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ORIGINAL ARTICLE

Involvement of matrix metalloproteinase-13 in stromal-cell-derived factor 1α-directed invasion of human basal cell carcinoma cells

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Basal cell carcinoma (BCC) is one of the most common skin neoplasms in humans and is usually characterized by local aggressiveness with little metastatic potential, although deep invasion, recurrence, and regional and distant metastases may occur. Here, we studied the mechanism of BCC invasion. We found that human BCC tissues and a BCC cell line had significant expression of CXCR4, which was higher in invasive than non-invasive BCC types. Further, of 19 recurrent tumors among 390 BCCs diagnosed during the past 12 years, 17/19 (89.5%) had high CXCR4 expression. We found that the CXCR4 ligand, stromal-cell-derived factor 1α (SDF-1α), directed BCC invasion and that this was mediated by time-dependent upregulation of mRNA expression and gelatinase activity of matrix metalloproteinase-13 (MMP-13). The transcriptional regulation of MMP-13 by SDF-1α was mediated by phosphorylation of extracellular signal-related kinase 1/2 and activation of the AP-1 component c-Jun. Finally, CXCR4-transfected BCC cells injected into nude mice induced aggressive BCCs that co-expressed CXCR4 and MMP-13. The identification of SDF-1α/CXCR4 as an important factor in BCC invasiveness may contribute insight into mechanisms involved in the aggressive potential of human BCC and may improve therapy for invasive BCCs.
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Keywords: collagenase-3; CXCR4; basal cell carcinoma; invasion; MMP-13; SDF-1α

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Introduction

Basal cell carcinoma (BCC) is one of the most common skin neoplasms in humans and is characterized by local aggressiveness and little metastatic potential (Lear *et al*., 1998), although BCC may behave aggressively with deep invasion, recurrence, and regional and distant metastasis (Walling *et al*., 2004). BCC can be classified as superficial, nodular, micronodular, infiltrative (including morpheaform) or mixed type in terms of histological growth pattern (Rippey, 1998). The latter three BCC types have been classified as aggressive because they more frequently exhibit deep invasion (Rippey, 1998; Walling *et al*., 2004).

The invasion of tumor cells is a complex, multistage process. Invading cells must change cell–cell adhesion, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons to facilitate cell motility (Woodhouse *et al*., 1997). Matrix metalloproteinases (MMPs) have important roles in these processes because their proteolytic activities assist in degradation of ECM and basement membrane (Egeblad and Werb, 2002; Kerkelä and Saarialho-Kere, 2003). Previous studies have shown that human BCC epithelium expresses MMP-3, MMP-7, MMP-10, MMP-12 and MMP-13 (Kerkelä and Saarialho-Kere, 2003). Of these MMPs, MMP-13 (collagenase-3) has been found to play a role in the ECM degradation associated with malignant epithelial growth in skin carcinogenesis (Airola *et al*., 1997; Pendás *et al*., 2000).

MMPs, cytokines, growth factors and chemokines have been shown to regulate tumor cell invasion through autocrine or paracrine pathways (Woodhouse *et al*., 1997). Chemokines are structurally related, small (8–14 kDa) polypeptide signaling molecules that bind to and activate a family of seven-transmembrane G-protein-coupled receptors, the chemokine receptors (Murphy, 1996; Zlotnik and Yoshie, 2000). Chemokines promote mitosis, modulate apoptosis, survival and angiogenesis, and are expressed by many tumor types (Zhou *et al*., 2002; Burger and Kipps, 2006). 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, breast cancer, melanoma, glioblastoma, pancreatic cancer, cholangiocarcinoma and BCC (Müller *et al*., 2001; Strieter, 2001; Bachelder *et al*., 2002; Kijima *et al*., 2002; Payne and Cornelius, 2002; Barbero *et al*., 2003; Neuhaus *et al*., 2003; Phillips *et al*., 2003; Fernandis *et al*., 2004; Bartolomé *et al*., 2004; Saur *et al*., 2005; Ohira *et al*., 2006; Chen *et al*., 2006). Although the mechanisms underlying SDF-1α/CXCR4-mediated tumor invasion have been studied in some cancers (Bachelder *et al*., 2002; Fernandis *et al*., 2004; Bartolomé *et al*., 2004, 2006; Brand *et al*., 2005; Zhang *et al*., 2005; Menu *et al*., 2006), the role of SDF-1α/CXCR4 in BCC invasiveness remains elusive.

This study aimed to examine the role of SDF-1α/CXCR4 in BCC invasion and elucidate the underlying mechanism. As a model for invasion, we tested a human BCC cell line, developed in our laboratory, in the Boyden chamber assay.

