

Suppressing serum response factor inhibits invasion in cervical cancer cell lines via regulating Egr-1 and epithelial-mesenchymal transition

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Abstract. Serum response factor (SRF) is a transcription factor that has important roles in tumor progression. However, its role in cervical cancer cell proliferation and invasion remains unclear. The present study revealed that SRF silencing constrained cervical cancer cell proliferation and invasion via controlling early growth response-1 (Egr-1). The results demonstrated that SRF was significantly increased in cervical cancer tissues and cell lines, compared with normal. Suppressing SRF, by using a loss-of-function experiment, constrained cervical cancer cell proliferation, invasion, and epithelial-mesenchymal transition. Furthermore, SRF knockdown significantly downregulated Egr-1 expression in cervical cancer cell lines, and overexpression of Egr-1 reversed the effect of SRF on cell proliferation, invasion, and epithelial-mesenchymal transition. Therefore, SRF may control cell proliferation and invasion by regulating Egr-1 in cervical cancer.

Introduction

Cervical cancer, also known as invasive cervical cancer, is the most common gynecological malignancy, and its incidence

rate is second only to breast cancer (1). Although screening, surgery, radiotherapy, and other treatments have improved prognoses for early cervical cancer, metastatic and recurrent cases are difficult to eradicate (2). Therefore, elucidating the molecular mechanism of cervical cancer invasion and metastasis has important scientific significance to improve prognosis for patients.

Serum response factor (SRF), a widely expressed transcription factor, belongs to the MADS-box gene family (3,4). SRF regulates cytoskeleton and cell motility, as well as gene expression of immediate early genes, muscle-related genes, and adhesion-related genes (5-8). SRF overexpression promotes tumor cell invasion and metastasis (9). It is associated with downregulation of E-cadherin expression and upregulation of N-cadherin expression in epithelial-mesenchymal transition (EMT) in gastric, peritoneal mesothelial, liver, and prostate cancer cells (10-12). EMT is a crucial process of tumor cell invasion and metastasis, and the loss of the polarity of epithelial cells and migration capacity are important features (13,14). The most important hallmark of EMT is decreased E-cadherin expression. Additionally, N-cadherin is upregulated in EMT and may promote tumor cell migration (15). Currently, the role of SRF in the proliferation and invasion of cervical carcinoma is unclear. The present study aimed to investigate the molecular mechanism of SRF in cervical cancer, and this knowledge could be useful in the future to improve treatment of the disease.

Early growth response-1 (Egr-1), a member of the zinc finger transcription factor family, acts as an early growth response gene (16,17). Egr-1 exerts a variety of biological functions that control synaptic plasticity, wound healing, cell growth, and apoptosis (18,19). The biological function of Egr-1 is associated with the development of human cancer. The absence or increase of abnormally expressed Egr-1 in tumors may be an important cause of tumorigenesis (20). Egr-1 acts as a tumor suppressor in lung cancer and liver cancer, and as a cancer-promoting gene in gastric carcinoma (19,21,22). The role of Egr-1 in EMT of cervical cancer remains unknown.

SRF reportedly activates Egr-1 expression (23). Therefore, it was hypothesized that SRF could affect proliferation and

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Abbreviations: SRF, serum response factor; EMT, epithelial-mesenchymal transition; Egr-1, early growth response-1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; BrdU, bromodeoxyuridine

Key words: serum response factor, suppression, cervical cancer, early growth response-1, epithelial-mesenchymal transition

invasion in cervical cancer by regulating Egr-1 and EMT. The present study investigated the molecular mechanism of SRF in cervical cancer by measuring SRF expression in cervical cancer cell lines. Cell proliferation and invasion were examined in cervical cancer cell lines following SRF knockdown, and the molecular mechanism underlying the effect of SRF was explored.

Materials and methods

Cells and tissues. The cervical cancer cell lines ME-180 and HeLa (American Tissue Type Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both from HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Human cervical epithelial HCerEpic cells (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in cervical epithelial cell medium (ScienCell Research Laboratories). All cells were cultured in an atmosphere of 5% CO₂ at 37°C.

