CXCR4 Promotes Oral Squamous Cell Carcinoma Migration and Invasion through Inducing Expression of MMP-9 and MMP-13 via the ERK Signaling Pathway

Tao Yu1,2, Yingying Wu2, Joseph I. Helman3, Yuming Wen1, Changmei Wang1, and Longjiang Li1,2

Abstract
The increased migration and invasion of oral squamous cell carcinoma cells are key events in the development of metastasis to the lymph nodes and distant organs. Although the chemokine receptor CXCR4 and its ligand, stromal cell–derived factor-1α, have been found to play an important role in tumor invasion, its precise role and potential underlying mechanisms remain largely unknown. In this study, we showed that knockdown of CXCR4 significantly decreased Tca8113 cells migration and invasion, accompanied with the reduction of MMP-9 and MMP-13 expression. Inhibition of ligand binding to CXCR4 by a specific antagonist TN14003, also led to reduced cancer cell migration and invasion. Because the degradation of the extracellular matrix and the basement membrane by proteases, such as matrix metalloproteinases (MMP) is critical for migration and invasion of cancer cells, we investigated the expression of several MMPs and found that the expression of functional MMP-9 and MMP-13 was selectively decreased in CXCR4 knockdown cells. More importantly, decreased cell migration and invasion of CXCR4 knockdown cells were completely rescued by exogenous expression of MMP-9 or MMP-13, indicating that the two MMPs are downstream targets of CXCR4-mediated signaling. Furthermore, we found the level of phosphorylated extracellular signal-regulated kinase (ERK) was significantly decreased in CXCR4-silenced cells, suggesting that ERK may be a potential mediator of CXCR4-regulated MMP-9 and MMP-13 expression in Tca8113 cells. Taken together, our results strongly suggest the underlying mechanism of CXCR4 promoting Tca8113 migration and invasion by regulating MMP-9 and MMP-13 expression perhaps via activation of the ERK signaling pathway.

Introduction
Oral cancer is identified as a significant public health threat worldwide because its treatment often produces dysfunction and distortions in speech, mastication and swallowing, dental health, and even in the ability to interact socially. It is 1 of the 10 most frequent cancers in human males worldwide, with approximately two thirds of all cases occurring in developing countries (1). The most common type of oral cancer is oral squamous cell carcinoma (OSCC).

Although local OSCC can be effectively controlled by surgical excision and radiotherapy, metastasis to the lymph nodes and distant organs significantly decreases the survival rate (2). As OSCC is a type of highly malignant tumor with a potent capacity to invade locally and distant metastasis, an approach decreasing the ability of invasion and metastasis may facilitate the development of effective adjuvant therapy. The invasion of tumor cells is a complex, multistage process. In this process, the degradation of the extracellular matrix (ECM) and the basement membrane surrounding the primary tumor by proteases, such as matrix metalloproteinases (MMP), is critical for migration and invasion of cancer cells. To facilitate the cell motility, invading cells need to change the cell–cell adhesion properties, rearrange the ECM environment, suppress anoikis and reorganize their cytoskeletons (3). Tumor cells secrete various ECM degrading enzymes, including MMP, to facilitate their migration and invasion (4–5). In particular, MMP2, MMP-9, and MMP-13 are highly expressed in OSCC compared with normal oral mucosa tissues, and their mRNA and protein levels are further increased on tumor progression (6–8). In addition, it is well established that MMP2, MMP-9, and MMP-13 are closely associated with tumor invasion and metastasis in a variety of human tumors (9–11).
Chemokines, a superfamily of small cytokine-like proteins, can bind to and activate a family of 7 transmembrane G-protein–coupled receptors, the chemokine receptors (12). Chemokines are expressed by many tumor types and can promote mitosis, modulate apoptosis, survival, and angiogenesis (13, 14). Interaction between the chemokine receptor CXCR4 and its ligand, stromal cell–derived factor-1α (SDF-1α or CXCL12), has been found to play an important role in tumorigenicity, proliferation, metastasis, and angiogenesis in many cancers, such as lung cancer, melanoma, esophageal cancer, ovarian cancer, glioblastoma, pancreatic cancer, cholangiocarcinoma, and basal cell carcinoma cells (15–22). Although several studies have implicated CXCR4 in tumor invasion, its precise role and potential underlying mechanisms remain largely unknown.

