

Original Article

PAR-2 promotes invasion and migration of esophageal cancer cells by activating MEK/ERK and PI3K/Akt signaling pathway

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Abstract: Protease-activated receptor 2 (PAR-2) has been demonstrated to promote invasion and metastasis of certain cancer cells. This study aimed to investigate the mechanism by which PAR-2 regulated invasion and migration of esophageal cancer (EC) cells EC109. A successfully constructed PAR-2 shRNA lentiviral vector (Lenti-PAR-2 shRNA) was stably transfected into EC109 cells, and the expression of PAR-2 in infected cells was detected by quantitative real-time PCR (qRT-PCR) and western blotting. Specific inhibitors, PD98059 (for MEK/ERK) and LY294002 (for PI3K/Akt), were used to confirm the role of MEK/ERK and PI3K/Akt signaling pathways, respectively, in PAR-2-regulated invasion and migration of EC109 cells. A significant decrease in PAR-2 mRNA and protein expression was detected in EC109 cells stably transfected with Lenti-PAR-2 shRNA. The PAR-2 agonist could dramatically promote cell invasion and migration, up-regulate the expression of MMP-9 and TM4SF3, and activate MEK/ERK and PI3K/Akt signaling pathways. However, PAR-2 gene silencing attenuated PAR-2-mediated enhancement of invasion and migration of EC109 cells, significantly down-regulated the mRNA and protein expression of MMP-9 and TM4SF3, and inhibited ERK (Try202/204) and Akt (Ser473) phosphorylation. An effect similar to PAR-2 silencing could be achieved with the two specific MEK/ERK and PI3K/Akt pathway inhibitors. Consequently, these results demonstrated that PAR-2 promoted invasion and migration of esophageal cancer cells EC109 by activating MEK/ERK and PI3K/Akt signaling pathways, which was accompanied by up-regulation of MMP-9 and TM4SF3 expression. Hence, PAR-2 may be a potential candidate for anti-metastasis treatment of EC.

Keywords: Protease-activated receptor 2, esophageal cancer, invasion, migration, MEK/ERK, PI3K/Akt

Introduction

Esophageal cancer (EC) is one of the main public health concerns in China, where it is the eighth most common malignancy, It is the sixth leading cause of cancer-related death in the world [1]. Insidious onset, rapid progression and low long-term survival rate are the main features of EC [2]. The poor prognosis of EC is largely due to its high invasiveness and early extensive metastasis; thus, blocking or preventing the invasion and migration of EC is essential to the development of effective therapy and new drugs.

Protease-activated receptor 2 (PAR-2) belongs to the family of G-protein coupled receptors

(GPCRs) expressed on the surface of cell membranes. GPCRs are irreversibly activated by serine-dependent proteolytic cleavage. This unique process exposes a N-terminal sequence called a tethered ligand which binds and activates the receptor trans-membrane domain, triggering a cellular cascade in response to multiple inflammatory and carcinogenic stimuli [3, 4]. The association of PAR-2 expression and cancer has long been reported. Recent studies found that PAR-2 was highly expressed in various digestive system tumors such as EC, liver, colon, and pancreatic cancer, and was related to the high morbidity, rapid progression, and poor prognosis of these tumors [5-8]. Inci et al. found that PAR-2 receptor was distinctly stained in the luminal part of the esophageal epitheli-

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um, where trypsin coming from duodenogastric/esophageal refluxate could cleave and activate the receptor [9]. Duodenogastroesophageal reflux was considered to be the main factor of EC development [10], which suggested that PAR-2 was closely related to the development of EC.

Our previous study confirmed that endogenous trypsin and SLIGKV, an artificial PAR-2 agonist peptide, could activate PAR-2, leading to EC cell proliferation, invasion, and metastasis [11, 12], and transfection of PAR-2 targeted shRNA plasmid into EC109 cells reversed this phenomenon [13]. However, the low transient transfection efficiency of common plasmids and the short duration of gene silencing greatly increase the difficulty of further investigating the underlying mechanisms. Lentivirus-mediated shRNA (Lenti-shRNA) can directly and efficiently infect cells, and compared with other vectors, Lenti-shRNA vector can be rapidly introduced into a specific cell line that can stably and continuously express the target gene for a long time [14]. In addition, PAR-2 regulated cell migration has been reported to involve several signaling pathways, including MEK/ERK and PI3K/Akt pathways [7, 15, 16].

In the present study, we used Lenti-shRNA to silence the expression of PAR-2 gene in EC109 cells, and explored whether it affected cell invasion and metastasis through the regulation of MEK/ERK and PI3K/Akt signaling pathways.

Material and methods

Lentiviral shRNA vectors and cell transfection

Three pairs of PAR-2 siRNA, targeting human PAR-2 gene (Gene ID: 2150) and a non-targeting siRNA, NC, were transfected respectively into conventional cultured EC109 cells (CCAS, Shanghai, China) with Lipofetamine™ 2000 (Hanbio Biotechnology, Shanghai, China) according to the protocol. Western blotting was used to detect PAR-2 protein level after 72 h, and the most efficient silencing sequence was screened. To construct lentivirus, the PAR-2-gene cDNA of the above selected silencing sequence cloned by PCR was inserted into pHB-U6-MCS-CMV-ZsGreen-PGK-puromycin lentiviral vectors. The recombinant lentivirus, PAR-2 shRNA vector, was produced by co-transfection of 293T cells with PSPAX2 and PMD-2G plasmids with Lipofetamine™ 2000. Later,

EC109 cells were transduced with lentiviral vector, and GFP expression was observed under a fluorescence microscopy 48 h after transfection. Puromycin was added to medium at the concentration of 1 µg/mL for stable cell line selection. The Lenti-NC vector was used as negative control. The stable PAR-2-gene-silencing cell line, EC109-PAR-2 shRNA, was obtained after antibiotic selection for 2 weeks. After cells were harvested, the expression level of PAR-2 gene was determined by western blotting and quantitative real-time PCR (qRT-PCR).