Cervical tumor samples (n=10) were collected from cervical cancerous area of patients in the Henan University of Chinese Medicine (Zhengzhou, China) undergoing hysterectomies without radiotherapy or chemotherapy. Normal tissues (n=10) were collected from patients undergoing surgery for myoma or adenomyoma. Normal tissue samples were nonmalignant and negative for human papilloma virus and ThinPrep cytological tests. All patients in the present study were 40-50 years old. Samples were collected under a protocol approved by the Institutional Review Board of the Henan University of Chinese Medicine (Zhengzhou, China) between December 2016 and August 2017. Informed consent was obtained from the patients.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA from cells or tissues was extracted by using TRIzol (Takara Biotechnology Co., Ltd., Dalian, China) and 5 µg RNA was synthesized into cDNA with a Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

The qPCR reaction (20 µl) contained 10 µl SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.) as the enzyme mixture. The reaction program was as follows: 94°C for 30 sec, 40 cycles at 94°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec. Primers were as follows: SRF, forward, 5'-TTC AAGGTAGAGAAGACTGGTTT-3' and reverse, 5'-CTGACC CCCATTCCCTGTGTC-3'; GAPDH, forward, 5'-GGAAGA TGGTGATGGGATT-3' and reverse, 5'-GGATTGGTTCGT ATT GGG-3'; and Egr-1, forward, 5'-AGCCCTACGAGCACC TGA C-3' and reverse, 5'-GGTTTGGCTGGGGTAACTG-3'. The relative levels of gene expression were estimated using the 2^{-ΔΔC_q} method (24).

Western blot analysis. Protein was extracted with RIPA lysis buffer and was quantified with a BCA kit (both from Beyotime Institute of Biotechnology, Haimen, China). Total protein samples (25 µg) were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by electrophoretic transfer. The membrane was then incubated with

5% skim milk for 2 h, followed by incubation overnight at 4°C with the following primary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA): anti-SRF (1:500; cat. no. 5147), anti-Egr-1 (1:500; cat. no. 4154), anti-E-cadherin (1:800; cat. no. 3195), anti-N-cadherin (1:800; cat. no. 13116), and anti-GAPDH (1:500; cat. no. 5174). Next, the membrane was incubated with secondary antibody (1:800; cat. no. 7074, Cell Signaling Technology, Inc.) diluted in the blocking buffer. Finally, the protein was detected using enhanced chemiluminescence. GAPDH was used as the protein loading control. Optical density of the bands was measured with the BandScan imaging analysis system (Glyko Inc., Hayward, CA, USA).

Construction of the recombinant plasmids and cell transfection. The full-length cDNA of Egr-1 (accession no., NM_001964) was amplified by RT-PCR and incubated with *Eco*RI and *Bam*HI enzymes. Subsequently, pcDNA.3.1/myc-His(-) Avector (Invitrogen; Thermo Fisher Scientific, Inc.) was also incubated with those two enzymes and the Egr-1 fragment was inserted at the *Eco*RI and *Bam*HI restriction sites. The recombinant plasmid was then amplified in DH5 *Escherichia coli*-competent cells (Takara Biotechnology Co., Ltd.), followed by extraction using a Takara MiniBEST Plasmid Purification kit version 4.0. Finally, the plasmid was sequenced, and the correct ones were selected as pcDNA.3.1-Egr-1.

Cell transfection was as follows: ME-180 and HeLa cells were separately cultured in 96-well plates under a humid atmosphere with 5% CO₂ at 37°C. The transfection program was based on TurboFect (Thermo Fisher Scientific, Inc.) and followed the manufacturer protocol. Cell transfection was conducted when cells are at ~80% confluency. The pcDNA.3.1-Egr-1 (0.3 µg), pcDNA.3.1 (0.3 µg), SRF small interfering (si) RNA (5'-AAC CACCCGCCACTCTTCCT-3', (0.3 µg), and non-specific siRNA (5'-ATTACACCGACTATCCAACAT-3', 0.3 µg) were transfected separately with 2 µl of TurboFect. The cells were then cultured in 5% CO₂ at 37°C for 24 h. Transfection efficiency was measured by RT-qPCR and western blot assays.

