ERK5 silencing inhibits invasion of human osteosarcoma cell via modulating the Slug/MMP-9 pathway

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Abstract. – **OBJECTIVE:** ERK5 is over expressed in a many of human cancers and this overexpression has been associated with metastasis and invasion. Furthermore, ERK5 silencing inhibits aggressive phenotypes of cancer cells. However, mechanisms by which ERK5 regulates tumour progression or metastasis have not been elucidated. In this study, using human osteosarcoma cell lines U2OS as a model, we explored the involvement of ERK5 silencing on invasiveness of U2OS cells.

MATERIALS AND METHODS: ERK5 siRNA targeting ERK5 was stably transfected into the human osteosarcoma cell lines U2OS. ERK5 knocked-down U2OS cells was then transfected with Slug cDNA or MMP-9 cDNA plasmid to re-express Slug or MMP-9. Cell proliferation was detected by MTT assay. Cell invasion and metastasis was detected by Matrigel invasion and wound healing assay. An orthotopic nude mouse model of U2OS was applied for in vivo lung metastasis experiments. ERK5, Slug, MMP-9 and E-cadherin were analyzed by real-time PCR, and Western blotting.

RESULTS: ERK5 silencing by siRNA in U2OS cells decreased Slug and MMP-9 expression. Compared with the vector-transfected cells, ERK5 knocked-down cells showed reduced migration and invasion *in vitro*, as well as decreased metastatic potential in experimental metastasis. Re-expression of Slug or MMP-9 in ERK5 knocked-down cells restored the invasive phenotypes. We also discovered that Re-expression of Slug in ERK5 knocked-down cells restored the MMP-9 expression, and re-expression of MMP-9 in ERK5 knocked-down cells did not affect Slug and ERK5 expression.

CONCLUSIONS: Our data suggest that ERK5 knockdown inhibits aggressive behaviour of human U2OS cells through modulating Slug signaling and MMP-9 expression.

Key Words:
   Osteosarcoma, Invasion, ERK5, Slug, MMP-9.

Introduction

The MEK5/ERK5 pathway is the least well studied mitogen-activated protein kinase (MAPK) signalling module. It has been proposed to play a role in the pathology of cancer[1]. Similar to other MAPKs, extracellular-signal-regulated kinase 5 (ERK5) is present in a wide variety of cell types and is believed to be ubiquitously expressed. Presumably, it serves to regulate diverse functions depending on the cellular context and circumstances[2]. ERK5 has been implicated in the survival response of pheocromocytoma 12 (PC12) cells to oxidative stress[3]. Further work demonstrated that ERK5 contributes to the survival response in neuronal dorsal root ganglia cells through a unique retrograde signaling system mediate by nerve growth factor (NGR)[4-5].

Work has been done to examine vascular integrity and endothelial failures in animal studies. ERK5 deletion was embryonically lethal in genetic knock-out mice and ERK5 deletion in adult mice lead to lethality within 2-4 weeks. Physiological analysis of the adult mice demonstrated abnormally leaky blood vessels. Histologically these mice demonstrated multiorgan hemorrhage and architectural irregularities in the endothelial lining of their blood vessels. This evidence suggests that ERK5 is critical for endothelial function and preserves the integrity of blood vessels[6]. Recent study found that ERK5 also plays a role in controlling cytoskeleton organization and motility of keratinocytes during cutaneous wound healing[7]. However, mechanisms by which ERK5 promotes tumour progression or metastasis have not been elucidated.

Epithelial to mesenchymal transition (EMT), which has been recognized for several decades as critical for embryogenesis[8], has recently been

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shown to also be relevant to cancer progression. During EMT of in situ cancer cells, expression of proteins that promote cell-cell contact such as E-cadherin can be lost, and mesenchymal markers such as vimentin, fibronectin, N-cadherin and the metalloproteinases MMP-2 and MMP-9 can be acquired, resulting in enhanced ability for cell migration and invasion[9]. Identified EMT-driving transcription factors include the zinc-finger-containing Snail family members (e.g. Snail and Slug), ZEB family members (e.g. ZEB1, SIP1), and basic helix-loop-helix (bHLH) factors (e.g. E2A, Id2, Id3, Twist); these factors serve as transcriptional repressor of the E-cadherin gene[9].

