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Enhancing Effect of Eps8 on Cervical Cancer Cell Motility by Increasing MMP-9 Expression through ERK1/2 activation

Eps8 經由活化 ERK1/2-MMP-9 訊息路徑而促進子宮頸癌細胞之移行能力

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Abstract

The oncoprotein Eps8 is known to facilitate colon and breast cancer cell motility. Our present study sought to determine whether FAK and MMP-9 were involved in Eps8-induced cervical cancer cell motility. Studies conducted in control and Eps8-attenuated HeLa and SiHa cervical cancer cells revealed that cell mobility was reduced in both Eps8-attenuated HeLa and SiHa cells. Diminished Eps8 not only led to reduced FAK Pi-Tyr-397 and Pi-Tyr-861, but also the expression of matrix metalloproteinase 9 (MMP-9). Restored expression of Eps8 in HeLa cells with *eps8* siRNA reversed all the biological events just mentioned. The simultaneous inhibition of cell motility, ERK activation and MMP9 expression in cells treated with PD98059 implicated the involvement of Eps8 in MMP9 enhancement, which was ERK-dependent. Indeed, suppressed ERK activation was observed in Eps8-

attenuated cells, which could be recovered by the restored expression of Eps8. MMP-9 specific inhibitor could decrease motility in HeLa and its derived cells without affecting ERK1/2 activity, indicating that MMP-9 expression was indeed downstream of ERK1/2. Taken together, our results demonstrate that, in addition to FAK, Eps8 can regulate the expression of MMP-9 by virtue of ERK activation. This novel finding broadens our understanding towards Eps8-mediated cell migratory process.

Keywords: Eps8, MMP-9, ERK1/2, cell motility

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摘要

致癌蛋白 Eps8 已知能促進結腸及乳癌細胞的移行能力。我們這項研究旨在確定 FAK 和 MMP-9 是否參與在 Eps8 誘導的子宮頸癌細胞移行能力。在控制組和 Eps8 表現減少的 HeLa 與 SiHa 子宮頸癌細胞上進行的研究顯示，Eps8 的減弱會降低子宮頸癌細胞的移行能力。Eps8 的減弱不僅減少 FAK Tyr-397 及 Tyr-861 的磷酸化，也降低基質金屬蛋白酶 9(MMP-9)的表現。而將 Eps 送回表現減低之 HeLa 細胞則可使上述這些因 Eps8 減弱所產生的變化有一定程度的回復，顯示 eps8 siRNA 的作用是具有專一性的。在以 PD98059 處理子宮頸癌細胞上發現細胞的移行能力，ERK 的活化和 MMP 9 的表現會同時被抑制，顯示 Eps8 所媒介的 MMP-9 蛋白的表達是有 ERK 依賴性的。的確，basal ERK 活性在 EPS8 減弱的 HeLa 與 SiHa 子宮頸癌細胞有降低的情形，而這現象可通過 EPS8 的還原表達來回復。MMP-9 專一性抑製劑可以在不影響 ERK1 / 2 的活性下而減少 HeLa 及其所衍生之細胞的移行能力，顯示 MMP-9 的表達的確在 ERK1 / 2 的下游。綜合以上結果，我們在子宮頸癌細胞中確立了 Eps8 可經由活化 ERK 而促進 MMP-9 的表現；同時，此條訊息傳遞路徑參與在部份 Eps8 所調控的子宮頸癌細胞移行能力中。這項發現拓寬了我們對 Eps8 媒介的細胞移行過程的理解。

關鍵詞：Eps8, MMP-9, ERK1/2, 細胞移行能力

Introduction

As an oncoprotein, Eps8 (EGFR pathway substrate number 8) has been known to contribute to Src-mediated transformation (Leu et al., 2004). Expression of dominant negative MEK1 is able to abrogate Eps8-induced cellular transformation (Maa. et al., 2001). Eps8 can also interact with Abi-1 and RN-tre, leading to actin cytoskeleton reorganization through Rac and receptor endocytosis through Rab5, respectively (Scita et al., 1999, Lanzetti et al., 2000). In addition, by capping the barbed end of actin, Eps8 can regulate actin-based motility (Disanza et al., 2004). Besides, our previous study indicates that by enhancing the activity and expression of focal adhesion kinase (FAK), Eps8 can promote proliferation and motility of colon cancer cells (Maa et al., 2007).

Focal adhesions are transient structures which allow cell protrusions to anchor to the ECM for a short period of time and are rapidly remodeled during migration. The rupture of these adhesive contacts are carried out by matrix metalloproteinases (MMPs) or serine proteinases of the plasmin system. MMPs play important roles in many normal physiological processes involving ECM remodeling. Of these,MMP-9 has been shown to participate in the migration of many cells (Buisson et al., 1996; Delclaux et al., 1996; Leppert et al., 1995; McCawley et al., 1998; Okada et al., 1997; Pluznik et al., 1992; Uhm et al., 1998). In the advancing lamellipodia of migrating human bronchial epithelial cells, MMP-9 accumulates through an actin-dependent pathway (Legrand et al., 1999). Besides, the expression of MMP-9 has been known to be regulated through the ERK pathway in cancer cells and endothelial cells (Genersch et al., 2000; Kaomongkolgit et al., 2008; Lakka et al., 2002).

Previously, we reported that Eps8 was elevated in cervical carcinoma and its level of expression was associated with parametrium invasion and lymph node metastasis (Chen et al., 2008). In the present study, we sought to examine the ability of Eps8 to modulate MMP-9 expression and investigate the signaling cascade behind this modulation in cervical cancer cells.

Material and Methods

Cell line generation

Two human cervical carcinoma cell lines (i.e. HeLa and SiHa) were used in this study and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin-streptomycin. The generation of control siRNA-, *eps8* siRNA- expressing HeLa or SiHa cells, and Eps8-attenuated HeLa cells reintroduced with Myc-tagged murine Eps8 were described previously (Chen et al., 2008).

Western blotting analysis

Lysis of the cells was carried out with modified RIPA buffer as described before (Maa et al., 1999) and protein concentration was determined by protein assay kit(Bio-Rad). Methods for imminoblotting analysis have been described (Maa et al., 1999). Membranes were probed with different primary antibodies specific for the following proteins: Eps8 (Upstate Biotechnology, Inc.), FAK (A-17), pi-Y397 FAK (Upstate), pi-Y861 FAK (BIOSOURCE International), ERK1/2 , pi-ERK1/2 and Actin (Santa Cruz Biotechnology). After washing, membranes were incubated with HRP-conjucated secondary antibodies, and results were detected by Enhanced Chemiluminescence method (Amersham, Arlington Heights, ILL, USA).

In Vitro Wound Healing Assay

Cells were grown to confluence, and a cell-free area was generated by scraping the monolayer with a pipette tip. Cells were incubated with fresh regular culture medium. To avoid a proliferative effect, cells were treated with 2 mM hydroxyurea (Sigma-Aldrich) from the time of scatching until the end of the experiment. Cells were photographed at 0 and 48 hrs after scratching to calculate the % of wound closure. In case of inhibitor treatment, PD98059 (10 μM; Calbiochem) or MMP-9 specific inhibitor 1 [N-Hydroxy-1-(4-methoxyphenyl)sulfonyl-4-(4-biphenylcarbonyl)piperazine-2-carboxamide] (5 nM; Calbiochem) was added at the time of plating and of medium change in the beginning

of wound-healing assay.