Cell viability assay. MTT was used to detect cell viability. Cells were cultured in 96-well plates at 37°C for 24 h. The culture medium was then changed to PBS containing MTT (20 µl/well) and incubated for another 4.5 h at 37°C. Afterwards, dimethyl sulfoxide (150 µl/well) was added to dissolve the formazan. Results were measured using a microplate reader (Thermo Fisher Scientific, Inc.) at 490 nm.

Bromodeoxyuridine (BrdU) assay. Cell proliferation was assessed using a BrdU kit (EMD Biosciences, Inc., Darmstadt, Germany) according to the specification sheets. Briefly, cells were cultured in a 96-well plate followed by 1 h of incubation with 10 µl of BrdU solution per well. After discarding the culture medium, a denaturing solution (180 µl) was added and incubated for 35 min. Cells were then incubated with an anti-BrdU antibody conjugated with peroxidase for 30 min. The results were measured at 450 nm using a SpectroFluor Plus multi-well plate reader (Tecan Group Ltd., Mannedorf, Switzerland).

Cell invasion. Cell invasion ability was tested using Bio-Coat cell migration chambers (Corning Incorporated, Toledo, NY, USA)

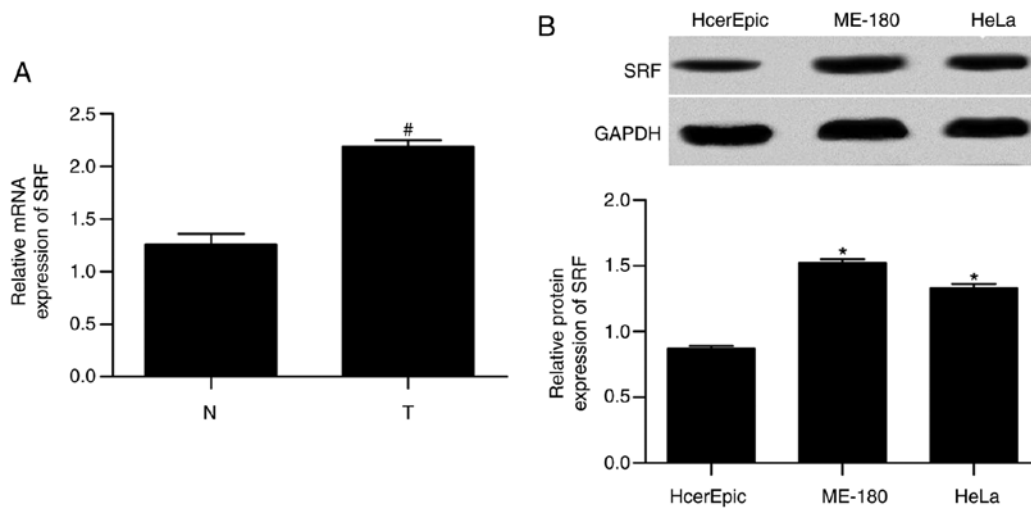


Figure 1. Expression of SRF in cervical cancer tissues and cell lines. (A) Relative SRF mRNA levels were measured in cervical cancer tissues and normal tissues from patients (n=10). [#]P<0.05 vs. N group. (B) SRF protein expression levels were measured in cervical cancer cell lines and in the normal cervical epithelial cell line HcErEpic, by western blotting (n=3). GAPDH was used as the internal control. ^{*}P<0.05 vs. HcErEpic group. SRF, serum response factor; N, normal; T, tumor.

coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Transfected cells (1×10^5 cells) were suspended in 200 μ l of serum-free medium and then plated in the upper chamber. Complete medium (300 μ l) was added to the lower chamber and incubated at 37°C for 48 h. Non-invading cells were gently removed with a cotton swab from the upper chambers. Invaded cells were fixed, stained, and observed using a light microscope. Ten visual fields in each membrane were randomly selected for cell number counting.