A study investigated the role of ERK5 in reepithelialization during cutaneous wound healing of human keratinocytes. The cells were treated with EGF to stimulate the EGF receptor. Treated cells showed increased levels of phosphorylated ERK5 which coincided with increased levels of Slug mRNA[7]; An ERK5 knockdown was also used in HaCaT cells, an immortalized human keratinocyte cell line. The treated HaCaT cells demonstrated decreased motility response and reduced Slug mRNA expression. The knockdown cells demonstrated a more compact morphology, disruption in desmosome organization and an altered ability to aggregate. This work suggests that ERK5 plays a role in controlling cytoskeleton organization and motility of keratinocytes by regulation of Slug during cutaneous wound healing[7].

It has recently found Slug promotes migration and invasion of pancreating cancer (PANC)-1 cells, which may correlate with the reorganization of MMP-9 and remodeling of the F-actin cytoskeleton, but not with E-cadherin expression[10]. Kim et al[11] has found ERK5 regulates the invasion of OS cells by inducing MMP-9 expression.

Osteosarcoma (OS) is a type of aggressive bone cancer of mesenchymal origin generally found in youths aged between 10 and 25 years. Any bone of the human body can be affected by this neoplasia and the general survival at five years is approximately 65 to 75%. The main causes of death are pulmonary metastases diagnosed by computed tomography (CT) in 35 to 45% of the patients[12]. In this study, aiming at further understanding the mechanisms underlying the progression and metastasis of OS, we employed an OS cell line and explored the role of ERK5 in modulating the invasive phenotype of cancer cells. We provide evidence linking ERK5 to the regulation the invasion and metastasis through the Slug /MMP-9 pathway in human OS cells.

Materials and Methods

Cell culture

Human osteosarcoma cell lines U2OS cell lines was purchased from the American Type Culture Collection and maintained in McCoy's 5A medium (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37°C in humidified air containing 5% v/v CO_2.

Plasmid constructs

MMP-9 cDNA was from Sino Biological Inc. (Bejing, China) BamH I and EcoR I enzyme were used to digest the MMP-9 cDNA gene and pcD-NA3.1 plasmid. MMP-9 cDNA gene was inserted (Invitrogen) into plasmids pcDNA3.1 to construct pcDNA3.1- MMP-9 cDNA. The structures and fidelity of the resulting constructs were confirmed by restriction mapping and sequencing. Plasmids were purified using the Plasmid Midi kit (Qiagen, Valencia, CA, USA). At least two independent plasmid preparations of each construct were used in reporter assays. The control pcDNA3.1 plasmid was construced as the method of above.

Cell transfection

The ERK5 siRNA was purchased from Dharmacon Inc. (Lafayette, CO, USA). A nontargeting siRNA (control siRNA; Dharmacon Inc.) was used as a negative control. To generate ERK5-silenced stable clones (U2OS-ERK5 siRNA 1 and 2), U2OS cells were transfected with 100 nM/L of control RNA or ERK5 siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer instructions. The cells were harvested 48 hours after siRNA transfection. siRNA knockdown efficiency was measured by real time RT-PCR and Western blot.

Table I. Oligonucleotide Sequences for Real Time RT-PCR.

Gene	Sequence 5'-3'
ERK5	CTGGCTGTCCAGATGTGAA ATGGCACCATCTTTCTTTGG
Slug	GCGAACTGGACACACACAGTTAT CCCCAGTGTGAGTTCTAATGTGTCC
E-cadherin	GGAAGTCAGTTCAGACTCCAGCC AGGCCTTTTGACTGTAATCACACC
MMP-9	CCTTCTACGGCCACTACT GCACTGCAGGATGTCATA
β-actin	CCTCTATGCCAACACAGTGC CATCGTACTCCTGCTTGCTG
18S	GTAACCCGTTGAACCCCATT CCATCCAACGGTAGTAGCG

For clones of U2OS-ERK5 siRNA cells expressing MMP-9 or Slug, U2OS-ERK5 siRNA 1/2 cells were transfected with pcDNA3.1- MMP-9 cDNA or pcDNA3.1-SLUG cDNA (kindly donated by Dr. Zhang, the Affiliated Hospital of Qing-Dao University), and pcDNA3.1 as a control using Lipofectamine 2000 (Invitrogen). For selection of stably transfected cell populations, G418 was added to the culture medium 48h after transfection at a concentration of 400 μg ml^{-1} for the above transfected U2OS cells. The cells were selected with 400 μg/mL G418 for 14 d.

