

## Original Article

# Effects of CXCR4 gene silencing by lentivirus shRNA on metastatic characteristics of EC9706 human esophageal carcinoma cell line

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**Abstract:** *Objective* CXCL12/CXCR4 has been studied as an important biomarker in numerous human malignancies, but studies are limited in esophageal squamous cell carcinoma (ESCC). CXCL12/CXCR4 may be involved in the development of ESCC. In the present study, we evaluated the effects CXCL12/CXCR4 on the metastatic potential of tumor cells *in vitro*. *Methods* An effective RNAi sequence targeting the CXCR4 gene was selected, and a lentivirus shRNA vector was constructed to specifically silence CXCR4 expression in the ESCC cell line EC9706. Then, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blot were used to evaluate its effects on the metastatic potential of tumor cells *in vitro*. *Results* CXCR4 expression in EC9706 was significantly decreased after transfection with lentivirus shRNA vector. Downregulation of CXCR4 was accompanied with reduction of MMP-9. In addition, after silencing CXCR4 expression, the metastatic potential of EC9706 cells was inhibited considerably. *Conclusion* The downregulation of CXCR4 expression upon transfection with a lentivirus shRNA vector could inhibit the metastatic potential of ESCC cells. Our results may provide useful information regarding gene therapy for ESCC patients.

**Keywords:** CXCL12, CXCR4, esophageal squamous cell carcinoma, RNA interference, metastasis

## Introduction

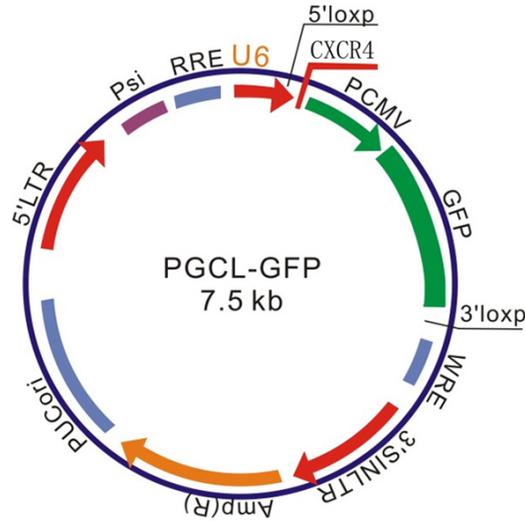
Esophageal squamous cell carcinoma (ESCC) is a common malignant gastro-enteric tumor. ESCC is prevalent in Eastern Asia, and patient survival is poor [1]. ESCC cells can invade the submucosa, muscular layers, and peripheral tissues of the esophagus, making radical resection difficult. Recently, new oncogenes and tumor suppressor genes associated with ESCC have been investigated and studied. It may be helpful for clinicians to better understand the unique biologic behavior of ESCC and to optimize individual therapeutic strategies [2-5].

Chemokines are chemotactic cytokines induced by inflammatory cytokines, growth factors and pathogenic stimuli. Chemokines directly cause the migration of leukocytes [6, 7]. Among them, C-X-C chemokine ligand 12 (CXCL12) and its receptor chemokine receptor

4 (CXCR4) play key roles in numerous immunological reactions. In recent studies, CXCL12/CXCR4 is expressed not only in immunocytes but also in many tumor cells [8-11]. The signaling pathway involving CXCL12 and CXCR4 may be closely correlated with the occurrence and progression of esophageal tumors, and CXCR4 expression is associated with poor clinical outcome in ESCC patients [12].

Recently, it has been suggested that the CXCL12/CXCR4 signaling pathway plays an important role in carcinogenesis and progression [13]. Inhibition of this pathway may improve prognosis. RNA interference (RNAi) has been widely used as an effective tool for gene research. RNAi could reduce the expression of target proteins through inhibition of their gene expression at the mRNA level. In this study, we used a lentivirus-based vector to transfect a CXCR4-specific short hairpin RNA (shRNA) into

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**Figure 1.** Lentiviral vector, pGCL-GFP vector encoding sustained RNA expression.

