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The Role of SDF-1/CXCR4 Axis in Ovarian Cancer Metastasis*

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Summary: This study was aimed to explore the role of stromal-derived factor 1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) axis in mediating the metastasis of ovarian cancer cells through activation of extracellular signal-regulated kinase-1/2 (ERK-1/2) signaling pathway. A highly metastatic ovarian cancer cell line, SKOV3, was used in the study. Intracellular calcium mobilization was detected by using laser scanning confocal fluorescence microscopy. Western blotting was used to detect the phosphorylation of ERK1/2 in SDF-1α-treated SKOV3 cells. Adhesion capability and matrix metalloproteinase (MMP) activity of ovarian cancer cells after exposure to SDF-1α were measured by adhesion assay and gelatin zymography. The results showed that SDF-1α induced rapid intracellular calcium mobilization in SKOV3 cells, as well as the phosphorylation of ERK-1/2. The adhesion of ovarian cancer cells to fibronectin and collagen Ⅳ was increased after SDF-1α treatment. An inhibitor of ERK-1/2 signaling, PD98059, could antagonize such effects of SDF-1α. SDF-1α could also increase the secretion of active MMP-2 and MMP-9. It was concluded that the SDF-1/CXCR4 axis played a critical role in the metastasis of human ovarian cancer by increasing the adhesion capability of cancer cells and the activity of MMP-2 and MMP-9 via ERK1/2 signaling pathway.
Key words: ovarian cancer; metastasis; CXC chemokine receptor 4; stromal-derived factor 1; extracellular signal-regulated kinase-1/2

Metastasis is a complex event involving many factors that can potentially affect tumor adhesion and extracellular matrix (ECM) degradation, of which, chemokines attract more attention[1]. The membranous CXC chemokine receptor 4 (CXCR4) and its ligand stromal-derived factor-1 (SDF-1, namely CXCL12) are members of chemokine family. Studies have demonstrated that SDF-1/CXCR4 plays a critical role in embryo development, immune response, inflammatory reaction, hematopoietic system modulation, HIV infection and angiogenesis[2-4]. Moreover, SDF-1/CXCR4 pathway has been recently reported to be implicated in the metastatic process of various malignancies[5-9].

Complex chemokine network exists in ovarian cancer. However, whether or how SDF-1/CXCR4 biological axis plays a role in ovarian cancer remains unclear. It had been reported that CXCR4 was the only one of the 14 chemokine receptors investigated that could be expressed on a subset of ovarian tumor cells[10]. In addition, SDF-1/CXCR4 axis might influence the spread of epithelial ovarian cancer[10, 11]. Porcile *et al* reported that there may exist an important "cross-talk" between SDF-1/CXCR4 and EGFR intracellular pathway that may link signals of cell proliferation in ovarian cancer[12].

In this study, we investigated the effects of SDF-1/CXCR4 on cell-matrix adhesion and MMP activity in ovarian cancer, observed the roles of the two chemokines in ovarian cancer metastasis, and preliminarily discussed the mechanism.

1 MATERIALS AND METHODS

1.1 Materials

Rabbit anti-extracellular signal-regulated kinase (ERK)-1 pAb, mouse anti-p-ERK mAb and rabbit anti-β-actin pAb were provided by Santa Cruz Biotechnology Inc., USA. Goat anti-rabbit horseradish peroxidase IgG and goat anti-murine horseradish peroxidase IgG were bought from Pierce Co., USA. Recombinant human SDF-1 was from Peprotech Co., UK. MAPK/ERK kinase-1 (MEK-1) inhibitor, PD98059, was from Promega Co., USA. Purified human plasma fibronectin (FN), laminin (LN), collagen Ⅳ (COL), and Fluo-3AM were purchased from Sigma Chemical Co., USA.

1.2 Cell Culture

A highly metastatic ovarian cancer cell line, SKOV3, was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). All the cells were subcultured after brief treatment with trypsin, and grew in a 5% CO_2 humidified atmosphere at 37°C. The culture medium was displaced every 3 days.