Gelatin Zymography

Cells were plated at a density of 6 x 10^5 cells per 6 cm dish and cultured in 10% FBS DMEM media for 24 h. Then the cells were subjected to serum-free media and incubated for another 48 h period. Conditioned media were collected and concentrated 30-fold and 15-fold for HeLa and SiHa and their derived cells, respectively. In case of inhibitor treatment, PD98059 (10 μM; Calbiochem) was added at the time of plating, and the cells were incubated for 24 h. Then the cells were subjected to serum-free media and incubated for another 48 h period. Conditioned media were collected and concentrated 20-fold and 10-fold for HeLa and SiHa cells, respectively in this experiment. 80 μl of concentrated media were electrophoresed in 8% SDS-polyacrylamide gel containing 0.1% gelatin (Sigma-Aldrich). Gels were washed with 2.5% Triton X followed by reacting in 250 ml buffer containing 40 mM Tris-HCl, 10 mM CaCl2, 0.15M NaCl, and 0.02% Brij-35 at 37^{o}C overnight. Then MMP-9 activity was visualized by staining with Coomassie R250 dye and imaged.

Reverse transcription polymerase chain reaction

Total RNA was extracted by using the Trizol reagent according to the manufacturer,s instruction (Life Technologies, Inc., Rockville, MD). Two micrograms of total RNA was used to generate cDNA in a reaction mixture containing oligo-dT, RNase inhibitor, dNTP, and M-MLV reverse transcriptase (Promega). cDNA was diluted 10-fold and 10 μl of it was amplified by PCR. The PCR reaction was carried out in a 50 ml mixture containing reaction buffer, Taq polymerase, 0.2 mM dNTP, 0.2 μM of forward and reverse primer for mmp-9 and gapdh in which gapdh served as an internal control. PCR reaction was run for 35 cycles, and PCR products were resolved in 2% agarose gel and detected by ethidium bromide staining. Sequences of the PCR primer pairs were as follows : mmp-9: forward, CCC GTC CTG CTT TGC AGT; reverse, ATC CAA GTT TAT TAG AAA CAC TCC A; gapdh: forward, CCA TCA CCA TCT TCC AGG AG; reverse: CCT GCT TCA CCA CCT TCT TG.

Statistical analysis

All values were expressed as mean ± S.D. Our experimental results were statistically

evaluated using Kruskal-Wallis one-way ANOVA on ranks followed by Student-Newman-Keuls pairwise multiple comparison test. Differences were considered significant if $P < 0.05$.

Results

Reduction of cervical cancer cell migratory ability following Eps8 attenuation

To address the role of Eps8 in cervical cancer cell migration, their movement was examined by scratch wound-healing assay. In this assay, scratch wounds were generated in confluent Eps8-attenuated HeLa and SiHa cells and their respective control cells using pipette tips, and wound closure was evaluated at 48 hrs after scratching. As shown in Fig. 1, Eps8 attenuation significantly reduced migratory ability in HeLa and SiHa cells at 48 hrs after scratching, and ectopically expressed Eps8 could rescue this effect (Fig. 1A). These results corroborate the importance of Eps8 in cervical cancer cell mobility.

Augmentation of FAK activity and expression in cervical cancer cells by Eps8

We have proved that the activity and expression of FAK can be regulated by Eps8 and is critical in Eps8-mediated colonocyte migration (Maa el al., 2007). Since Eps8 was also involved in cervical cancer cell motility, its effect on the activity and expression of FAK were examined. As depicted in Fig. 2, Eps8 knockdown significantly reduced FAK Tyr-397 and Tyr-861 phosphorylations in both HeLa and SiHa cells. FAK expression was also reduced in Eps8-diminished cells. Furthermore, ectopic expression of Eps8 in Eps8-attenuated HeLa cells could recover FAK activity and expression, indicating that the effect of eps8-siRNA was specific. These results implicate the participation of FAK in Eps8-mediated mobility in cervical cancer cells.

Enhancement of MMP-9 expression in cervical cancer cells by Eps8

Various evidences have indicated that MMP-9 is involved in the migration of many cells (Buisson et al., 1996; Delclaux et al., 1996; Leppert et al., 1995; McCawley et al., 1998; Okada et al., 1997; Pluznik et al., 1992; Uhm et al., 1998). To examine whether MMP-9 expression can be regulated by Eps8, HeLa and SiHa cells were cultured in regular medium for 24 h, then mRNA were extracted for RT-PCR. For gelatinase activity analysis, cells

cultured in regular medium for 24 h were subjected to serum-free medium for a further 48 h period, then conditioned media were collected and concentrated. As revealed by gelatin zymography in Fig.3A, MMP-9 expression was reduced in Eps8-attenuated HeLa and SiHa cells. RT-PCR analysis further confirmed that Eps8 regulated MMP-9 expression at the transcriptional level (Fig.3B). To further verify this result, rescue experiment was performed. By restoring Eps8 expression, MMP-9 expression could be recovered in Eps8-attenuated HeLa cells (Fig. 3).

Contributing effect of Eps8-elicited ERK1/2 activation and MMP-9 expression in Eps8-mediated cellular motility

MMP-9 expression is known to be regulated through the ERK1/2 signaling cascade (Genersch et al., 2000; Kaomongkolgit et al., 2008; Lakka et al., 2002). To examine whether Eps8 regulated MMP-9 expression through the ERK1/2 cascade in cervical cancer cells, basal ERK acivity was compared first in Eps8-attenuated and control HeLa and SiHa cells. As shown in Fig.4A, basal ERK phosphorylation was indeed decreased in Eps8-diminished cells. Ectopically expressed Eps8 in Eps8- diminished HeLa cells could partially restore ERK activity, indicating the effect of eps8 siRNA was specific. To further verify this signaling cascade, PD98059 (10 μM), which proved to be a specific ERK1/2 inhibitor, was added to parental HeLa and SiHa cells for 24 h and assayed for MMP-9 expression and ERK activity. As depicted in Fig.4B and 4C, gelatin zymographs of conditioned media and RT-PCR result of cellular extracts demonstrated a great reduction of MMP-9 expression following PD98059 treatment. ERK1/2 phosphorylation was also greatly diminished in these cells treated with PD98059. To evaluate to what extent could the ERK1/2 signaling pathway affect Eps8-mediated cellular motility, PD98059 (10 μM) was added to HeLa and its derived cells. As shown in Fig. 4D, inhibition of ERK1/2 activity could decrease cell motility in HeLa, Eps8-attenuated HeLa and Eps8-reintroduced HeLa cells. The results indicated that the ERK1/2 signaling pathway played a significant role in Eps8-mediated cellular motility.

