca Ski cells were called Ca Ski-sh1 and Ca Ski-sh2, and EFEMP2-overexpression-lentivirus transfected HT-3 cells were called HT-3-cDNA. Then, the transfected cells were amplified, and the transfection effect was determined by western blotting (Figure 1G), real-time RT-qPCR (Figure 1H), and ICC (Figure 1I). The results showed that at protein and mRNA levels, the expressions of EFEMP2 in Ca Ski-sh1 and Ca Ski-sh2 were significantly lower than that in negative control cells, at the same time, the expression of EFEMP2 in HT-3-cDNA was significantly higher than that in negative control cells.

Effects of EFEMP2 up- or downregulation on cervical cancer cell proliferation, cell cycle, and clonogenicity. The cell growth curve is one of the basic parameters of the biological characteristics of cultured cells and also an important index to determine the viability of cells. The results of growth curves revealed that EFEMP2 downregulation significantly hampered the proliferation capacity of cervical cancer cells, while EFEMP2 upregulation remarkably improved the proliferation capacity of cervical cancer cells (Figure 2A). Further flow cytometry cell cycle test results showed that the downregulated expression of EFEMP2 inhibited cells from entering the DNA replication phase, increased the proportion of cells in the G1 phase (prophase of DNA synthesis), and decreased the proportion of cells in the S phase (DNA synthesis), on the other hand, EFEMP2 upregulation promoted cells entering the DNA replication phase (Figure 2B). Effects of upregulation or downregulation of EFEMP2 on the clone formation ability of cervical cancer cells were detected by the plate cloning formation test. The results revealed that EFEMP2 downregulation inhibited the clonogenicity of cervical cancer cells, meanwhile, the number of cell clones formed in the EFEMP2 upregulation group was markedly increased (Figure 2C). In conclusion, EFEMP2 downregulation inhibited the growth, proliferation, and clonogenicity of cervical cancer cells, while EFEMP2 upregulation promoted cell growth, proliferation, and clonogenicity.

Effects of EFEMP2 up- or downregulation on cervical cancer cell migration and invasion. Using the Transwell chambers, we examined the effect of the upregulation or downregulation of EFEMP2 on the migration and invasion activity of cervical cancer cells. By comparing the average number of cells migrated through the PVPF membrane or invaded through the Matrigel between different groups, the following results could be concluded. In the cell migration experiment (Figure 2D), the number of EFEMP2 shRNA1 and shRNA2 transfected cells migrating through the PVPF membrane was significantly less than that of the control group, and the cell migration ability of the EFEMP2 down-expression group was decreased. When EFEMP2 expression was upregulated, the number of EFEMP2 cDNA transfected cells migrating through the PVPF membrane was significantly higher than that of the control group, therefore the migration ability of the cells was also upregulated accordingly. Similarly, in the cell invasion experiment (Figure 2E), when EFEMP2 expression was downregulated, the average number of EFEMP2 shRNA1 and shRNA2 transfected cells invading through Matrigel was significantly lower than that of negative control cells, and the cell invasion ability of the EFEMP2 down-expression group was decreased. On the contrary, the average number of EFEMP2 cDNA transfected cells invading through Matrigel was significantly higher than that of negative control cells, with the upregulated expression of EFEMP2, the invasion ability of cervical cancer cells was significantly enhanced. In conclusion, the expression of EFEMP2 is positively correlated with the migration and invasion ability of cervical cancer cells.

Effects of EFEMP2 up- or downregulation on the growth of xenotransplantation tumor *in vivo*. The xenotransplantation model of nude mice was established to further evaluate the effect of up- or downregulation of EFEMP2 on tumor growth *in vivo*. The experiment was divided into 5 groups, 5 nude mice in each group were subcutaneously inoculated with EFEMP2 shRNA1 and shRNA2 transfected cells, EFEMP2 cDNA transfected cells, and corresponding negative control cells, respectively. All mice were fed in the SPF (specific pathogen-free) environment for 8 weeks. The EFEMP2 shRNA1 and shRNA2 transfection groups showed significant lag in tumor size and growth rate, while tumors formed by cells transfected with EFEMP2 cDNA grew faster *in vivo* than those of the negative control group (Figure 3A). EFEMP2 shRNA1 and shRNA2 transfected cells downregulating EFEMP2 had significantly smaller mean tumor volume than the negative control group, while EFEMP2 cDNA transfected cells upregulating EFEMP2 had significantly larger subcutaneous tumor volume than the negative control group (Figures 3B, 3C). In general, EFEMP2 downregulation inhibited tumor formation and growth, while EFEMP2 upregulation significantly promoted tumor growth *in vivo*.

Effects of EFEMP2 up- or downregulation on the epithelial to mesenchymal transition (EMT)-related genes and matrix metalloproteinases (MMPs). In this study, we found that up- or downregulation of EFEMP2 can affect the proliferation and invasion ability of cervical cancer cells *in vitro* and