ESCC cells to stably inhibit CXCR4 expression. In addition, we investigated the biological effects of CXCR4 inhibition on ESCC.

### Methods

#### Cell culture

The human ESCC cell line EC9706 was obtained from the laboratory of Sun Yat-sen University Cancer Center (Guangzhou, China) and maintained in DMEM medium (Gibco Biocult, Paisley, UK) supplemented with 10% calf bovine serum (Sijiqing Biotechnology, Hangzhou, China), 100 µg/mL streptomycin and 100 U/mL penicillin (North China Pharmaceutical Group Corporation, Shijiazhuang, China) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. When the EC9706 cells grew to 75% confluence, the culture medium was removed and replaced with free-serum medium for 24 hours. In addition, 293T cells were obtained from Shanghai GeneChem Co. Ltd. and maintained in DMEM medium (Gibco Biocult, Paisley, UK) supplemented with 10% calf bovine serum (Sijiqing Biotechnology, Hangzhou, China).

#### Construction of plasmid targeting the CXCR4 mRNA

Based on principles of shRNA design and the human CXCR4 structure (NM\_001008540.1), the preparation, synthesis and a preliminary experiment for screening for a positive oligonu-

cleotide fragment targeting CXCR4 mRNA were conducted. Briefly, three plasmid vectors encoding shRNA directed against CXCR4 mRNA were constructed. Of these vectors, one resulted in greater than 75% CXCR4 inhibition. The shRNA targeting sequence was 5'-TTCAAGAGA-3'. The 5'-TTCTCCGAACGTGTCACGT-3' scrambled sequence was used as a negative control that does not target any known human mRNA. shRNA was synthesized and cloned into the pGCL-GFP vector via double-enzyme digestion with Age I (New England Biolabs, Inc., USA) and EcoR I (New England Biolabs, Inc., USA) followed by T4 ligation. The vector was then transformed into DH5α competent cells (TaKaRa Biotechnology Co., Ltd., Japan) for AMP-resistant plasmid screening. Positive plasmids were selected for clonal expansion.

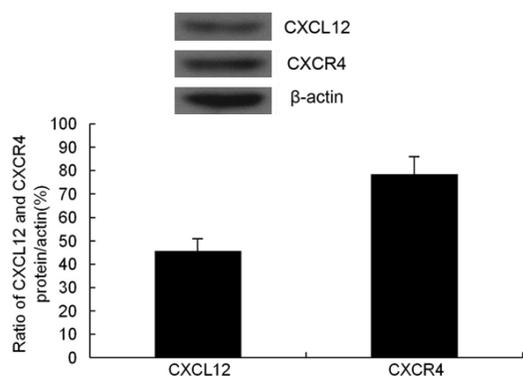
#### Transfection and assessment of the transfection rate

Three groups were established for the cell transfection: the control group without the transfection, the NC-GFP-RNAi-LV group with negative sequence transfection, and the CXCR4-RNAi-LV group with lentivirus target sequence interference. The CXCR4-specific shRNA-encoding pGC-LV vector (**Figure 1**) (Genechem, Shanghai, China) was cotransfected into the 293T cell line using lipofectamine 2000 (Invitrogen, Carlsbad, CA) to produce lentivirus stocks, and a negative construct served as a negative control. Forty-eight hours after transfection, 293T cells were harvested, and the cell debris was removed by centrifugation at 4°C. The crude viral extract was filtered, and centrifuge enrichment was conducted. The virus concentration was determined using a single/uniporous tracer dilution. A total of 2 µL of human CXCR4-RNAi-LV with a titer of 2E+09T U/ml was added to the EC9706 cell suspension and cultured for 72 hour. The expression of the GFP lentivirus reporter gene was observed by fluorescence microscopy. Five visual fields (200×) were chosen to count the number of the cells presenting with green fluorescence, which were considered to represent the infected proportion of total cells in the field.

