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RESEARCH PAPER

Strong expression of chemokine receptor CXCR4 by renal cell carcinoma cells correlates with metastasis

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Abstract The chemokine receptor CXCR4 is involved in the metastasis of many cancers. Recent evidence suggests that CXCR4 may be also involved in the metastasis of renal cell carcinoma. We analyzed the expression of CXCR4 in primary carcinomas, metastatic tissues and normal tissues using immunohistochemistry. We further investigated the migration of renal carcinoma cells in response to stimulation by CXCL12 in vitro. We also studied the subcellular localization of CXCR4 in renal cell carcinoma cells in response to CXCL12 by confocal microscopy. We observed the highest percentage of CXCR4 expression in renal cell carcinoma metastases compared with that in renal cell carcinomas and normal renal tissues. We further found that CXCR4 was localized predominantly in the membrane in primary renal cell carcinomas and predominantly in the cytoplasm in renal cell carcinoma metastases. Moreover, we found that CXCR4 was translocated from the cytoplasmic membrane to the cytoplasm upon stimulation by its ligand CXCL12. Renal cell carcinoma metastasis was associated with higher expression of CXCR4 and interaction of CXCR4 and its ligand CXCL12 resulted in the internalization of CXCR4 from the cytoplasmic membrane. These findings implicate the CXCR4-CXCL12 axis in the metastasis of renal cell carcinoma.

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Introduction

Renal cell carcinoma accounts for 3% of all adult malignancies and is the most aggressive urologic cancer. More than, 40% of patients with renal cell carcinoma will die from the disease. Approximately, one-third of patients with renal cell carcinoma have tumor metastasis at the time of initial presentation, and as many as 40% of them eventually develop distant metastases [1, 2]. Metastasis is a highly organized, nonrandom and organ-selective process [3]. Mechanisms involved in the homing of leukocytes and hematopoietic progenitors may be appropriated for the dissemination of tumors via the bloodstream and lymphatics [4]. Although a number of molecules have been implicated in the metastasis of renal cell carcinoma, the precise mechanisms of the directional migration and invasion of renal cell carcinoma cells into specific organs remain undefined [5].

Chemokines are 8–10 kDa chemo-attractive cytokines that possess a wide range of biological activities, including induction of cell differentiation, proliferation and directional migration of several cell types [4, 6–8]. The chemokine receptor CXCR4 and its corresponding ligand CXCL12 (SDF-I, stromal derived factor-I) are involved in the metastasis of many cancers, such as breast cancer [9], epithelial ovarian cancer [10], and colorectal cancer [11]. Recent evidence also suggests that the CXCR4 and CXCL12 axis is involved in the metastasis of renal cell carcinoma [12].

In the current study, we examined the expression of CXCR4 in renal cell carcinoma samples, their metastases

and normal renal tissues. We also investigated the migration of renal cell carcinoma cells and the subcellular localization of CXCR4 in response to stimulation by CXCL12 in vitro. Our findings indicate that expression of CXCR4 in renal cell carcinoma is associated with renal cell carcinoma metastasis.

Materials and methods

Patients and tissue samples

Twenty patients, 13 males and 7 females with age ranging from 28 to 73, with conventional or clear-cell renal cell carcinoma were admitted into Department of Urology, Changhai Hospital, Shanghai, China, from October 01 to December 31, 2006. Fresh tissue specimens and adjacent normal tissues were collected immediately following radical nephrectomy and were formalin-fixed and paraffin-embedded. The tumors were staged according to the TNM staging system and the Fuhrman grading system. The marginal normal part tissue of surgically removed kidney specimen (approved by pathologists) was used as normal control. In addition, we also studied archived formalin-fixed and paraffin-embedded tumor specimens from 23 patients with renal cell carcinoma, 15 males and 8 females with age ranging from 21 to 72 years, who were admitted into our hospital from January 1, 2000 to December 31, 2005. Written informed consent was obtained from all patients who participated in the study. The study protocol was approved by the Institution Review Board, Changhai Hospital, Second Military Medical University, Shanghai, China.

Immunohistochemical staining

Immunohistochemical staining of fresh and archived tumor specimens and normal renal tissues was performed using anti-human CXCR4 antibody (Chemicon International, Temecula, CA) as previously described. The expression of CXCR4 was evaluated independently by two pathologists who were blind to the patients' clinicopathological data. The intensity of staining was scored semi-quantitatively as follows: +, weak; ++, moderate; +++, strong; ++++, very strong. Samples with a score of ++ and above were considered CXCR4-positive. Membrane and cytoplasmic staining were also recorded.