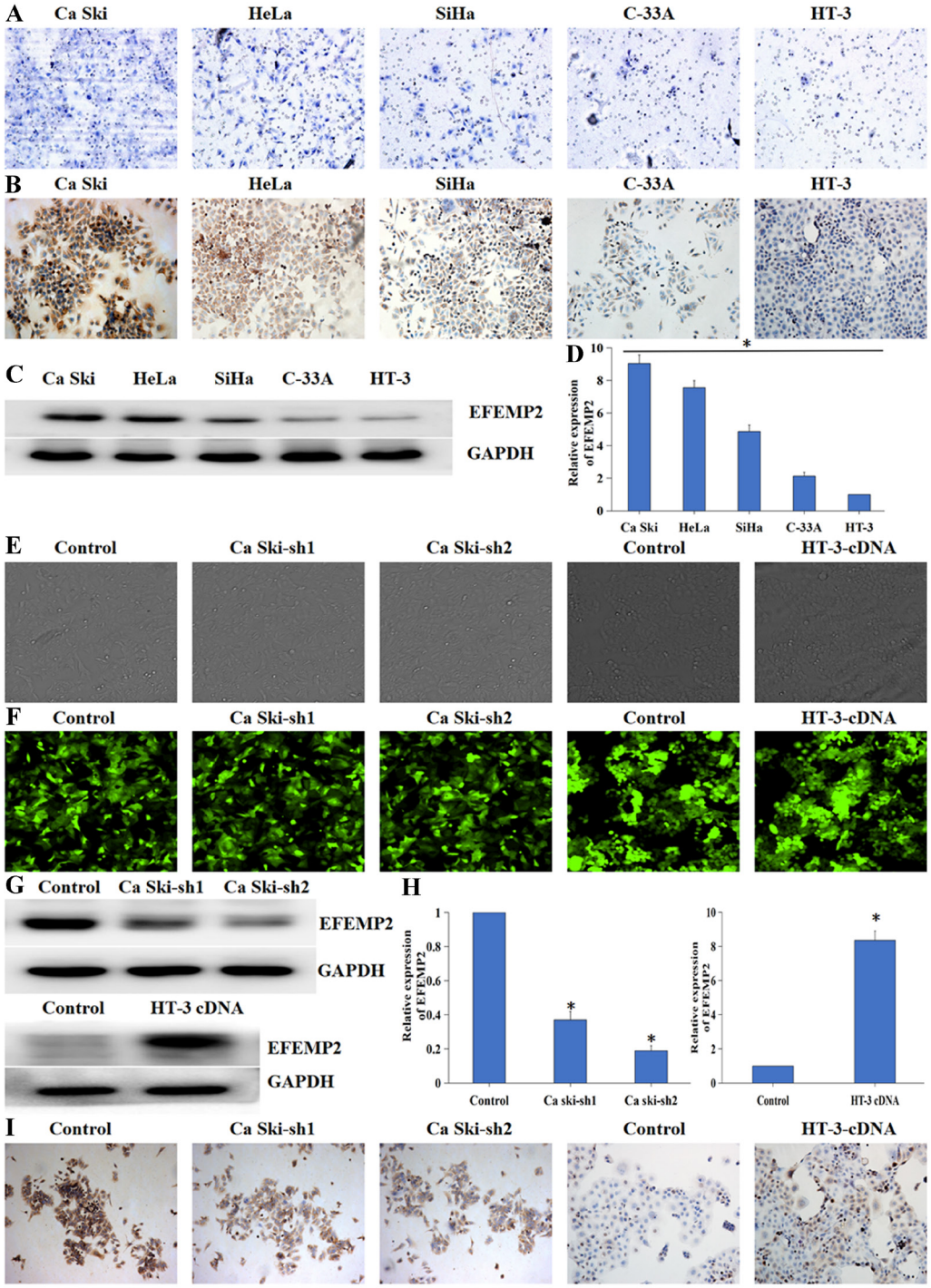
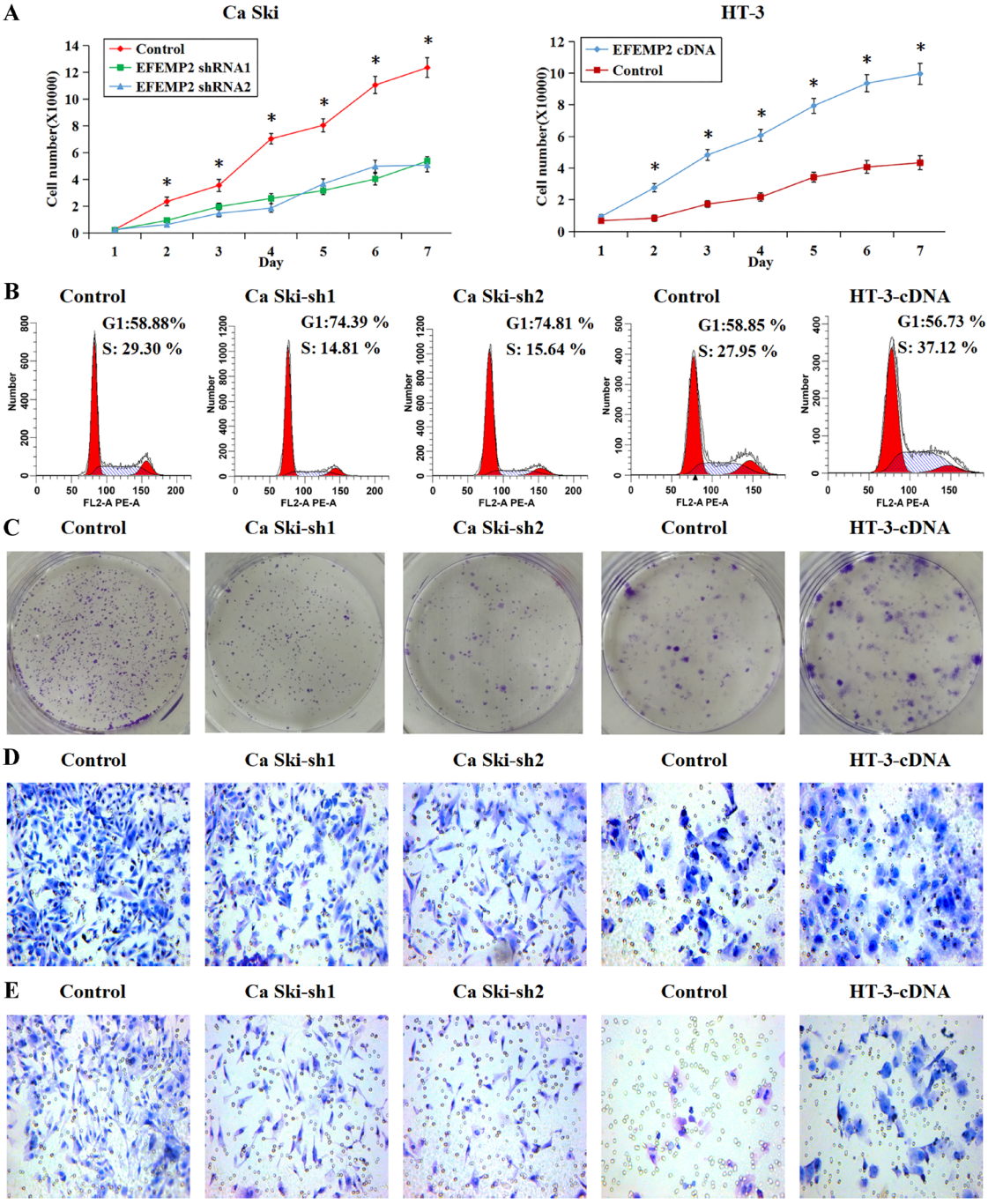