In this study, we hypothesize that CXCR4 regulates OSCC cell migration and invasion by altering the expression and function of MMPs. First, several MMPs, including MMP2, MMP-7, MMP-9, and MMP-13, directly or indirectly interact with CXCR4, which could alter MMP-mediated pericellular proteolysis (23–25). Second, several studies have shown that CXCR4, and MMP2, MMP-9, and MMP-13 were highly upregulated in human OSCC, suggesting that their expression levels are likely coupled (26, 27). Herein, we present evidence that CXCR4 regulates tumor cell migration and invasion by altering the expression of MMP-9 and MMP-13.

Materials and Methods

Cell line and reagents

SCC-4, SCC-9, SCC-15, SCC-25, and Tca8113 human tongue squamous cell carcinoma cell lines were obtained from ATCC and State Key Laboratory of Oral Diseases at Sichuan University, respectively. Recombinant human SDF-1α was obtained from Research Diagnostics, Inc. MMP inhibitors, OA-Hy, and inhibitor II were obtained from Calbiochem. Antibody to human CXCR4, MMP1, MMP2, MMP7, MMP-9, and MMP-13 were obtained from Santa Cruz Biotechnology. Rabbit anti-ERK/pERK, anti-AKT/pAKT, anti-P38/pP38, anti-JNK/pJNK, and anti-GAPDH primary antibodies were purchased from Cell Signaling Technology. The real-time PCR primers for CXCR4, MMP1, MMP2, MMP7, MMP-9, MMP-13, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized commercially (Takara). All primers sequence were shown in Table 1. The relative expression levels were quantified and analyzed by using Bio-Rad iCycler iQ software. The comparative threshold cycle (Ct) method was used to calculate amplification fold. The migration and invasion assay kit were from Dojindo Laboratories.

Construction of plasmid vector expressing siRNAs for CXCR4

Two siRNAs were designed based on the CXCR4 sequence (NM_001008540): siRNA 1, 5′-GATCAGGTCTATGTCGTAAGCTTGGTAGATGA-3′; siRNA 2, 5′-GGCACTGTCATGTCATGTC-3′. Oligonucleotides with a sequence predicted to induce efficient RNAs of CXCR4 (containing sense and antisense sequences) were synthesized (siRNA 1 sense: 5′-GATCCGGTGTGTTCTATGTTGGCGTCT-3′; antisense: 5′-CTAGAAAAAGGTGGCTCTATGTGGCGTCTGCAAGCTTCCAGACGCCAACATAGACCACCGA-3′; siRNA 2 sense: 5′-GATCAGGTCTATGTCGTAAGCTTGGTAGATGA-3′; antisense: 5′-CTAGAAAAAGGTGGCTCTATGTGGCGTCTGCAAGCTTCCAGACGCCAACATAGACCACCGA-3′). These oligonucleotides were annealed in STE buffer [10 mmol/L Tris (pH 8.0), 50 mmol/L NaCl, and 1 mmol/L EDTA] at 94°C for 5 minutes and cooled gradually. The double-stranded products were cloned downstream to the human U6 promoter of the pRNAi/U6 vector (Genscripts) and designated as CXCR4-shRNA1 and CXCR4-shRNA2. A control vector (Control) was constructed (5′-GAAGCAAGCAGCATTCTTTCTT-3′) with no significant homology to any mammalian gene sequence and, therefore, served as a nonsilencing control.