Cell treatment and experimental grouping

EC109 cells in the logarithmic growth phase were treated with various reagents for 24 hours and then grouped. PAR-2 agonist group (50 µmol/L SLIGKV-NH₂, Abcam, Cambridge, MA, USA), PAR-2 anti-agonist group (50 µmol/L VKGILS-NH₂, R&D Systems, Minneapolis, MN, USA), MEK/ERK inhibitor group [EC109 cells were pretreated with 10 µmol/L PD98059 (Abcam, Cambridge, MA, USA) for 1 h, then 50 µmol/L SLIGKV-NH₂ was added], PI3K/Akt inhibitor group [EC109 cells were pre-treated with 20 µmol/L LY294002 (Abcam, Cambridge, MA, USA) for 1 h, then 50 µmol/L SLIGKV-NH₂ was added]. The optimal concentrations of SLIGKV-NH₂ and VKGILS-NH₂ were chosen according to data obtained in preliminary experiments. The same batch of untreated EC109 cells and EC109-PAR-2 shRNA cells served as control group and PAR-2 shRNA group, respectively.

Cell viability assay

To determine the final concentration of both PD98059 and LY294002, EC109 cells (5×10^4 /mL) were plated in 96-well plates and divided into a control group, a drug group, and a blank group not seeded with cells, and 5 replicate wells in each group were incubated overnight. Then, fresh medium (100 µL/well) was added into the blank group and the control group, and cells in the drug group were incubated with various concentrations of PD98059 (0.1, 1, 10, 20, 30 µmol/L) or LY294002 (10, 20, 30, 40, 50 µmol/L) for 24 h. Cell viability was then detected using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturers' instructions. The cell viability ratio was calculated by the following formula: cell viability (%) = average absorbance of (drug-blank)/average absorbance of (control-

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Table 1. Sequences

Gene	Sequence (5'-3')
siRNA1	GCAAAGAACGCUCUCCUUU
siRNA2	CCAUGUACCUGAUCUGCUU
siRNA3	GCACCAUCCAAGGAACCAA
NC	UUCUCCGAACGUGUCACGUAA
PAR-2 shRNA	FP: TTCTCCGAACGTGTCACGTAA RP: TTACGTGACACGTTCCGGAGAA
NC shRNA	FP: GCACCATCCAAGGAACCAA RP: TTGGTTCCTGGATGGTGC
PAR-2	FP: AATATGGCTGCTGATTCTGCTGGTC RP: ATAGGCAGAGGCTGTGAGGAAGG
MMP-2	FP: CACCTACACCAAGAACTCCGCTCTG RP: GTGCCAAGGTCAATGTCAGGAGAG
MMP-9	FP: TCCTGGTGCTCCTGGTGCTG RP: CTGCCTGTCGGTGAGATTGGTTC
TM4SF3	FP: GCTATAAAGAAAGTCGCTGCA RP: TTCATTACAATGCGATCAGAC
β -actin	FP: TGTTGAGACCTTCAACACCC RP: AGCACTGTGTTGGCGTACAGG

blank) \times 100%, and the IC₅₀ (half-inhibitory concentration of drug) value was calculated using GraphPad PRISM. 6.0 (GraphPad Software, CA, USA).

Cell growth curve

To assess cell proliferation, EC109 cells treated with different drugs were seeded in 96-well culture dishes (3000 to 5000 cells/well) and measured by recording time lapse images of the cells over 7 days. During this study, 5 fixed sites per well were monitored using a JuLI™ Stage Real-Time cell recorder (NanoEnTek, Seoul, Korea) at 24 h intervals, and confluences were also measured by the system software to plot the cell survival curve. The experiments were carried out in triplicate.

Transwell invasion and migration assay

To detect the invasion ability of EC109 cells, transwell chambers with a pore size of 8.0 μ m were coated evenly with 60 μ l Matrigel, which was diluted 1:4 in serum-free RPMI-1640 medium and allowed to gel in an incubator for 1 h. 100 μ l cell suspensions (5×10^4 cells/well) were seeded in the upper chambers, while 600 μ l RPMI-1640 medium containing 15% FBS was added into the lower chambers, and then cultured for 24 h at 37°C in a CO₂ incubator. After invasion, the upper chamber was removed with tweezers, and cells were fixed in 100%

methanol for 30 min and stained with 0.1% crystal violet for 30 min. Cells that did not pass through the membrane were carefully wiped with a wet cotton swab. Under the microscope, we randomly selected five fields of view: upper, middle, lower, left and right, and the average of cells were calculated for statistical analyses.

In the migration experiments, all procedures followed the transwell invasion assay, except for the coating of the transwell chamber with Matrigel. All experiments in every group were performed in triplicate.