1.3 Calcium Mobilization Assay

SKOV3 cells were seeded onto the coverslips in a culture dish, and incubated with Fluo-3AM working solution (the final concentration of Fluo-3AM: 10 μmol/L) at 37°C for 30 min to reach 90% confluence. The cells

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were washed thrice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to remove extracellular Fluo-3AM, and re-cultured in DMEM. The fluorescence signals were detected by using laser scanning confocal fluorescence microscopy (OLYMPUS, Japan). SDF-1α at the concentration of 50–200 ng/mL was added to each well. An argon laser was used to excite Fluo-3AM at 488 nm and fluorescence was emitted at 530 nm. Intracellular calcium changes were defined as fluorescence intensity (FI).

1.4 Western Blot Analysis

SKOV3 cells were serum-starved in DMEM+0.1% BSA+1% penicillin-streptomycin overnight, then exposed to SDF-1α (100 ng/mL) for different time lengths (0–30 min). The cells were washed with 1×PBS, lysed with lysis buffer for 20 min at 4°C, and centrifuged at 14 000 r/min for 15 min at 4°C. The protein concentration of cell lysates was measured by using the Bradford protein assay. Proteins were re-suspended in 2×reducing sample buffer, denatured at 100°C for 10 min, and then separated by 12% SDS-PAGE. The protein bands were transferred to nitrocellulose membranes at 90 mA for 90 min at 4°C. Blots were blocked with TBST containing 5% nonfat dry milk for 2 h at room temperature, and probed with the following antibodies: (1) rabbit anti-ERK-1 pAb (1:400); and (2) mouse anti-p-ERK mAb (1:400). Rabbit anti-ERK-1 pAb was used in conjunction with goat anti-rabbit horseradish peroxidase at 1:1000 dilution, and mouse anti-p-ERK mAb was used to react with goat anti-murine horseradish peroxidase at 1:1000 dilution. Protein concentration equivalence was confirmed after probing by amido black staining and β-actin antibody. After washing with TBST buffer, immunoreactive protein bands were detected using Quantity One Software (Bio-Rad, USA).

1.5 ECM Coating and Adhesion Assay

FN and LN (1 mg/mL) were diluted to the concentration of 50 μg/mL with PBS. COL at 2 mg/mL was diluted to 50 μg/mL with PBS. The 96-well flat-bottomed plates were coated with FN, LN and COL (40 μL each well) respectively at room temperature overnight. Wells were washed with PBS, incubated with 2% BSA at 37°C for 2 h to block nonspecific binding of cells to the plastic. Briefly, SKOV3 cells were detached by trypsinization, washed in PBS, suspended in DMEM and seeded into ECM-coated 96-well plates. Experimental wells contained SDF-1α (0–200 ng/mL). For ERK inhibition, cells were pretreated with PD98059 (30 μmol/L) for 15 min at 37°C prior to incubation with SDF-1α. Then the cells were incubated for 2 h at 37ºC in 5% CO_2/95% air to allow cell attachment. Afterwards, the cells were washed gently with PBS 3 times to remove detached cells. Then 200 μL FBS (20%) and 20 μL of stock MTT solution (5 mg/mL) were added, and co-cultured with cells at 37°C for 4 h. The culture medium was carefully removed after the addition of 200 μL of DMSO to each well and vibration of culture wells for 10 min. The absorbance (A) of each sample was measured at a wavelength of 490 nm with a microtiter plate reader. Cell-free culture medium served as negative control, and each assay was performed in triplicate.

1.6 Gelatin Zymograph

MMPs were assessed under non-reducing conditions by using a modified SDS-PAGE. SKOV3 cells were incubated with SDF-1α (0–600 ng/mL) in serum-free medium for 24 h, and then conditioned media were collected and stored at –70°C until analysis. Conditioned media were diluted with non-reducing sample buffer. The proteins from the conditioned media were separated by SDS-PAGE by using 10% (w/v) acrylamide gels containing 0.1% (w/v) gelatin at 120 V, 4°C for 3 h. After the gel was washed for 2×45 min at room temperature with 2.5% Triton X-100 to remove SDS and to allow the electrophoresed enzymes to renature, the gel was washed for 2×20 min at room temperature in developing buffer, and then incubated in incubation buffer at 37°C for 42 h. Gelatinases were identified by staining the gels with 0.25% (w/v) Coomassie brilliant blue R-250 and destaining with 10% (w/v) methanol and 5% (w/v) acetic acid. Prestained standard high-range protein marker was used to determine the molecular weight of the gelatinase. Enzymatic activity was characterized by the presence of clear zones in a background of blue staining. Gelatinolytic bands were quantified by gel scanning and densitometry with the aid of Quantity One Software (Bio-Rad, USA).