#### RT-PCR

RNAs from different groups were extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase (Tiangen Biotech,

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**Figure 2.** CXCL12 and CXCR4 expression in EC9706 cells. CXCL12 and CXCR4 expression was determined by Western blot.  $\beta$ -actin was used as a loading control.

Beijing, China). cDNA was synthesized from 2  $\mu$ g of total RNA according to the manufacturer's instructions in a total volume of 25  $\mu$ L (Fermentas, MD, USA). Negative control reactions were performed without reverse transcriptase. An equal volume of product was subjected to PCR. The primer sequences were specific to an updated version of the GenBank sequences. Primer sequences were used to detect human CXCR4 (NM\_001008540.1) [14], MMP-9 (NM\_000633.2) [15], and  $\beta$ -actin (NM\_001101.3; internal control). Primers for CXCR4 sense (5'-ATGTAGACACTGGCGGAAATGG-3') and antisense (5'-AGGTGGGCGAAA-GGAAAC-3'), MMP-9 sense (5'-CCTGGAGACCTGAGAACCAATCT-3') and antisense (5'-CCACCAGTGTACCATAGC-3'), and  $\beta$ -actin sense (5'-AAGATGACCCAGATCATGTTTGAG-3') and antisense (5'-AGGAGGAGCAATGATCTTGATCTT-3') were used. The amplification conditions were as follows: 40 cycles of 95°C for 5 min, 95°C for 15 s, and 60°C for 1 min for CXCR4 and  $\beta$ -actin; 94°C for 2 min followed by 31 cycles of 94°C for 30 min, 54°C for 20 s, and 63°C for 30 min followed by final extension at 72°C for 5 min for MMP-9. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired using a Cohu High-Performance CCD camera (Cohu Inc. San Diego, CA), and quantification of the bands was performed using Image Tool 3.0 (The University of Texas Health Science Center, San Antonio, TX, USA). Band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be determined and  $\beta$ -actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean

and standard deviation of all experiments performed were calculated after normalization to  $\beta$ -actin.

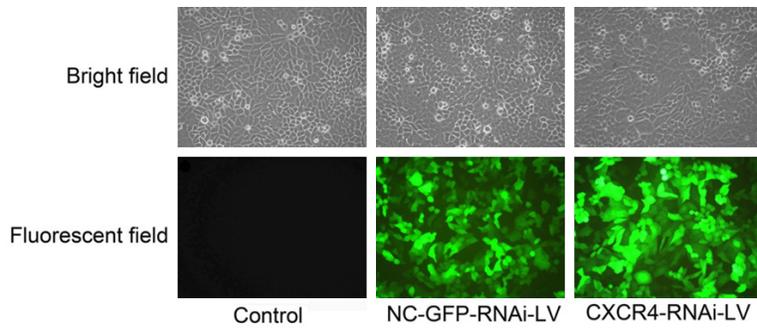
### Western blot analysis

Seventy-two hours after transfection, cells in each group were lysed in 400  $\mu$ L of ice-cold buffer A (1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) by gentle pipetting. The cells were allowed to swell on ice for 25 min. Then, 40  $\mu$ L of a 10% solution of Nonidet P-40 (Amresco, OH, USA) was then added. The tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 45 s in a microcentrifuge. Equal amounts of protein, which was quantified using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA), were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 [TBST; 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween-20] for 2 hours at room temperature and incubated with primary antibody (anti CXCR4, 1:1,000, Abcam, Cambridge, UK; anti CXCL12, 1:1,000, R&D System, USA; anti MMP-9: 1:2,000, Abcam, Cambridge, UK; and  $\beta$ -actin, 1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer at 4°C overnight. Following a wash with TBST, the CXCR4, CXCL12 and MMP-9 membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:4,000, Dako, Glostrup, Denmark) for 3 hours. The  $\beta$ -actin membrane was incubated with a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (1:1,000, Dako, Glostrup, Denmark) for 3 hours. The membranes were washed with TBST, and protein bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (KPL, Gaithersburg, USA).  $\beta$ -actin bands were used as a loading control. The protein quantity was analyzed using UTHSCSA Image Tool 3.0. The formula for determining the relative expression of CXCR4 was as follows: CXCR4 protein relative intensity ratio/loading control.