Cell migration assays

The clear-cell renal cell carcinoma-derived cell line A-498 (Cell Center, Beijing Institute of Basic Medical Sciences, Beijing, China) was used for cell migration assays. Atni-CXCR4 antibody and/or 200 ng/ml CXCL12β (SDF-1β; R & D Systems, Canandaigua, NY) were added to the lower chamber of 24-well transwell inserts (BD Biosciences, Bedford, MA) and 1×10^5 A-498 cells were loaded into the upper chamber and allowed to migrate for 22 h at 37°C. Cell migration was quantified by determining the number of A-498 cells that had migrated into the filters.

Confocal microscopy

For measurement of CXCR4 internalization, A-498 cells were seeded at a density of 1×10^4 cells per coverslip in a six-well slide chamber 24 h prior to serum-starvation. Following overnight serum starvation with 0.5% FBS, the cells were treated with CXCL12β at a concentration of 100 and 200 ng/ml at 37°C for 24 h. After removal of CXCL12β, the cells were fixed and immunostained with rabbit anti-CXCR4 antibody followed by incubation with FITC-conjugated anti-rabbit IgG (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The cells were then counterstained with iodized pyridine to visualize the nuclei. The immunofluorescence images were acquired using Leica TCS SP2 confocal software and a Leica confocal laser fluorescence scanning microscope.

Cancer cell isolation

Surgical removed renal cancer tissue was cut into small pieces and treated with predigestion solution (1 × HBSS containing 5 mM EDTA and 1 mM DTT) at 37°C for 30 min under slow rotation. The tissue was collected by centrifugation (×1,000 rpm, 10 min) and incubated in digestion solution (Dissolve 0.05 g of collagenase D, 0.05 g of DNase I and 0.3 g of dispase II in 100 ml of 1 times PBS) at 37°C for 60 min under slow rotation. Cells were filtered with a cell strainer using for further study. (Reagents were purchased from Sigma).

Flow cytometry

Cells were collected and incubated with fluorescently labeled primary antibodies on ice for 30 min (For the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permealization reagents for 30 min on ice). The stained cells were analyzed using a FACSarray (BD Bioscience, San Jose, CA). Data were analyzed with software FlowJo.

Western blotting

Total proteins were extracted from surgical removed renal cancer tissue. Equal amounts protein extracts of each sample were separated on a precast NuPAGE gel system

and blotted onto nitrocellulose membrane. The membranes were then blotted with primary antibodies against target proteins. The proteins were detected using horseradish peroxidase conjugated second antibodies. Horseradish peroxidase enzymatic reaction was detected with enhanced chemiluminescent reagents and recorded with X-ray films.

Statistical analysis

Statistical analysis was determined by one-way ANOVA and Chi-Square test, with the P value less than 0.05 regarded as statistically significant. Data are expressed as mean $\pm$ SD.

Results

We analyzed the expression of CXCR4 in 43 primary renal cell carcinomas and 21 metastases to the lymph nodes (14 cases), the adrenal glands (four cases), the bone (two cases) and the brain (one case; Table 1). Nineteen patients had synchronous metastases and two had metachronous metastases. Histological typing identified conventional renal cell carcinoma in 20 cases and papillary renal cell carcinoma in one case. Fuhrman grading further revealed grade 1 tumor in two cases, grade 2 tumor in seven cases, grade 3 tumor in nine cases, and grade 4 in three cases.

Renal cell carcinoma metastases were associated with higher levels of CXCR4 expression and exhibited predominantly cytoplasmic CXCR4 staining