Real-Time RT-PCR

Real-time RT-PCR (QPCR) was performed using the LightCycler as the manufacture's instruction. For a final reaction volume of 20 μl, the following reagents were added: 10 μl SYBR Advantage qPCR Premix (Clontech, Mountain View, CA, USA), 1 μL of each forward and reverse 10 μM primers (Table I), 7 μL H$_2$O, and 1 μL cDNA template. Messenger RNA (mRNA) levels were quantified using the standard curve method. Standard curves were constructed by using serially diluted standard template. The data were normalized to 18S ribosomal RNA or μ-actin RNA to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

Western blot analysis

Cells were washed twice with phosphate buffered saline (PBS) and lysed in the RIPA buffer (Upstate, Billerica, MA, USA) at 4 °C for 20 min. Lysates were cleared at 12,000 g for 12 min at 4 °C and supernatants were subjected to western analysis. Equal-amount (40 □g) cell lysates were separated by SDS-PAGE (10% gel), transferred to polyvinylidene membranes (Millipore, Billerica, MA, USA), and probed with antibodies against proteins of interest, including Phospho-ERK5 antibody (pERK5) and ERK5 antibody (Cell Signaling Techn, Beverly, MA, USA), E-cadherin (Biosciences, Hudson, NH, USA), MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Abcam, Burlingame, CA, USA). Blots were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.). Bound antibodies were visualized by enhanced chemiluminescence.

Cell proliferation assay

Cells were seeded at 5×10^3 per well on 96-well plates in growth medium supplemented with 10% serum, and were cultured in a humidified chamber at 37°C for up to 3 days. Viable cells were identified using the MTT assay as the manufacture's instruction. The contents of the plates were mixed for 5 min, and the absorbance was read at 540 nm using a plate reader.

Matrigel invasion assay

Cell invasion assays were performed using Transwell membrane filter inserts with 8-mm pore size (Corning Costar, Cambridge, MA, USA). Cells were trypsinized, washed and resuspended in RPMI-1640+10% fetal bovine serum (FBS). Samples of 50,000 cells were placed in the upper chamber of each Transwell device (Nucleopore) with 8-μm Matrigel-coated polycarbonate membrane filter insert in 24-well plates, and the same medium was placed in the lower chamber. After 24 h of incubation, non-invading cells were removed by wiping the upper surface of the filter with a cotton swap; the remaining cells were fixed in 100% methanol for 20 min and stained with Giemsa (Sigma, St Louis, MO, USA). The degree of invasion was quantified by counting the cells on the underside of filters under a microscope. Experiments were repeated at least three times in duplicates for each cell line.

Wound healing assay

Cells were seeded at 1×10^6 cells per well in 6-well plates and cultured for approximately 24 h to be nearly confluent. A scratch "wound", i.e. a cell-free line of ~20-cell diameters in width, was made using a p200 pipette tip and the medium was changed to remove detached cells. Phase contrast micrographs were taken immediately and 24 h after making the wound. Numbers of cells which have migrated were counted comparing the micrographs. For each sample, cells from three representative fields were counted.

In vivo experimental lung metastasis

For experimental lung metastasis assay, 1×10^6 cells resuspended in 100 μl PBS buffer were injected into the tail vein of 7- to 10-week-old male non-obese diabetic severe combined immunodeficient (NODSCID) mice. Mice were sacrificed 8 weeks after injection. Lungs were removed and fixed in 10% formalin. Paraffin-embedded lung tissues were sectioned and stained with hematoxylin and eosin for histological examination. The extent of lung metastasis was scored by counting total tumour nodules in serial 5-μm sections.

Statistical analysis

Data are shown as the mean±S.D. where possible and statistical analysis was obtained using SPSS version 11.0, computer software (SPSS Inc., Chicago, IL, USA). A p-value of < 0.05 was taken to indicate statistical significance. Statistical significance of difference between groups was tested using Student's t-test or if there were more than two groups, using one way analysis of variance (ANOVA) followed by post-hoc analysis.