Reduction of motility in HeLa-based cells without affecting ERK1/2 activity following the treatment of MMP-9 specific inhibitor

To determine whether MMP-9 played a role in Eps8-mediated cervical cancer cell motility, MMP-9 specific inhibitor (5 nM) was added to HeLa and its derived cells. As shown in Fig. 5A, MMP-9 inhibitor reduced cellular motility in these cells, indicating that MMP-9

contributed to Eps8-mediated cellular motility. To further eluciadate the upstream-downstream relationship of ERK1/2 and MMP-9 in Eps8-mediated cellular motility, MMP-9 specific inhibitor of various concentrations was added to HeLa cells for 48 hr, followed by examination of ERK1/2 activity. As shown in Fig. 5B, inhibition of MMP-9 expression did not affect ERK1/2 activity, indicating that MMP-9 expression is downstream of ERK1/2 in Eps8-mediated motility. Taken together, our results establish a signaling cascade in which Eps8 can upregulate MMP-9 expression by enhancing ERK1/2 activation.

Discussion

In this study, we aimed to investigate the ability of Eps8 to modulate cervical cancer cell motility. We observed that Eps8 attenuation reduced cell motility in both HeLa and SiHa cells. Diminished Eps8 not only led to reduced FAK Pi-Tyr-397 and Pi-Tyr-861, but also the expression of matrix metalloproteinase 9 (MMP-9). Restored expression of Eps8 in HeLa cells with *eps8* siRNA reversed all the biological events just mentioned. The simultaneous inhibition of cell motility, ERK activation and MMP9 expression in cells treated with PD98059 implicated the involvement of Eps8 in MMP9 enhancement, which was ERK-dependent. Indeed, MMP-9 specific ihhibitor 1 decreased mobility in HeLa-based cells without affecting ERK1/2 activity. With these data, we disclosed the role of Eps8 in upregulation of MMP-9 required for cell motility.

FAK Tyr397 residue, while being autophophorylated upon integrin engagement, mediates SH2-dependent binding to Src family tyrosine kinases. Src can phosphorylate FAK Tyr861 while associating with FAK, thereby further activating FAK (Calalb et al., 1995; Calalb et al., 1996). Our previous studies conducted in colon cancer cells revealed that by virtue of Src, Eps8 could induce the activity and expression of FAK involved in Eps8-evoked colonocyte migration (Maa et al., 2007). Consistent with decreased Src activation (as reflected by Pi-Tyr-416) by diminished Eps8 in our previous study in cervical cancer cells (Chen et al., 2008), we observed a significant reduction of FAK activity (as reflected by Pi-Tyr-397 and Pi-Tyr-861) in Eps8-attenuated HeLa and SiHa cells, and restoration of Eps8 rescued this defect. Thus, activation and up-regulation of FAK by Eps8 seemed to be a universal phenomenon that was applicable to both colon and cervical cancer cells.

With the ability to degrade the underlying matrix, MMP-9 can regulate cell mobility, angiogenesis and tumor growth (Egeblad & Werb, 2002). Accumulation of MMP9 is detected in the advancing lamellipodia of migrating cells (Legrand et al., 1999). The modulation of

MMP-9 expression by Eps8 implicated that MMP-9 was also an Eps8 target. The concomitantly reduced MMP-9 expression and cell mobility by PD98059 indicated that the expression of MMP-9 was ERK-dependent in cervical cancer cells. While Eps8 provoked ERK activation and caused transformation in murine fibroblasts (Maa et al., 2001), we declared that Eps8-induced ERK activation resulted in MMP-9 expression and contributed to cell mobilization. Figure 6 represents a simple model proposed to illustrate the mechanisms of Eps8-elicited migration in cervical cancer cells.

A previous study demonstrated that a positive correlation between Eps8 expression and migratory capability was detected in pancreatic cancer cells (Welsch et al., 2007). In this study, in addition to upregulation of activity and expression of FAK, we established the Eps8-ERK1/2-MMP-9 signaling cascade which contributed to Eps8-mediated cell motility in cervical cancer. These results perhaps gave more insights into understanding of the mechanisms involved in migration triggered by Eps8.

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Figure Legends

Fig. 1　Promotion of cervical cancer cell migration by Eps8

Confluent HeLa (A) and SiHa (B) cells were wounded with 200 μl and 10 μl pipette tips, respectively, followed by incubating in fresh regular culture media containing hydroxyurea (2mM). Four wounded regions were marked on each dish, and photographs were taken at 0 and 48 h after wounding under 40X microscopy to evaluate % of wound closure. One set of representative pictures were shown. Values represented mean % wound closure ± S.D of three independent experiments with similar results. **P* < 0.05 compared with control cells; $^{\#}P$ < 0.05 compared with Eps8-attenuated cells.

Fig. 2　Reduction of FAK avtivity and expression in cervical cancer cells following Eps8 attenuation

Lysates (100 μg) prepared from (A)HeLa-based control, eps8 siRNA-expressing and Myc-tagged Eps8-reexpressed cells and (B)SiHa and its derived cells were resolved in SDS-PAGE and immunoblotted with the indicated antibodies to determine FAK activities and expression. Similar results were obtained in two independent experiments.

Fig. 3　Effect of Eps8 on MMP-9 expression in cervical cancer cells

(A)Conditioned media were collected, concentrated 30-fold and 15-fold for HeLa-based and SiHa-based cells, respectively, and cell-equivalents were subjected to gelatin zymography. The data were representative of two independent experiments showing similar results. (B) HeLa, SiHa and their derived cells were cultured in regular media for 24 h, then total RNA were extracted for RT-PCR. GAPDH was used as an internal control. Similar results were obtained in two independent experiments.

Fig. 4　Effect of PD98059 on MMP-9 expression and cellular motility in cervical cancer cells

Basal ERK activity was examined by resolving protein lysates (100 μg) of 24 h-cultured (A)HeLa-based control, eps8 siRNA-expressing, Myc-tagged Eps8-reexpressed cells and SiHa-based control and eps8 siRNA-expressing cells in SDS-PAGE and immunoblotted with anti-phospho-ERK antibody. HeLa and SiHa cells cultured with or without 10 μM of PD98059 (MEK1 inhibitor) for 24 h were subjected to (B) gelatin zymography and (C) RT-PCR. The condition of gelatin zymography for this part of the experiment was described under "Methods and Materials". The data were representative of two independent experiments showing similar results. (D) 10 μM of PD98059 was added at the time of plating and of medium change in the beginning of wound-healing assay. Confluent HeLa-based cells were wounded with 200 μl pipette tips, followed by incubating in fresh regular culture media containing hydroxyurea (2mM).　Four wounded regions were marked on each dish, and photographs were taken at 0 and 48 h after wounding under 40X microscopy to evaluate % of wound closure. Data from one representative experiment of three with similar results were shown, and values represented mean % wound closure ± S.D. **P* < 0.05.

