Abstract. Epidermal growth factor receptor pathway substrate 8 (Eps8) has been identified as a novel substrate for epidermal growth factor receptor (EGFR) kinase and is involved in EGFR-mediated signaling pathways correlated with tumorigenesis, proliferation and metastasis in various cancer types. However, the precise role of Eps8 in cervical cancer metastasis remains to be elucidated. Immunohistochemistry revealed that Eps8 was significantly increased in cervical cancer specimens compared with squamous intraepithelial lesion and normal cervical tissues. Additionally, it was revealed that Eps8 expression not only correlated with cervical cancer progression, but also exhibited a close correlation with the epithelial-mesenchymal transition (EMT) markers, E-cadherin and vimentin. Furthermore, the present study focused predominantly on the EMT-associated role of Eps8 in the EMT, migration and invasion of cervical cancer cells. Eps8-short hairpin (sh) RNA was transfected into HeLa and SiHa cells to deplete its expression, and reverse transcription-quantitative polymerase chain reaction and western blot analyses were performed to confirm Eps8-knockdown and to investigate the influence of Eps8 on EMT markers. The present findings have revealed that Eps8 silencing led to the upregulation of the epithelial marker E-cadherin, while expression of the mesenchymal marker vimentin and the transcription factor snail was decreased at both mRNA and protein expression levels. Transwell cell migration and Matrigel invasion assays showed that down-regulation of Eps8 significantly inhibited cell migration and invasion of HeLa and SiHa cells. Taken together, these results suggested that Eps8 promotes cervical cancer metastasis by orchestrating the EMT.

Introduction

Despite the great progress that has been made in early screening of cervical cancer programs, cervical cancer remains the leading cause of gynecological cancer-associated mortalities worldwide (1). Persistent high-risk human papilloma virus infection is suggested to be the prerequisite for cervical cancer (2); however, the virus alone is insufficient for the development of cervical cancer, suggesting that other molecular events are involved in the initiation and progression of cervical cancer (3). The expression of molecules associated with tumor invasion and metastasis indicate an unfavorable prognosis (4). The present understanding of the molecular mechanisms contributing to the invasion-metastasis of cervical cancer remains limited, although accumulating evidence suggests that the epithelial-mesenchymal transition (EMT) serves a pivotal role in the initial step of tumor invasion-metastasis (5). Consequently, the EMT has become the focus of cervical cancer metastasis research.

The EMT is a process of switching the epithelial cell phenotype to a more loosely fibroblast-like phenotype (6). This is coupled with the loss of cell polarity and intercellular junctions, remodeling of the cytoskeleton and alteration of various EMT-related markers, including downregulation of epithelial markers, including E-cadherin, and upregulation of the mesenchymal markers N-cadherin and vimentin (6). These changes provide cancer cells with elevated invasive/metastatic capacities and therapeutic resistance (7).

Epidermal growth factor receptor pathway substrate 8 (Eps8) was initially identified as a novel substrate for epidermal growth factor receptor (EGFR) kinase (8). Overexpression of Eps8 facilitates enhanced mitogenesis and malignant transduction via EGFR-mediated activation of downstream signaling pathways (9). Previous studies have revealed that Eps8 is highly expressed in various human tumor types, including colorectal, pituitary, oral, esophageal, pancreatic, ovarian, lung, breast, thyroid and cervical cancer (10-17). Eps8 overexpression has also been shown to be involved in numerous signaling pathways associated with...
tumorigenesis, metastasis and proliferation of cancer, and represents an unfavorable prognostic biomarker in patients with cancer (10,12-14,17).

Epithelial growth factor (EGF) is suggested to be one of the most potent EMT regulatory factors in cervical cancer. Overexpression of EGF in cervical cancer indicates poor clinical prognosis and EGF-mediated EMT may account for this (18-20). More recent studies have shown that chemoresistance is associated with the EMT in cancer cells, and the acquired chemotherapeutic resistance is often accompanied by the transformation from the epithelial to the mesenchymal phenotype in cancer cells (21-23). Chen et al (17) found that Eps8 knockdown contributes to increased sensitivity to chemotherapy. Based on these findings, it was hypothesized that Eps8 is associated with the process of EMT in cervical cancer.