Immunohistochemical staining of tumor specimens and normal renal tissues with anti-CXCR4 antibody revealed that the primary renal cell carcinoma samples and their metastases at the lymph node, the adrenal gland or the bone showed noticeably higher intensity of CXCR4 staining than normal renal tissues (Fig. 1). Tissue sections in which CXCR4 expression was $\geq 2+$ and $\geq 50\%$ of the cells were stained by the anti-CXCR4 antibody were defined as positive. We found that 81.40% of the primary renal cell carcinoma tissue samples were stained positive for CXCR4 while only 50% of normal renal tissues were CXCR4 positive (Table 2). However, the highest percentage of positive CXR4 staining was seen in the renal cell carcinoma metastases, which were 95.24% positive for CXCR4. These findings suggested that renal cell carcinoma metastases were associated with higher levels of CXCR4 expression. We further examined the expression patterns of CXCR4 in the renal cell carcinoma tissues and their metastases and found that CXCR4 was predominantly expressed in the cytoplasmic membranes of the primary renal cell carcinoma cells and predominantly expressed in the cytoplasm in the metastatic cells (Fig. 1). Altogether, 67% of the primary renal cell carcinoma samples displayed predominantly membranous staining while 81% of the metastatic carcinoma samples showed predominantly cytoplasmic staining (Table 3). Since the hematopoietic stem cells also express CXCR4, we further detected the phenotypes of $CXCR4^+$ cells from surgical removed renal cancer cells. The results showed very few dendritic cells, T cells or eosinophils were labeled by anti-CXCR4 antibody (Fig. 1b).

Considering if metastasis of cancer still kept the expression of CXCL12, we analyzed the CXCL12 protein in surgically removed renal cancer tissue. The results showed that although less CXCL12 protein was detected in metastasis cancer tissue, the difference did not reach significance (Fig. 1c).

CXCR4 mediated the migration of renal cell carcinoma cells A-498

We also tested the hypothesis that expression of CXCR4 was associated with renal cell carcinoma metastasis by measuring the migration of renal cell carcinoma cells A-498 in vitro using cell migration assays. We treated A-498 cells with CXCL12β, the only known physiological ligand for CXCR4.

Table 1 Source and characteristics of tissue specimens

	Total	Source		Sex		Age	
		Fresh tissues	Archived tissues	Male	Female	<50	≥50
Primary carcinoma	43	20	23	29	14	9	34
Metastatic carcinoma							
Lymph node metastases	14	0	14	5	9	4	10
Adrenal gland metastases	4	0	4	3	1	1	3
Back bone metastases	2	0	2	1	1	0	2
Brain metastases	1	0	1	1	0	0	1
Normal renal tissues	22	20	2	15	7	4	18
Total	86	40	46	54	32	18	68