RNA extraction and qRT-PCR

Cells of each group were collected and the total RNA was extracted by Trizol reagent according to typical protocol. RNA concentration and quality were measured by a NanoPhotometer® Spectrophotometer (IMPLEN, CA, USA) [17]. Primers are listed in **Table 1**. cDNA synthesis and qRT-PCR used commercial kit according to manufacturer's protocol, and 2 μ l reverse transcription product was added as template into a 20 μ l reaction. A PikoReal™ TCRO096 (Thermo Fisher Scientific, Waltham, MA, US) was used for the reaction, and the Ct values per well were obtained as raw data for analyses of results. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of the target gene in each group, and GAPDH or β -actin was selected as endogenous control gene to normalize samples. All reactions were performed in triplicate.

Western blotting assay

Cells were washed three times with pre-cooled PBS and lysed in protein extraction reagent (RIPA: PMSF = 100:1) for 30 min. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was collected as total cell protein and stored at -80°C. The protein concentration was determined by the bicinchoninic acid method. Total proteins were boiled at 100°C for 10 min for denaturation, loaded onto a 10% polyacrylamide gel for electrophoresis, and then transferred to a PVDF membrane. Membranes were incubated in blocking buffer (5% non-fat milk diluted with TBST) at room temperature for 1 h, and washed with TBST (3 \times ; 5 minutes each time). Later, membranes incubated with mouse anti-human β -actin and GAPDH monoclonal antibodies (Proteintech, Chicago, IL, USA), and rabbit anti-human PAR-2, ERK1, p-ERK (Try202/204), Akt, p-Akt (Ser473), MMP-9 and MMP-2 monoclonal antibodies, and mouse anti-human TM4SF3 polyclonal

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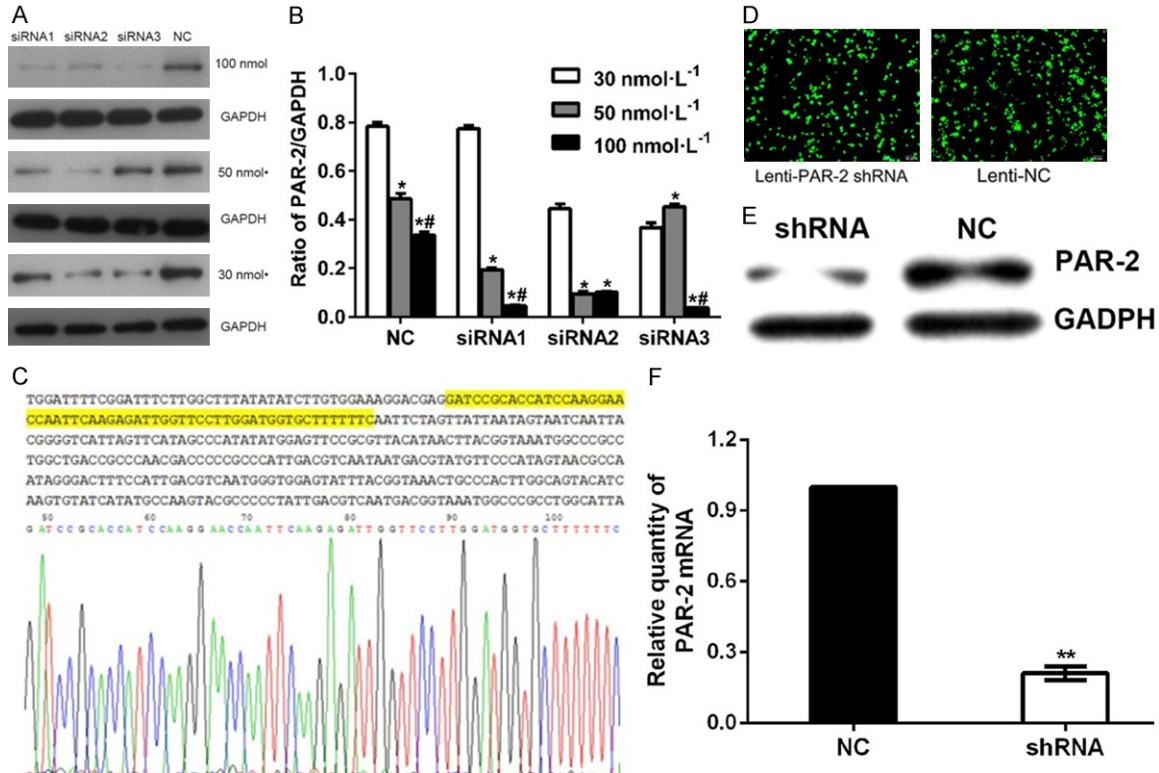


Figure 1. Construction of lentiviral vector and identification of Lentivirus-mediated PAR-2 gene silencing. (A, B) Western blot assay detected the expression level of PAR-2 protein in EC109 cells transfected with different concentrations of siRNA for 72 h. (C) PAR-2 shRNA plasmid sequencing peak. (D) Lenti-PAR-2 shRNA and Lenti-NC transfected EC109 cells show green fluorescence for 48 h ($\times 100$). (E) Western expression of PAR-2 in EC109 stable cell lines. $**P < 0.01$ vs NC group (F) The relative expression levels of PAR-2 mRNA in EC109 stable cell lines. $*P < 0.05$ vs 30 nmol/L group, $\#P < 0.05$ vs 50 nmol/L group.

antibody (Abcam, Cambridge, MA, USA) (1:1,000) prepared with 5% non-fat milk co-incubated overnight at 4°C. After washing with TBST (3 \times ; 5 min each time), membranes were incubated with the corresponding secondary antibody (1:5,000) diluted with TBST at room temperature for 1 h. Following incubation in ECL reagent for 1 minute, membranes were exposed and scanned by a ChemiDoc™ XRS + System with Image Lab™ Software (Bio-RAD, Hercules, CA, USA). The gray value of western blotting signals was assessed by image analysis software. The protein levels were normalized using GAPDH or β -actin as the endogenous control, and the ratio of target protein signal values to those of the corresponding endogenous control signal value were used for statistical analysis.