Fig. 5　The importance of ERK1/2-MMP-9 signaling cascade for Eps8-mediated cellular motility

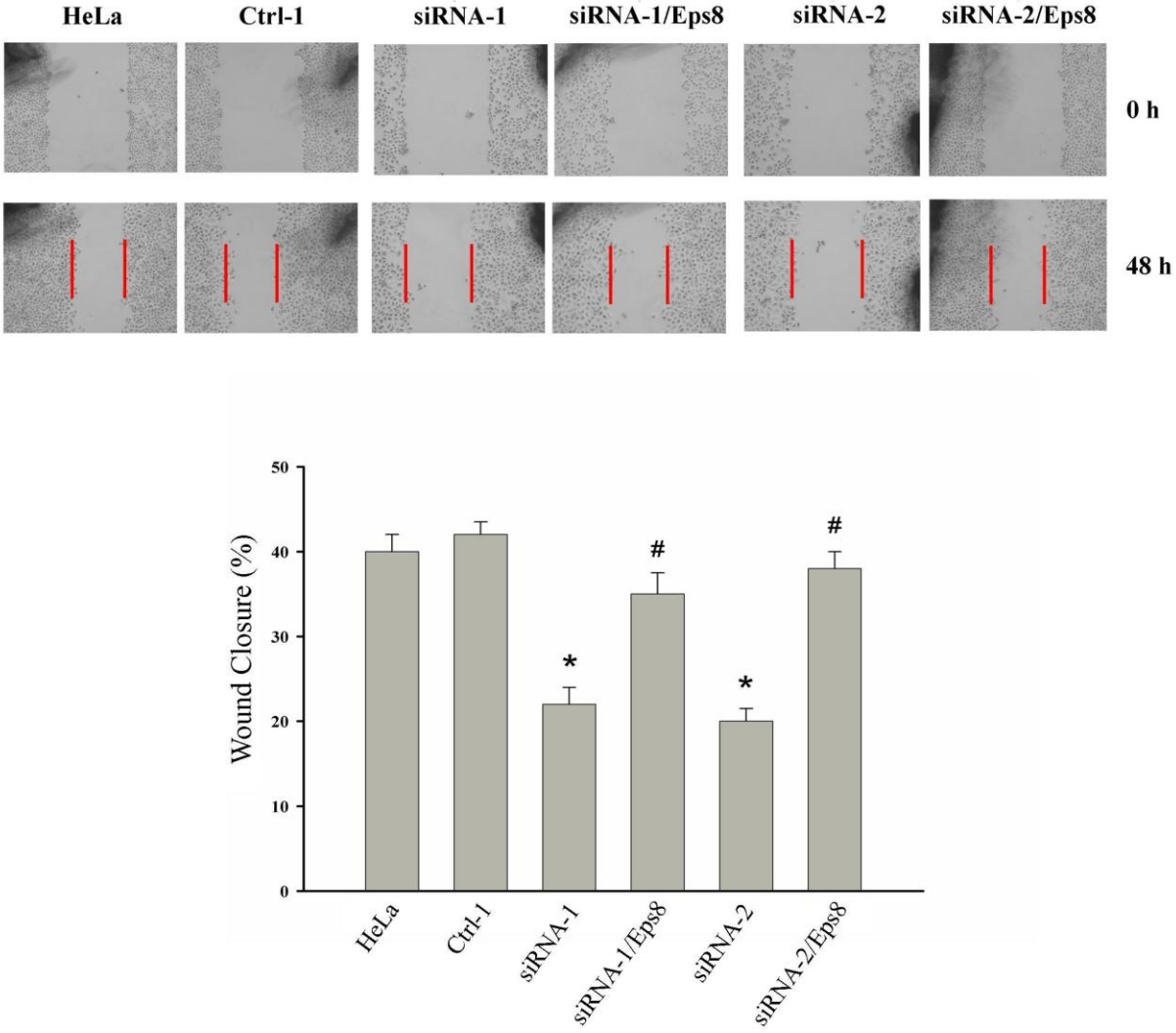
(A) 5 nM of inhibitor 1 was added at the time of plating and of medium change in the beginning of wound-healing assay. Confluent HeLa-based cells were wounded with 10 μl pipette tips, followed by incubating in fresh regular culture media containing hydroxyurea (2mM).　Four wounded regions were marked on each dish, and photographs were taken at 0 and 48 h after wounding under 40X microscopy to evaluate % of wound closure. Note that

higher overall % of wound closure in this part of the study was attributed to relatively smaller starting wounded area. One set of representative pictures were shown. Values represented mean % wound closure ± S.D of three independent experiments with similar results. *P < 0.05. (B) Inhibitor 1 of various concentrations was added to HeLa cells for 48 h, and protein lysates were collected for examination of ERK1/2 activity.

Fig. 6　Diagrammatic model illustrating Eps8-elicited cervical cancer migration

Figures

Figure 1A


HeLa
Ctrl-1
siRNA-1
siRNA-1/Eps8
siRNA-2
siRNA-2/Eps8
0 h
48 h
Wound Closure (%)
50
40
30
20
10
0
*
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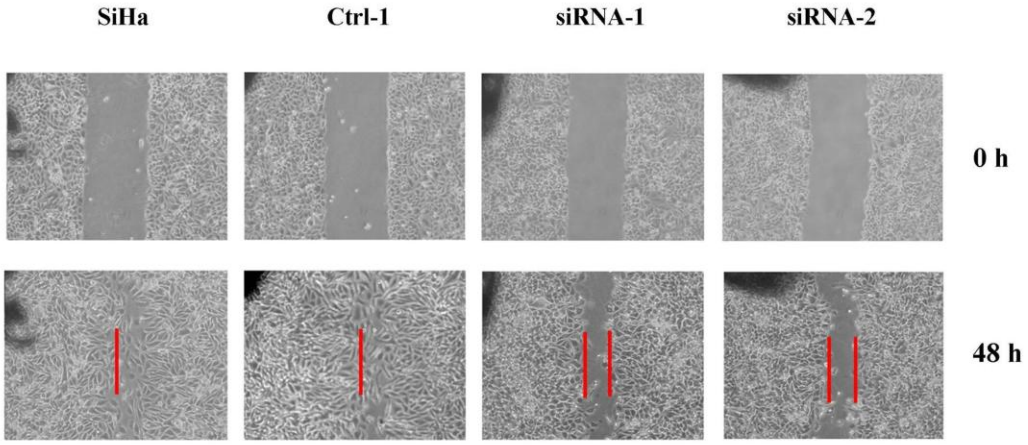
HeLa
Ctrl-1
siRNA-1
siRNA-1/Eps8
siRNA-2
siRNA-2/Eps8