Results

Human BCCs express CXCR4 but not its ligand, SDF-1α

Immunohistochemical examination of human BCC tissues demonstrated significant CXCR4 cell-surface expression but no SDF-1α expression (Figure 1A). SDF-1α expression was found in the infiltrating mononuclear cells and dermal fibroblasts, suggesting a paracrine effect between BCC and stromal tissues. The human BCC cell line expressed both CXCR4 mRNA and protein without evident expression of its ligand, SDF-1α (Figure 1B and C). In contrast, human dermal fibroblasts had significant expression of SDF-1α (Figure 1C). CXCR4 surface expression by human BCC cells was also confirmed by flow cytometry analysis (Supplementary Figure 1).

Correlation of CXCR4 expression and aggressiveness of human BCC tumors

To examine the biological significance of CXCR4, we studied its expression in benign human skin tumors as well as non-invasive and invasive human BCCs. We also

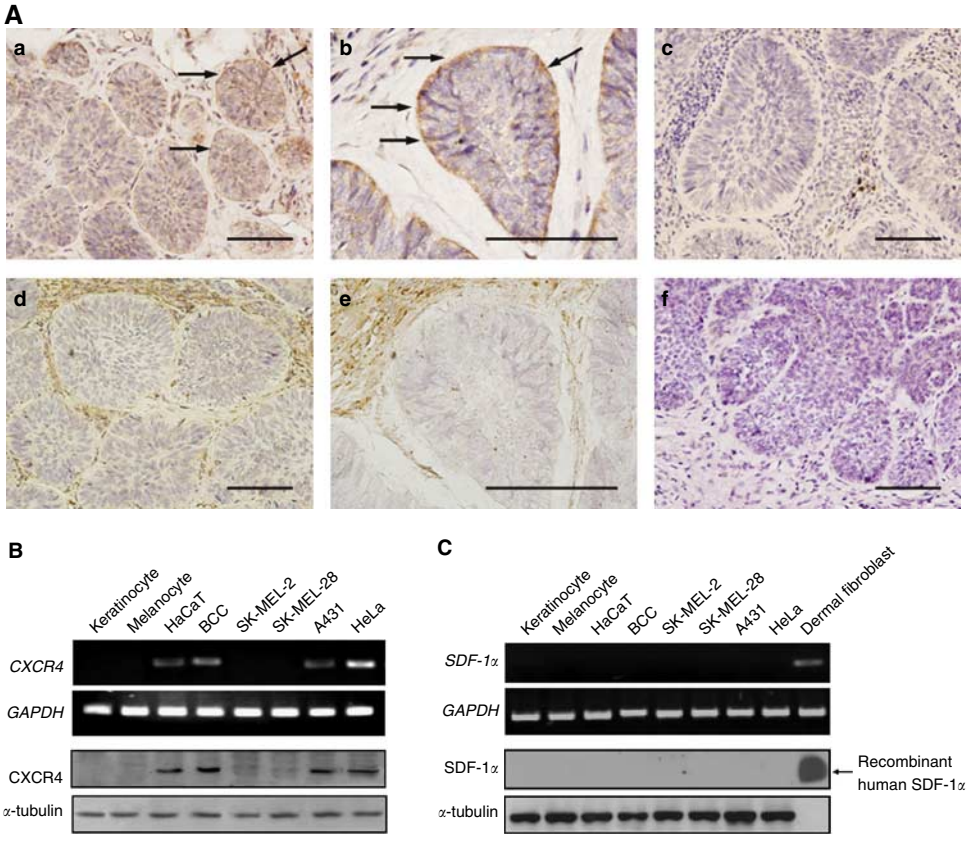


Figure 1 Human BCCs express CXCR4 but not SDF-1α. (**A**) Immunohistochemical examination of BCCs from patients showed cytoplasmic (a) and/or cell surface (b) expression of CXCR4 in tumor cells (arrows) and in infiltrating lymphocytes. (c) A nodular BCC expressed no CXCR4 expression. (d, e) SDF-1α was not expressed in BCCs but was expressed in infiltrating mononuclear cells and dermal fibroblasts surrounding the tumors. (f) Isotype control did not stain for CXCR4. Bar, 10 μM. (**B**, **C**) RT–PCR (in upper two panels) and Western blot analyses (in lower two panels) of (**B**) CXCR4 and (**C**) SDF-1α expression in various human cells showed expression of CXCR4 but not SDF-1α in the BCC cell line. Positive controls were (**B**) HeLa cells, (**C**, upper two panels) dermal fibroblasts and (**C**, lower two panels) recombinant human SDF-1α.