Statistical analysis

All experimental data are presented as $\bar{x} \pm s$ and statistically analyzed using GraphPad

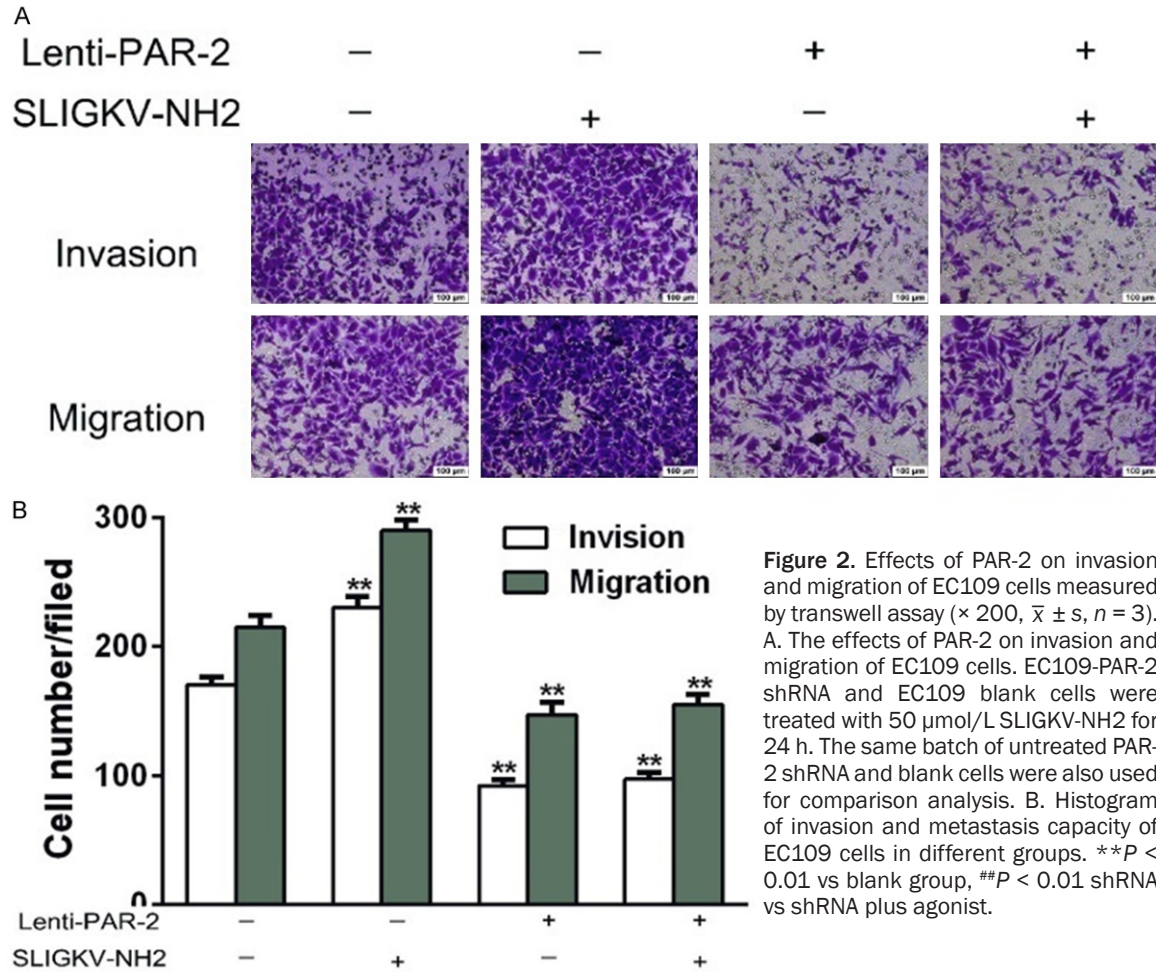
PRISM software version 6.0 (GraphPad Software, CA, USA). Student's t test was used to compare the differences between two groups. One-way ANOVA with Tukey's post-hoc test was used to investigate the differences among multiple groups. In all analyses, $P < 0.05$ was considered statistically significant.

Results

Identification of PAR-2 gene silencing-stable cell lines

We designed three different PAR-2 specific targeting sequences to investigate the function of PAR-2 in the biological progression of EC. Fortunately, different concentrations of siRNA targeting human PAR-2 gene could significantly down-regulate the expression of PAR-2, especially siRNA3 at 100 nmol/L (**Figure 1A, 1B**); therefore, it was chosen for subsequent lentivirus vector construction and lentivirus packaging (**Figure 1C**). After transfection for 48 h, GFP

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expression was observed in EC109 cells transfected with PAR-2 shRNA or NC lentivirus under a fluorescence microscope (Figure 1D). The expression of PAR-2 protein was also significantly lower than that of NC group ($P < 0.01$, Figure 1E), and the relative expression level of PAR-2 mRNA in EC109-PAR-2 shRNA cell line was decreased by 0.211 time that of EC109-NC cells ($P < 0.01$, Figure 1F). These results showed that a PAR-2 gene silencing-stable cell line was successfully constructed.