1.7 Statistical Analysis

The data were expressed as $\bar{x}\pm s$. Multiple group comparison was performed by using ANOVA. Two-group comparison was conducted by using Student's t test. All statistical analyses were performed with SPSS 12.0 software package. A $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Intracellular Calcium Mobilization in Ovarian Cancer Cells

SDF-1α at the concentration of 50–200 ng/mL induced a rapid and robust mobilization of intracellular calcium in SKOV3 cells (fig. 1A). The concentration of intracellular calcium was quickly elevated after SDF-1α was added, and the result was recorded as a pulse wave. The peak was followed by a quite long plateau and afterward returned to the resting level at 470 s. Well-distributed green fluorescence was observed in the cells when intracellular calcium was rapidly increased (fig. 1B).

2.2 Activation of ERK-1/2 in SKOV3 Cells

Western blot showed that phosphorylated ERK-1/2 (phosph-ERK-1/2) was found in SKOV3 cells after 5-min exposure to SDF-1α (fig. 2). There was a gradual increase in the ratio of phospho-ERK to total ERK at different time points from 0 to 30 min.

2.3 The Results of Cell Adhesion Assay

SDF-1α treatment (50–200 ng/mL) augmented SKOV3 cell adhesion to COL and FN, but not to laminin (LN). And PD98059 reduced SDF-1α-mediated SKOV3 cell adhesion to FN and COL (fig. 3A and B).

2.4 The Effects of SDF-1α on the Activation of MMP-2 and MMP-9

Gelatin zymography revealed that with the increases in the concentrations of SDF-1α, active MMP-2 (62 kD) and MMP-9 (92 kD) were increased (fig. 4). Densitometry showed that differently active MMP-2 (62 kD) and MMP-9 (92 kD) were induced by SDF-1α.

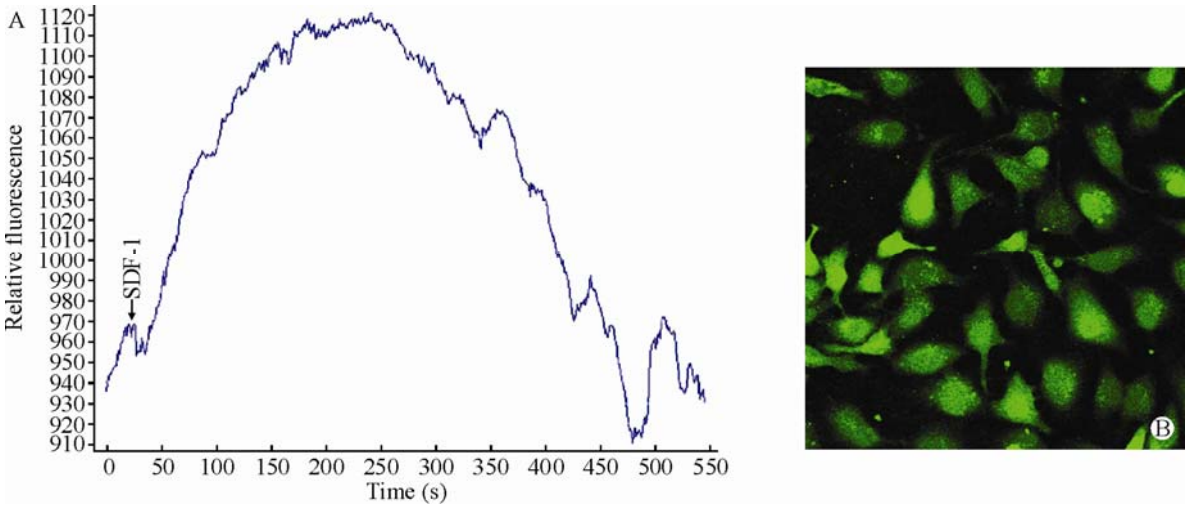

Fig. 1 The dynamic changes of intracellular calcium in SKOV3 cells at different time points after SDF-1α (200 ng/mL) treatment (A) (SDF-1α was added at the time (20 s) as indicated by the arrow), and the fluorescent picture of the cells when intracellular calcium was rapidly increased (B)