Figure 1. Expression of EFEMP2 in 5 cervical cancer cell lines Ca Ski, HeLa, SiHa, C-33A, and HT-3 and the verification of lentiviral transfer efficiency for EFEMP2 up- or downregulation. A) Images of cell invasion assay performed in 5 cervical cancer cell lines Ca Ski, HeLa, SiHa, C-33A, and HT-3 by the Boyden chambers. Ca Ski cells had the highest invasive ability with the most cells penetrating the Matrigel and membrane, while HT-3 cells had the lowest invasion capability with the less penetrating cells. The expressions of EFEMP2 in these cervical cancer cell lines were measured by B) ICC staining, C) western blotting (cropped blot), and D) real-time RT-qPCR. EFEMP2 was highly expressed in highly invasive Ca Ski cells and lowly expressed in less invasive HT-3 cells. E) The phase contrast images and F) GFP fluorescence images showed effective lentiviral transfer efficiencies, when EFEMP2 was up- or downregulated, the fluorescent cell count >80% indicated good transfection efficiency. The expression of EFEMP2 in EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, EFEMP2 cDNA transfected HT-3 cells, and negative control cells were measured by G) western blotting (cropped blot), H) real-time RT-qPCR, and I) ICC staining. EFEMP2 expressions of Ca Ski-sh1 and Ca Ski-sh2 were significantly lower than that of negative control cells, at the same time, the expression of EFEMP2 in the HT-3 cDNA group was significantly higher than that in the negative control group (magnification ×200; *p<0.05).



◂ **Figure 2. Effects of EFEMP2 up- or downregulation on cervical cancer cell proliferation, clonogenicity, and migration and invasive activity *in vitro*. The proliferation, clonogenicity, and migration and invasive abilities of cervical cancer cells were evaluated by growth curve method, flow cytometry cell cycle assay, plate cloning formation test, and Boyden chamber. A) EFEMP2 downregulation significantly hampered the proliferation capacity of Ca Ski cells, while EFEMP2 upregulation remarkably improved the proliferation capacity of HT-3 cells. B) The downregulated expression of EFEMP2 inhibited cells from entering the DNA replication phase, and the proportion of the G1 phase cells increased and the proportion of the S phase cells decreased, while the effect of EFEMP2 upregulation was just the opposite. C) The number of colonies formed by Ca Ski-sh1 and Ca Ski-sh2 cells was significantly lower than that of the negative control cells, meanwhile the number of clones formed in the HT-3 cDNA group was markedly increased, compared to the negative control group. D) The images of cell migration assay performed in EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, EFEMP2 cDNA transfected HT-3 cells, and negative control cells. E) The images of cell invasion assay performed in EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, EFEMP2 cDNA transfected HT-3 cells, and negative control cells. The number of EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells migrating through the PVPF membrane or invading through the Matrigel was significantly lower than that of negative control cells. When EFEMP2 was upregulated, more cells migrated through the PVPF membrane or invaded through the Matrigel in the EFEMP2 cDNA transfected HT-3 group than that in the control group (magnification ×200; *p<0.05).**