Cell culture and transfection

The cells were cultured at 37°C in 5% CO2 in RPMI 1640 containing 10% FBS (Gibco Life Technologies), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 100 U/mL penicillin G, and 100 mg/mL streptomycin (Sigma). The human tongue squamous cell carcinoma cell lines were transfected in Opti-MEM 1 medium with Lipofectamine LTX with plus reagent (Invitrogen) in accordance with the manufacturer’s instructions. For stable transfection, selection was performed with neomycin (500 mg/ml; Invitrogen) in RPMI 1640 medium containing 20% FBS for approximately 2 weeks from the day after transfection. The cells with knockdown of CXCR4 were collected for cell proliferation and cytotoxicity assay, migration and invasion assays, real-time PCR, and Western blotting.

Quantitative real-time PCR

Quantitative reverse transcription-PCR was carried out using real-time PCR with the SYBR Green reporter. Total RNA isolated using the RNeasy Mini kit (Qiagen) was subsequently reverse transcribed to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). The quantitative real-time RT-PCR was performed using the SYBR premix Ex Taq II Kit (Takara) according to the manufacturer’s instructions. The reaction mix was subjected to quantitative real-time PCR to detect levels of the corresponding GAPDH, and several MMPs. All primers sequence were shown in Table 1. The relative expression levels were quantified and analyzed by using Bio-Rad iCycler iQ software. The comparative threshold cycle (Ct) method was used to calculate amplification fold. GAPDH gene was used as a reference control gene to normalize the expression value of each gene. Triplicate replicates were performed for each gene and average expression value was computed for subsequent analysis. The relative
expression level of the genes was calculated using the 
\(2^{-\Delta\Delta C_t}\) method (28).

**Western blotting**

The cells were lysed in lysis buffer (PBS containing 1% Triton X-100, protease inhibitor cocktail, and 1 mmol/L phenylmethylsulfonyl fluoride) at 4°C for 30 minutes. Equal quantities of protein were subjected to SDS-PAGE. After transfer to Immobilon-P transfer membrane, successive incubations with anti-CXCR4, MMP1, MMP2, MMP7, MMP-9, MMP-13, ERK/pERK, AKT/pAKT, P38/pP38, and JNK/pJNK antibody or anti-GAPDH antibody and horseradish peroxidase–conjugated secondary antibody were carried out. The immunoreactive proteins were then detected using the ECL system. Bands were scanned using a densitometer (GS-700; Bio-Rad) and quantification was performed using Quantity One 4.6.3 software (Bio-Rad).

**Cell proliferation assay**

Cell proliferation assay was performed using Cell Counting Kit-8 (Dojindo). Cells were plated in 96-well plates at 1 \(\times 10^4\) cells per well and incubated for 5 days. Every day, at one time, 10 \(\mu\)L of Cell Counting Kit-8 solution were added to each well, then the cells were incubated for another 2 hours. The value of optical density (OD) was obtained by the differences in absorbance at a wavelength of 450 nm by using a microplate reader (VARI OSKAN FLASH 3001 Thermo). The amount of the formazan dye, generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells.

**Cell migration assay**

This cell migration assay was performed in a migration chamber with an 8-\(\mu\)m pore size polycarbonate membrane (Millipore), based on the Boyden chamber principle. Briefly, cells were resuspended in serum-free RPMI 1640, and 3 \(\times 10^5\) cells were added to the interior of the inserts. RPMI 1640 (500 \(\mu\)L) containing 10% FBS were added to the lower chamber as chemoattractant. Cells were incubated for 24 hours at 37°C in a CO2 incubator (5% CO2). Then, the upper surface of the chamber was scraped to remove noninvasive cells. Invaded cells were fixed and stained with 0.1% crystal violet and photographed under a light microscope (\(\times 200\)). The stained inserts were transferred to a clean well containing 500 \(\mu\)L of 10% acetic acid for 15 minutes at room temperature. The OD of the stained cell was measured at 560 nm.