In the present study, immunohistochemistry was used to detect the expression of Eps8 in different cervical samples. Correlations between Eps8 expression and the clinicopathological characteristics, as well as EMT associated proteins in cervical cancer tissues were further analyzed. RNA interference (RNAi) was applied to inhibit the expression of Eps8 in cervical cancer cells, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were performed to investigate the influence of Eps8 knockdown on EMT markers at the mRNA and protein expression levels. Transwell cell migration and Matrigel invasion assays were used to investigate the effects of Eps8-knockdown on the capacity of invasive and migration in HeLa and SiHa cells.

Materials and methods

Cell culture and clinical specimens. Human HeLa and SiHa cervical cancer cell lines were purchased from the Cell Bank of China (Shanghai, China) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

A total of 134 paraffin-embedded cervical specimens, including 16 normal cervical epithelia (NC), 25 low-grade squamous intraepithelial lesion (LSIL), 30 high-grade squamous intraepithelial lesion (HSIL) and 63 squamous cervical cancer (SCC) specimens, were obtained from the Pathology Department at the International Peace Maternity and Child Health Hospital of the China Welfare Institute. The main clinicopathological characteristics of the patients with cervical cancer are summarized in Table I.

Immunohistochemical analysis. Immunohistochemical staining was performed on 4 μm thick paraffin-embedded tissue sample sections. Briefly, the sections were dewaxed by xylene twice for 15 min and rehydrated through a graded alcohol series (100, 90 and 70%) at room temperature. Antigen retrieval was performed using citrate buffer (pH 6.0) at 95°C for 15 min and endogenous peroxidase activity was quenched by immersion in 3% H₂O₂ for 10 min at room temperature. Subsequently, tissue sections were blocked by incubation in 1% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Beijing, China) for 30 min at room temperature to prevent non-specific binding. The tissue sections were then incubated at 4°C overnight with anti-Eps8 (1:200; ab203272; Abcam, Cambridge, UK), anti-E-cadherin (1:300; 3195S; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-vimentin (1:200; 5741S; Cell Signaling Technology, Inc.) primary rabbit monoclonal antibodies. Following incubation with the primary antibodies, the tissue sections were incubated at 37°C for 30 min with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:500; GTX26721; GeneFex Inc., Irvine, CA, USA). The 3,3′-diaminobenzidine substrate was applied to visualize the immunoreactivity prior to counterstaining the slides with 10% hematoxylin, dehydrating in graded ethanol and cleared in xylene. For negative controls, phosphate-buffered saline was substituted for the primary antibodies. The expression levels were evaluated by two pathologists in a blinded manner. The protein expression levels of Eps8, E-cadherin and vimentin were scored semi-quantitatively by multiplying the 'percent positivity score' by the 'staining intensity score'. The percent positivity was scored according to the following criteria: 0, <10%; 1, 10-24%; 2, 25-50%; 3, >50% positive tumor cells. Staining intensity was graded as 0, no staining; 1, weak staining; 2, moderate staining; or 3, strong staining. The final score was defined as follows: 0, negative; 1-4, low expression; 5-12, high expression.

Transient transfection. The short hairpin (sh)RNA sequence targeting Eps8 (Eps8-shRNA: 5'-GCTGTGAGCCTGTATTGATTGTTA-3') and the negative control shRNA were synthesized and cloned into the pGPU6/6 green fluorescent protein (GFP)/Neo vector (GenePharma, Shanghai, China). The HeLa (2x10⁵) or SiHa (4x10⁵) cells were grown in 6-well plates at 70-80% confluence prior to transient transfection with a mixture of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and Eps8 shRNA or control shRNA, according to the manufacturer's instructions. Following incubation for 6 h, the transfection medium was replaced with regular medium and after 48 h, GFP expression was evaluated by fluorescent microscopy (Zeiss Axioskop 40; Zeiss, Oberkochen, Germany), for the qualitative assessment of the transfection efficiency. The cells were harvested at 48 h post-transfection for RNA analyses and at 72 h post-transfection for protein analyses to confirm the downregulation of Eps8 expression.