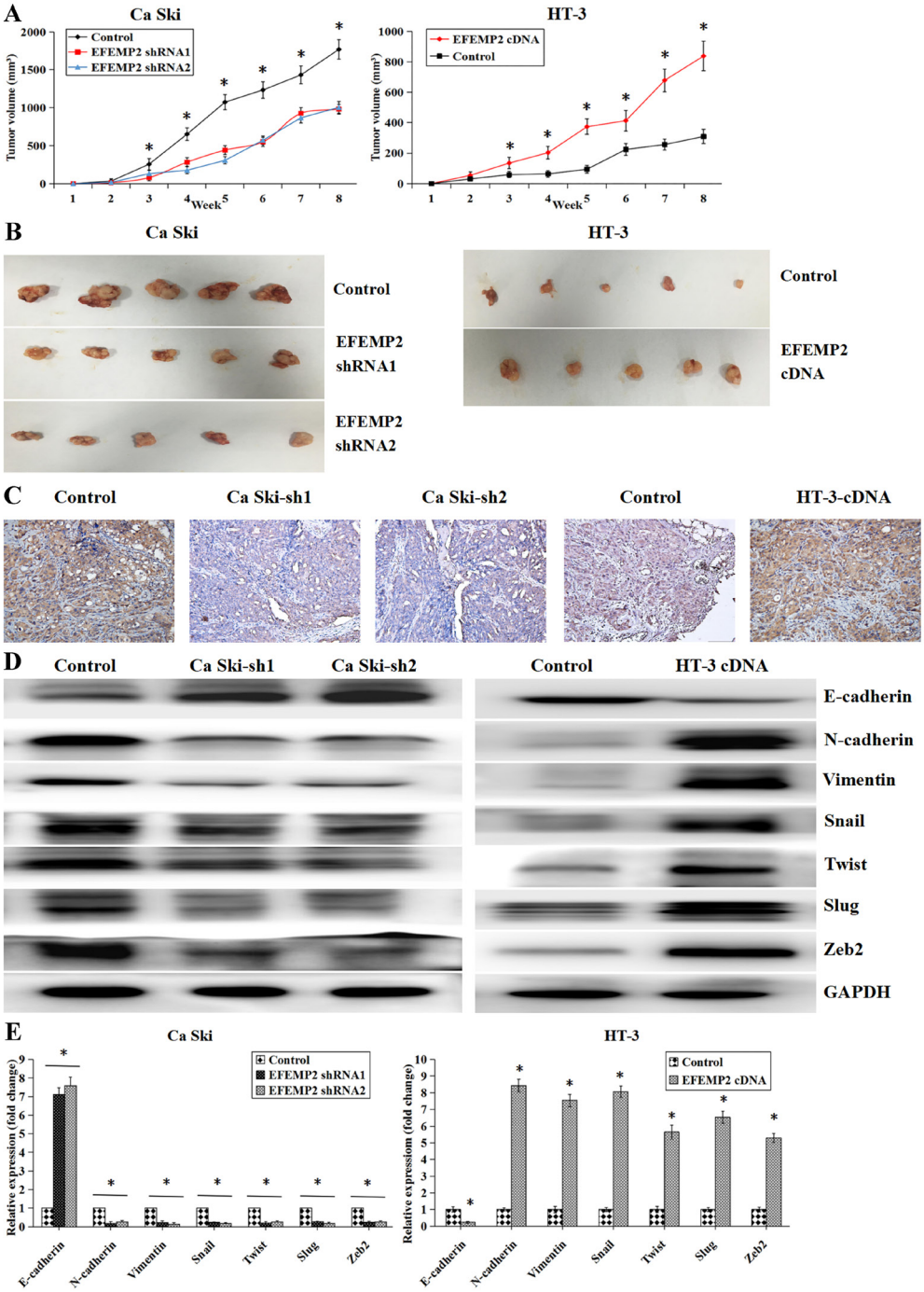


Figure 3. Effects of EFEMP2 up- or downregulation on tumor growth *in vivo* and the process of EMT. A) The growth tumors profile of EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, EFEMP2 cDNA transfected HT-3 cells, and negative control cells were observed continuously for 8 weeks. Tumor growth in the Ca Ski shRNA1 and shRNA2 groups was slower than that in the negative control group, while tumors formed by EFEMP2 cDNA transfected HT-3 cells grew faster *in vivo* than those of negative control cells. B) The images of xenotransplantation tumor after subcutaneous inoculation of EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, EFEMP2 cDNA transfected HT-3 cells, and negative control cells. EFEMP2 knockdown could inhibit tumor formation and growth in the nude mice, whereas EFEMP2 upregulation could promote tumor growth *in vivo*. C) Compared to the control group, the RNAi group exhibited a lower EFEMP2 level and the overexpression group revealed a higher EFEMP2 level by immunohistochemical assay. By D) western blotting (cropped blot) and (E) real-time RT-qPCR, EMT hallmarks, including E-cadherin, N-cadherin, Vimentin, Snail, Slug, Twist, and Zeb2 were measured in EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, EFEMP2 cDNA transfected HT-3 cells, and negative control cells. EFEMP2 downregulation increased the expression of the epithelial hallmark E-cadherin and reduced the expression of mesenchymal hallmarks N-cadherin and Vimentin, and transcription factors Snail, Slug, Twist, and Zeb2. Meanwhile, EFEMP2 upregulation decreased the expression of E-cadherin and increased the expression of N-cadherin, Vimentin, Snail, Slug, Twist, and Zeb2. *p<0.05.