Table 1 Immunohistochemical analysis of the CXCR4 expression in benign skin lesions, and non-invasive, invasive and recurrent BCCs

Tissue	*Tumor type*	*CXCR4 expression (no. of lesions/tumors (%))*		
		Negative	+	+ + – + + +
Seborrheic keratosis ($n=10$)	N/A	10 (100)	0 (0)	0 (0)
BCC, non-invasive ($n=31$)		16 (51.6)	3 (9.7)	12 (38.7)*
	Nodular	10 (50)	1 (5)	9 (45)
	Superficial	5 (50)	2 (20)	3 (30)
	Metatypical	1 (100)	0 (0)	0 (0)
BCC, invasive ($n=11$)		0 (0)	3 (27.3)	8 (72.7)*
	Micronodular	0 (0)	1 (33.3)	2 (66.7)
	Infiltrative (including morpheaform)	0 (0)	2 (40)	3 (60)
	Mixed[a]	0 (0)	0 (0)	3 (100)
BCC, recurrent ($n=19$)		2 (10.5)	0 (0)	17 (89.5)
	Nodular	0 (0)	0 (0)	2 (100)
	Micronodular	1 (25)	0 (0)	3 (75)
	Infiltrative (including morpheaform)	1 (25)	0 (0)	3 (75)
	Mixed[a]	0 (0)	0 (0)	9 (100)

Abbreviations: BCC, basal cell carcinoma; N/A, not applicable. Lesions and tumors were diagnosed in and removed from patients in 2004, except for the recurrent BCCs. The recurrent BCCs were found among patients whose original BCCs ($n=390$) were surgically removed at National Taiwan University Hospital during 1993–2004. [a]Mixed tumor type includes combined nodular and infiltrative types or nodular and micronodular types. *$P<0.05$, χ^2 test for comparison of high CXCR4 expression between non-invasive and invasive BCCs.

collected 19 recurrent BCCs from 390 human BCCs diagnosed during the past 12 years. None of the 10 seborrheic keratosis lesions had CXCR4 expression, whereas 12/31 (38.7%) of non-invasive BCCs, 8/11 (72.7%) of invasive BCCs and 17/19 (89.5%) of recurrent BCCs had high CXCR4 expression (Table 1). The rate of high CXCR4 expression was significantly higher in invasive histological types (micronodular, infiltrative and mixed) of BCC compared to non-invasive types (72.7 vs 38.7%, $P<0.05$), indicating that CXCR4 may be involved in BCC invasiveness.

SDF-1α/CXCR4 interaction directs chemoinvasion of BCC cells

The importance of the interaction between SDF-1α and CXCR4 for BCC invasiveness was examined using the Boyden chamber assay with correction of SDF-1α-induced proliferation effects on human BCC cells (Chen *et al*., 2006, Supplementary Information and Supplementary Figure 2). SDF-1α dose-dependently directed BCC chemoinvasion (Supplementary Figure 3), which could be inhibited by pretreatment of cells with the CXCR4-neutralizing antibody (12G5) or a specific inhibitor, AnorMED (AMD) 3100 (Figure 2a). In addition, a CXCR4 highly expressing cell line exhibited an increase in chemoinvasive ability compared to control cells (Figure 2b). Transient transfection of small interfering RNA (siRNA) against *CXCR4*, but not a mutant form of siCXCR4 (siCXCR4-mut), effectively inhibited BCC chemoinvasion directed by SDF-1α (Figure 2c).

SDF-1α/CXCR4-directed BCC chemoinvasion involves MMP-13

Previous studies have shown significant expression of MMP-3, -7, -10 and -13 in BCCs (Kerkelä and Saarialho-Kere, 2003). We, therefore, hypothesized that any of these BCC-associated MMPs may be involved in SDF-1α/CXCR4-directed BCC chemoinvasion. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis showed that SDF-1α-induced mRNA expression of *MMP-13* only, starting at 2 h and peaking at 12 h (Figure 3a). MMP-2 and MMP-9 were undetectable in BCC cells (Supplementary Figure 4). SDF-1α further increased both the 59 kDa pro-form and the active 48 kDa form of MMP13 in BCC cells in a time-dependent manner (Figure 3b). Active MMP-13 was also increased in the supernatant, and its enzyme activity was upregulated at 4 h and peaked at 12 h. *MMP-13* transcription and gelatinase activity was abolished by SDF-1α inhibitors, including AMD 3100, 12G5 and siCXCR4, whereas a control antibody and siCXCR4-mut had no effect (Figure 3c), confirming SDF-1α involvement in MMP-13 regulation.

SDF-1α/CXCR4-directed BCC chemoinvasion was significantly inhibited by a selective MMP-13 inhibitor, CL82198 (100 μM; Figure 3d) and transiently transfected siMMP13 but not by the control small interfering glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Figure 3e), demonstrating MMP-13's role in this process.