### Cell invasion assay

Cell invasion was assayed according to the methods described by Yang *et al.* [16]. Seventy-two hours after transfection, cells were har-

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**Figure 3.** Representative graphs of EC9706 cells infected with the indicated lentiviruses are shown (200 $\times$ ).

Matrigel (25 mg/50 mL; BD Biosciences, MA, USA) was applied to the bottom chamber that contained standard medium. Filters were then air-dried for 5 hours in a laminar flow hood. The cells that invaded were fixed with 100% methanol and stained with 5% Giemsa. Cell numbers were counted under a light microscope.

### Wound-healing assay

Different groups cells were grown to 90% confluence in a 6-well plate at 37°C in an incubator with 5% CO<sub>2</sub>. A wound was created by scratching cells with a sterile 200- $\mu$ L pipette tip. Then, cells were incubated with DMEM medium containing 0.5% FBS for 0, 24 and 48 hours. Cells were photographed using a phase-contrast microscope ( $\times$ 100).

### Statistical analysis

All statistical analyses were performed by Statistical Package of Social Sciences 13.0 software. A two-sided probability value of less than 0.05 was considered statistically significant. All of the results are presented as the mean  $\pm$  standard deviation. All data were statistically analyzed using the Student's *t* test.

## Results

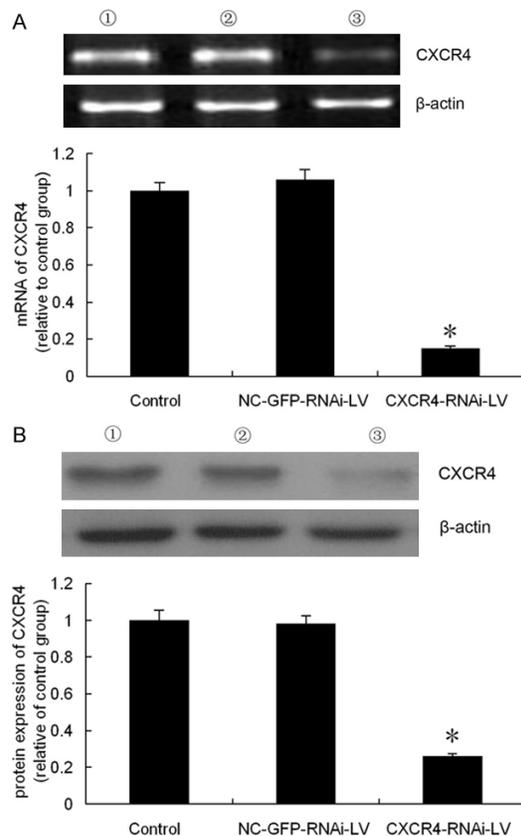
### CXCR4 and CXCL12 expression in EC9706 cells

CXCL12 and CXCR4 expression in EC9706 cells was examined by Western blot (**Figure 2**).

The expression of CXCR4 was higher than the expression of CXCL12 in EC9706 cells.

### Plasmid construction and transfection

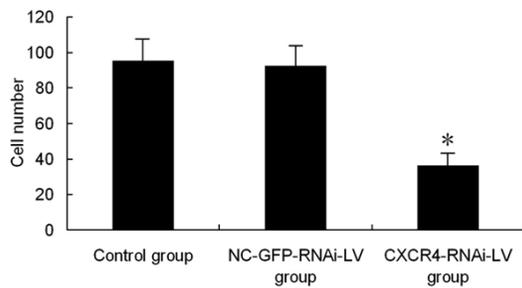
The coded target sequences of the obtained recombinant plasmids were constructed including the CXCR4-shRNA recombinant and negative control plasmids. The transfection outcome was observed using a fluorescence microscope 72 hours after experimental treatment. The transfection rate was (87.3 $\pm$ 1.2)% for the CXCR4-RNAi-LV group and (90.1 $\pm$ 1.4)% for the NC-GFP-RNAi-LV group (**Figure 3**).