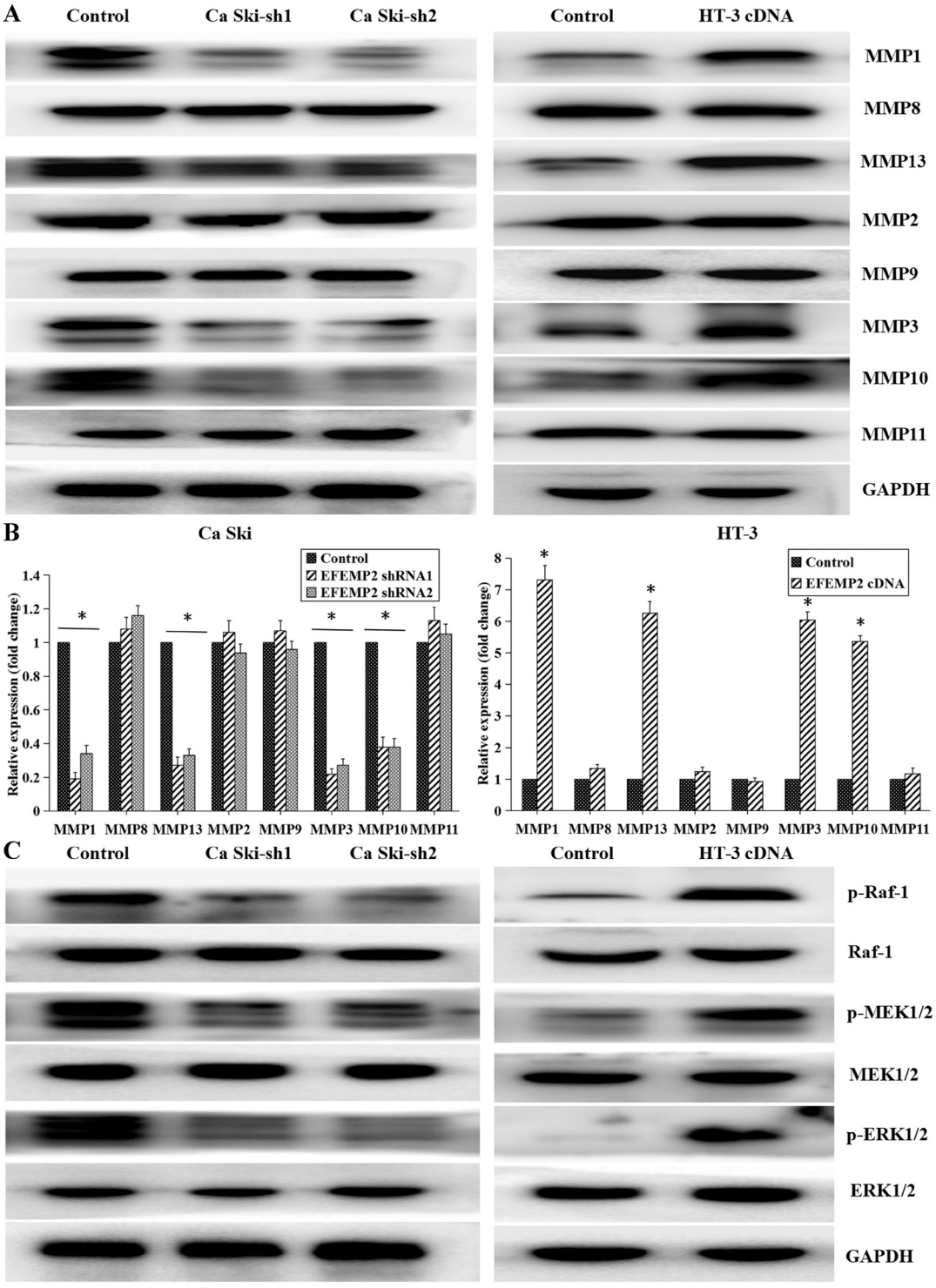


Figure 4. Effects of EFEMP2 up- or downregulation on the expression of MMPs and the Raf/MEK/ERK signaling pathway. By A) western blotting (cropped blot) and B) real-time RT-qPCR, the expression of MMP-1, MMP-8, MMP-13, MMP-2, MMP-9, MMP-3, MMP-10, and MMP-11 were measured in EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, EFEMP2 cDNA transfected HT-3 cells, and negative control cells. The downregulation of EFEMP2 weakened the expression of MMP-1, MMP-13, MMP-3, and MMP-10 but had no effect on the expression of MMP-2, MMP-9, MMP-8, and MMP-11, at the same time, the upregulation of EFEMP2 enhanced the expression of MMP-1, MMP-13, MMP-3, and MMP-10 but did not change the expression of MMP-2, MMP-9, MMP-8, and MMP-11. C) In EFEMP2 shRNA1 and shRNA2 transfected Ca Ski cells, the downregulated expression of EFEMP2 significantly reduced the phosphorylation levels of Raf-1, MEK1/2, and ERK1/2; in contrast, in EFEMP2 cDNA transfected HT-3 cells, upregulation of EFEMP2 expression remarkably increased the phosphorylation levels of Raf-1, MEK1/2, and ERK1/2.

in vivo. Therefore, we doubted whether up- or downregulation of EFEMP2 would affect EMT-related genes and MMPs. By western blotting (Figure 3D) and RT-qPCR (Figure 3E), we got the same conclusion that the downregulated expression of EFEMP2 reduced the expression of mesenchymal hallmarks N-cadherin and Vimentin and transcription factors Snail, Slug, Twist, and Zeb2, and increased the expression of the epithelial hallmark E-cadherin in Ca Ski cells. Meanwhile, upregulated EFEMP2 increased the expression of N-cadherin, Vimentin, Snail, Slug, Twist, and Zeb2, and decreased the expression of E-cadherin in HT-3 cells. About the relationship between EFEMP2 and MMPs, we got similar results as above at the protein and mRNA levels (Figures 4A, 4B), the downregulation of EFEMP2 weakened the expression of MMP-1, MMP-13, MMP-3, and MMP-10, but had no effect on the expression of MMP-2, MMP-9, MMP-8, and MMP-11. At the same time, the upregulation of EFEMP2 enhanced the expression of MMP-1, MMP-13, MMP-3, and MMP-10, but did not change the expression of MMP-2, MMP-9, MMP-8, and MMP-11. In conclusion, the downregulated expression of EFEMP2 inhibited the EMT process of cervical cancer cells and weakened the expression of some kinds of MMPs, but had no effect on gelatinases MMP-2 and MMP-9; while the upregulation of EFEMP2 expression could promote the EMT process of cervical cancer cells and enhance the expression of some MMPs, but still had no effect on gelatinases MMP-2 and MMP-9.