ERK1/2 and AP-1 signaling pathways are involved in SDF-1α-mediated MMP-13 upregulation and BCC chemoinvasion

As SDF-1α/CXCR4 interaction has been shown to activate several signaling pathways, including phosphatidylinositol 3-kinase/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK), in various cancer cell lines (Kijima *et al*., 2002; Barbero *et al*., 2003; Phillips *et al*., 2003; Fernandis *et al*., 2004), we performed Western blot analysis to elucidate the

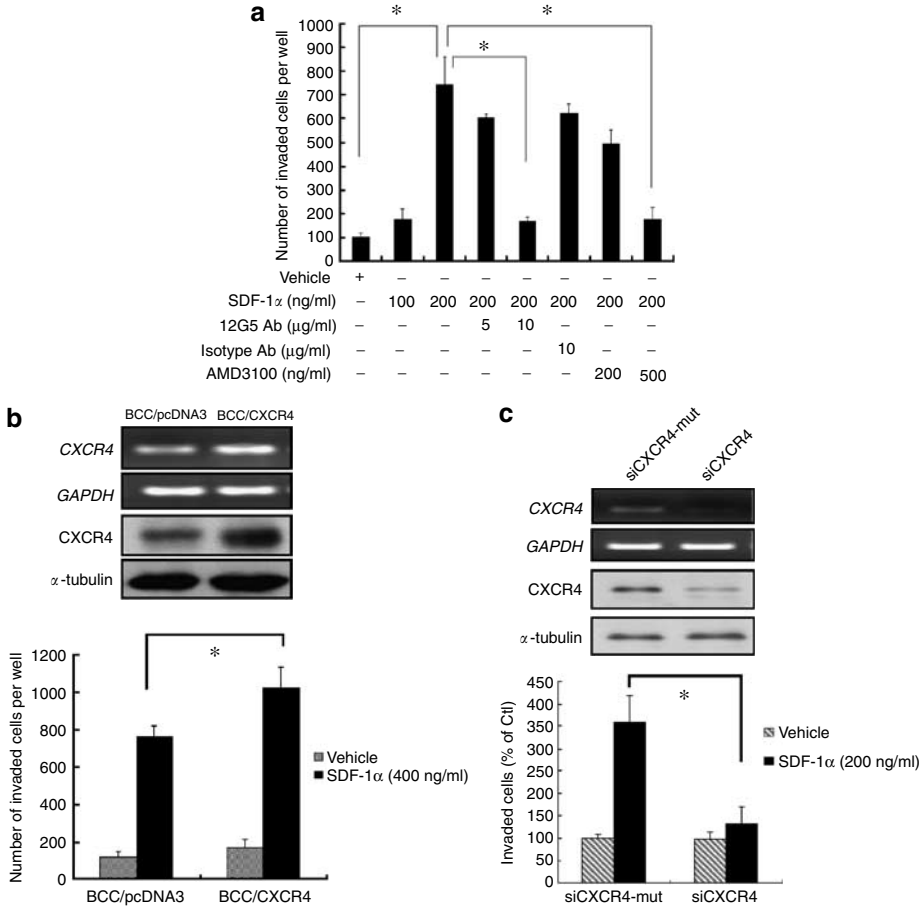


Figure 2 SDF-1α/CXCR4-directed chemoinvasion of human BCC cells. (**a**) SDF-1α (200 ng/ml) induced BCC chemoinvasion, which was inhibited by 30-min pretreatment of cells with the CXCR4-neutralizing 12G5 antibody (10 μg/ml) or AMD 3100 (500 ng/ml) but not the isotype control antibody (10 μg/ml) (*$P<0.01$, Student's *t*-test). (**b**) SDF-1α (400 ng/ml) increased more strongly the chemoinvasion of BCC/CXCR4 cells than that of BCC/pcDNA3 control cells (*$P<0.05$, Student's *t*-test). (**c**) SDF-1α-directed chemoinvasion was decreased by *CXCR4* gene silencing but was unaffected by siCXCR4-mut. Invaded cells are shown as percentage of vehicle control cells (*$P<0.05$, Student's *t*-test). Data are shown as mean ± s.d. of three independent experiments.

signal-transduction mechanisms involved in the SDF-1α-induced upregulation of MMP-13. SDF-1α activated the extracellular signal-related kinase 1/2 (ERK1/2) pathway in BCC cells, as evidenced by the increase in phosphorylated p42 and p44 (p-ERK1/2) at 15 min, which then rapidly declined to basal levels (Figure 4A). Other signaling pathways including c-Jun NH_2-terminal kinase (JNK), p38 MAPK and Akt were not activated up to 4 h after treatment. SDF-1α-induced mRNA expression and gelatinase activity of MMP-13 were greatly reduced by treatment with PD98059, a specific ERK inhibitor, but was not affected by either SP600125 (a JNK inhibitor) or SB203580 (a p38 MAPK inhibitor; Figure 4B).