**Cell invasion assay**

The cell invasion assay was performed with QCM 24-well Invasion Assay kit (Chemicon International). This cell invasion assay was performed in an invasion chamber, based on the Boyden chamber principle. This kit contains 24 inserts and each insert contains an 8-\(\mu\)m pore size polycarbonate membrane coated with a thin layer of ECM. Briefly, cells were resuspended in serum-free RPMI 1640, and 3 \(\times 10^5\) cells were added to the interior of the inserts, which had been previously rehydrated at room temperature for 30 minutes. RPMI 1640 (500 \(\mu\)L) containing 10% FBS were added to the lower chamber as chemoattractant. Cells were incubated for 48 hours at 37°C in a CO2 incubator (5% CO2). Then, the upper surface of the chamber was scraped to remove noninvasive cells. Invaded cells were fixed and stained with 0.1% crystal violet and photographed under a light microscope (\(\times 200\)). The stained inserts were transferred to a clean well containing 500 \(\mu\)L of 10% acetic acid for 15 minutes at room temperature. The OD of the stained cell was measured at 560 nm.

**Data statistics**

All quantified data represent an average of at least 3 samples. Error bars represent SD. Statistical significance was determined by Student’s t-test, and differences at probability of less than 0.05 were considered statistically significant.

**Results**

CXCR4 silencing inhibits Tca8113 cells migration and invasion by regulating MMP-9 and MMP-13 expression

We investigated whether CXCR4 is required for the invasive phenotypes of oral squamous cell carcinoma cells in vitro by expressing short hairpin RNAs (shRNA) to knock down CXCR4 expression. We expressed shRNA from 2 different CXCR4 sequences (shRNA1 or shRNA2) in the highly malignant Tca8113 cell line and SCC-9 cell line (Fig. 1A). Expression of CXCR4 protein and its

| Table 1. The primers for quantitative real-time RT-PCR |
|-----------------|-----------------|-----------------|
| **Genes** | **Forward primer (5’–3’)** | **Reverse primer (5’–3’)** |
| CXCR4 | AGACCCACAGATCATCCTCATCCT | GTTCTCAAACCTCACAACCCCTTGC |
| MMP1 | GGGAGATCATCAGGGAACAACCTC | GGCGCTGTGGTAAGAAGCAT |
| MMP2 | CTGACCCCGAGTATCTGCG | TGTGGGAGCAGCTGACTTCA |
| MMP7 | GAGATGCTCACTTCGAGAGGGA | GGATCAGAGGAATGTCCCATAC |
| MMP-9 | CTTTGGACAGGCGAAGAAGTG | GCCACTGGAGGATGATCA |
| MMP-13 | TTGTGTGCTGCGGATGATTCG | GGGTGTCATAGCCAGCATCA |
| GAPDH | CTTTGGTATCCTGGGAAGACTC | GTAGAGGCAAGGATGATTTCT |
mRNA levels were substantially reduced by 90%, 82% and 88%, 74% in CXCR4-shRNA1 expressing Tca8113 and SCC-9 cells, respectively (Fig. 1B–D). However, the expression of CXCR4 protein and its mRNA levels were substantially reduced by 20% and 30% in CXCR4-shRNA2 expressing Tca8113 and SCC-9 cells, respectively (data not shown). Therefore, 1 of the 2 shRNAs (CXCR4-shRNA1) was chosen for further study. Furthermore, the quantitative real-time RT-PCR and Western blotting results showed that the mRNA and protein expression of CXCR4 in both Tca8113 and SCC-9 cells were significantly inhibited in vitro. We also selected the 1 of the 2 cell lines (Tca8113) for further study. In addition, we examined the effect of CXCR4 silencing on the survival and proliferative activity of Tca8113 cells because a decrease in survival or proliferation after CXCR4 silencing may also contribute to the observed decrease in migration and invasion of Tca8113 cells. The results showed that CXCR4 silencing decreased the proliferation rate of Tca8113 cells slightly compared with Tca8113 and control cells (Supplementary Fig. S1). To investigate whether CXCR4 silencing affects Tca8113 cell migration and invasion, we conducted 3-dimensional cell migration assays using migration chambers and invasion assays with matrigel-coated invasion chambers. The results showed that knockdown of CXCR4 in Tca8113 cells decreased cell migration and invasion at approximately 75% and 85%, respectively, compared with
controls (Fig. 2A–B). To further confirm whether CXCR4 regulates Tca8113 cell migration and invasion by modulating MMP expression, we analyzed the mRNA and protein levels of MMPs on CXCR4 silencing. Quantitative real-time PCR results showed that the mRNA levels of MMP-9 and MMP-13 were decreased by approximately 70% and 60%, respectively, in CXCR4-silenced cells, compared with control cells (Fig. 2C). The Western blotting results showed that the protein levels of MMP-9 and MMP-13 were decreased by approximately 75% and 65%, respectively, in CXCR4-silenced cells, compared with control cells (Fig. 2D–E). As controls, the mRNA and protein levels of the MMP1, MMP2, and MMP7 did not alter under these conditions. To further confirm that the effect of CXCR4 silencing on the decrease of migration and invasion of Tca8113 cells is specific, we restored CXCR4 function by coculturing the ligand of CXCR4 SDF-1α (100 ng/mL, 12 hours), which can significantly promote the upregulation of CXCR4 expression (Fig. 3A; ref. 29). We also observed that the upregulation of CXCR4 expression restored Tca8113 cell migration and invasion and the protein levels of MMP-9.