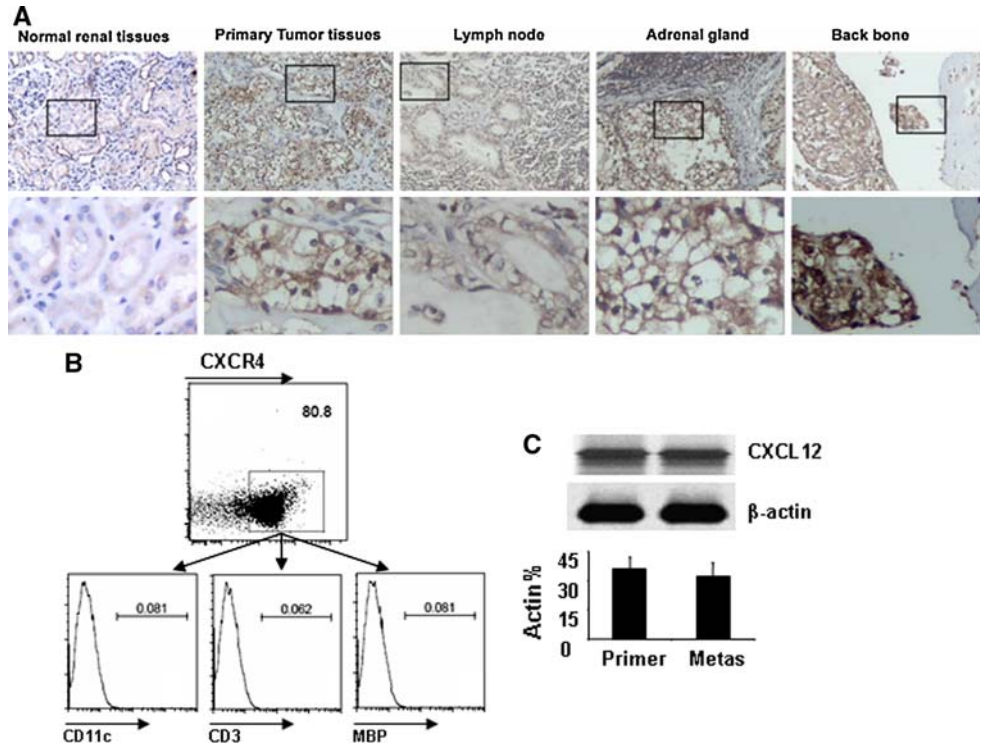

Fig. 1 Expression of CXCR4 and CXCL12 in renal cell carcinoma, metastases and normal renal tissues. **a** Sections of normal renal tissues, primary renal cell carcinoma tissues, and metastatic tissues including the lymph node, the adrenal gland and the bone were examined by immunohistochemical staining with anti-CXCR4 antibody. The images in the *lower panel* (×400) were from inserts in the images in the *upper panel* (×100). **b** Single cells were prepared from surgically removed renal cancer tissue and examined for CXCR4 expression by flow cytometry. Representative flow cytometry data show CXCR4 positive cells (the gated cell population) in *panel a*. The gated cells in panel were further analyzed for positive cells of dendritic cells (*b*), T cells (*c*) and eosinophils (*d*). **c** Renal cancer tissue protein extracts were analyzed for CXCL12 with Western blotting. The immune blots show CXCL12 in renal cancer tissue. *Bars* indicate the data densitometry analysis of the bands. Data were presented as mean ± SD, from 3 separate experiments

Table 2 Renal cancer cells CXCR4-staining (case number)

	−	+	++	+++	++++	Total
Primary	0	8 (18.6)*	29 (67.4)	4 (9.3)	2 (4.7)	43
Metastases	0	1 (4.8)	4 (19)	5 (23.8)	11 (52.4)	21
Normal	11	11 (50)	–	–	–	22

* Percentage $P = 0.001$ (χ^2 test). Primary: primary tumor, metastases: paired metastases, normal: normal renal tissues

Table 3 CXCR4 was localized predominantly in the cytoplasm in RCC metastases

	M > C[a]	C > M	*P* value
Primary carcinoma samples	29(67%)	14 (33%)	<0.0005
Metastases samples	4 (19%)	17 (81%)	

[a] *M* membranous CXCR4 staining; *C* cytoplasmic CXCR4 staining

We found that only 23.6 ± 5.6 cells migrated in the absence of CXCL12β (Fig. 2a). The addition of CXCL12β, however, led to an approximately fivefold increase in the chemotaxis of A-498 cells ($P < 0.001$). Moreover, this increased chemotaxis by CXCL12β was abolished by anti-CXCR4-antibody, indicating that CXCR4 mediated the enhanced chemotaxis of A-498 cells. Examination of the subcellular localization of CXCR4 revealed that, in the absence of its physiological ligand CXCL12β, CXCR4 remained localized on the cell membrane (Fig. 2b). However, with the addition of CXCL12β, CXCR4 translocated from the cell membrane to the cytoplasm.

Discussion

Renal cell carcinoma is among the most resistant of tumors to therapy. Renal cell carcinoma metastasis poses even greater challenges for treatment. Chemokines and their receptors are known to play an important role in leukocyte trafficking and homing, especially at sites of inflammation, infection, tissue injury and malignant tumor growth [10]. These proteins also regulate non-leukocyte cell functions such as angiogenesis, cell migration, activation of apoptotic cell death, and proliferation [10]. Chemokine receptor