Because the promoter region of human MMP-13 contains an AP-1 binding site and phosphorylation of ERK can lead to AP-1 activation (Eferl and Wagner, 2003; Ala-aho and Kähäri, 2005), we further examined activation of the AP-1 component c-Jun after SDF-1α treatment. SDF-1α-activated c-Jun was evidenced by nuclear accumulation of phosphorylated c-Jun (p-c-Jun) in the nucleus, starting at 60 min and peaking at 120–240 min (Figure 4C). The SDF-1α-induced c-Jun activation was inhibited by PD98059 but not SP600125 or SB203580 (Figure 4D). SDF-1α-induced mRNA expression and gelatinase activity of MMP-13 were inhibited dose-dependently by c-*jun* antisense oligonucleotides but not by control c-*jun* sense oligonucleotides (Figure 4E).

SDF-1α/CRCX4-directed BCC chemoinvasion was effectively inhibited by PD98059 and c-*jun* antisense oligonucleotides (3 μg), but not by SB203580 or c-*jun* sense oligonucleotides, whereas the inhibitory effect of SP600125 was smaller than that of PD98059 (Figure 4F).

After confirming the involvement of c-Jun, we wanted to assess the importance of the AP-1 binding site in SDF-1α-induced MMP-13 upregulation. Hereto, we used four different human MMP-13 promoter constructs: p182 contained the osteoblast-specific element 2 (OSE-2), polyomavirus enhancer A-binding protein-3 (PEA-3) and AP-1 sites; p83 had PEA-3 and AP-1 sites; p56 contained the AP-1 site only; and p56-mut contained a double mutation in the AP-1 consensus sequence. After SDF-1α treatment, p56, p83 and p182