Figure 1B


SiHa
Ctrl-1
siRNA-1
siRNA-2
0 h
48 h

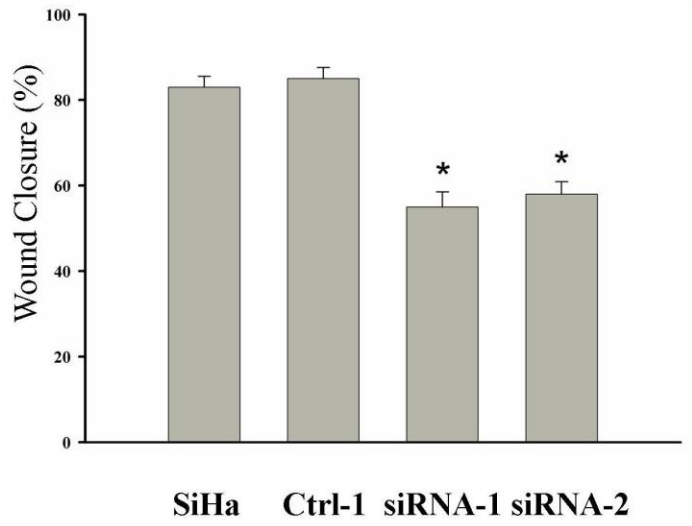
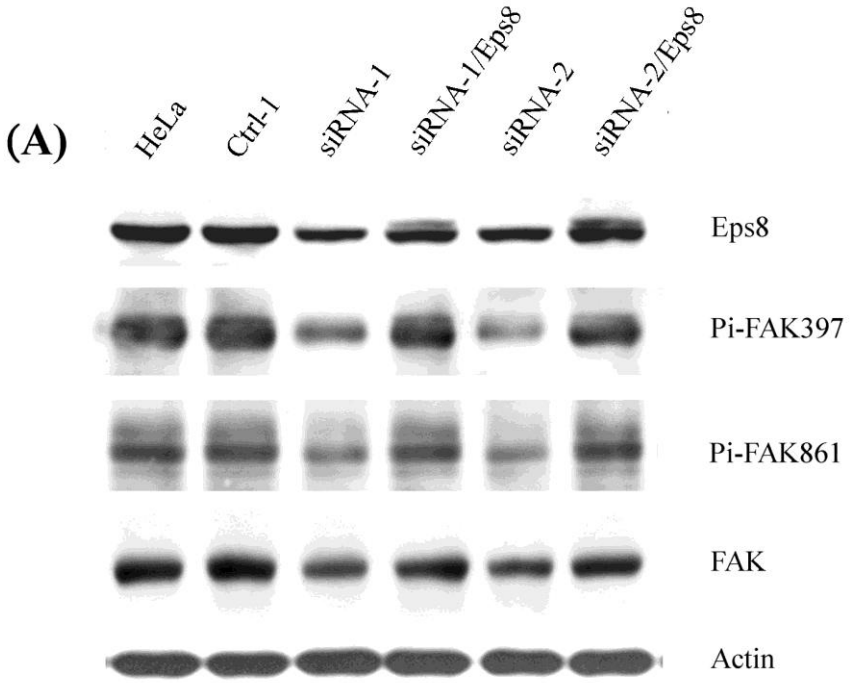

Wound Closure (%)
100
80
60
40
20
0
*
*
SiHa
Ctrl-1
siRNA-1
siRNA-2

Figure 2


(A)
HeLa
Ctrl-1
siRNA-1
siRNA-1/Eps8
siRNA-2
siRNA-2/Eps8
Eps8
Pi-FAK397
Pi-FAK861
FAK
Actin