Effects of PAR-2 on invasion and migration of EC109 cells

The number of EC109 cells that migrated through the Matrigel barrier in the PAR-2 shRNA group was significantly lower than that in the blank group (40.8 ± 4.6 vs 184.5 ± 9.4 , $P < 0.01$) and agonist group (40.8 ± 4.6 vs 255.3 ± 8.3 , $P < 0.01$) (Figure 2B). Transwell cell migration assays showed the same pattern; the num-

ber of EC109 cells that migrated through the artificial basement membrane by deformation in the PAR-2 shRNA group was significantly decreased when compared with blank group (122 ± 9.6 vs 158.0 ± 6.1 , $P < 0.01$) and agonist group (122 ± 9.6 vs 224.2 ± 8.8 , $P < 0.01$). At the same time, the PAR-2 agonist dramatically enhanced the invasion and migration ability of EC109 cells, but had no significant effect on the ability of the PAR-2 shRNA group (Figure 2A). In summary, these results suggested that PAR-2 low expression could inhibit the capability of EC109 cell migration and invasion.

Effects of PAR-2 on the mRNA and protein expression levels of cell invasion related molecules

In the blank group, agonist group, PAR-2 shRNA group, and PAR-2 shRNA plus agonist group, the relative expression levels of PAR-2 mRNA were 1.000 ± 0.000 , 2.036 ± 0.095 , $0.211 \pm$

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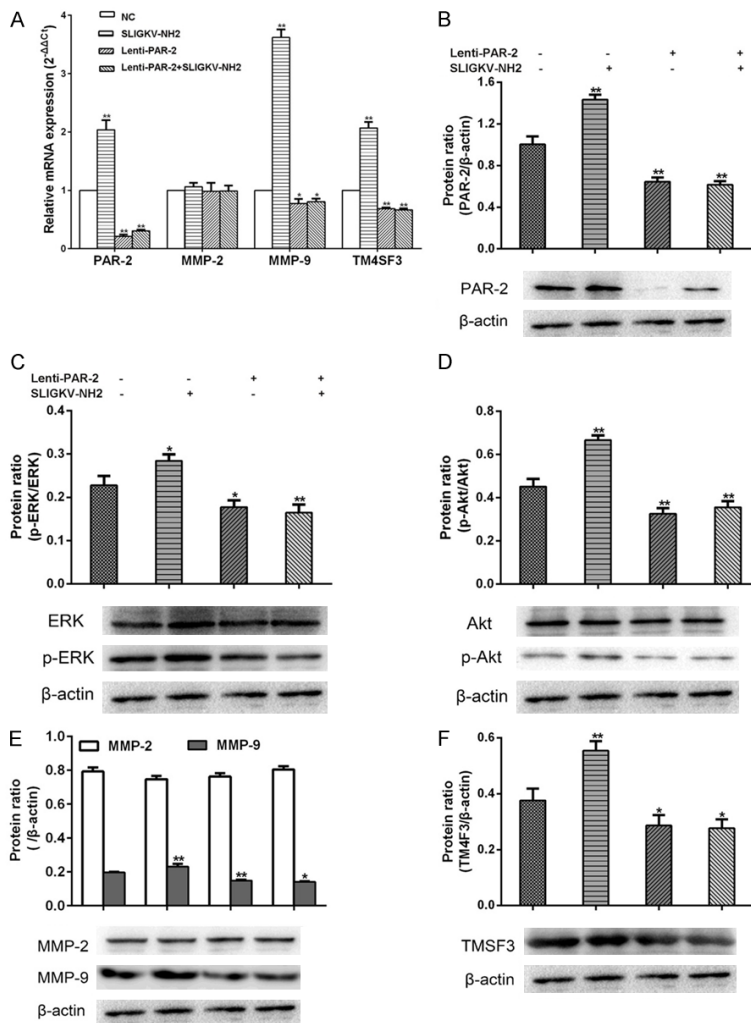


Figure 3. Effects of PAR-2 on the mRNA and protein expression levels of cell invasion related molecules ($\bar{x} \pm s$, $n = 3$). A. The relative mRNA expression levels of PAR-2, MMP-2, MMP-9, and TM4SF3. B-F. Protein expressions of PAR-2, MMP-2, MMP-9, TM4SF3, ERK, p-ERK, Akt, and p-Akt. * $P < 0.05$ vs blank group, ** $P < 0.01$ vs blank group.

0.017, and 0.305 ± 0.011 ; the relative expression levels of MMP-2 mRNA were 1.000 ± 0.000 , 1.059 ± 0.059 , 0.980 ± 0.020 , and 0.989 ± 0.011 ; the relative expression levels of MMP-9 mRNA were 1.000 ± 0.000 , 7.619 ± 0.136 , 0.872 ± 0.77 , and 0.873 ± 0.028 ; the relative expression levels of TM4SF3 mRNA were 1.000 ± 0.00 , 0.809 ± 0.0139 , 1.708 ± 0.014 , and 1.760 ± 0.045 (Figure 3A).

The results of qRT-PCR and western blotting presented a parallel trend (Figure 3B-F). The protein expression of PAR-2, p-ERK (Try202/204), p-Akt (Ser473), MMP-9, and TM4SF3 in the PAR-2 shRNA group were significantly decreased ($P < 0.05$), but MMP-2 did not

change obviously between blank and other treatment groups. However, PAR-2 activation significantly promoted the expression of the above-mentioned proteins ($P < 0.05$), whereas the PAR-2 agonist had no effect on the PAR-2 shRNA group.