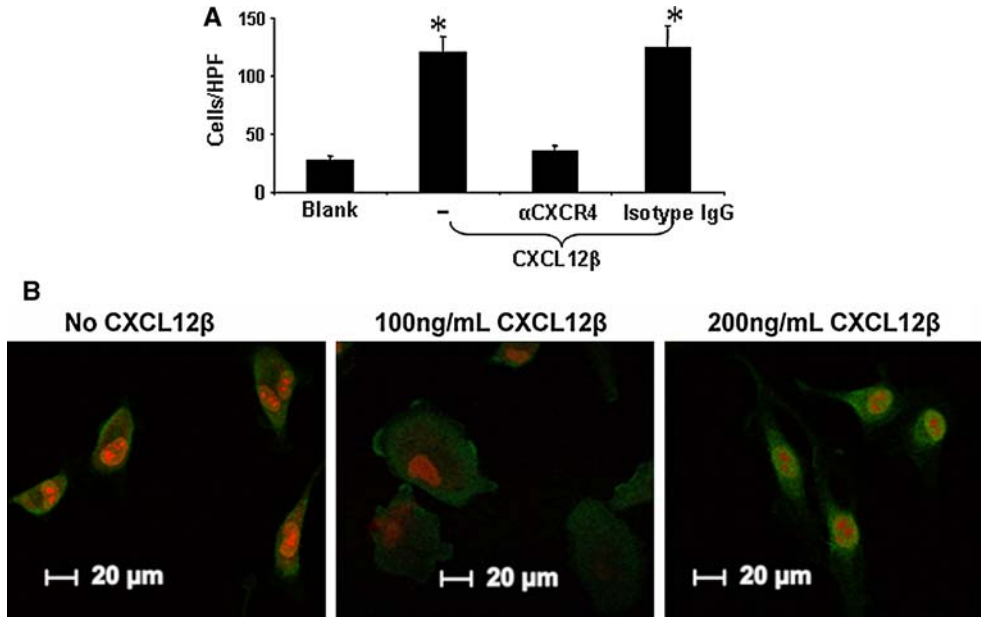

Fig. 2 **a** CXCL12β stimulated the migration of renal cell carcinoma cells A-498 in vitro. The migration of A-498 in response to medium, CXCL12β, anti-CXCR4 antibody, anti-CXCR4 antibody (αCXCR4) or isotype IgG was studied using a transwell system. The data was expressed as mean $\pm$ SD from six independent experiments. * $P < 0.05$ versus blank. **b** CXCL12β triggered the internalization of CXCR4 in renal cell carcinoma cells A-498 in vitro. A-498 cells were incubated with 0, 100 or 200 ng/ml CXCL12β for 24 h and subsequently immunostained with anti-CXCR4 antibody and FITC-conjugated antibody. Fluorescent images were obtained by a Leica confocal microscope. The nucleus (red) was visualized using iodized pyridine and CXCR4 was shown in green. Original magnification was 63 $\times$ 10 (Color figure online)

CXCR4 is constitutively expressed in bone marrow stromal cells as well as in various organs, including the brain, heart, lung, thymus, spleen, and liver. Moreover, CXCR4 and its physiological ligand CXCL12 have been implicated in stimulating the metastasis of many different neoplasms [13].

CXCR4 expression has been shown to be heterogeneous in malignant cells of different origin. It was reported to be markedly decreased in hepatocellular carcinomas [14] and increased in glioblastoma multiforme, breast and uterine cancer as well as Burkitt's lymphoma [15, 16]. The expression of CXCR4 was found to be significantly upregulated in malignant renal tissue while the expression of its ligand CXCL12α was markedly reduced compared to normal tissue [17]. Additionally, the CXCR4/CXCL12 biological axis could induce renal cell carcinoma metastasis [12]. Our results are in line with previous study [12] that renal cancer tissue had high expression of CXCR4 and CXCL12. It was also found that CXCL12 could induce the internalization of CXCR4 [18–21]. However, the distribution pattern of CXCR4 in primary renal cell carcinomas, metastases and normal renal tissues has not been previously elucidated.

The roles of CXCR4 in normal renal tissues have not yet been fully understood, but it appears that it mediates a large variety of functions including leukocyte development and trafficking, correct foetal vascularisation, cell division, tumourigenesis and growth. In the current study, we have found that both primary renal cell carcinomas and their metastases express higher levels of CXCR4 than normal renal tissues, suggesting a possible role of CXCR4 in mediating metastasis of renal cell carcinoma. Additionally, CXCR4 was found to be localized both in the cytoplasm and the nucleus in non-small-cell lung cancer [22]. We have also found that CXCR4 is localized in different parts of the renal cell carcinoma cells, including the membrane, the cytoplasm and the nucleus. We have further found that CXCR4 is predominantly localized in the cytoplasmic membrane in the primary renal cell carcinoma cells, but mainly in the cytoplasm and the nucleus in the renal cell carcinoma metastases. We have also demonstrated that CXCR4 is translocated from the cytoplasmic membrane in response to CXCL12 in renal cell carcinoma cells A-498. This is similar to the epidermal growth factor receptor, which, upon binding to the epidermal growth factor, translocates from the cytoplasmic membrane to the nucleus [23].

CXCR4 has been implicated in the metastasis of many cancers and the CXCR4/CXCL12 axis was also shown to be involved in renal cell carcinoma metastasis. The up-regulation of CXCR4 especially in metastatic renal cells may involve many different mechanisms; one of those has been reported is the CXCR4 promoter, which has been identified as a candidate for renal cell carcinoma targeting [24]. We have shown that renal cell carcinoma cells A-498

exhibit higher chemotaxis in response to CXCL12, which, however, can be abolished by anti-CXCR4 antibody. Together with our findings that renal cell carcinoma metastases exhibit a more extensive expression of CXCR4 and show predominantly cytoplasmic and nuclear expression of CXCR4, this result indicates that CXCR4 is intimately involved in the metastasis of renal cell carcinoma. Our results lend further support to the proposition that targeting CXCR4 could inhibit the metastatic progression of renal cell carcinomas [12], in which the CXCL12-CXCR4 pathway might be an interesting therapeutic target, CXCL12 as a promising molecule may be useful for the presensibilisation of tumor cells in a combination-therapy of renal cell carcinomas [25].