Results

No effect of ERK5 knockdown on cell proliferation

Both qRT-PCR and Western blot analyses revealed higher ERK5 expression levels in U2OS cell. The degree of inhibition of ERK5 mRNA expression induced by siRNA against ERK5 is shown in Figure 1A. SiRNA 1 and siRNA 2 targeting ERK5 inhibited ERK5 mRNA levels in siRNA stably transfected U2OS cell to < 10% of control cells, respectively (Figure 1A). Similar results were observed in ERK5 protein levels after siRNA 1 and siRNA 2 stable transfection by western blot assay (Figure 1B).

Inhibition of ERK5 expression in U2OS cells did not suppress cell proliferation compared to the control after 4 days after stable transfection (Figure 2C). The information indicated that ERK5 knockdown did not affect the growth curves of U2OS cells.

ERK5 knockdown on migration, invasion, and metastasis of U2OS cells

ERK5 knockdown in U2OS did not affect the growth curves of cells .We next examined the effect of ERK5 knockdown on the invasive behaviour of cells. When compared to control cells, ERK5 siRNA cells showed less migration in the wound-healing assay (Figure 2A) and exhibited decreased invasion in the Matrigel assay (Figure 1B). To check if the reduced *in vitro* migration and invasiveness in ERK5 siRNA clones could affect the *in vivo* metastatic potential of cells, we performed an experimental metastasis assay. Severe combined immunodeficiency (SCID) mice were injected with ERK5 siRNA 1 and ERK5 siRNA2, vector transfected U2OS cells respectively via the tail veins, and examined 7 weeks after injection for metastatic nodules formed in lungs. While most mice injected with the parental or vector transfected U2OS cells formed pulmonary tumour nodules, none of the mice inject-
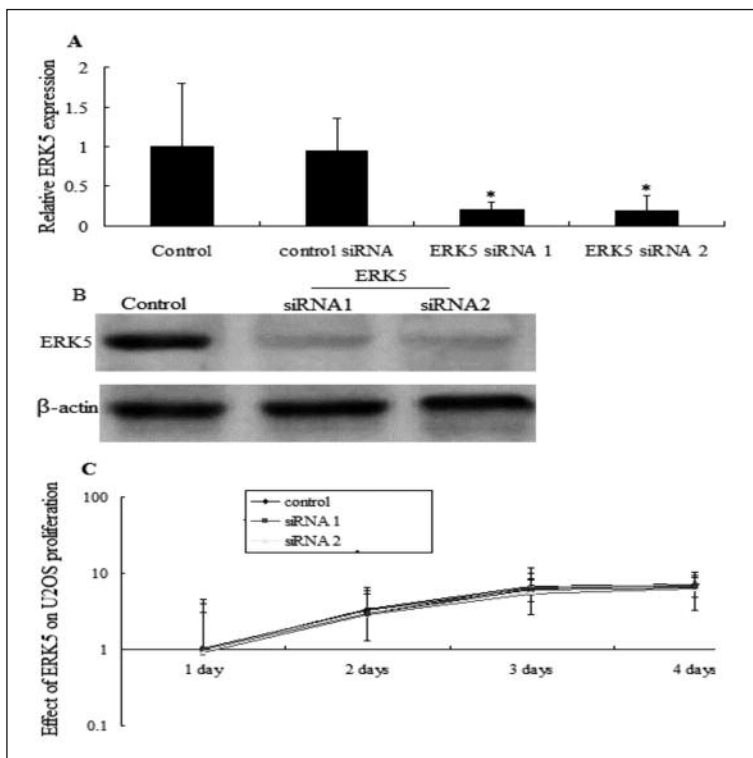

Figure 1. Effect of ERK5 knockdown using a siRNA-expressing vector on ERK5 and proliferation in U2OS cells. **A**, Real-time PCR showed decreased ERK5 mRNA expression in U2OS cells stably expressing a control or ERK5-specific siRNA 1 and 2. **B**, Western blot showed decreased ERK5 protein expression in U2OS cells stably expressing a control or ERK5-specific siRNA 1 and 2. **C**, Proliferation in U2OS cells stably expressing a control or ERK5-specific siRNA 1 and 2 by MTT assay ($^*p$ < 0.05).