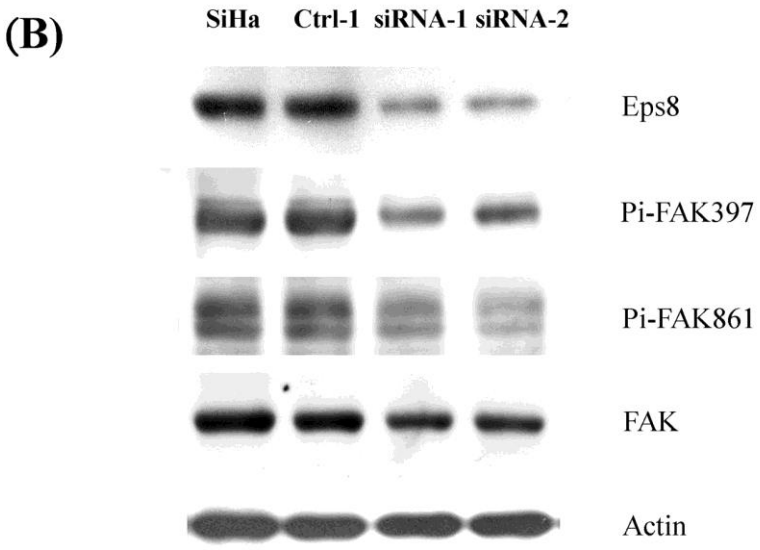

(B)
SiHa
Ctrl-1
siRNA-1
siRNA-2
Eps8
Pi-FAK397
Pi-FAK861
FAK
Actin

Figure 3

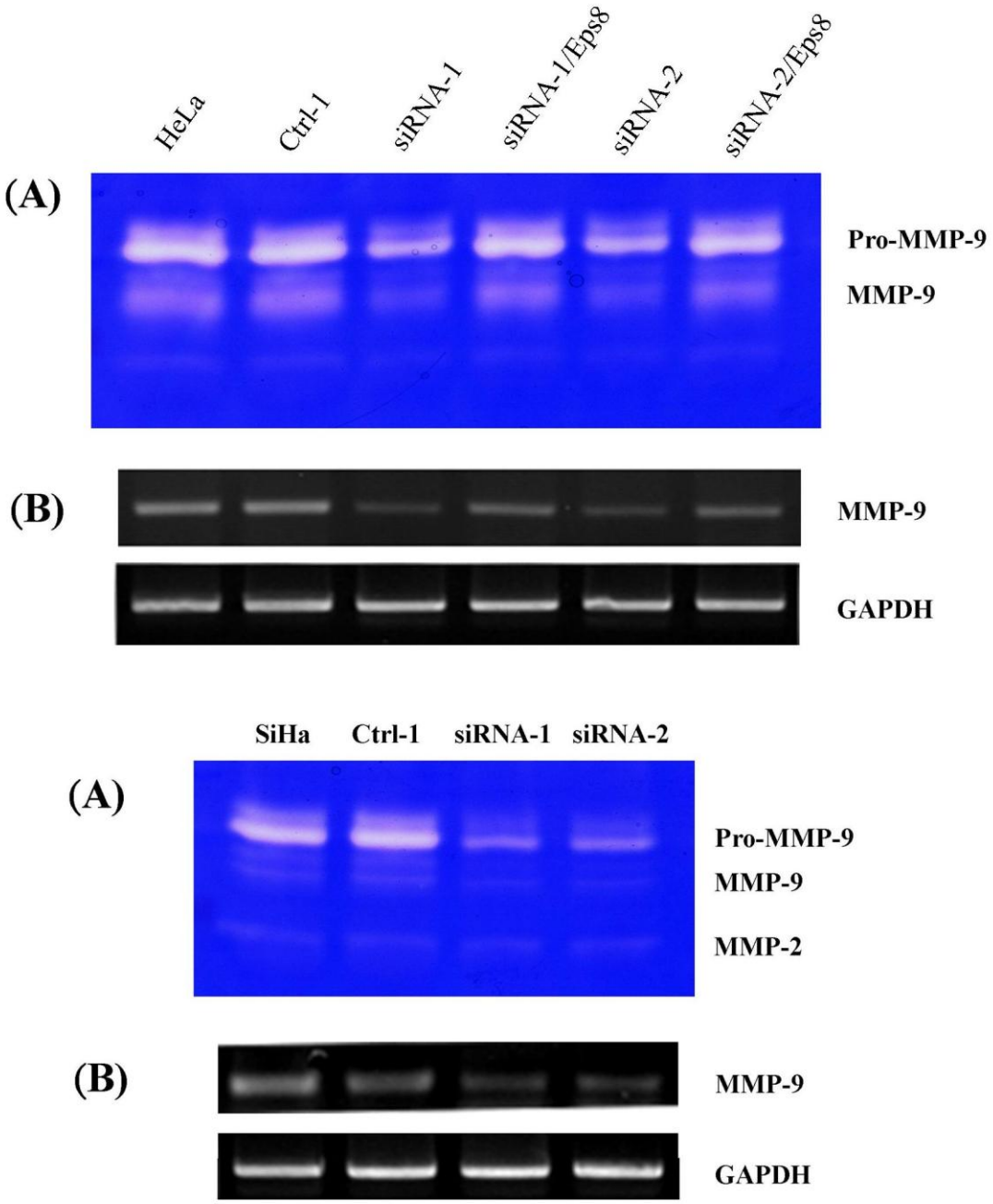

HeLa
Ctrl-1
siRNA-1
siRNA-1/Eps8
siRNA-2
siRNA-2/Eps8
(A)
Pro-MMP-9
MMP-9
(B)
MMP-9
GAPDH
SiHa
Ctrl-1
siRNA-1
siRNA-2
(A)
Pro-MMP-9
MMP-9
MMP-2
(B)
MMP-9
GAPDH

Figure 4A

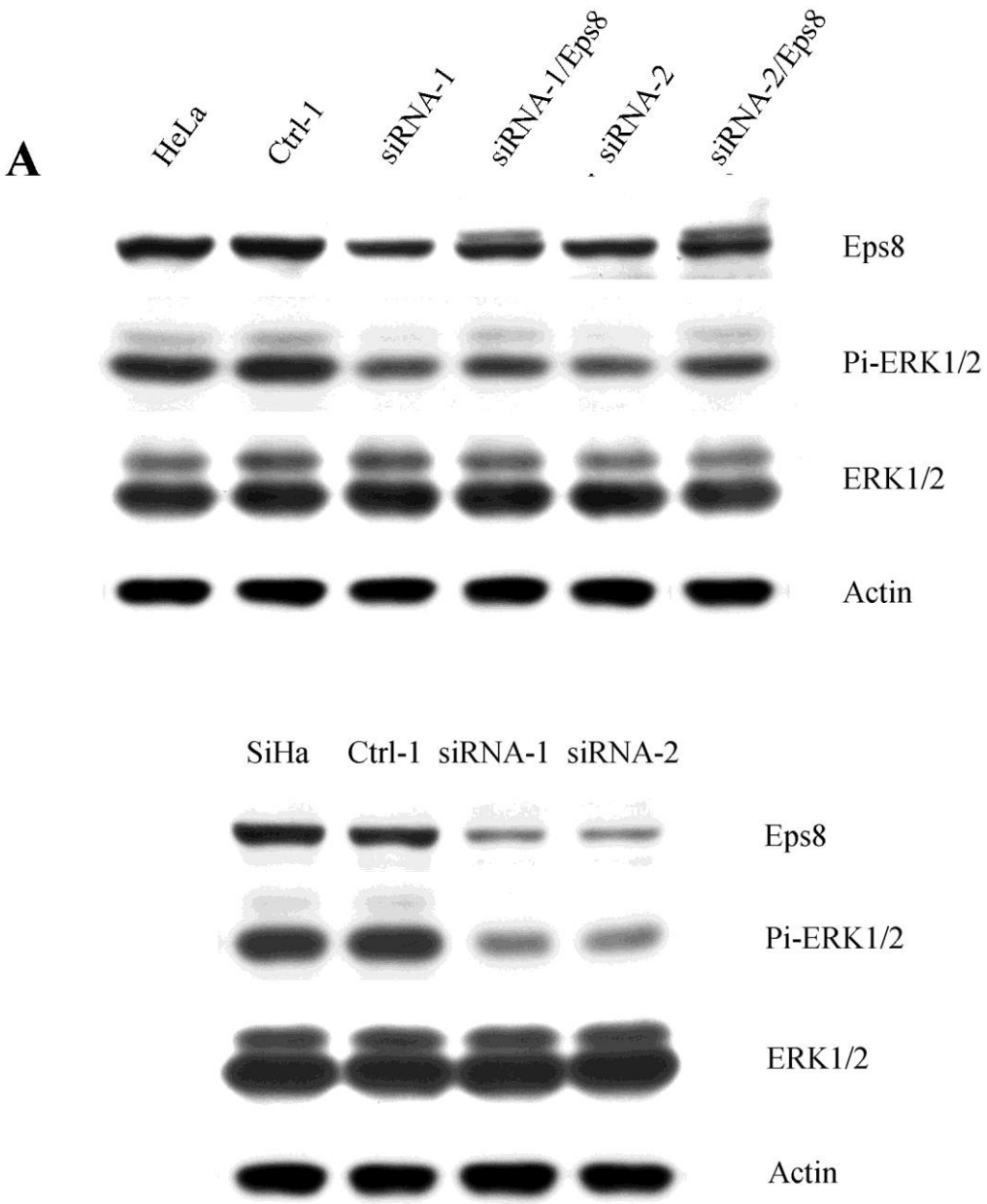

A
HeLa
Ctrl-1
siRNA-1
siRNA-1/Eps8
siRNA-2
siRNA-2/Eps8
Eps8
Pi-ERK1/2
ERK1/2
Actin
SiHa
Ctrl-1
siRNA-1
siRNA-2
Eps8
Pi-ERK1/2
ERK1/2
Actin