Effects of EFEMP2 up- or downregulation on the Raf/MEK/ERK signaling pathway. In order to explore the mechanism of EFEMP2 inhibiting or promoting the EMT process, we investigated whether the Raf/MEK/ERK signaling pathway was inhibited or activated during EFEMP2 downregulation or upregulation. As shown in Figure 4C, the downregulated expression of EFEMP2 significantly reduced the phosphorylation levels of Raf-1, MEK1/2, and ERK1/2 in Ca Ski cells transfected with EFEMP2 shRNA1 and shRNA2. In contrast, upregulation of EFEMP2 expression remarkably increased the phosphorylation levels of Raf-1, MEK1/2, and ERK1/2 in HT-3 cells transfected with EFEMP2 cDNA. Collectively, the downregulation of EFEMP2 inactivated the Raf/MEK/ERK signaling pathway, while the upregulation of EFEMP2 activated it. In addition, using RAF-1 inhibitor Sorafenib (3, 6, and 12 μmol/l), and ERK1/2 inhibitor U0126 (10, 20, and 40 μmol/l), we treated the EFEMP2 cDNA transfected HT-3 cells for 48 h to further evaluate whether signaling pathway inhibitors could inhibit the increased invasiveness of cancer cells due to increased EFEMP2 expression. As shown in Fig. 5A, Sorafenib and U0126 could significantly weaken cervical cancer cell migration and invasion capacities, which were increased by EFEMP2 upregulation. We observed this inhibition increased with the increase in the dose (Figure 5B). At the same time, Sorafenib inhibited the phosphorylation of RAF-1, and its downstream effector molecules MEK1/2 and ERK1/2 phosphorylation, further decreased the expression of N-cadherin and Vimentin, and increased the expression of E-cadherin in HT-3 cells transfected with EFEMP2 cDNA. Meanwhile, U0126 could remarkably reduce the phosphorylation levels of MEK1/2 and ERK1/2, but had no effect on Raf-1 phosphorylation, and also hampered the process of EMT. In conclusion, EFEMP2 upregulation could promote the migration and invasion ability and the EMT process of cervical cancer cells by activating the Raf/MEK/ERK signaling pathway.

Discussion

In this study, we examined the effect of altered expression of EFEMP2 on the biological behavior of cervical cancer cells. Ca Ski cells have strong proliferation and invasion ability because they contain complete HPV-16 genome and HPV-18 related sequence, in contrast, C-33A and HT-3 cells have weak proliferation and invasion ability because they do not contain HPV-16 and HPV-18 virus particles. EFEMP2 was highly expressed in highly invasive cancer cells and lowly expressed in lowly invasive cancer cells. Further lentivirus transfection and cell function detection *in vitro* and *in vivo* revealed that EFEMP2 downregulation could inhibit cervical cancer progression by blocking EMT and inactivating the Raf/MEK/ERK signaling pathway, at the same time, EFEMP2 upregulation could promote cervical cancer development by inducing EMT and activating the Raf/MEK/ERK pathway.

Human papillomavirus (HPV) infection is the leading cause of cervical cancer, its early regions encode E5, E6, and E7 oncoproteins, which have made major contributions to the development of cervical cancer by having an important effect on the various functional proteins of cancer cells [27]. Therefore, Ca Ski cells had highly invasive ability and corresponding high expression of EFEMP2, while HT-3 cells had less invasive ability and low expression of EFEMP2. The high expression of EFEMP2 was positively correlated with the invasive ability of cancer cells. In further experiments, we demonstrated that EFEMP2 could promote the development of cervical cancer. EFEMP2 played an obvious tissue-specific role in tumor development, with promotive or inhibitory effects. In osteosarcoma, EFEMP2 was overexpressed in highly invasive cell lines and subclones, its knockdown inhibited osteosarcoma cell proliferation and invasion capacities [28]. In glioma, the most aggressive type of brain tumor, EFEMP2 also exerted tumorigenic potential, the expression of EFEMP2 was significantly increased in glioma tissues and cells, and the downregulated expression of EFEMP2 significantly inhibited the proliferation and invasion capacities of the two glioma cells (U87 and U373) [17]. Similarly, EFEMP2 was highly expressed in colon cancer cells, its knockdown suppressed the growth and invasion of colon cancer cells LoVo and SW620 [18]. The inhibitory role of EFEMP2 on tumor progression was mainly shown in the following cancers. In bladder cancer, the expression of EFEMP2 is significantly lower than that in normal tissue, and the deficiency of EFEMP2 dramatically promoted cancer