Statistical analysis. Statistical analyses were processed with SPSS version 22.0 software (IBM Corporation, Armonk, NY, USA). Differences between multiple groups were evaluated with one-way analysis of variance followed by a Bonferroni test. Differences between normal and tumor samples were evaluated with the Mann-Whitney's U test. Data are expressed as the mean \pm standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

SRF is upregulated in cervical cancer cell lines and tissues. First, the mRNA expression levels in tissues and the protein expression levels in cell lines were measured for SRF by RT-qPCR and western blotting, respectively. The results demonstrated that mRNA expression (Fig. 1A) in tissues and protein expression (Fig. 1B) in cell lines were significantly increased in cervical cancer compared with normal, implying an important role of SRF in cervical cancer.

SRF silencing suppresses proliferation in cervical cancer cells. To investigate the role of SRF in cervical cancer cell proliferation, MTT and BrdU assays were used to detect proliferation in the cervical cancer cell lines ME-180 and HeLa, following SRF knockdown. First, SRF siRNA was transfected into ME-180 and HeLa cells in order to knockdown SRF expression. The data demonstrated that both the mRNA (Fig. 2A) and protein (Fig. 2B) levels of SRF were

successfully decreased by >50%. Next, ME-180 and HeLa cell proliferation was measured. In the MTT assay (Fig. 2C), cell proliferation was significantly decreased in the cells following SRF knockdown, compared with the control cells. Similarly, in the BrdU assay (Fig. 2D), cell proliferation also appeared to be markedly downregulated following SRF knockdown.

SRF silencing suppresses invasion in cervical cancer cells. Cell invasion of ME-180 and HeLa cells following SRF knockdown by siRNA was measured by Transwell assays. Expression of the established EMT markers E-cadherin and N-cadherin was also investigated by western blotting. As presented in Fig. 3A, the results from the Transwell assays revealed that cell invasion was significantly decreased following SRF knockdown in ME-180 and HeLa cells. The western blotting results demonstrated that E-cadherin protein expression was upregulated 2-fold, while N-cadherin was markedly downregulated in ME-180 and HeLa cells following SRF knockdown (Fig. 3B).

SRF silencing downregulates Egr-1 expression. Egr-1 has been reported to be regulated by SRF and it affects EMT progression. Thus, it was speculated that SRF may control Egr-1 expression in cervical cancer cell lines. The mRNA (Fig. 4A) and protein (Fig. 4B) expression levels of Egr-1 were detected in ME-180 and HeLa cells following SRF knockdown. Egr-1 expression was significantly decreased in cervical cancer cell lines following SRF silencing.

SRF silencing controls cervical cancer cell line proliferation and invasion by regulating Egr-1. To explore the molecular mechanism of SRF in regulating cervical cancer cell proliferation and invasion, a gain-of-function experiment was performed for Egr-1 in SRF-knockdown ME-180 and HeLa cell lines. When the SRF-knockdown cells were transfected with an Egr-1-overexpressing plasmid, Egr-1 was upregulated by 3-fold compared with cells transfected with an empty plasmid, suggesting that overexpression was successful (Fig. 5A). Next, the effect of Egr-1 overexpression