**Figure 4.** CXCR4 shRNA inhibits CXCR4 gene expression in EC9706 cells. A. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of CXCR4 RNA levels. B. Western blot analysis of the CXCR4 protein levels in the three groups,  $\beta$ -actin was used as a loading control. ①: Control group; ②: NC-GFP-RNAi-LV group; ③: CXCR4-RNAi-LV group. \*,  $P < 0.01$  as compared with the Control and NC-GFP-RNAi-LV groups.

vested and seeded in a Boyden chamber (Neuro Probe, Cabin John, MD, USA) at 10<sup>4</sup> cells/well in serum-free medium. Cells were incubated for 24 hours at 37°C. Briefly, 10  $\mu$ L

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**Figure 5.** Cell invasion was measured using a Boyden chamber for 16 hours and 24 hours with polycarbonate filters, respectively. The invasion abilities of EC9706 cells were quantified by counting the number of cells invade the underside of the porous polycarbonate. The value represented the means  $\pm$  SD of at least three independent experiments. a-Control group; b-NC-GFP-RNAi-LV group; c-CXCR4-RNAi-LV group. \*,  $P < 0.05$  as compared with the control and NC-GFP-RNAi-LV groups.

### *Inhibition of CXCR4 mRNA and protein expression using CXCR4 shRNA*

RT-PCR was used to determine CXCR4 mRNA levels (**Figure 4A**). Western blot analysis was used to determine CXCR4 protein expression (**Figure 4B**). These results indicated that CXCR4-RNAi-LV could efficiently silence CXCR4 in EC9706 cells.

### *Silencing of CXCR4 expression inhibits EC9706 cell invasion in vitro*

A cell invasion assay was employed to study the impact of downregulating CXCR4 expression on EC9706 cell invasion *in vitro*. CXCR4 interference significantly decreased membrane-penetrating cells ( $P < 0.05$ ; **Figure 5**).

### *Effect of CXCR4 interference on wound closure of EC9706 cells in vitro*

A wound-healing assay was employed to study the impact of downregulating CXCR4 expression on EC9706 cell migration *in vitro*. CXCR4 interference induced marked reductions in the migration of EC9706 cells ( $P < 0.05$ ; **Figure 6**).

### *Effect of CXCR4 interference on MMP-9 mRNA and protein*

RT-PCR was used to determine MMP-9 mRNA levels (**Figure 7A**). Western blot analysis was used to determine MMP-9 protein expression (**Figure 7B**). CXCR4 interference significantly reduced MMP-9 mRNA and protein levels com-