Figure 2. CXCR4 silencing inhibits Tca8113 cells migration and invasion. A, 3-dimensional migration and invasion assay were performed using Boyden chambers in CXCR4-shRNA1 Tca8113 cells. B, the quantitative analysis of migrated and invaded cells, which were eluted using 10% acetic acid and measured optical density value at 560 nm. Scale bars, 100 μm. C, transcript levels of CXCR4, MMP1, MMP2, MMP7, MMP-9, and MMP-13 in CXCR4-silenced Tca8113 cells, relative to GAPDH, determined by quantitative RT-PCR. D, Western blotting analysis of CXCR4 MMP1, MMP2, MMP7, MMP-9, and MMP-13 in CXCR4-shRNA1 Tca8113 cells. GAPDH loading control is also shown. E, densitometric analysis of protein levels of CXCR4, MMP1, MMP2, MMP7, MMP-9, and MMP-13, relative to GAPDH, determined by Western blotting and compared with Tca8113 cells. Representative photos of migration and invasion are presented, which were taken at a magnification of ×200 under inverted microscopy. Error bars indicate SD; n = 3 experiments; **, P < 0.01.
Figure 3. CXCR4 modulates cell migration and invasion by regulating MMP-9 and MMP-13 expression. A, Western blotting analysis of the ligand of CXCR4 SDF-1α was cocultured with CXCR4-shRNA1 Tca8113 cells. The cells were serum starved for 12 hours and treated with SDF-1α (100 ng/mL) for 12 and 24 hours. CXCR4 silencing and SDF-1α overexpression were determined by Western blotting (GAPDH levels as loading control). B, the quantitative analysis of migrated and invaded cells treated with SDF-1α (100 ng/mL) for 12 hours, which were eluted using 10% acetic acid and measured optical density value at 560 nm. C, Western blotting analysis of MMP1, MMP2, MMP7, MMP-9, and MMP-13 in the ligand of CXCR4 SDF-1α cocultured with CXCR4-shRNA1 Tca8113 cells. GAPDH loading control is also shown. D, densitometric analysis of protein levels of MMP1, MMP2, MMP7, MMP-9, and MMP-13, relative to GAPDH, determined by Western blotting. E, Tca8113 cell migration and invasion were analyzed with or without 100 nmol/L TN14003 in both chambers. F, transcript levels of MMP1, MMP2, MMP7, MMP-9, and MMP-13 in Tca8113 cells with or without 100 nmol/L TN14003, relative to GAPDH, determined by quantitative RT-PCR. G, Western blotting analysis of MMP1, MMP2, MMP7, MMP-9, and MMP-13 protein levels of Tca8113 cells with or without 100 nmol/L TN14003. GAPDH loading control is also shown. H, densitometric analysis of MMPs protein levels for Tca8113 cells with or without 100 nmol/L TN14003, relative to GAPDH, determined by Western blotting. Error bars indicate SD; n = 3 experiments; *, P < 0.05; **, P < 0.01.
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and MMP-13 were increased by approximately 60% and 70%, respectively, in CXCR4-restored cells, compared with control cells. As controls, the protein levels of the MMP1, MMP2, and MMP7 did not alter under these conditions (Fig. 3B-D). We have further tested the levels of CXCR4 silencing on the invasion of several OSCC cell lines (SCC-4, SCC-9, SCC-15, SCC-25) and found that the invasion of these human tongue squamous cell carcinoma cells positively correlated with CXCR4 expression levels (Supplementary Fig. S2A). To further confirm whether CXCR4 regulates Tca8113 cell migration and invasion by modulating MMP expression, we analyzed the protein levels of MMPs in different cell lines on CXCR4 silencing. The Western blotting results showed that the protein levels of MMP-9 and MMP-13 were decreased significantly in CXCR4-silenced cells except SCC-25 cell line, compared with control cells (Supplementary Fig. S2B). As controls, the protein levels of the MMP1, MMP2, and MMP7 did not alter on CXCR4 silencing. These results showed that the expression levels of CXCR4 positively correlated with the migration and invasion of human tongue squamous cell carcinoma cells.