RNA extraction and RT-qPCR. The total RNA from HeLa and SiHa cells was extracted using TRIzol reagent (Invitrogen;
Table I. Correlation between the clinicopathological characteristics, and the expression levels of Eps8, E-cadherin and vimentin in patients with cervical cancer.

| Characteristic                  | Eps8 expression | E-cadherin expression | Vimentin expression |
|---------------------------------|------------------|-----------------------|--------------------|
|                                 | No. patients     | Negative Low High     | P-value            |
|                                 |                  |                       |                    |
| Age                             |                  |                       |                    |
| ≤45 years                       | 26               | 2 6 18                | 0.503              |
| >45 years                       | 37               | 8 5 24                | 0.816              |
| FIGO stage                      |                  |                       |                    |
| I                               | 41               | 8 9 24                | 0.072              |
| II                              | 22               | 2 2 18                | 0.491              |
| Histological grade              |                  |                       |                    |
| Well                            | 12               | 6 3 3                 | 0.001              |
| Moderate                        | 41               | 3 7 31                | 0.104              |
| Poor                            | 10               | 1 1 8                 |                    |
| Invasive interstitial depth     |                  |                       | <0.001             |
| <1/2                            | 16               | 8 4 4                 |                    |
| ≥1/2                            | 47               | 1 1 8                 |                    |
| Lymph node metastases           |                  |                       | 0.009              |
| Negative                        | 46               | 9 11 26               |                    |
| Positive                        | 17               | 1 0 16                |                    |

FIGO, International Federation of Gynecology and Obstetrics.
Western blot analysis. HeLa and SiHa cells were harvested and washed twice with ice-cold PBS. The cells were subsequently lysed on ice for 30 min with radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Beijing, China), containing 1 mM phenylmethylsulfonyl fluoride. The lysates were subsequently centrifuged at 12,000 x g at 4°C for 15 min. The protein concentrations were measured using bicinchoninic acid protein assays (Beyotime Biotechnology). Equivalent quantities (60 µg) of total protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel stained for 15 min with 0.1% crystal violet. A total of 10 reactions were fixed for 20 min in 4% paraformaldehyde, and the chamber surface were wiped off using a cotton swab and for 24 h, the non-migratory to the upper chambers, while 500 µl DMEM with 20% FBS serum-free DMEM medium with 0.1% BSA and were added after transfection, 2x10^4 cells were resuspended on ice in 200 µl DMEM medium with 0.1% BSA and were added to the upper chambers, while 500 µl DMEM with 20% FBS was placed in the lower chambers. Following incubation for 24 h, the non-migratory/non-invasive cells on the upper chamber surface were wiped off using a cotton swab and the cells that had migrated/invasive to the lower membrane surface were fixed for 20 min in 4% paraformaldehyde, and stained for 15 min with 0.1% crystal violet. A total of 10 random visual fields were selected under an inverted microscope (magnification, x200) to capture images and count the number of migrated/invasive cells. Each assay was performed in triplicate.