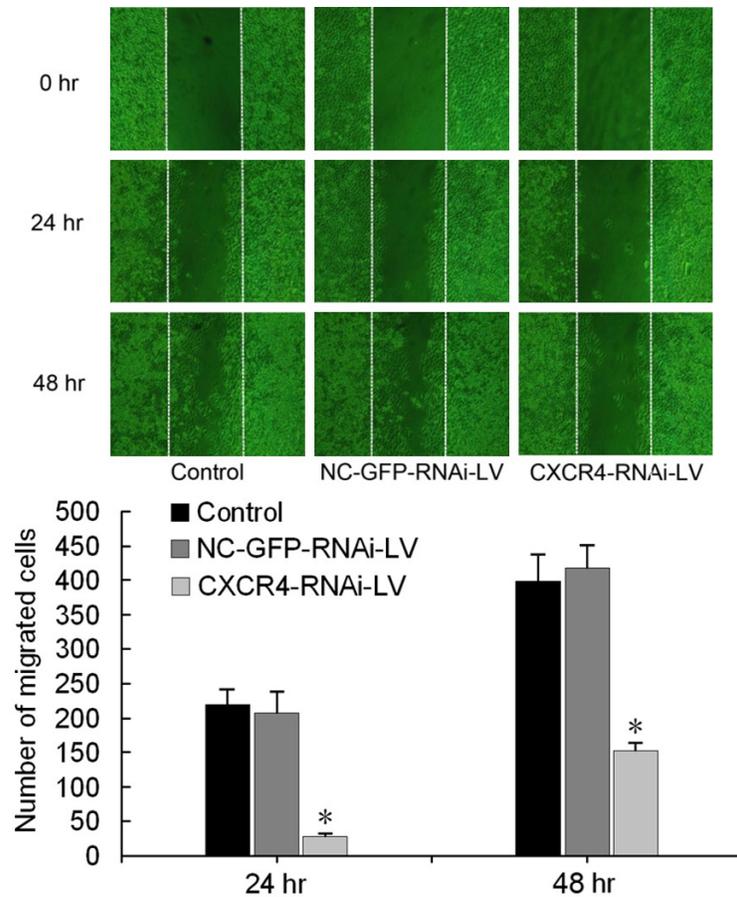
pared with the Control group ( $P < 0.01$ ) and NC-GFP-RNAi-LV group ( $P < 0.01$ ) (**Figure 7**).

## Discussion

Esophageal cancer exhibits poor prognosis given its aggressiveness. It is important to understand the role of molecular factors in the acquisition of malignant potential. Chemokines are small proteins that stimulate and attract leukocytes to sites of inflammation [17]. CXCL12, which is also named stromal cell-derived factor-1, is a chemokine of the CXC sub-family. CXCL12 was first discovered among the chemokines secreted by mouse bone marrow stromal cells. CXCR4 is its specific receptor. CXCL12 and CXCR4 genes are expressed not only in immunocytes but also in esophageal cancer [18], and the CXCL12/CXCR4 pathway might closely correlate with ESCC tumorigenesis and progression. Whether and how the CXCL12/CXCR4 pathway influences the metastatic characteristics of the ESCC cells may be useful and provide significant information for ESCC treatment and research. In this study, we targeted the CXCL12-specific CXCR4 receptor using lentivirus-induced shRNA silencing in EC9706 ESCC cells to inhibit the biological activity of the CXCL12/CXCR4 pathway. The effect of silencing CXCR4 and its inhibition of the metastatic characteristics in ESCC cells were discussed.

Current approaches to study CXCL12/CXCR4-mediated tumor metastasis are based on the functional inhibition of CXCR4 with CXCR4 antagonists and neutralizing antibodies [19, 20]. T140, an antagonist of CXCR4, could potentially replace neutralizing antibodies as anti-metastatic agents for breast cancer treatment and reduced the metastatic potential of the human breast cancer cell line MDA-MB-231 in SCID mice [21]. Ehtesham *et al.* [22] also reported that invasive populations of glioma cells overexpress CXCR4 at the mRNA and protein levels, and 25- to 89-fold increased expression was noted compared with noninvasive tumor cells. A previous study demonstrated that CXCL12 and CXCR4 were highly expressed in ESCC tissues, and the expression of CXCR4 was significantly associated with prognosis in ESCC patients [23]. These results suggested that CXCR4 expression might be involved in ESCC progression.

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**Figure 6.** Effect of CXCR4 interference on wound closure of EC9706 cells in vitro. Three groups of cells were wounded for 0 hour, 24 hours and 48 hours in 0.5% FBS-containing medium. (100 $\times$ ) At 0 hour, 24 hours and 48 hours, phase-contrast pictures of the wounds at three different locations were taken.