MEK/ERK and PI3K/Akt signaling pathway inhibitors attenuated PAR-2-mediated enhancement of invasion and migration in EC109 cells

Two pharmacological inhibitors, PD98059 (for MEK/ERK pathway) and LY294002 (for PI3K/Akt pathway), were used to verify whether PAR-2-induced enhancement of invasion and metastasis was regulated by MEK/ERK and PI3K/Akt signaling pathways in EC109 cells. PD98059 and LY294002 significantly inhibited the proliferation of EC109 cells ($P < 0.05$), which was assessed by CCK8 assay after 24 h incubation (Figure 4A, 4B). The IC_{50} values of PD98059 and LY294002 were $10 \mu\text{mol/L}$ and $20 \mu\text{mol/L}$, respectively. Subsequently, EC109 cells were exposed to different drugs for 7 days. The ability of cell proliferation was stronger

in PAR-2 agonist group, when compared with blank control, while slower in PAR-2 shRNA group, the MAPK inhibitor group, and the PI3K/Akt inhibitor group (Figure 4C). Finally, PAR-2-induced cell invasion and metastasis were abolished by treatment with the two specific inhibitors (Figure 4D, 4E). As with the PAR-2 shRNA group, the ability of "three-dimensional" plane migration and erosion of Matrigel were significantly weaker after inhibitor treatment than those in the control group ($P < 0.01$), which suggested that the positive effect of PAR-2 on cell invasion and migration was regulated by MEK/ERK and/or PI3K/Akt signaling pathways in EC109 cells.

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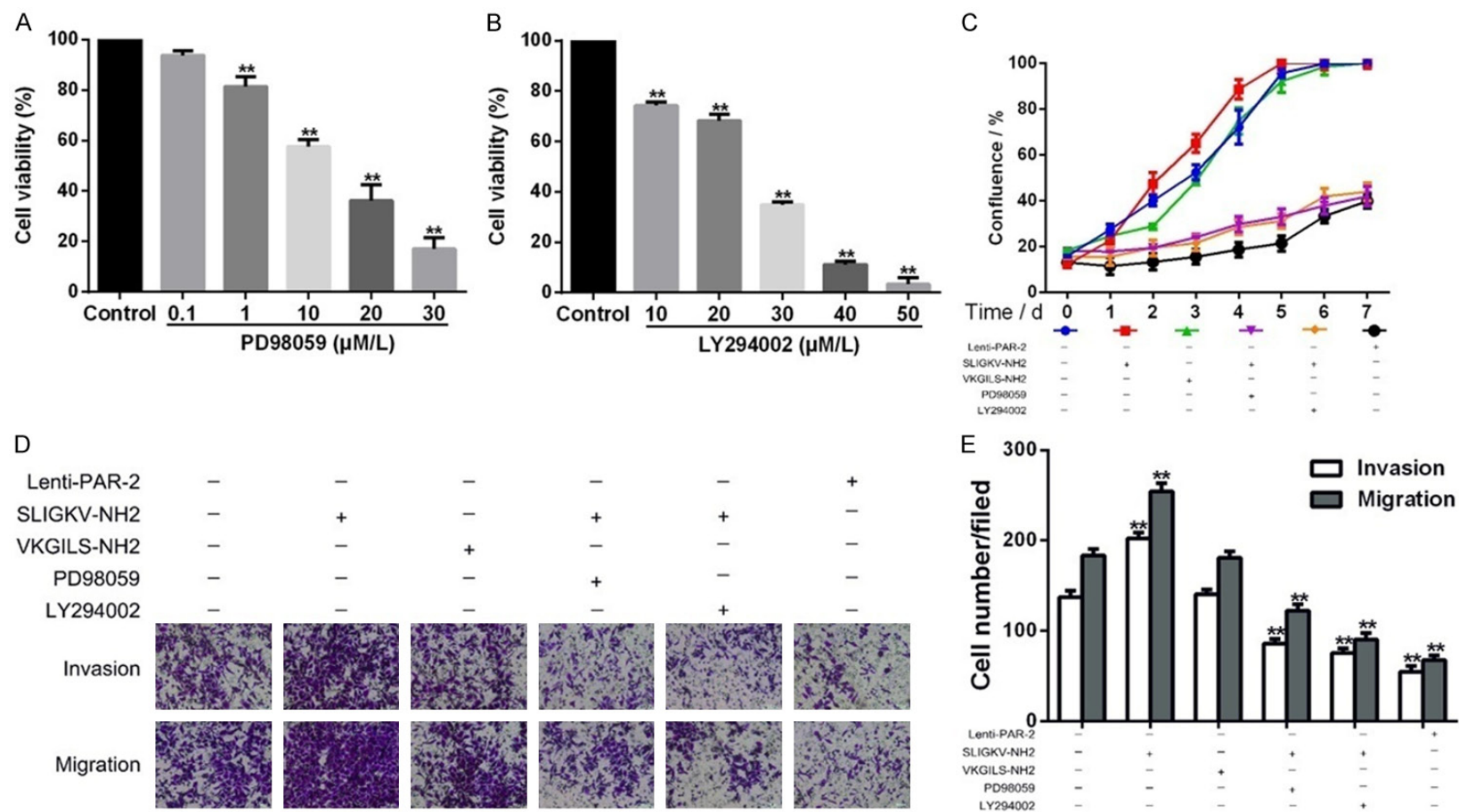


Figure 4. MEK/ERK and PI3K/Akt signaling pathway inhibitors attenuated PAR-2-mediated enhancement of invasion and migration in EC109 cells ($\bar{x} \pm s$, $n = 3$). CCK-8 assay is used to measure the effect of two pharmacologic inhibitors, PD98059 (for MEK/ERK pathway, A) and LY294002 (for PI3K/Akt pathway, B), on EC109 cells. (C) Cell growth curve in each group. (D) Cells passing through in every group in the transwell invasion and migration experiment. (E) Data summary of the invasion and migration capacity of EC109 cells in different groups. ** $P < 0.01$ vs control group.