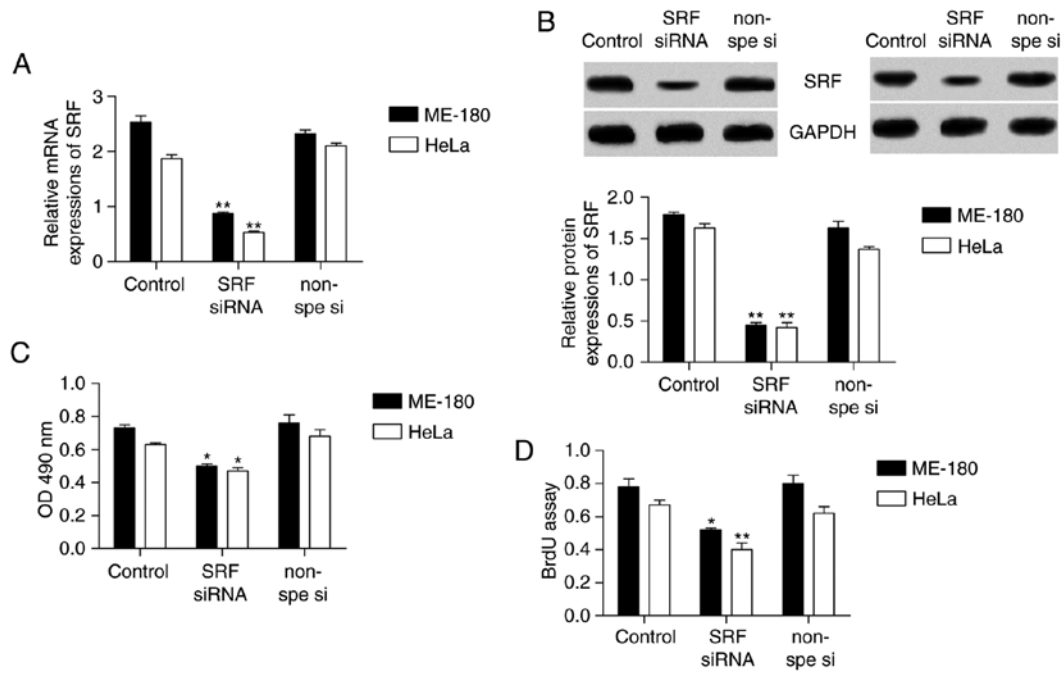


Figure 2. Effect of SRF silencing on cervical cancer cell proliferation. (A) Relative mRNA levels and (B) protein levels of SRF in cervical cancer cell lines following transfection with SRF siRNA or non-specific siRNA. (C) Proliferation of cervical cancer cells with SRF knockdown was measured by MTT and (D) BrdU assays. * $P < 0.05$ and ** $P < 0.01$ vs. the non-specific si group ($n = 3$). SRF, serum response factor; si, small interfering; BrdU, bromodeoxyuridine.