Figure 2. ERK5 knockdown reduces cell migration and invasion. **A**, U2OS cells migration analysis by the wound healing assay. A scratch "wound" was made in a cell monolayer and numbers of cells having migrated were counted comparing phase contrast micrographs taken immediately and 24 h after making the wound. Averages from three experiments are shown. $^*p < 0.01$, Student's t-test. **B**, Matrigel invasion assay. The assay was done in modified Boyden chambers consisting of Transwell with Matrigel-coated 8-μm filter insert in 24-well plates for 24 h. Average numbers from three experiments are shown. $^{**}p < 0.001$, Student's t-test.
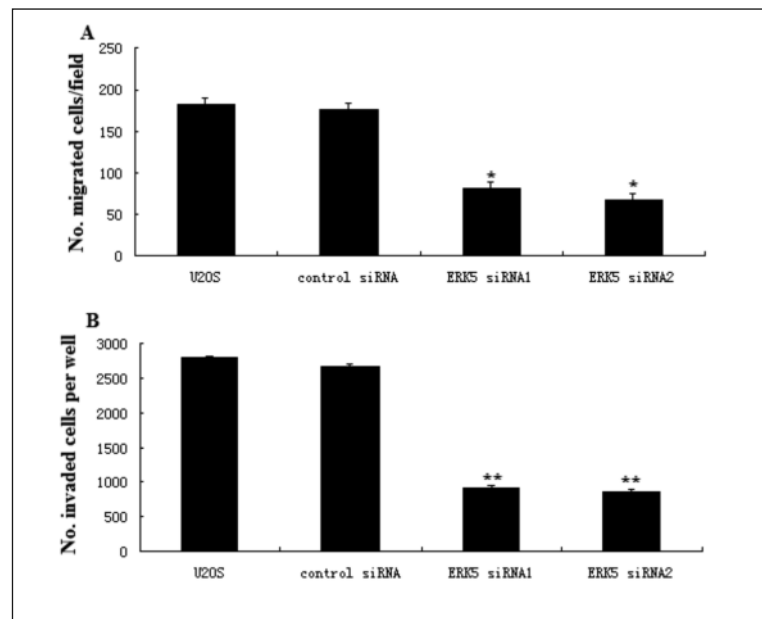
ed with ERK5 siRNA cells developed metastatic lesions in lungs (Table II), demonstrating that ERK5 knockdown reduced the metastatic potential of U2OS cells.

ERK5 knockdown suppresses the Slug-dependent MMP-9 expression in the U2OS cells

qRT-PCR analysis revealed that ERK5 knockdown inhibits Slug (Figure 3 A) and MMP-9 mRNA (Figure 3 B) levels, but ERK5 knockdown did not induce E-cadherin upregulation (Figure 3 C). Similar results was found by western blot assay (Figure 3 D).

We next investigates whether MMP-9 was dependent on Slug. A Slug-expressing plasmid was transfected into ERK5 siRNA cells. The results showed MMP-9 mRNA and protein (Figure 3 D) expression was significantly increased. But Slug re-expression did not induce E-cadherin down-

regulation. Vector transfection did not affect the MMP-9 levels (data not shown).

We, then, investigate whether MMP-9 re-expression could affect Slug and ERK5 levels. A MMP-9-expressing plasmid was transfected into ERK5 siRNA cells. The results showed MMP-9 re-expression did not affect the Slug, E-cadherin and ERK5 levels (data not shown). We, therefore, suggested that ERK5 knockdown suppresses the Slug-dependent MMP-9 expression in the U2OS cells.

Re-expression of Slug or MMP-9 in ERK5-silenced U2OS restores invasive phenotypes

It has shown above that ERK5 siRNA cells showed decreased migration and invasion ability in U2OS cells. At the same time, Slug and MMP-9 expression at both mRNA and protein levels was significantly inhibited in the U2OS cells sta-

Table II. ERK5 knockdown decreases the metastatic potential of U2OS cells.