CXCR4 ligand-binding function is required for efficient cancer cell migration and invasion

To determine whether the ligand-binding ability of CXCR4 affected Tca8113 cell migration and invasion, we pretreated Tca8113 cells with TN14003, an antagonist that blocks ligand binding to CXCR4 (30). Treating Tca8113 cells with 100 nmol/L TN14003 resulted in a significant decrease in migration and invasion at approximately 85% and 70%, respectively, compared with control cells, suggesting that ligand binding to CXCR4 is required for the CXCR4 function in tumor cell migration and invasion (Fig. 3E). We also investigated the mRNA and protein levels of MMP1, MMP2, MMP7, MMP-9, and MMP-13 in Tca8113 cells with or without 100 nmol/L TN14003. Quantitative real-time PCR results showed that the mRNA levels of MMP-9 and MMP-13 were decreased by approximately 60% and approximately 75% in Tca8113 cells treated with TN14003, compared with control cells (Fig. 3F). The Western blotting results showed that protein levels of MMP-9 and MMP-13 were decreased by approximately 50% and approximately 65%, respectively, in Tca8113 cells treated with TN14003, compared with...
control cells (Fig. 3G–H). So the antagonist of CXCR4, TN14003, may inhibit Tca8113 cancer cells migration through downregulating expression levels of MMP-9 and MMP-13 in CXCR4-related pathway.