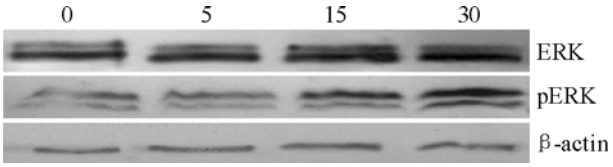

Fig. 2 ERK-1/2 phosphorylation in SKOV3 cells treated with SDF-1α (100 ng/mL) for different periods (0, 5, 15, and 30 min)

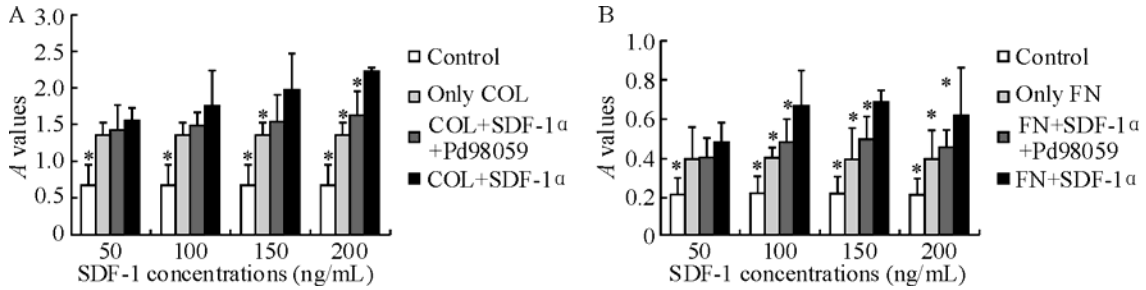

Fig. 3 Increased cell adhesion of SKOV3 cells to COL (A) and FN (B) after SDF-1α at different concentrations was added and the antagonizing effects of PD98059 (asterisks indicative of the statistically significant differences when compared with FN+SDF-1α or COL+SDF-1α)

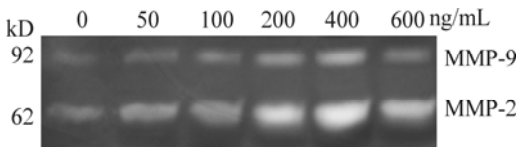

Fig. 4 The increased activity of MMP-9 (92 kD) and MMP-2 (62 kD) in SKOV3 cells treated with different concentrations of SDF-1α

3 DISCUSSION

Tumor metastasis was once viewed as a passive consequence of a single tumor cell simply "escaping" from a primary tumor and traveling great distances through draining lymph nodes and blood, lodging in small blood vessels and thereby forming micrometastasis[13]. Some findings, however, have demonstrated that tumor metastasis is an active process involving multiple molecular and cellular mechanisms[14, 15]. The interaction between tumor cells and stroma is crucial for tumor metastasis[16].

Increased cell migration and invasion are major features of malignant tumor. The results of this study suggested that cell signaling induced by binding of SDF-1α to CXCR4 leads to the activation of MAPK signaling pathways, and increased cell-matrix adhesion and secretion of active MMP-2 and MMP-9 into the local environment. From this, we speculate that the SDF-1/CXCR4 biological axis plays a critical role in mediating ovarian cancer migration and invasion, and blocking this process can become one of the promising biological treatment options for ovarian cancer.

Chemotactic SDF-1 gradients have been implicated in organ-specific metastasis as important determinants. The findings by Muller *et al* first provided new insights into the potential mechanisms of SDF-1 related to the

organ-specific metastases of breast cancer[7]. Kryczek *et al* showed that human ovarian epithelial tumor cells expressed high levels of SDF-1[17]. Some reports indicated that the expression of the chemokine SDF-1 was related to the malignant transformation of the ovarian surface epithelia[10, 11]. Moreover, recently, Jiang *et al* reported that CXCR4 could be an independent prognostic factor for epithelial ovarian cancer patients[18]. Nevertheless, the specific mechanism of ovarian cancer metastasis remains poorly understood.