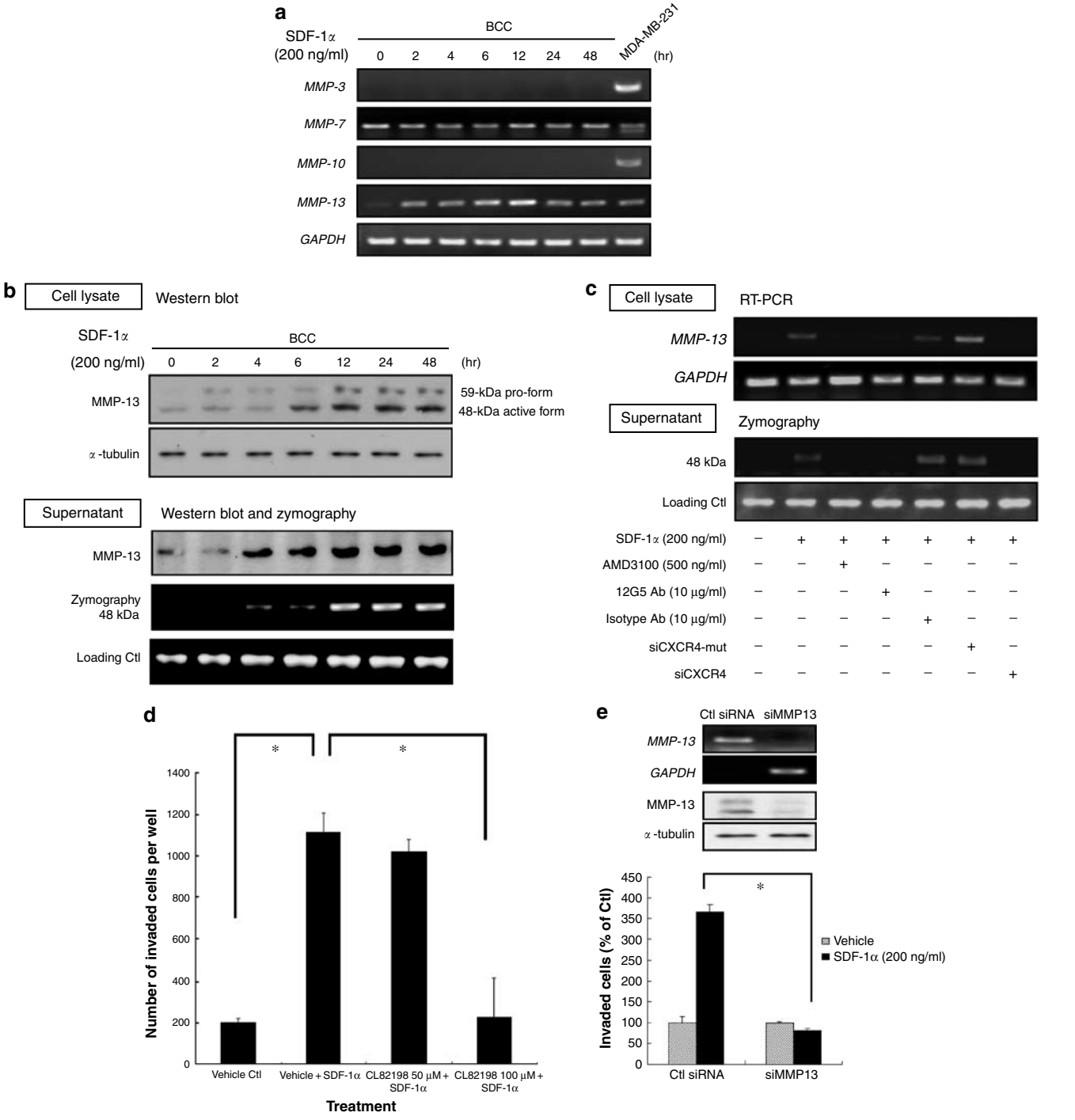
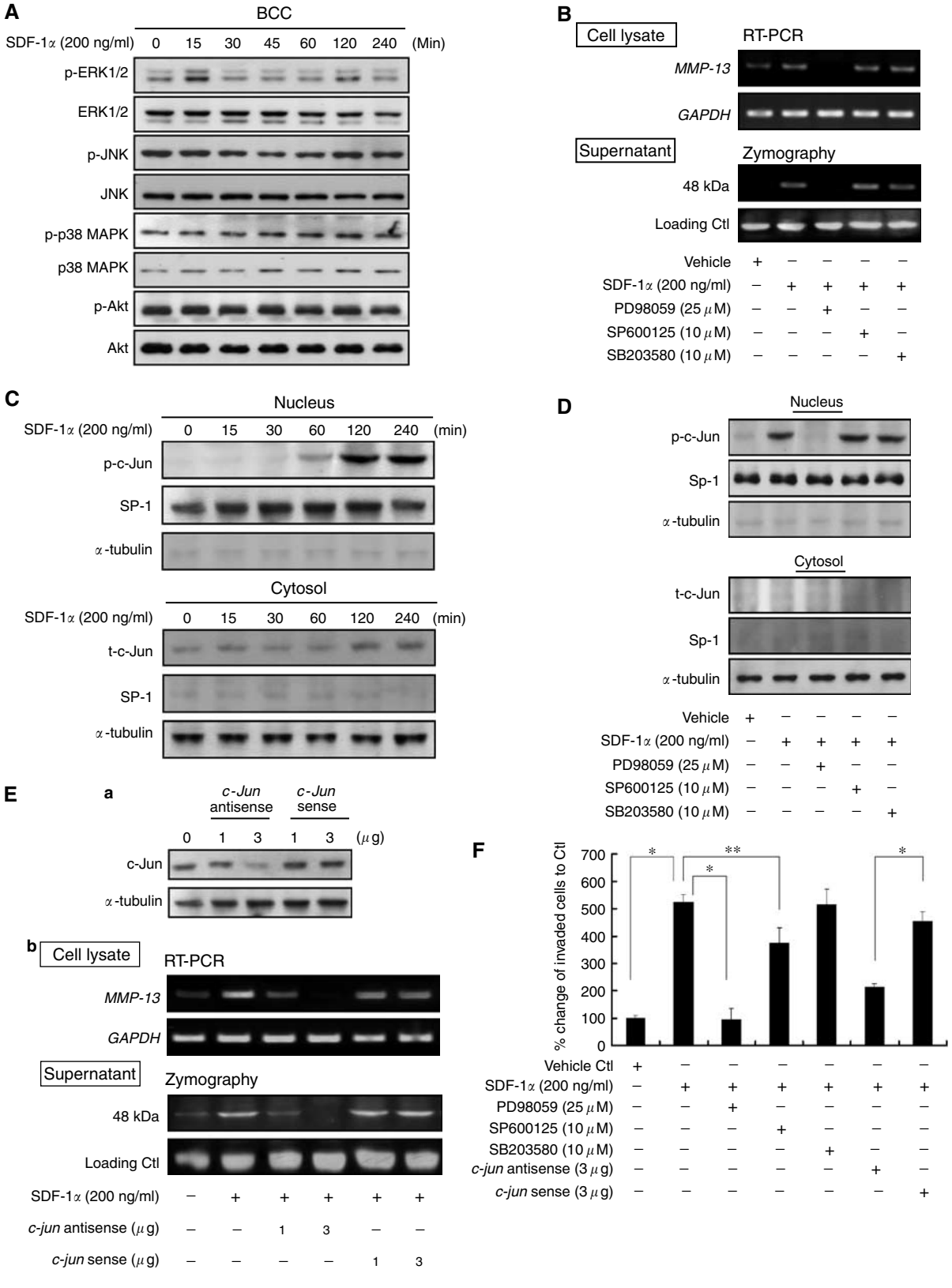