MMP-9 and MMP-13 are downstream targets of CXCR4

To investigate whether MMP activity is required for Tca8113 cell migration and invasion, we tested the effect of OA-Hy, a broad spectrum MMP inhibitor, and inhibitor II, a specific inhibitor of MMP-9 and MMP-13 (Fig. 4A). Both inhibitors significantly blocked Tca8113 cells migration and invasion, showing that MMP activity is necessary for efficient migration and invasion of Tca8113 cells. In our previous data, we have analyzed the mRNA and protein levels of MMPs on CXCR4 silencing. Both quantitative real-time PCR and Western blotting results showed that the mRNA and protein levels of MMP-9 and MMP-13 were decreased significantly in CXCR4-silenced cells, respectively, compared with control cells (Fig. 2C–E). Herein, we investigated the protein levels of MMPs on CXCR4 silencing and MMP inhibitors. The protein levels of MMP-9 and MMP-13 were decreased by approximately 80% and 70%, respectively, in CXCR4-silenced cells, compared with control cells. The inhibition effect of protein expression of MMP-9 and MMP-13 on CXCR4 silencing was similar with MMP inhibitors, indicating that CXCR4 is involved in the transcriptional regulation of MMP-9 and MMP-13 in Tca8113 cells (Fig. 4B–C). To confirm the finding that CXCR4 regulates MMP-9 and MMP-13 expression, we investigated the mRNA levels of MMP-9 and MMP-13 in CXCR4-deficient mouse embryonic fibroblasts (CXCR4-deficient MEF cells) and CXCR4-expressing MEF cells (MEF cells) by quantitative real-time PCR. Consistent with findings in CXCR4 knockdown cells, mRNA levels of MMP-9 and MMP-13 in CXCR4-deficient MEF cells are only approximately 25% and 40%, respectively, of MEF cells (Fig. 4D). Furthermore, the mRNA levels of MMP-9 and MMP-13 in the brain of CXCR4-deficient forebrain mice were also significantly lower than those in the brain of littermate control mice (ref. 31; Fig. 4E). In both cases, the levels of MMP1, MMP2, and MMP7 were not changed, suggesting that CXCR4 specifically regulates MMP-9 and MMP-13 expression. Additionally, to further confirm that the effect of CXCR4 silencing on the decrease of MMP-9 and MMP-13 expression levels in Tca8113 cells is specific,
we restored CXCR4 function by coculturing the ligand of CXCR4 SDF-1α (100 ng/mL, 12 hours). We found that SDF-1α significantly restored mRNA levels of MMP-9 and MMP-13 in CXCR4-silenced cells (Fig. 4F). Additionally, we tested whether ectopic expression of MMP-9 or MMP-13 can restore cell migration and invasion or not in CXCR4-silenced Tca8113 cells. We found that MMP-9 or MMP-13 overexpression successfully restored both migration and invasion in these cells (Fig. 5A). We also detected the protein levels of MMP-9 and MMP-13 during this process. The restoration of protein expression of MMP-9 and MMP-13 increased the invasiveness of CXCR4-silenced cells. Furthermore, SDF-1α restored protein levels of MMP-9 and MMP-13 in CXCR4-silenced cells and significantly increased invasiveness of these cells, indicating MMP-9 and MMP-13 are downstream targets of CXCR4 (Fig. 5B–D).

**Extracellular signal-regulated kinase is a potential mediator of CXCR4 regulation of MMP-9 and MMP-13 expression**

To understand the mechanism that CXCR4 regulates MMP-9 and MMP-13 expression, we analyzed the effects of CXCR4 silencing on the activation of several potential signaling pathways. We found that the level of phosphorylated extracellular signal-regulated kinase (ERK) was significantly decreased in CXCR4-silenced Tca8113 cells, which was restored by coculturing the ligand of CXCR4 SDF-1α (Fig. 6A–B). Phosphorylated Akt, p38, and c-jun NH kinase (JNK) were not significantly changed in CXCR4-silenced Tca8113 cells. These results show that ERK is likely a downstream target of CXCR4-mediated signaling that regulates MMP-9 and MMP-13 expression and their functions in cell migration and invasion in Tca8113 cells.

**Discussion**

Although local OSCC can be effectively controlled by surgical excision and radiotherapy, in tongue squamous cell carcinoma, the incidence of occult cervical lymph nodes metastases is relatively high. Of the patients with T1 and T2 squamous cell carcinoma of the oral tongue clinically staged N0, 13% to 33% and 37% to 53%, respectively, have occult metastases at the time of diagnosis (32, 33). The metastasis of tumor cells is a very complex process. Tumor cells must migrate through ECM and basement membranes to invade local tissues and metastasize to distal sites. Muller and colleagues (34) showed that injecting the antibody that neutralizes CXCR4 activity leads to inhibition of metastasis to the bone marrow and lungs in vivo. Recently, it has been proposed that chemokine receptors are critical in determining the metastatic destination of tumor cells (35–37). Uchida and colleagues (38) showed a possible role for CXCR4 in mediating the dissemination of OSCCs to the lymph nodes. In this study, we show that CXCR4 silencing leads to a significant decrease of Tca8113 cells' migration and invasion and provide evidence that CXCR4 regulates the transcriptional levels of MMP-9 and MMP-13 in Tca8113 cells. We
further show that CXCR4 regulation of MMP-9 and MMP-13 expression is likely mediated by ERK signal pathways.