In conclusion, we show here that strong expression of CXCR4 in renal cell carcinomas correlates with metastasis, which exhibits predominantly cytoplasmic and nuclear expression of CXCR4. We further show that CXCL12 triggers the migration of renal carcinoma cells in vitro and the internalization of CXCR4. Our study suggests that analysis of the subcellular localization and expression of CXCR4 staining in primary tumors and metastases may enhance our ability to predict the biologic behavior of renal cancer cells and to develop novel strategies to prevent metastases.

References

1. Landis SH et al (1999) Cancer statistics. CA Cancer J Clin 49(1):8–31
2. Jones J, Libermann TA (2007) Genomics of renal cell cancer: the biology behind and the therapy ahead. Clin Cancer Res 1(2 Pt 2):685s–692s
3. Nicolson GL (1993) Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. Cancer Metastasis Rev 12(3–4): 325–343
4. Kucia M et al (2004) CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. J Mol Histol 35(3):233–245
5. Cohen HT, McGovern FJ (2005) Renal-cell carcinoma. N Engl J Med 353(23):2477–2490
6. Butcher EC et al (1999) Lymphocyte trafficking and regional immunity. Adv Immunol 72:209–253
7. Campbell JJ, Butcher EC (2000) Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. Curr Opin Immunol 12(3):336–341
8. Zlotnik A, Yoshie O (2000) Chemokines: a new classification system and their role in immunity. Immunity 12(2):121–127
9. Muller A et al (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410(6824):50–56
10. Jiang SH et al (2008) Alternate arrangement of PpL B3 domain and SpA D domain creates synergistic double-site binding to VH3 and Vkappa regions of fab. DNA Cell Biol 27(8):423–431
11. Brand S et al (2005) CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. Exp Cell Res 310(1): 117–130
12. Struckmann K et al (2008) pVHL co-ordinately regulates CXCR4/CXCL12 and MMP2/MMP9 expression in human clear-cell renal cell carcinoma. J Pathol 214(4):464–471
13. Murphy PM (2001) Chemokines and the molecular basis of cancer metastasis. N Engl J Med 345(11):833–835
14. Begum NA et al (1999) Reduced expression of the CXCR4 receptor mRNA in hepatocellular carcinoma and lack of inducibility of its ligand alpha-chemokine hIRH/SDF1alpha/PBSF in vitro. Int J Oncol 14(5):927–934
15. Sehgal A et al (1998) CXCR-4, a chemokine receptor, is overexpressed in and required for proliferation of glioblastoma tumor cells. J Surg Oncol 69(2):99–104
16. Rempel SA et al (2000) Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. Clin Cancer Res 6(1):102–111
17. Schrader AJ et al (2002) CXCR4/CXCL12 expression and signalling in kidney cancer. Br J Cancer 86(8):1250–1256
18. Signoret N et al (1998) Differential regulation of CXCR4 and CCR5 endocytosis. J Cell Sci 111(Pt 18):2819–2830
19. Kraitchman DL et al (2005) Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. Circulation 112(10):1451–1461
20. Nagasawa T, Kikutani H, Kishimoto T (1994) Molecular cloning and structure of a pre-B-cell growth-stimulating factor. Proc Natl Acad Sci USA 91(6):2305–2309
21. Signoret N et al (1997) Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. J Cell Biol 139(3):651–664
22. Spano JP et al (2004) Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome. Ann Oncol 15(4):613–617
23. Pleass RJ et al (2001) Streptococcal IgA-binding proteins bind in the Calpha 2-Calpha 3 interdomain region and inhibit binding of IgA to human CD89. J Biol Chem 276(11):8197–8204
24. Haviv YS et al (2004) Transcriptional targeting in renal cancer cell lines via the human CXCR4 promoter. Mol Cancer Ther 3(6):687–691
25. Schrader AJ et al (2002) CXCR4/CXCL12 expression and signalling in kidney cancer. Br J Cancer 86:1250–1256