Figure 4B,C

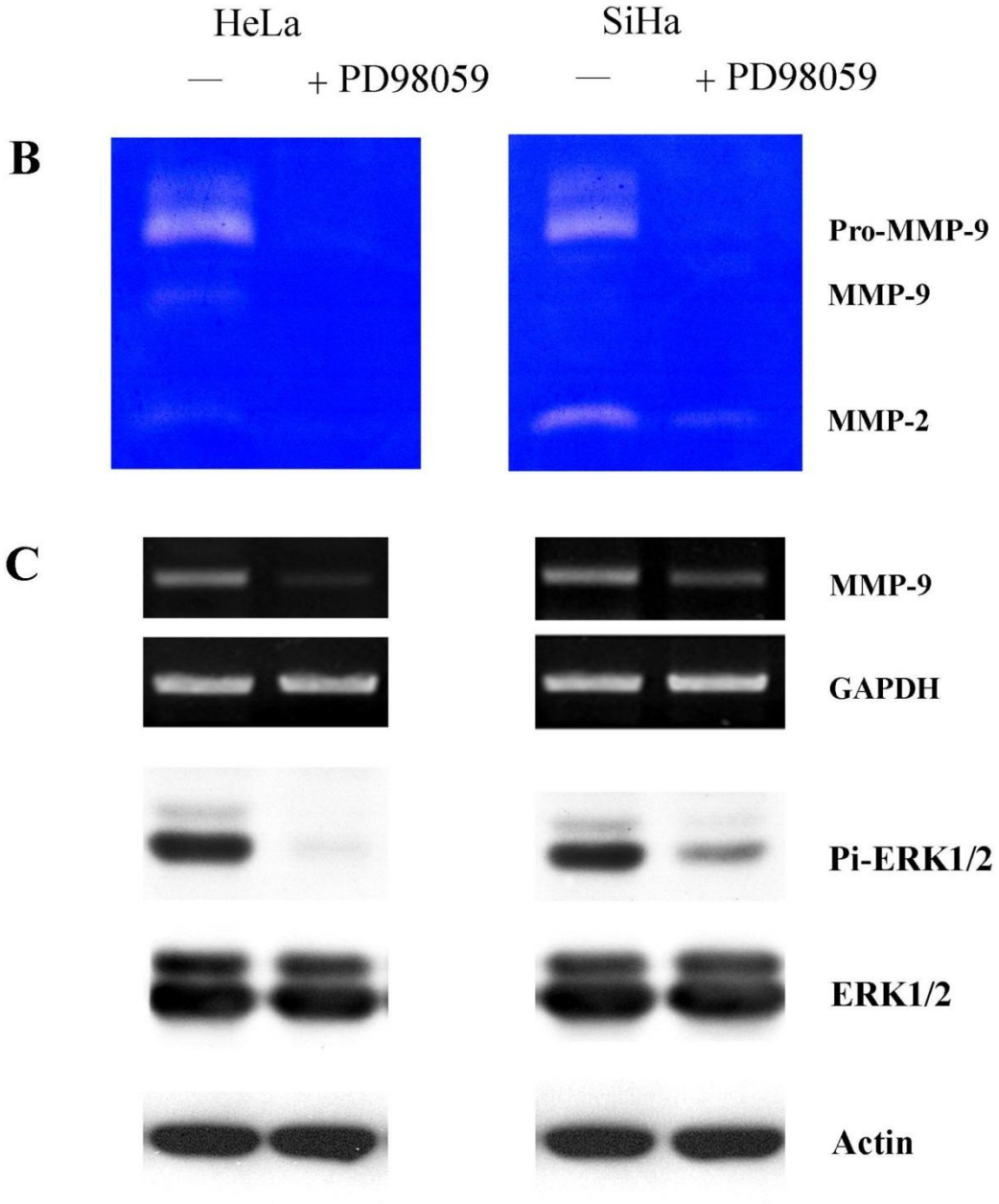

HeLa
SiHa
—
+ PD98059
—
+ PD98059
B
Pro-MMP-9
MMP-9
MMP-2
C
MMP-9
GAPDH
Pi-ERK1/2
ERK1/2
Actin

Figure 4D

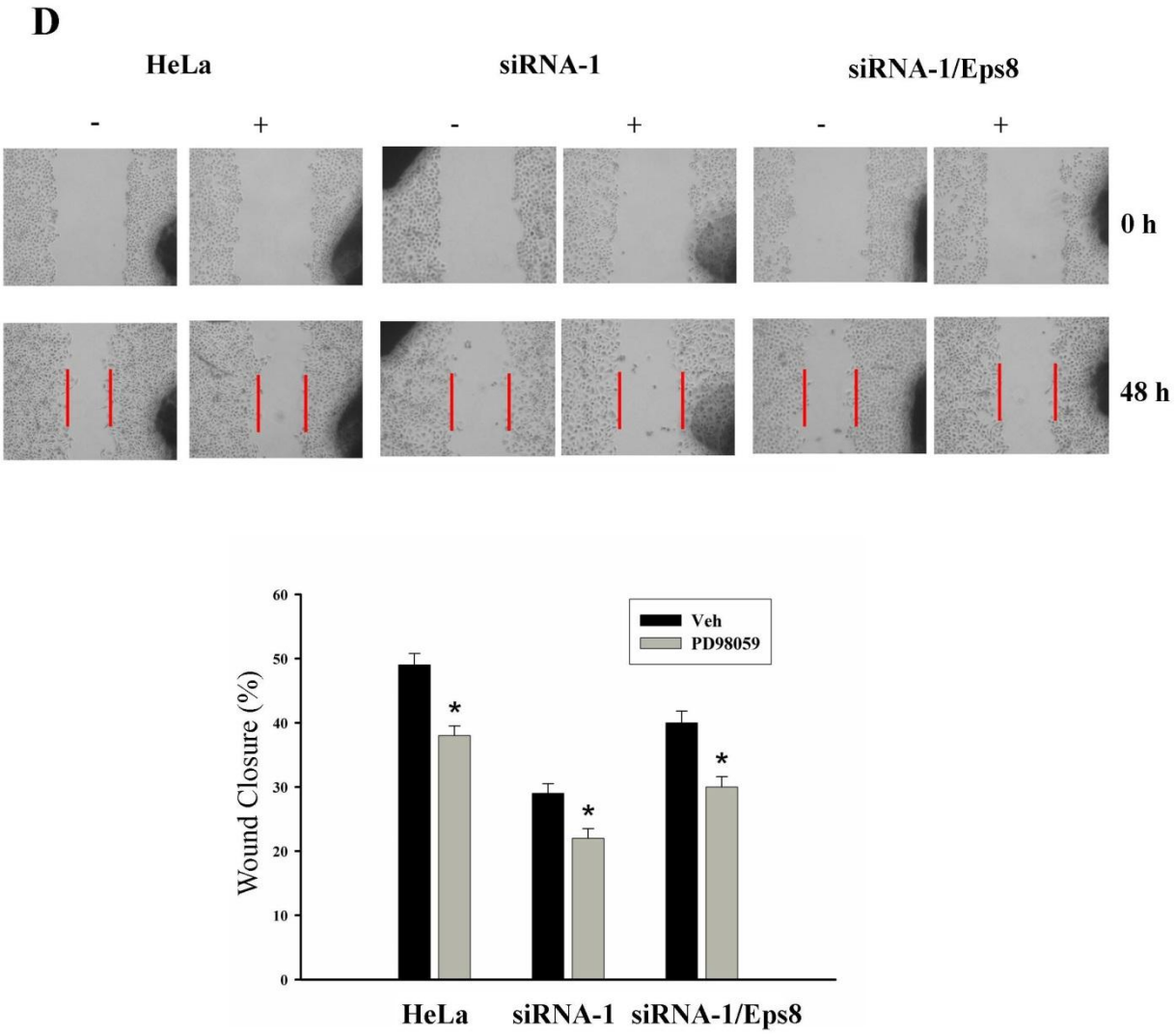

D
HeLa
siRNA-1
siRNA-1/Eps8
-
+
-
+
-
+
0 h
48 h
Wound Closure (%)
60
50
40
30
20
10
0
Veh
PD98059
*
*
*
HeLa
siRNA-1
siRNA-1/Eps8