Statistical analysis. Each experiment was performed in triplicate. SPSS 17.0 software was used to perform statistical analysis. The data are presented as the mean ± standard deviation. Kruskal-Wallis one-way analysis of variance or Student's unpaired t-test was applied to evaluate significant differences. Correlations were assessed using the Pearson's correlation coefficient test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of Eps8, E-cadherin and vimentin in the normal cervix, LSIL, HSIL and SCC. To investigate the expression of Eps8 and its association with EMT-related proteins, immunohistochemistry was performed using Eps8, E-cadherin and vimentin antibodies in NC, LSIL, HSIL and SCC (Fig. 1A). Eps8 and vimentin were predominantly localized in the cytoplasm of cervical cancer tissues, and were absent from the 16 normal cervix samples. Weak expression of Eps8 and vimentin was observed in 12/25 (48.0%) LSIL specimens, and 13/30 (43.3%) and 15/30 (50.0%) of the 30 HSIL specimens, respectively. Of the 63 SCC cases, the staining intensity of Eps8 was weak and strong in 11 (17.5%) and 42 (66.7%) specimens, respectively. In addition, vimentin was expressed weakly in 16 cases (25.4%) and strongly in 41 cases (65.1%). E-cadherin was expressed predominantly in the plasma membrane of epithelial cells in the NC. Based on the expression scores, the percentage of high E-cadherin-expressing samples decreased gradually.

Table II. Quantitative polymerase chain reaction primer sequences.

| Gene     | Sequence (5’-3’)          |
|----------|---------------------------|
| Eps8     | TGAATGGCTACGGATCATCACC    |
| Forward  | CACTGTCCCGTGCTTAATTCT     |
| Reverse  | GACGCCATCAACACCGAGTT      |
| E-cadherin| CGAGAGCTACAGGTTCACCGG    |
| Forward  | GGGTTGTCAGGGGAAAAATAGG    |
| Reverse  | CTTTGTCTGTTGTTAGCTGGT     |
| Vimentin | GACGCCATCAACACCGAGTT      |
| Snail    | TCGGAAGCTAACTACAGCGA      |
| Forward  | AGATGAGCATTGCGACGG       |
| Reverse  | GGGTGTCAGGGGAAAAATAGG     |
| GAPDH    | GGAGCCGATCCCTCCTCAAAT     |
| Forward  | GGCTTGTGGTCATATTCTCATGG   |

Eps8, epidermal growth factor receptor kinase substrate 8; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
from 87.5% (14/16) in NC, >72.0% (18/25) in LSIL and >60.0% (18/30) in HSIL to 30.2% (19/63) in SCC (Fig. 1B).

**Association of the expression levels of Eps8, E-cadherin, vimentin with the clinicopathological characteristics of patients with SCC.** As shown in Table II, Eps8 expression was significantly associated with histological grade (P=0.001), invasive interstitial depth (P<0.001) and lymph node metastases (P=0.009). However, little association was demonstrated between the expression of Eps8 and age (P=0.503), and FIGO stage (P=0.072). The expression levels of E-cadherin and vimentin demonstrated a marked correlation with invasive interstitial depth (P=0.015 and P=0.005, respectively) and lymph node metastases (P=0.006 and P=0.015, respectively).

**Expression of Eps8 correlates with EMT-related proteins in cervical cancer samples.** The present study further explored correlations between the expression of Eps8 and EMT marker proteins in 63 samples of cervical SCC tissues. In accordance with the changes in the expression of proteins during the EMT process, the reduced expression of E-cadherin was significantly associated with the increased expression of vimentin (P=0.001; r=−0.398). Furthermore, a significant negative correlation was observed between the expression levels of E-cadherin and Eps8 (P=0.004; r=−0.355), and the expression of Eps8 correlated positively with that of vimentin (P<0.001; r=0.562) (Table III). These analyses suggested that Eps8 is associated with the expression of the EMT-related proteins and may be an important indicator of the EMT process in cervical cancer.