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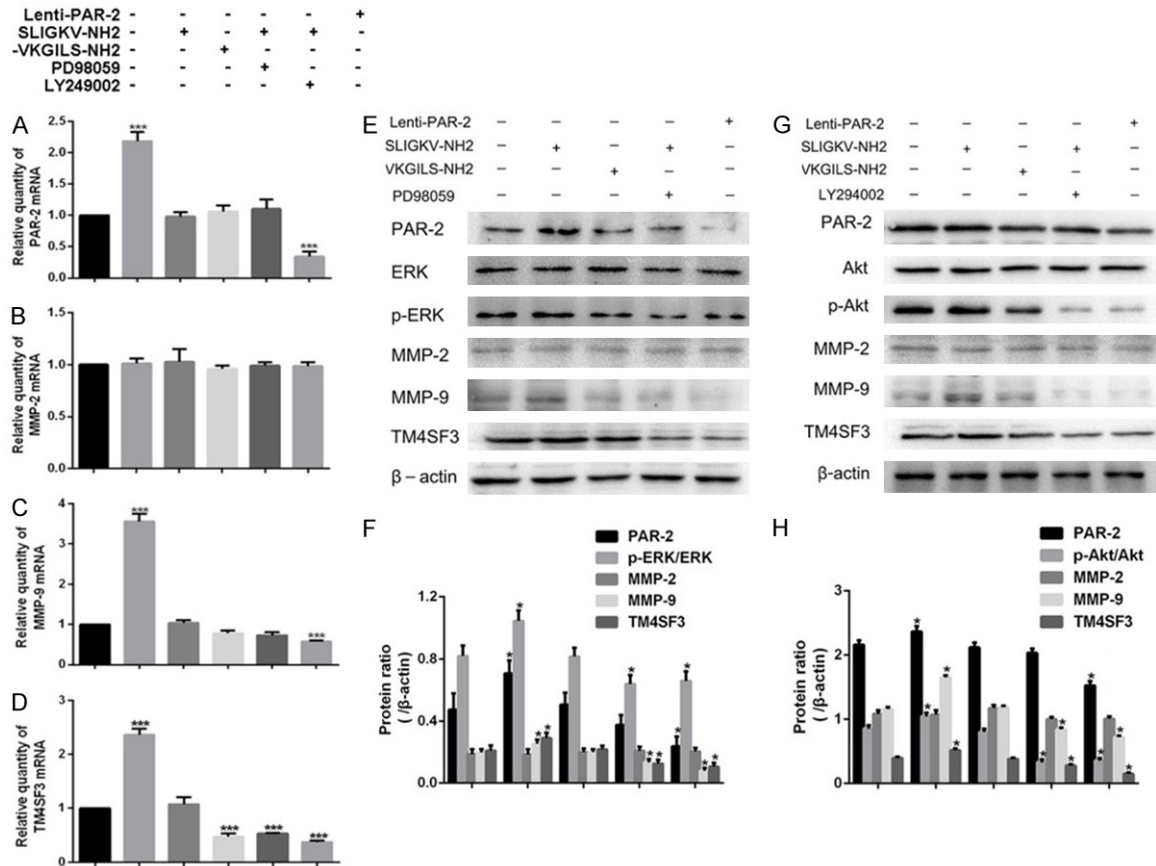


Figure 5. MEK/ERK and PI3K/Akt signaling pathway inhibitors attenuated PAR-2-mediated up-regulation of invasion related molecules in EC109 cells ($\bar{x} \pm s$, $n = 3$). PAR-2 agonist group (50 $\mu\text{mol/L}$ SLIGKV-NH2, Abcam, Cambridge, MA, USA), PAR-2 anti-agonist group (50 $\mu\text{mol/L}$ VKGILS-NH2, R&D Systems, Minneapolis, MN, USA), MEK/ERK inhibitor group [EC109 cells were pretreated with 10 $\mu\text{mol/L}$ PD98059 (Abcam, Cambridge, MA, USA) for 1 h, then 50 $\mu\text{mol/L}$ SLIGKV-NH2 was added], PI3K/Akt inhibitor group [EC109 cells were pre-treated with 20 $\mu\text{mol/L}$ LY294002 (Abcam, Cambridge, MA, USA) for 1 h, then 50 $\mu\text{mol/L}$ SLIGKV-NH2 was added]. The optimal concentration of both SLIGKV-NH2 and VKGILS-NH2 was selected according to data obtained in preliminary experiments. The same batch of untreated EC109 cells and EC109-PAR-2 shRNA cells served as control group and PAR-2 shRNA group, respectively. A-D. The relative mRNA expression levels of PAR-2, MMP-2, MMP-9, and TM4SF3. E, F. Protein expressions of PAR-2, MMP-2, MMP-9, TM4SF3, ERK and p-ERK in blank control, PAR-2 agonist group, PAR-2 anti-agonist group, MEK/ERK inhibitor group, PAR-2 shRNA group. G, H. Protein expressions of PAR-2, MMP-2, MMP-9, TM4SF3, Akt and p-Akt in blank control, PAR-2 agonist group, PAR-2 anti-agonist group, PI3K/Akt inhibitor group, PAR-2 shRNA group. * $P < 0.05$ vs control group.