	U2OS	ControlsiRNA	ERK5 siRNA1	ERK5 siRNA2
No. of mice with PN / No. of mice injected	8/10	7/9 (one mouse died)	0/10*	0/9* (one mouse died)
Average No. of PN per mouse±SD	7.1±3.2	7.8±3.4	0**	0**

PN: pulmonary nodules.
Asterisks indicate statistically significant difference between the Control siRNA and ERK5 siRNA1/2 groups ($^*p < 0.05$, one-way ANOVA; $^{**}p < 0.01$, Student's t-test).
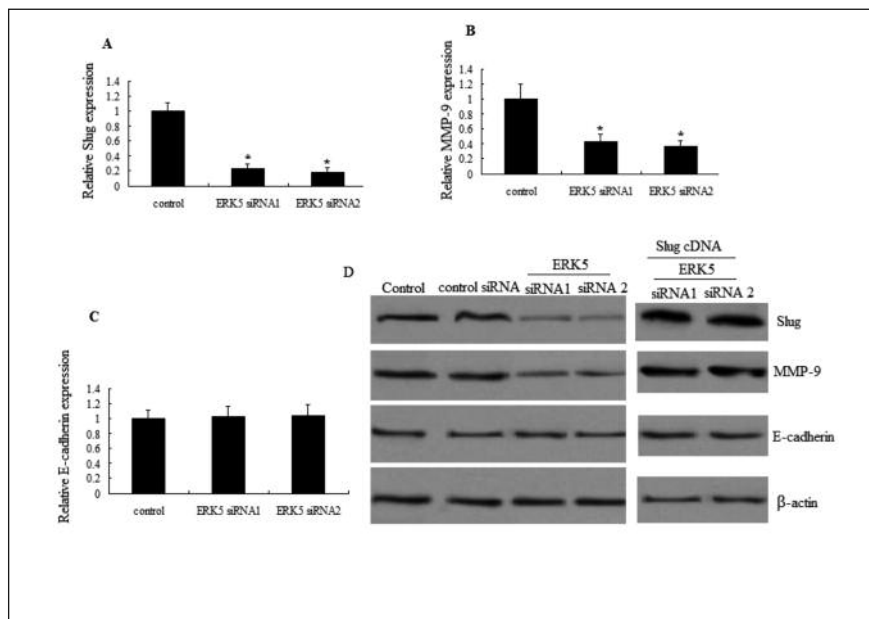
Figure 3. Expressing Slug, E-cadherin and MMP-9 in ERK5-silenced U2OS cells. **A**, Real-time PCR showed decreased Slug mRNA expression in U2OS cells stably expressing a control or ERK5-specific siRNA 1 and 2. **B**, Real-time PCR showed decreased MMP-9 mRNA expression in U2OS cells stably expressing a control or ERK5-specific siRNA 1 and 2. **C**, Real-time PCR showed no significant effect on E-cadherin expression in U2OS cells stably expressing a control or ERK5-specific siRNA 1 and 2. **D**, Western blot showed decreased Slug and MMP-9 protein expression in U2OS cells stably expressing a control or ERK5-specific siRNA 1 and 2. Restoration of Slug expression in ERK5-silenced U2OS cells increased the MMP-9 protein levels but not the E-cadherin levels. β-Actin was a loading control. (Vs control, $^*p < 0.05$).

bly expressing a control or ERK5-specific siRNA 1 and 2 (Figure 3). Compared to control cells, ERK5 siRNA 1/2 cells with reconstituted Slug or MMP-9 expression displayed more migration (Figure 4A) and increased invasion (Figure 4B). The results suggest that ERK5 expression associates with aggressive phenotypes of U2OS cells by regulation of Slug and MMP-9.

Discussion

In the present study, we observed that the ERK5 knockdown inhibits the *in vitro* invasiveness and in vivo lung metastasis. *In vitro*, ERK5 knockdown inhibits the invasiveness through the transcriptional down-regulation of Slug and MMP-9 in human U2OS cells.

The Snail family, which includs Slug, Snail, Snail-like, Scratch1, and Scratch2, has been shown to participate in mesoderm formation, neural crest cell formation and migration, cell differentiation, cell adhesion, cell invasion, cell cycle regulation, and antiapoptosis[13-18]. Slug is expressed in several carcinomas with increased invasion through regulation of E-cadherin[19]. Arnoux

et al[7] has found that ERK5 regulats the cytoskeleton by Slug signals, and activated ERK5 was the most potent activator of a Slug promoter-driven reporter gene. We, therefore, suggested that ERK5 might be a upstream gene of Slug. In our study, we found that ERK5 and Slug was overexpressed in the U2OS cells at gene and protein levels. Knockdown of ERK5 inhibits invasion and metastasis of U2OS cells, followed by decreased Slug expression. However, when a Slug-expressing plasmid was transfected into the ERK5 siRNA cells, the invasive phenotypes of ERK5 siRNA transfected cells was restored.