**Knockdown of Eps8 led to increased expression of epithelial markers and reduced expression of mesenchymal markers.** To further characterize the role of Eps8 in the process of the EMT in cervical cancer, Eps8-targeting shRNA and control shRNA plasmids were designed and transiently transfected into HeLa and SiHa cells. To confirm Eps8-knockdown and to investigate the influence of Eps8 on EMT markers, RT-qPCR and western blot analyses were performed in Eps8-inhibited cell lines and control cells. As shown in Fig. 2, Eps8 expression in Eps8-inhibited cell lines was significantly downregulated at both the mRNA and protein levels.
expression levels compared with the control cells. RT-qPCR analysis demonstrated that knockdown of Eps8 led to increased expression of the epithelial marker, E-cadherin, and decreased expression of the mesenchymal marker, vimentin, as well as a key EMT inducer, snail, at the mRNA level (Fig. 2A). Additionally, western blot analysis revealed that the inhibition of Eps8 resulted in downregulated protein expression of vimentin and snail (Fig. 2B). Taken together, these results revealed that Eps8 is involved in EMT progression and that inhibition of Eps8 impairs the process of the EMT in cervical cancer.

**Eps8 silencing represses cell migration and invasion.** The present study subsequently performed Transwell cell migration and Matrigel invasion assays to investigate the effects of Eps8 knockdown on the regulation of cell migration and invasion in HeLa and SiHa cells. As shown in Fig. 3, the Transwell migration assay demonstrated that Eps8 knockdown notably inhibited the migration capacity of the Eps8 shRNA transfected cells compared with that of the control cells (P<0.01; Fig. 3). Matrigel invasion assays also demonstrated that transfection with Eps8 shRNA plasmids effectively impaired the invasive capacity of HeLa and SiHa cells (P<0.01; Fig. 3). These results suggested that Eps8 knockdown decreases the migration and invasiveness of HeLa and SiHa cell.

**Discussion**

The progression of malignant tumors is a sophisticated process involving multiple factors and molecular events. The EMT, which is believed to be a crucial step in tumor metastasis (25), allows cancer cells to invade adjacent tissues and metastasize to distance sites. It is important to understand the molecular mechanisms underlying the EMT pathway and identification of ways to prevent or reverse the process are critical to the development of novel therapeutic strategies against cancer. Several signaling pathways, including the Wnt, transforming growth factor-β, EGF, Notch and fibroblast growth factor pathways, have been shown to be involved in regulating the process of the EMT by modulating the expression of certain key transcription factors, including snail, ZEB and twist, which suppress the expression of E-cadherin and activate the mesenchymal transcriptomes (26,27).
Eps8 is a novel phosphorylation substrate for EGFR, which enhances the cellular transformation of EGFR-dependent mitogenic signals upon overexpression (28). As an actin-capping protein, the role of Eps8 in cancer metastasis has aroused widespread concern, resulting in a number of studies to elucidate the mechanisms by which Eps8 influences tumor migration. Eps8 overexpression is responsible for enhanced Rac-induced actin cytoskeleton remodeling and cell migration by formation of Eps8-Abi-1-Sos-1 and Eps8-IRSp53 complexes, which are involved in transducing signals to activate the Rho-GTPase family members Rac and Cdc42 (29‑31). In addition to the role of Eps8 in tumor metastasis mediated via Rac‑dependent pathways, Eps8 is also involved in tumor migration mediated via Rac-independent pathways, including the the Eps8/AKT/FOXM1/matrix metalloproteinase-9 cascade (32). Although considerable efforts have been made to clarify the functions of Eps8 in the metastasis of numerous cancer types, the role of Eps8 in the development of cervical cancer remains to be elucidated. Chen et al (17) analyzed the expression levels of Eps8 in 110 cervical cancer samples by immunohistochemistry and observed that increased Eps8 expression was associated with shorter disease-free survival and overall survival (17). These observations suggested that Eps8 serves a role in the progression of cervical cancer. Therefore, the present study hypothesized that Eps8 is also involved in cervical cancer metastasis. The present study focused predominantly on the EMT-related role of Eps8 in this process.