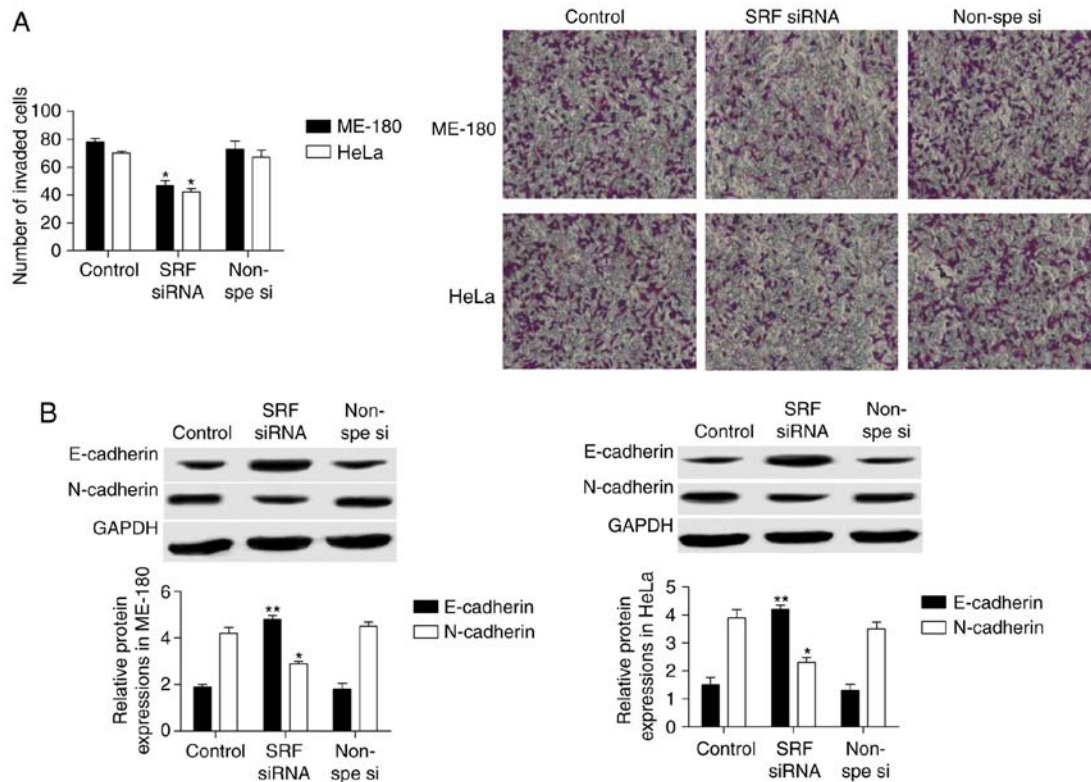


Figure 3. Effects of SRF silencing on invasion and EMT in cervical cancer cells. (A) Invasion was assessed by Transwell invasion assays in cervical cancer cell lines. (B) Protein expression levels of established EMT markers were assessed by western blot analysis. * $P < 0.05$ and ** $P < 0.01$ vs. the non-specific si group ($n = 3$). SRF, serum response factor; EMT, epithelial-mesenchymal transition; si, small interfering.

was investigated on cell proliferation and invasion. The results demonstrated that Egr-1 overexpression in ME-180 and HeLa cells reversed the inhibitory effect of SRF knockdown on proliferation (by MTT assay; Fig. 5B) and on invasion (by

Transwell assay; Fig. 5C). Furthermore, E-cadherin protein expression was significantly decreased, and N-cadherin increased (Fig. 5A). Therefore, SRF controlled cervical cancer cell proliferation and invasion through Egr-1.

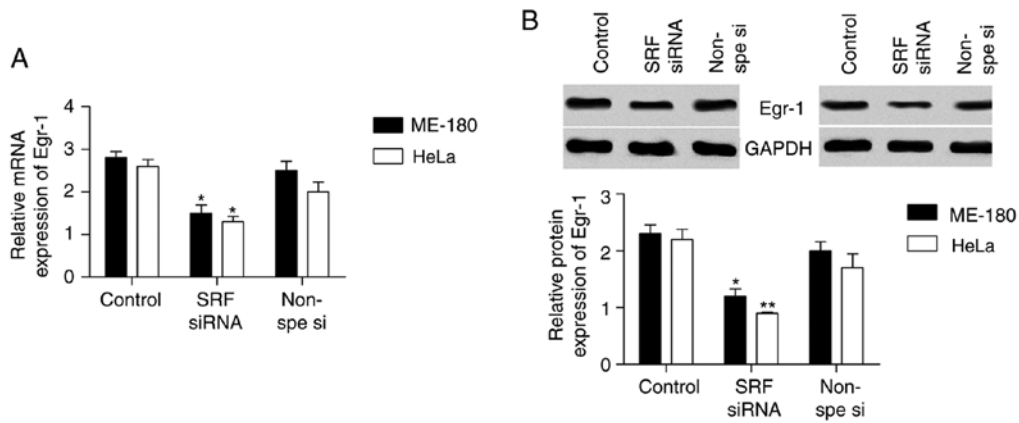


Figure 4. Egr-1 expression following SRF silencing. (A) The relative mRNA levels and (B) protein levels of Egr-1 were measured in cervical cancer cell lines following SRF silencing by siRNA. * $P<0.05$ and ** $P<0.01$ vs. the non-specific si group ($n=3$). Egr-1, early growth response-1; SRF, serum response factor; si, small interfering.