Slug is a E-cadherin repressor, which has recently been demonstrated to be important for cancer cells to down-regulate epithelial markers and up-regulate mesenchymal markers in order to become motile and invasive. Birgit et al[20] has found Slug did not repress E-cadherin, neither at the RNA nor at the protein level. Shuji et al[21] has found using siRNA for Slug demonstrated no effects on cancer cell migration or invasion *in vitro*. In the present study, we found ERK5 knockdown or Slug re-expression did not induce E-cadherin expression. E-cadherin appears to have insignificant role in this process.

Figure 4. Re-expressing Slug and MMP-9 in ERK5-silenced U2OS cells increases cell migration and invasion. **A**, *In vitro* wound healing assay showing the effect of Slug and MMP-9 over-expression on migration of ERK5 siRNA1/2 cells. **B**, *In vitro* Matrigel assay showing the effect of Slug and MMP-9 over-expression on invasiveness of ERK5 siRNA1/2 cells. Vs Slug or MMP-9 groups, $^*p < 0.05$, Student's *t*-test.
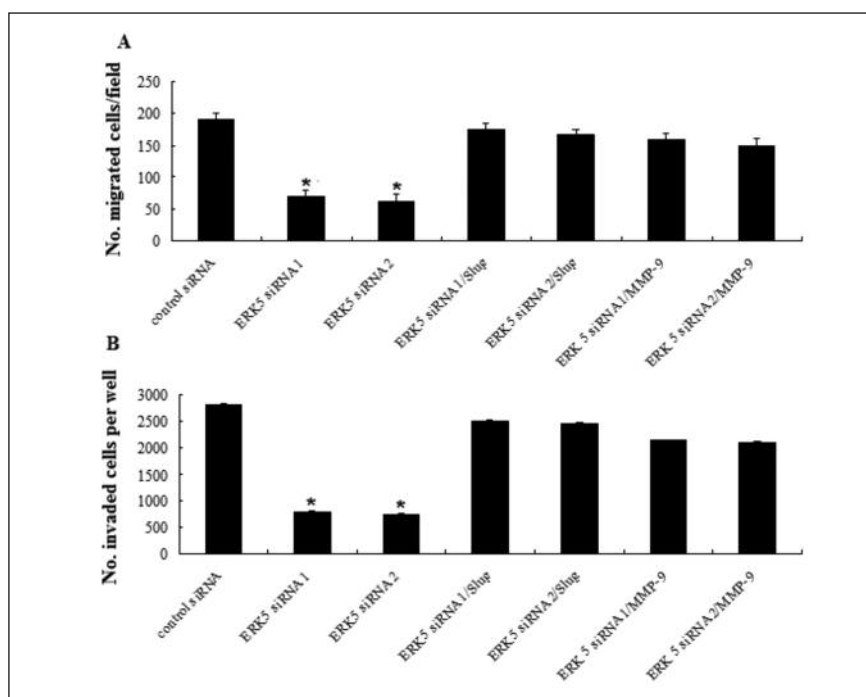
MMPs play an important role in degradation of extracellular matrix, which is an essential step in the cascade of metastasis[22]. In the present study, the western blot and QRT-PCR all showed that ERK5 siRNA transfected cells had decreased Slug and MMP-9 RNA and protein production, and Slug-transfected cells had increased MMP-9 RNA and protein production, which is a major component of basement membrane. We have also provided evidence suggesting that ERK5 signals through the Slug cascade to regulate MMP-9 expression. However, it remains to be determined how ERK5 links to Slug activation. Relatively little is known about the upstream signaling events that regulate MMP-9 function in cancer cells. More investigation is needed to delineate the signaling mechanism underlying the Slug activation by ERK5.

Conclusions

ERK5 silencing inhibits invasiveness of human OS cells. The mechanism underlying the ERK5-mediated invasiveness involves the activation of Slug and MMP-9 regulation. Further characterization of this ERK5-Slug-MMP-9 signaling pathway to discover its molecular components or regulators should bring about additional insights concerning the molecular action of ERK5 and the complex regulation of the invasion during cancer progression.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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