The present data demonstrated that Eps8 expression in cervical cancer samples was significantly higher compared with that in NC, LSIL and HSIL samples; thus, indicating the involvement of Eps8 in the development of cervical cancer. The analysis with regard to its clinical significance revealed that high Eps8 expression correlated with histological grade and lymph node metastases, suggesting that Eps8 serves a pivotal role in cervical cancer progression, and may be an independent biomarker for an unfavorable clinical prognosis. This is in agreement with previous observations that Eps8 overexpression is associated with poor prognosis in patients with oral squamous cell carcinoma, mixed lineage leukaemia and ovarian cancer (33‑35). It is generally accepted that decreased expression of E-cadherin, coupled with increased expression of vimentin, is characteristic of EMT (36). Therefore, the present study analyzed the possible association between the expression levels of Eps8 and EMT-associated proteins (E-cadherin and vimentin) in cervical carcinoma tissues. Correlation analysis revealed that high Eps8 expression was negatively correlated with the expression of E-cadherin, coupled with increased expression of vimentin, implying the involvement of Eps8 in the EMT phenotype. Taken together, it was hypothesized that Eps8 makes a vital contribution to the progression of cervical cancer, partly through its influence on the EMT.

Given the results of the immunohistochemical analysis, the present study further investigated the role of Eps8 in the process of the EMT using shRNA-mediated knockdown of Eps8 in HeLa and SiHa cells. Eps8 knockdown appeared
to cause a reversal of the EMT process in HeLa and SiHa cells, with changes observed in the expression of certain EMT markers (Fig. 2), including the E-cadherin upregulation and vimentin downregulation at both the mRNA and protein expression levels. E-cadherin is a major cell adhesion molecule involved in epithelial cell adhesion. Its loss or decreased expression has been suggested to be a key event in the EMT process. Reduced expression of E-cadherin serves an pivotal role in cancer progression and is associated with metastasis (37-42). E-cadherin is considered to be a prognostic biomarker in certain solid malignant tumor types (43,44). In addition, vimentin, as a canonical marker of the EMT, is widely expressed in mesenchymal cells. It has been previously reported that vimentin is overexpressed in various epithelial-derived tumor cells and tissues (45), and its overexpression is associated with enhanced tumor cell invasiveness (46). The present finding that Eps8 knockdown resulted in E-cadherin upregulation and vimentin downregulation suggested that Eps8 is involved in the EMT in HeLa and SiHa cells.

The present study also revealed that knockdown of Eps8 led to the downregulation of the transcription factor snail, which is hypothesized to be a key regulator of the EMT (47). The expression of snail has been implicated in suppressing the expression of E-cadherin and is involved in the initial migratory phenotype (48). Snail is expressed at the invasive front of tumors and is associated with tumor recurrence and lymph node metastasis, suggesting a role for snail in tumor progression (49). Other previous studies have indicated that Eps8 activates AKT by stimulating phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) (16), and that the PI3K/AKT-snail pathway is involved in the EMT in breast and lung cancer cells (50,51). Furthermore, it was speculated that downregulation of snail by Eps8 silencing represents a plausible mechanism by which Eps8 is involved in the process of the EMT in cervical cancer cells. Furthermore, the present observation that Eps8 depletion in cell invasion and migration assays significantly impaired the invasive and migratory capacities of cervical cancer cells indicated that Eps8 serves a vital role in tumor progression.

In conclusion, the results of the present study demonstrated that the expression of Eps8 was significantly associated with the expression of EMT-related proteins in cervical cancer samples. Furthermore, these results indicated that Eps8 overexpression was correlated with lymphatic and distant metastasis of cervical cancer. Additionally, decreased expression of Eps8 impaired the EMT process and suppressed the migration and invasion of HeLa and SiHa cells. These results provided an insight into the important function of Eps8 in cervical cancer progression by orchestrating the EMT, and indicated that Eps8 is a novel potential target for developing therapeutic strategies for cervical cancer. However, the specific regulatory role of Eps8 in the EMT remains unknown and further in vitro and in vivo investigations are required to elucidate the detailed mechanism by which Eps8 regulates the EMT.

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