Figure 3 SDF-1α/CXCR4-directed chemoinvasion of human BCC cells involves upregulation of MMP-13. (**a**) RT–PCR analysis of MMP-3, 7, 10 and 13 in human BCC cells showed that SDF-1α increased mRNA expression of MMP-13 only. (**b**) Western blot analysis found upregulation of the 59-kDa pro-form (at 12 h) and the active 48-kDa form (at 6 h) of MMP-13 in the lysate of SDF-1α-treated BCC cells. Western blot analysis and zymography of the supernatant of these cells revealed increased secretion of active MMP-13 and gelatinase activity at 4 h after treatment, with a peak at 12 h. Data shown are representative of three independent experiments. (**c**) RT–PCR analysis and zymography showed that SDF-1α-induced mRNA expression and gelatinase activity of MMP-13 in human BCC cells were inhibited by 12G5 (10 μg/ml), AMD 3100 (500 ng/ml) and siCXCR4, but not by the isotype control antibody (10 μg/ml) or siCXCR4-mut. (**d**, **e**) SDF-1α-directed BCC chemoinvasion was inhibited dose dependently by selective MMP-13 inhibitor, CL82198 (**d**) and by *MMP-13* gene silencing (**e**), but was unaffected by the control GAPDH siRNA (Ctl siRNA). The relative number of invading cells is shown as percentage of control cells. Data are shown as mean ± s.d. of three independent experiments (*$P<0.05$, Student's *t*-test).

stimulated luciferase activity 4.2-, 3.6- and 4.8-fold, respectively (Figure 5a). In contrast, p56-mut inhibited luciferase activity (0.6-fold change from basal levels). Of the MMP-13 promoter sequences studied, only those containing the AP-1 site permitted luciferase activity stimulation, thus confirming the involvement of the AP-1 site in SDF-1α-upregulated MMP-13 transcription. Furthermore, SDF-1α-stimulated luciferase



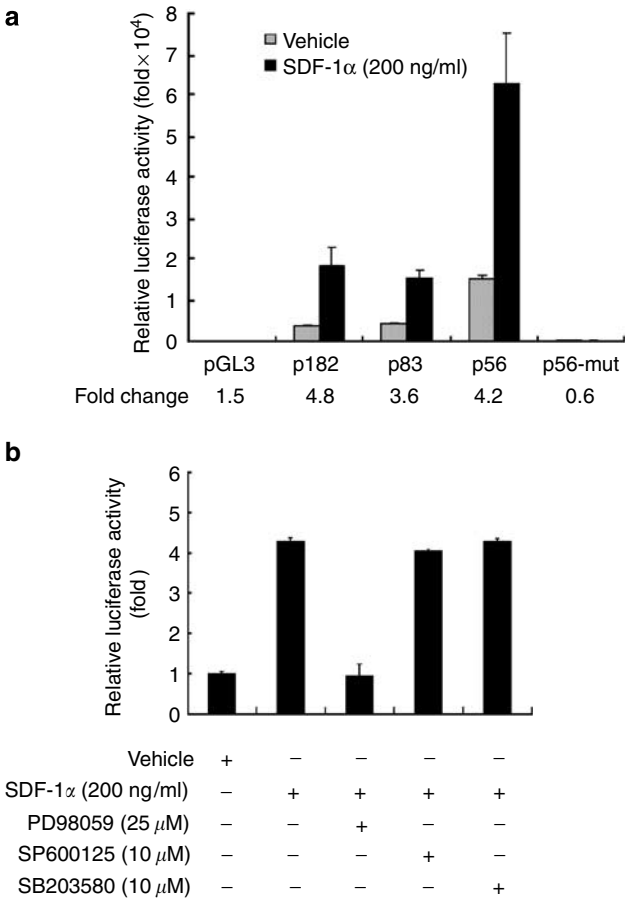


Figure 5 SDF-1α induces MMP-13 upregulation through AP-1 site. (**a**) Human BCC cells were co-transfected with pGL3 (control) or one of several pGL3-based MMP-13 promoter constructs along with, as an internal control of transfection efficiency, pRL-TK containing the renilla luciferase gene. The promoter activities were calculated as the firefly-renilla luciferase activity ratios and normalized to the control. In BCC cells transfected with an MMP-13 promoter construct containing the AP1 site only (p56), AP-1 and PEA-3 sites (p83), or AP-1, PEA-3 and OSE-1 sites (p182), SDF-1α after 4 h markedly stimulated luciferase activity relative to basal levels (vehicle). Mutation of the AP-1 site in the MMP-13 promoter (p56-mut) dramatically decreased its inducibility by SDF-1α. (**b**) In BCC cells transfected with p56, SDF-1α-stimulated luciferase activity was inhibited by PD98059 (25 μM) but not SP600125 (10 μM) or SB203580 (10 μM). The promoter activities were calculated as the firefly-renilla luciferase activity ratios and normalized to the control (vehicle). Data are shown as mean ± s.d. of three independent experiments.

activity was inhibited by PD98059 (25 μM) but not SP600125 (10 μM) or SB203580 (10 μM) in BCC cells transfected with p56 (Figure 5b), which confirmed that activation of ERK1/2 is involved in SDF-1α-induced MMP-13 promoter activity.