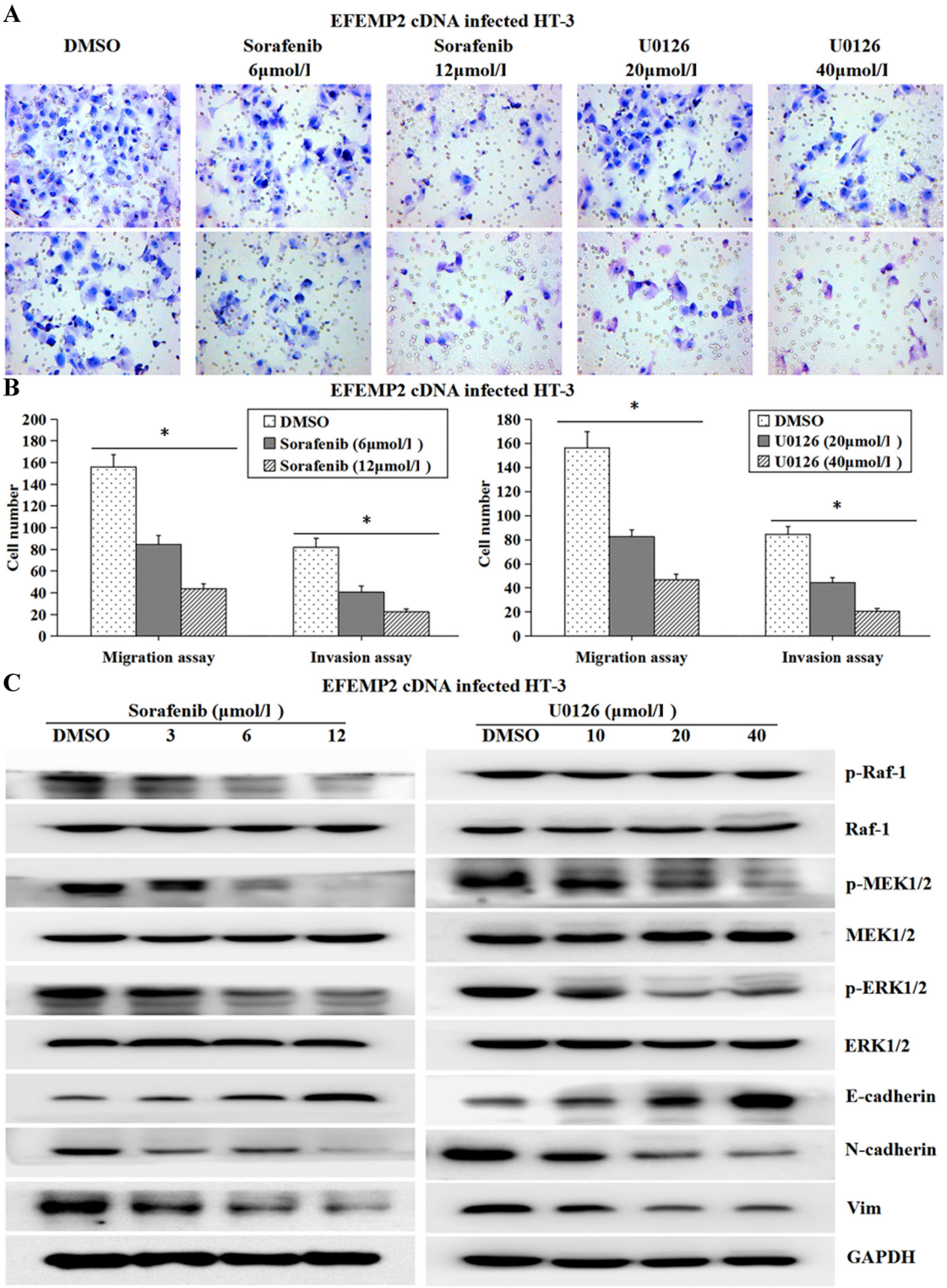


Figure 5. Effects of RAF-1 inhibitor Sorafenib and ERK1/2 inhibitor U0126 on the migration and invasion ability of cervical cancer cells and the process of EMT. A) Sorafenib and U0126 could significantly weaken cervical cancer cell migration and invasion capacities, which were increased by EFEMP2 upregulation. B) We observed this inhibition increased with the increase in the dose. C) The RAF-1 inhibitor Sorafenib, could significantly hinder RAF-1 phosphorylation and the EMT process, both of which were activated by EFEMP2 upregulation. In the same way, the ERK1/2 inhibitor U0126 could remarkably reduce the phosphorylation levels of MEK1/2 and ERK1/2 but had no effect on Raf-1 phosphorylation, and also hampered the process of EMT. EFEMP2 downregulation could inactivate the Raf/MEK/ERK signaling pathway, while EFEMP2 upregulation could activate it.

cell proliferation, migration, and metastasis [19]. In breast cancer [20, 21], lung carcinoma [22], and endometrial cancer [23], similar conclusions were drawn that the expression of EFEMP2 was reduced in cancer tissues and cells, and its expression was negatively related to the invasion and metastasis ability of cancer cells. EFEMP2 participated in tumor-related processes in an environmentally dependent manner by exhibiting either pro- or anti-tumor properties. Emerging studies have suggested that members of the fibulin family could co-act with many other ECM ingredients to form or adjust the molecular environment [29, 30].