Previous studies have shown that SDF-1 induced adhesion of most circulating lymphocytes and $CD34^+$ progenitor cells[19]. Binding of SDF-1 to CXCR4 has been shown to activate ERK and phosphatidylinositol 3-OH kinase (PI3K), induce mobilization of Ca^{2+} and phosphorylate focal adhesion components including Crk and paxillin in several cancer cell lines[20]. Integrin-mediated binding to ECM ligands is known to be up-regulated by chemokines or cytokines via intracellular activation pathways. Adhesion of lymphocytes, $CD34^+$ stems cells, as well as prostate carcinoma cells to ECM proteins or endothelia is stimulated by SDF-1, likely through activation of integrins or other cell surface molecules. ERK phosphorylation has been associated with cell adhesion and invasion via downstream signaling events, including phosphorylation of Ser/Thr residues on paxillin and activation of the myosin light chain. SDF-1 was recently found to up-regulate β1 integrin expression in ovarian carcinoma cells[11]. Brand *et al* demonstrated that CXCR4 activation results in the migration of intestinal epithelial cells, which could be blocked using MEK-1 inhibitors PD98059[21].

In this study, the intracellular calcium flux of ovarian cancer cells was increased with the concentration of SDF-1α. The capability of the ovarian cancer cell lines to elicit an intracellular calcium flux and to migrate in response to SDF-1α indicated the functionality of the CXCR4 cell surface receptors. The SDF-1/CXCR4 axis could have some effects.

Several research groups have shown that SDF-1 can activate MAP kinases such as ERK-1/2 that plays an important role in regulation of cell proliferation and migration[22]. The MAPK pathways are thought to be activated via PI3kγ and can lead to a variety of cell-type specific effects and gene induction. ERK1 and ERK2 are important components of this MAPK cascade. In this study, ERK phosphorylation of human ovarian cancer cells was found by Western blot after SDF-1α treatment, suggesting that the MAPK pathway was activated by SDF-1/CXCR4.

In addition, we examined the effects of SDF-1α on ovarian cancer cell adhesion to ECM proteins such as COL, FN and LN, which is an integral part of the metastasis process. Our results showed that the activation of ERK signaling pathways by SDF-1 led to the increased adhesion of ovarian cancer cells to FN and COL. Previously, it was shown that ovarian cancer chemotaxis toward SDF-1α is mediated by the ERK signaling pathway. Thus, we examined the role of ERK in SDF-1-mediated ovarian cancer cell adhesion. Blockage of MEK with PD98059 markedly decreased the SDF-1α-induced adhesion of ovarian cancer cells to FN and COL. These results suggested that SDF-1α binding to CXCR4 on ovarian cancer cells stimulated a signaling cascade involving ERK phosphorylation, which led to the increased cell adhesion and migration.

Many proteinases are capable of degrading ECM components. MMPs have extracellular matrix-degrading activity, and are involved in cancer progression. The increase in MMP activity contributes to the acquisition of malignant properties. SDF-1 has been shown to induce the secretion of latent MMPs in several cell types, including cancer cells[23]. Up-regulation of MMP secretion represents an important step in the ability for tumor cells to invade and metastasize.

We examined the effects of SDF-1 on MMP secretion in ovarian cancer cells, and found that active MMP-9 (92 kD) and MMP-2 (62 kD) existed in the supernatant of SKOV3 cells. We observed that SDF-1 treatment increased the release of MMP-9 and MMP-2. The active MMP-2 (62 kD), and MMP-9 (92 kD) were increased with the concentration of SDF-1α, as confirmed by densitometry. Moreover, when the concentration of SDF-1α was up to 400 ng/mL, the levels of active MMP-2 and MMP-9 reached the peak. The SDF-1α ligand promoted migration and adhesion processes of ovarian cancer cells. The expression of the chemokine receptor CXCR4 could drive the migration of tumor cells across lymphatic and vascular system corresponding to an active form of MMP-9 and MMP-2, as described by other authors[21].