The finding that CXCR4 silencing led to a significant decrease of Tca8113 cells migration and invasion was consistent with many previous reports, which showed that high levels of CXCR4 expression promoted OSCC cell invasion (26). Moreover, functional blocking of CXCR4 by TN14003 also led to decreased cell migration and invasion, which was also consistent with reports that TN14003 treatment decreased migration of breast cancer cells and pancreatic cancer cells in vitro and reduced pulmonary metastasis of breast cancer in SCID mice (30, 39). Additionally, we rescued cell migration and invasion in CXCR4-silenced Tca8113 cells by coculturing the ligand of CXCR4 SDF-1α, which suggested that CXCR4 expression levels were positively correlated with cancer cell migration and invasion.

We further observed that the number of invaded cells positively correlated with endogenous levels of CXCR4 in different human tongue squamous cell carcinoma cell lines, suggesting that CXCR4 regulates cancer cell migration and invasion via a similar mechanism among different tongue squamous cell carcinoma cell types. To determine how CXCR4 promotes Tca8113 cells migration and invasion, we focused on delineating the relationship between CXCR4 and MMPs, particularly MMP-9 and MMP-13. In human cancer cells, MMP1, MMP2, MMP-9, and MMP-13 have been found to correlate with malignant grade and metastasis (40, 41). MMPs have been implicated to facilitate cancer cell invasion and metastasis through degradation of surrounding ECM proteins. Expression of MMPs and other extracellular proteinases have been shown to positively correlate with the progression of cancer in patients (10, 42). It is interesting to note that several studies have shown that the expression levels of CXCR4 were also highly upregulated in human head and neck squamous cell carcinoma and OSCC, suggesting that MMPs and CXCR4 expression levels are likely coupled (26, 43, 44). However, it is unknown how these genes regulate each other during tumorigenesis. Herein, we found that CXCR4 silencing significantly decreased the mRNA and protein levels of both MMP-9 and MMP-13, but not the mRNA and protein levels of the MMP1, MMP2, and MMP7, indicating that CXCR4 specifically modulates the expression of MMP-9 and MMP-13.

Although more than 90% of CXCR4 expression was silenced by shRNA, the migration and invasion rates of Tca8113 cells and the expression levels of MMP-9 and MMP-13 were reduced by only approximately 50% to 80%. A potential explanation for this discrepancy is the paradoxical function of some MMPs. Indeed, MMP-9 has a function of host resistance in cancer, as well as protumorigenic action (45). Additionally, other modulators may regulate MMP expression and Tca8113 cell migration and invasion independent of CXCR4. Further investigation will be needed to reveal the relationship of CXCR4-dependent and CXCR4-independent mechanisms in cancer cell migration and invasion. We further showed that the expression of MMP-9 and MMP-13 in both CXCR4-deficient MEF cells and the brain of CXCR4-deficient forebrain mice were significantly lower than those in the control samples. These results suggest that CXCR4 is involved in the transcriptional regulation of MMP-9 and MMP-13 in Tca8113 cells. We also showed that the ectopic expression of MMP-9 or MMP-13 successfully restored both migration and invasion of CXCR4-silenced Tca8113 cells, indicating that MMP-9 and MMP-13 are indeed downstream targets of CXCR4.

The signal transduction pathways that modulate the activity of MMP transcription factors are highly diverse. Mitogen-activated protein kinase signal transduction pathways, including p38, ERK, and JNK, are well known mediators that stimulate or inhibit MMP expression depending on cell types (46–48). In this study, we showed that downregulation of CXCR4 expression by shRNA could significantly reduce the phosphorylation of ERK. Thus, ERK signaling pathway is likely associated with several mechanisms of cell motility mediated by CXCR4, including regulation of the transcriptional levels of MMP-9 and MMP-13.

Taken together, in this study we showed that the down-regulation of CXCR4 by shRNA may well serve as a potential therapeutic strategy to treat cancer, particularly cancer metastasis. Further studies aimed at identifying the precise signal transduction pathways and transcription factors that mediated CXCR4 regulation of MMPs expression may provide a molecular understanding of the roles of CXCR4 in cancer biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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