EMT is a process in which epithelial cells acquire mesenchymal characteristics and the ability to migrate, which is involved in tumor initiation, invasion, metastasis, and therapeutic resistance [31, 32]. Whether EFEMP2 promotes or inhibits the progression of EMT is mainly determined by its role in different tumors. In our study, EFEMP2 induced EMT to promote cervical cancer progression. When EFEMP2 was overexpressed, cervical cancer cells HT-3 showed increased proliferation and invasion ability, meanwhile, the expression of epithelial marker E-cadherin decreased, and the expression of interstitial marker N-cadherin and Vimentin increased. The expression of Snail, Slug, Twist, and Zeb2, key transcription factors that block E-cadherin transcription, was also increased, and the EMT process was activated. On the contrary, when EFEMP2 was downregulated, the proliferation and invasion ability of cervical cancer cells Ca Ski was weakened, and EMT was inhibited. In osteosarcoma, EFEMP2 acted as an oncogene to induce EMT and promote osteosarcoma development [28]. However, in breast cancer [20], lung carcinoma [22], and endometrial cancer [23], EFEMP2 suppressed the invasion of cancer cells by inhibiting EMT. As one of the important members of the ECM protein, the fibulin family is involved not only in the maintenance of the structural integrity of the basement membrane and elastic fibers but also in the organization and reconstruction of the tumor microenvironment [29, 33]. Fibulins participate in the development of a variety of tumors but the exact mechanism is not well understood. More data will facilitate the development of new targeted drugs that can effectively inhibit invasion and metastasis and fully improve the prognosis of patients.

MMPs play a crucial role in the progression and metastasis of many cancers, which are associated with uncontrolled cell growth, ECM degradation, and enhanced cell migration ability [34, 35]. Collagenases, MMP-1 [36], MMP-8 [37], and MMP-13 [38], were considered to be related to the prognosis of cervical cancer patients. Among stromelysins, MMP-3 [39] and MMP-11 [40] were identified to be involved in cervical tumorigenesis, and MMP-10 [41] could regulate the angiogenesis and apoptosis pathway and promote cervical cancer progression. Gelatinases, MMP-2 and MMP-9, have been proven very early to be associated with tumor cell invasion and metastasis in human carcinomas, including cervical cancer [42]. In view of the importance of MMPs in the development and progression of cervical cancer, this study examined the effect of downregulation or upregulation of EFEMP2 on the expression of these MMPs at mRNA and protein levels. The results showed that EFEMP2 knockdown remarkably reduced the expressions of MMP-1, MMP-13, MMP-3, and MMP-10, but had no effect on the expression of MMP-8, MMP-11, MMP-2, and MMP-9, while the upregulation of EFEMP2 significantly enhanced the expressions of MMP-1, MMP-13, MMP-3, and MMP-10, and also had no impact on the expression of MMP-8, MMP-11, MMP-2, and MMP-9. In colon cancer, EFEMP2 could promote cancer cell growth and invasion by regulating MMP-3 expression [18]. This conclusion was consistent with ours. In gliomas [17] or lung cancer [22], EFEMP2 could promote or inhibit the invasive ability of cancer cells, which was associated with the upregulated or downregulated expressions of MMP-2 and MMP-9. However, no association between EFEMP2 and MMP-2 and MMP-9 was found in our cervical cancer trial data. Since fibulin family is associated with multiple tumor invasion and metastasis, it should be closely related to the expression regulation of MMPs. Our study revealed that in cervical cancer, the increased expression of MMP-1, MMP-13, MMP-3, and MMP-10 was involved in the process of EFEMP2 enhancing the invasion ability of cancer cells.

The RAF/MEK/ERK signaling pathway is the most well-studied of the MAPK cascade and is involved in cell proliferation, differentiation, and survival. Abnormal regulation of this pathway plays an important role in a large proportion of human cancers [43, 44]. This signaling pathway controls the switching on and off of EMT and facilitates migration, invasion, and metastasis when activated [45]. In our study, EFEMP2 knockdown significantly reduced the phosphorylation levels of Raf-1, MEK1/2, and ERK1/2, so inactivated the Raf/MEK/ERK signaling pathway. In contrast, EFEMP2 upregulation activated this pathway.

The RAF-1 inhibitor Sorafenib and the ERK1/2 inhibitor U0126 could hamper the signaling pathway activation and EMT progression caused by EFEMP2 overexpression. All in all, EFEMP2 promoted the EMT process by activating the Raf/MEK/ERK signaling pathway in cervical cancer cells. In colon cancer, EFEMP2 was also found to activate the ERK1/2 signaling pathway to promote cancer cell invasion and growth. But in bladder cancer [19], breast cancer [20], and endometrial carcinoma [23], EFEMP2 acted as a tumor suppressor gene and inhibited EMT through the Wnt/β-catenin signaling pathway. Fibulins are closely related to EMT, during which multiple signaling pathways are involved. How these signaling pathways interact with each other and the downstream effector proteins of fibulins remain unclear. It is important to further investigate the role of fibulins as a friend or foe in tumor progression, which has far-reaching significance for inhibiting the invasion and metastasis of cancer cells.

In conclusion, the expression of EFEMP2 was positively correlated with the invasion ability of cervical cancer cells, and the stronger the invasion ability, the higher the expres-

sion. In addition, EFEMP2 could promote cervical cancer invasion and metastasis by upregulating the expression of MMP-1, MMP-13, MMP-3, and MMP-10 and inducing the EMT process through the Raf/MEK/ERK pathway. We believe that EFEMP2 provides a new therapeutic target for inhibiting the invasion and metastasis of cervical cancer and fully improving the prognosis of patients, which is of great significance.

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