RNA interference (RNAi) has emerged as a powerful tool to induce loss-of-function phenotypes by post-transcriptional silencing of gene expression [24], which is now widely used in gene analysis and gene therapy studies [25, 26]. In our study, CXCL12 and CXCR4 mRNA and protein expression was examined by RT-PCR and Western blot, respectively. A lentiviral vector was chosen to deliver shRNA expression cassettes into ESCC cells, offering sustained expression and potent function of the encoded shRNA. The results revealed that CXCR4-shRNA transfection into EC9706 cells significantly suppressed CXCR4 expression in Western blot and RT-PCR assays, in which highly efficient gene transfection and decreased expression of CXCR4 protein inhibited the biological effect of CXCL12/CXCR4. Our previous study found that CXCR4 levels correlate with ESCC progression and prognosis [23]. In the current study, inva-

sion and wound-healing assays revealed that lentivirus-mediated shRNA could efficiently silence CXCR4, and the metastatic characteristics of the human ESCC cell line EC9706 were inhibited.

We also found that MMP-9 expression was decreased at the protein and mRNA levels. A previous study found that CXCR4 was highly expressed in ESCC and correlates with MMP-9 expression and patient prognosis [27]. A regulatory mechanism between CXCR4 and MMP-9 might exist in ESCC. We hypothesized that the change in the metastatic characteristics could possibly be explained by mechanisms of silencing genes downstream of CXCL12/CXCR4, such as MMP-9. MMP-9 is one of the gelatinases that mainly degrades type IV collagen, which is the main component of the basement membrane [28]. MMP-9 expression could possibly offer additional information about the aggressiveness and activity of esophageal carcinoma lesions. Further investigations are need-

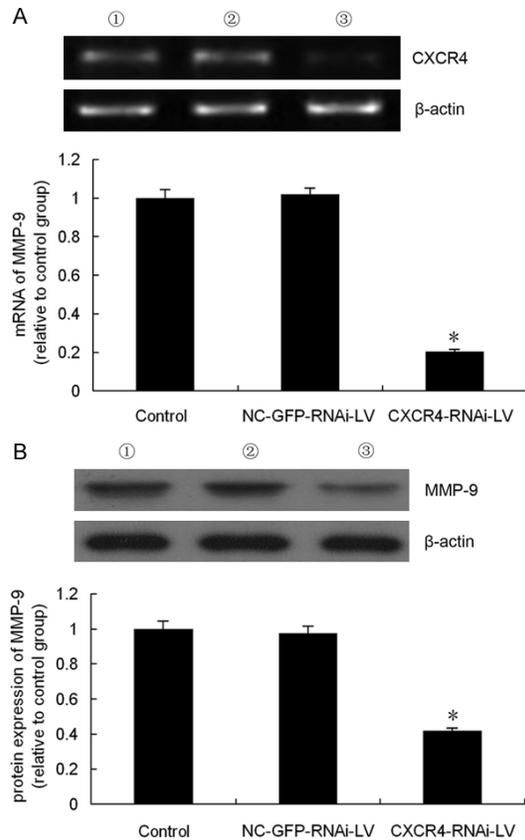
ed to determine whether other pathways participate and alter the metastatic characteristics of ESCC cells.

In summary, our study demonstrates that CXCR4 might be a key molecule in ESCC progression. Regarding tumor metastatic characteristics, downregulation of CXCR4 expression by lentivirus-mediated shRNA could effectively inhibit the metastatic characteristics in ESCC cells. We propose that lentivirus-mediated shRNA holds great promise as a novel approach in human ESCC.

### Acknowledgements

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**Figure 7.** RNA interference inhibits MMP-9 gene expression in EC9706 cells. A-Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the MMP-9 RNA levels. B-Western blot analysis of MMP-9 protein levels in the three cell groups.  $\beta$ -actin was employed as a loading control. ①: Control group, ②: NC-GFP-RNAi-LV group, ③: CXCR4-RNAi-LV group. \*,  $P < 0.01$  as compared with the Control and NC-GFP-RNAi-LV groups.

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### Disclosure of conflict of interest

None.

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