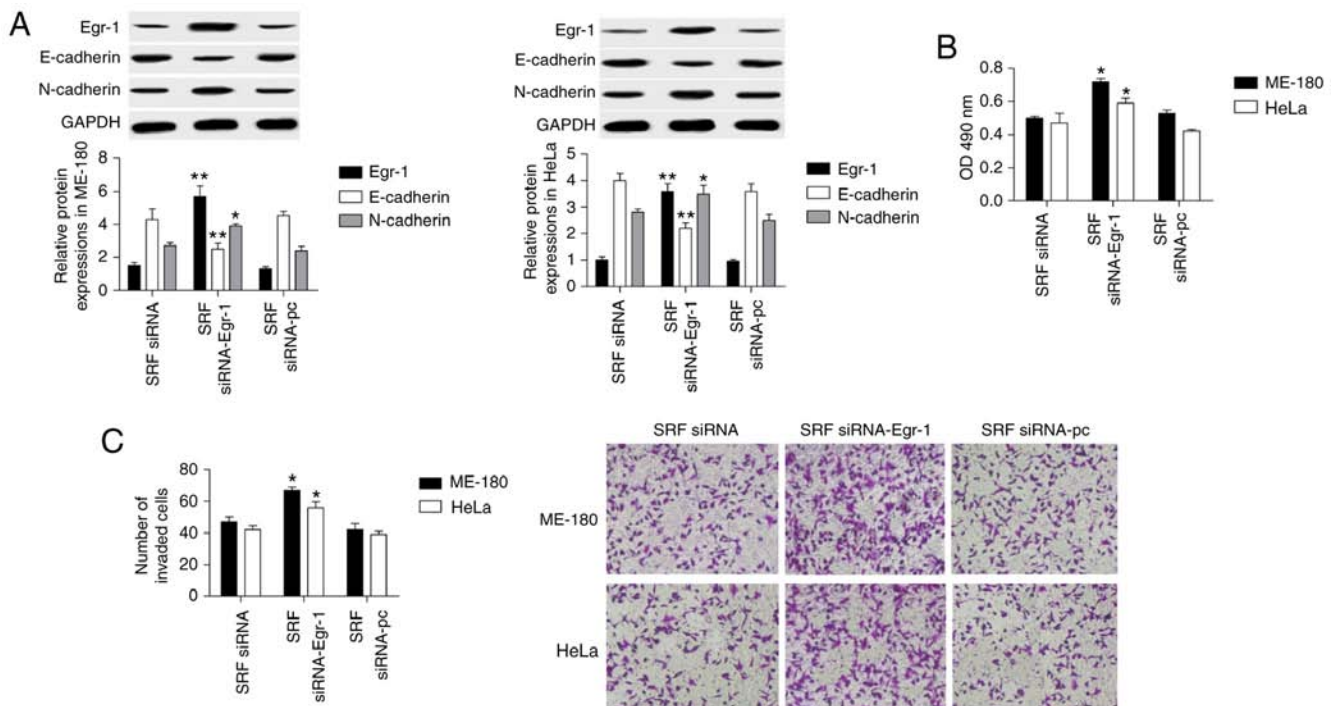


Figure 5. Effect of Egr-1 overexpression on cell proliferation and invasion following SRF silencing. SRF knockdown cervical cancer cells were transfected with either an Egr-1 overexpressing vector or empty vector control. (A) The protein expression levels of Egr-1, E-cadherin and N-cadherin were detected by western blotting. (B) Proliferation was measured by MTT assay. (C) Invasion was measured by Transwell invasion assay. * $P<0.05$ and ** $P<0.01$ vs. the SRF siRNA-pc group ($n=3$). Egr-1, early growth response-1; SRF, serum response factor; si, small interfering; pc, empty plasmid control.