MEK/ERK and PI3K/Akt signaling pathway inhibitors attenuated PAR-2-mediated up-regulation of invasion related molecules in EC109 cells

The expression of PAR-2 mRNA in PAR-2 agonist group was increased, while decreased in PAR-2 shRNA group when compared with blank control (**Figure 5A**); The expression of MMP-9 (**Figure 5C**) and TM4SF3 (**Figure 5D**) was higher in PAR-2 agonist group but lower in PAR-2 shRNA group, MEK/ERK inhibitor group and PI3K/Akt inhibitor compared with blank con-

trol. However, the MMP-2 protein expression (**Figure 5B**) in each group did not change significantly.

As shown in **Figure 5E-H**, we observed same tendencies of the above-mentioned protein expression in each group. In the PAR-2 agonist group, p-ERK (Try202/204) and p-Akt (Ser473) were significantly increased, and TM4SF3 and MMP-9 expressions were dramatically up-regulated, while they were decreased in PAR-2 shRNA group when compared with control group ($P < 0.05$). In addition, the expression of

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MMP-9 and TM4SF3 were inhibited by two pharmacologic inhibitors, accompanied by blockade of MEK/ERK and PI3K/Akt signaling pathways.

Discussion

RNA interference (RNAi) is a specific gene silencing phenomenon induced by dsRNA homologous to the target gene sequence [18]. The viral shRNA vector can also be used for RNA interference. Since lentivirus belongs to retrovirus group, it is feasible to inhibit continuous and stable expression of a target gene in transfected cells by using a Lenti-shRNA vector with antibiotic label [14]. In this experiment, we successfully established a cell line with PAR-2 gene silencing, and in this cell the mRNA and protein expression of PAR-2 were down-regulated to 21% and 30%, respectively, fully meeting the need for further study of PAR-2 function.

It has been reported that PAR-2 has the ability to promote cell invasion of colon cancer [7], breast cancer [15], and melanoma [19]. Knocking out PAR-2 in melanoma animal models limited the growth of primary tumors and reduces the amount of spontaneous metastases [19]. Wang et al. [5] revealed higher expression of PAR-2 in EC tissues than that in normal tissues. Meanwhile, our previous studies confirmed that high expression of PAR-2 was involved in EC invasion and metastatic phenotype [11]. In this study, we found that silencing PAR-2 inhibited the migration behavior of EC109 cells moving through transwell chambers, and arrested the invasion process of cells penetrating Matrigel, and these effects were enhanced in EC109 cells treated with PAR-2 agonist. Previous reports have shown that PAR-2 can inhibit MMP-9 activity in colon cancer cells [7]. TM4SF3 binds to prostaglandin F2a receptor regulatory protein (FPRP) in the presence of CD9 and CD81, thereby regulating the binding of GPCRs to their corresponding ligands [20]. There are also reports that MMP-2, MMP-9 [21, 22], and TM4SF3 [23, 24] play important roles in the invasion and migration of EC. Therefore, we proposed a hypothesis that MMP-2, MMP-9, or TM4SF3 may be involved in PAR-2-promoted cell invasion and metastasis. Consistent with phenotypic observations, we found that mRNA and protein expression of these metastasis-associated proteins, MMP-9 and TM4SF3, were significantly reduced in

EC109-PAR-2 shRNA cells, whereas increased in EC109 cells treated with PAR-2 agonist. Based on the above results, we initially considered that Lentivirus-mediated RNA knockdown of PAR-2 restricted EC cell invasion and migration, and the molecular mechanism may be associated with expression up-regulation of MMP-9 and TM4SF3.

Investigating which downstream signaling pathways PAR-2 regulates will help us better elucidate the molecular mechanisms of invasion and metastasis in EC cells. In this study, we found that the MEK/ERK and PI3K/Akt pathways were suppressed in PAR-2 gene silenced cells, but were enhanced in PAR-2 activated cells. Moreover, two pathway-specific inhibitors resulted in attenuation of the enhanced invasion and migration induced by PAR-2 activation in EC cells, and the increased expression levels of MMP-9 and TM4SF3. Our previous studies have revealed that PAR-2 promoted proliferation of EC109 cells by activating the MEK/ERK signaling pathway [12]. In addition, several studies in the past have shown that activation of MEK/ERK and PI3K/Akt signaling pathways could significantly promote malignant tumor invasion and metastasis, such as in esophageal [25], gastric [26], pancreatic [27], and breast cancers [28]. Li et al. found that activation of MEK/ERK and PI3K/Akt signaling pathways in breast cancer increased MMP-9 degradation of fibronectin and binding to β 6 integrin, enhancing the aggressiveness of breast cancer cells [29]. Xia et al. found that miR302a was significantly down-regulated in EC cell lines, and up-regulation of miR302a markedly reduced phosphorylation of ERK1/2 and Akt and inhibited proliferation and invasion of EC cells [30]. These findings, together with results of the current study, indicated that PAR-2 may promote EC cell invasion and migration via MEK/ERK and PI3K/Akt pathway regulation.

In conclusion, the present study indicated that PAR-2 promoted invasion and migration of EC109 cells via MEK/ERK and PI3K/Akt pathway, and MMP-9 and TM4SF3 took part in this process.

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Disclosure of conflict of interest

None.

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