Figure 5A

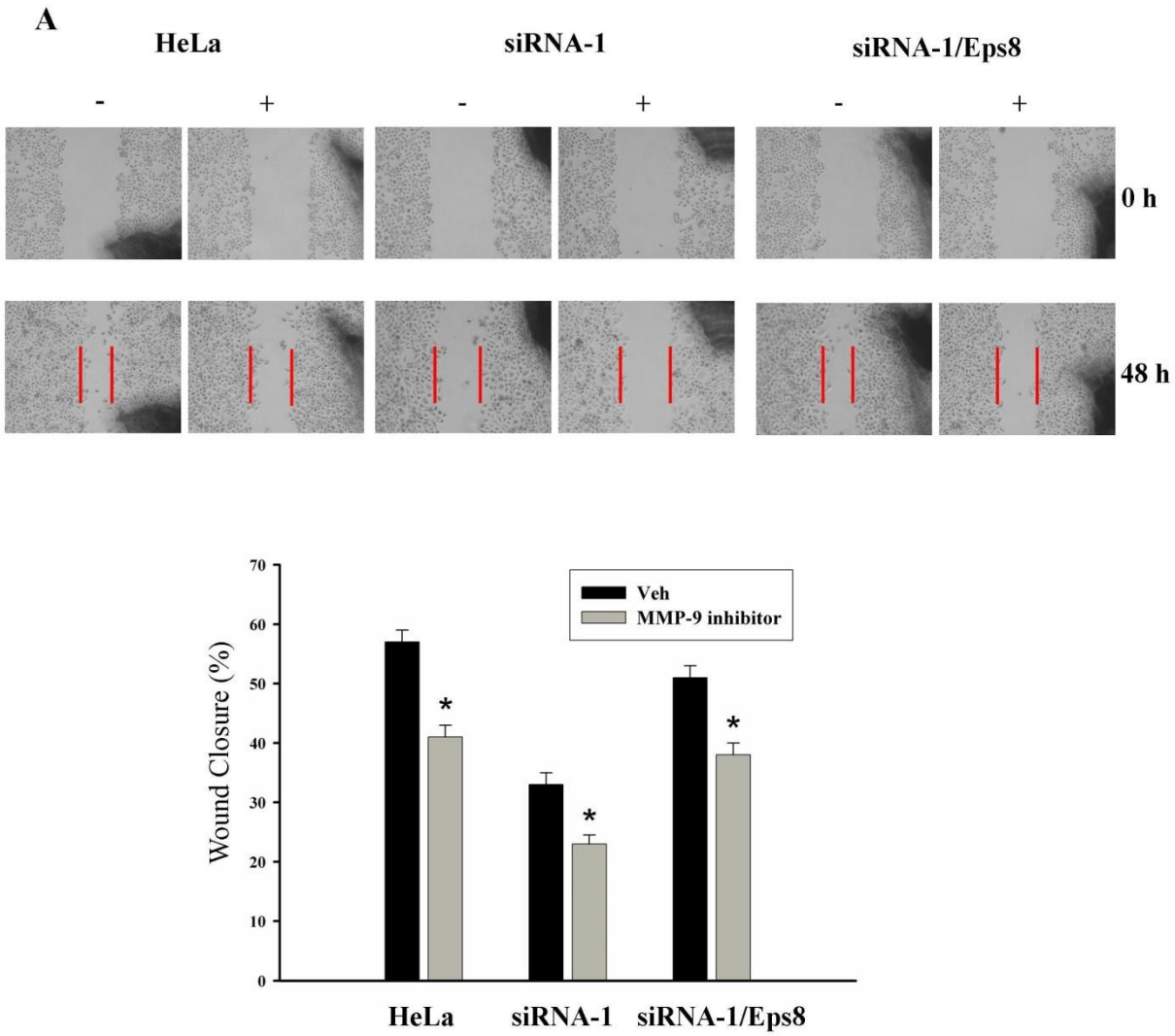

A
HeLa
siRNA-1
siRNA-1/Eps8
-
+
-
+
-
+
0 h
48 h
Wound Closure (%)
70
60
50
40
30
20
10
0
Veh
MMP-9 inhibitor
*
*
*
HeLa
siRNA-1
siRNA-1/Eps8

Figure 5B

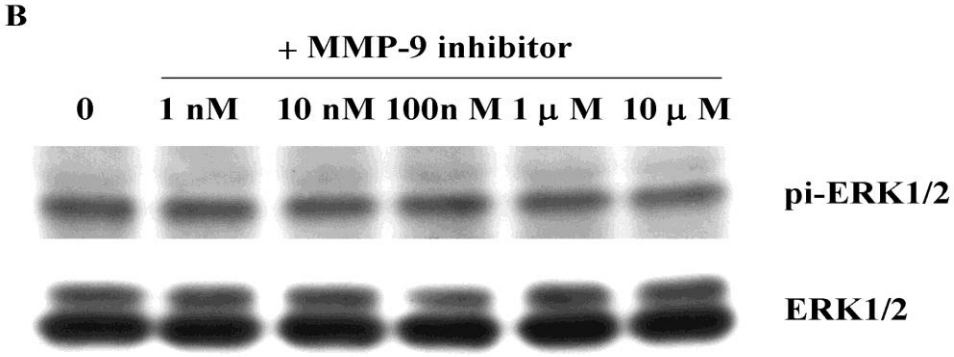
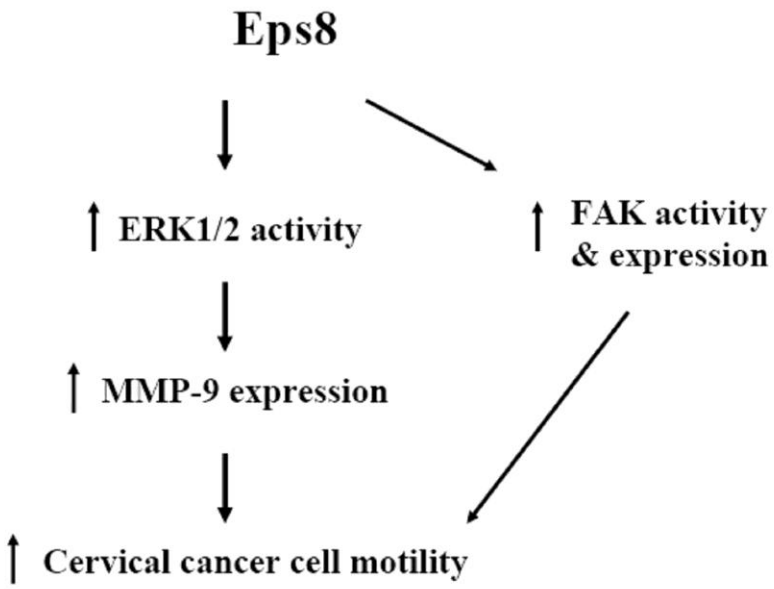


Figure 6


Eps8
ERK1/2 activity
FAK activity
& expression
MMP-9 expression
Cervical cancer cell motility