The present study suggested that the SDF-1/CXCR4 axis plays a role in ovarian cancer tumor progression. SDF-1α may induce events as following: intracellular calcium mobilization; phosphorylation of mitogen-activated ERK1/2; adhesion to COL and FN; increased secretion of MMP-2 and MMP-9. The MEK inhibitor, PD98059, blocked, at least in part, the responses of SKOV3 cells to SDF-1α. In conclusion, SDF-1/CXCR4 biological axis could contribute to the metastasis of ovarian cancer cells by increasing the cell adhesion and secretion of MMPs by activating ERK-1/2 pathway.

REFERENCES

1 Balkwill F. The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol, 2004,14(3):171-179

2 Grunewald M, Avraham I, Dor Y, *et al*. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell, 2006,124(1):175-189

3 Katayama Y, Battista M, Kao WM, *et al*. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell, 2006,124(2): 407-421

4 Zou YR, Kottmann AH, Kuroda M, *et al*. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature, 1998,393(6685):595-599

5 Begley LA, MacDonald JW, Day ML, *et al*. CXCL12 activates a robust transcriptional response in human prostate epithelial cells. J Biol Chem, 2007,282(37): 26767-2674

6 Kucia M, Jankowski K, Reca R, *et al*. CXCR4-SDF-1 signaling, locomotion, chemotaxis and adhesion. J Mol Histol, 2004,35(3):233-245

7 Muller A, Homey B, Soto H, *et al*. Involvement of chemokine receptors in breast cancer metastasis. Nature, 2001,410(6824):50-56
8 Orimo A, Gupta PB, Sgroi DC, *et al*. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell, 2005,121(3):335-348
9 Marchesi F, Monti P, Leone BE, *et al*. Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. Cancer Res, 2004,64(22):8420-8427
10 Scotton CJ, Wilson JL, Milliken D, *et al*. Epithelial cancer cell migration: a role for chemokine receptors? Cancer Res, 2001,61(13):4961-4965
11 Scotton CJ, Wilson JL, Scott K, *et al*. Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Cancer Res, 2002,62(20):5930-5938
12 Porcile C, Bajetto A, Barbieri F, *et al*. Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation. Exp Cell Res, 2005,08(2):241-253
13 Zlotnik A. Chemokines in neoplastic progression. Semin Cancer Biol, 2004,14(3):181-185
14 Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer, 2002,2(8):563-572
15 Christopherson KW II, Hangoc G, Mantel CR, *et al*. Modulation of hematopoietic stem cell homing and engraftment by CD26. Science, 2004,305(5686):1000-1003
16 Chung LW. Prostate carcinoma bone-stroma interaction and its biologic and therapeutic implications. Cancer, 2003, 97(3):772-778
17 Kryczek I, Lange A, Mottram P, *et al*. CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. Cancer Res, 2005,65(2):465-472
18 Jiang YP, Wu XH, Shi B, *et al*. Expression of chemokine CXCL12 and its receptor CXCR4 in human epithelial ovarian cancer: an independent prognostic factor for tumor progression. Gynecol Oncol, 2006,103(1):226-233
19 Peled, A, Grabovsky V, Habler L, *et al.* The chemokine SDF-1 stimulates integrin-mediated arrest of CD34 (+) cells on vascular endothelium under shear flow. J Clin Invest, 1999,104(9):1199-1211
20 Murakami T, Maki W, Cardones AR, *et al*. Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. Cancer Res, 2002,62(24): 7328-7334
21 Brand S, Dambacher J, Beigel F, *et al*. CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. Exp Cell Res, 2005,310(1):117-130
22 Uchida D, Begum NM, Almofti A, *et al*. Possible role of stromal-cell-derived factor-1/CXCR4 signaling on lymph node metastasis of oral squamous cell carcinoma. Exp Cell Res, 2003,290(2):289-302
23 Yu X, Huang Y, Collin-Osdoby P, *et al*. Stromal cell-derived factor-1 (SDF-1) recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase-9 (MMP-9) activity, and collagen transmigration. J Bone Miner Res, 2003,18(8):1404-1418

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