Discussion

According to the World Health Organization, 500,000 new cases of cervical cancer occur worldwide each year, ~80% in developing countries (25). Cervical cancer rates continue to rise and affect younger women (26). SRF is closely associated with cancer metastasis. SRF expression is elevated in prostate and gastric cancers (27). Zhao *et al* (10) reported that SRF modulates EMT and promotes metastasis in human gastric cancer. Wang *et al* (28) reported that SRF regulates non-small-cell lung cancer invasion and proliferation via the miR29b/matrix metalloproteinase 2 axis. SRF siRNA has been indicated to reduce the invasion potential of prostate cancer cells *in vitro* (12).

SRF is an important transcription factor that regulates EMT (29). EMT is a process of cytoskeletal rearrangement that increases cell migration and invasion abilities (30). Studies generally agree that EMT is associated with embryonic development, tissue regeneration, and cancer metastasis (31,32). During tumorigenesis, EMT is characterized by downregulation of E-cadherin, which causes differentiated epithelial tumor cells to become tumorigenic cells with migration and invasion abilities (33). Upregulation of N-cadherin promotes EMT (34). He *et al* (35) demonstrated that SRF promotes EMT in human peritoneal mesothelial cells. SRF also provokes EMT in renal tubular epithelial cells of diabetic nephropathy (36), and it induces EMT

in hepatocellular carcinoma, prostate cancer, and gastric cancer (37). Therefore, SRF has a significant role in EMT and cancer metastasis. Nevertheless, its role in cervical cancer remains unclear. In the present study, it was demonstrated that SRF expression in cervical cancer tissues and cell lines was highly increased compared with normal. A loss-of-function experiment was performed by transfecting SRF siRNA in cervical cancer cell lines, and SRF silencing significantly decreased N-cadherin and increased E-cadherin expression in cervical cancer cell lines. In addition, cell proliferation and invasion were suppressed following SRF silencing.

Silverman and Collins (23) demonstrated that Egr-1 gene transcription could be activated by SRF. Egr-1 gene expression can be regulated by a variety of extracellular signals, subsequently modulating cell proliferation and invasion (38). Egr-1 also directly affects cell proliferation in astrocytes, glioma cells, and mesangial cells (39-41). Additionally, it is closely related to EMT and cell invasion in nasopharyngeal cancer, human ovarian cancer, colon cancer, and thyroid cancer cells (20,42,43). Egr-1 serves different roles in different cancer cells. Previous studies have demonstrated that Egr-1 decreases the malignancy of human non-small-cell lung carcinoma by regulating type I intermediate filament chain keratin 18 expression, whereas Egr-1 promotes growth in prostate cancer cells (44,45).

The effect of Egr-1 on cervical cancer is unclear. The present study demonstrated that Egr-1 decreased when SRF was silenced in cervical cancer cell lines. Notably, Egr-1 overexpression abolished the effect of SRF silencing on cell proliferation, invasion, and E-cadherin and N-cadherin expressions in cervical cancer cells. Therefore, SRF inhibition may control cervical cancer metastasis by modulating Egr-1 expression. The data in the present study demonstrated that SRF was highly expressed in clinical cervical cancer tissues and cell lines compared with normal. SRF knockdown restrained Egr-1 expression, resulting in repression of proliferation, invasion and N-cadherin expression and induction of E-cadherin expression. Thus, the current study provides a novel insight into the molecular mechanism of SRF in cervical cancer and provides a potential target for treatment. Because the present study was performed mostly *in vitro*, further studies with additional tissue samples or *in vivo* will be required in the future to fully characterize the role of SRF in human cervical cancer.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

LM and YY designed and prepared the experiments. LM and YY performed the experiments. LM, XQ and YY contributed

reagents/materials/analysis tools. LM and YY wrote the manuscript. XQ modified and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Patient samples were collected under a protocol approved by the Institutional Review Board of the Henan University of Chinese Medicine (Zhengzhou, China), and informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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