BCC/CXCR4 cells have higher tumorigenic potential than BCC/pcDNA3 cells

Animal studies confirmed that BCC/CXCR4 cells are more tumorigenic and developed more invasive tumors than control BCC/pcDNA3 cells (Supplementary Information and Supplementary Figure 5).

Co-expression of CXCR4 and MMP-13 in BCC xenografts and human BCCs

See Supplementary Figure 6.

Discussion

Although CXCR4 expression was shown in a previous study to enhance tumorigenesis and angiogenesis of BCC, its role in BCC invasion was not elucidated (Chen *et al*., 2006). This study showed for the first time that the SDF-1α/CXCR4 interaction mediates invasion of BCC. We found that human BCCs express CXCR4 but not SDF-1α, whereas SDF-1α is expressed by stromal cells. One of the mechanisms underlying SDF-1α-CXCR4 directed chemoinvasion was transcriptional upregulation of MMP-13 and activation of ERK and AP-1 pathways.

Human skin has increased expression of SDF-1α during wound healing (Avniel *et al*., 2006). As the stromal reactions surrounding neoplasms mimic a wound healing process (Mueller and Fusenig, 2004), the secretion of SDF-1α by surrounding fibroblasts might contribute to the progression of tumor. The identification of SDF-1α as a potential paracrine factor that can mediate BCC cell invasion may contribute to better knowledge of the mechanisms involved in the aggressiveness of human BCC, as well as a potential therapeutic approach of aggressive BCC.

MMP-13 expression has been detected in several pathologic conditions that are characterized by the destruction of normal collagen tissue architecture (Ala-aho and Kähäri, 2005). In cancer cells, the constitutive

Figure 4 Signal-transduction mechanisms involved in SDF-1α-mediated MMP-13 upregulation in human BCC cells. (**A**) SDF-1α-induced transient upregulation of ERK1/2 phosphorylation, which peaked at 15 min, without significant changes of JNK, p38 MAPK or Akt phosphorylation. (**B**) SDF-1α-induced upregulation of mRNA expression and gelatinase activity of MMP-13 in human BCC cells was abolished by MEK inhibitor PD98059 (25 μM), but not by JNK inhibitor SP600125 (10 μM) or p38 MAPK inhibitor SB203580 (10 μM). (**C**) Nuclear and cytosolic extracts of SDF-1α-treated human BCC cells were extracted as described in Materials and methods. p-c-Jun accumulated in nuclei of SDF-1α-treated cells, but total-c-Jun (t-c-Jun) in cytosol did not change markedly. (**D**) SDF-1α-induced nuclear accumulation of p-c-Jun was inhibited by PD98059 (25 μM), but not SP600125 (10 μM) or SB203580 (10 μM). (**C** and **D**) SP-1 and α-tubulin served as internal controls for nuclear and cytosolic fractions. (**E**) Antisense c-*jun* oligonucleotide (3 μg), but not sense c-*jun* oligonucleotide (3 μg), abolished SDF-1α-induced upregulation of MMP-13 mRNA and gelatinase activity in human BCC cells. (**F**) SDF-1α-directed BCC chemoinvasion was inhibited by PD98059 (25 μM) and antisense c-*jun* oligonucleotide (3 μg), but not by SP600125 (10 μM), SB203580 (10 μM) or sense c-*jun* oligonucleotide (3 μg). Data are shown as mean ± s.d. of three independent experiments (*$P<0.01$, **$P=0.03$, Student's *t*-test).

expression of MMP-13 has been detected in 27.3% of breast carcinomas, 85.7% of squamous cell carcinomas in head and neck, 75% of cell lines established from invasive squamous carcinomas of the vulva and 52.2% of malignant melanomas (Leeman *et al*., 2002). The expression of MMP-13 has also been detected in malignant squamous epithelium of the skin including human BCCs and SCCs (Airola *et al*., 1997).

Previous studies have shown that SDF-1α/CXCR4 interactions modulate cell migration and invasion in several cancer cells (Fernandis *et al*., 2004; Bartolomé *et al*., 2004, 2006; Singh *et al*., 2004; Brand *et al*., 2005; Saur *et al*., 2005; Zhang *et al*., 2005; Menu *et al*., 2006; Ohira *et al*., 2006). SDF-1α-mediated invasion may involve activation and secretion of MMP-2 and/or MMP-9 (Fernandis *et al*., 2004; Brand *et al*., 2005), membrane-type 1 MMP (MT1-MMP; MMP-14) (Bartolomé *et al*., 2004, 2006) and MT2-MMP (Zhang *et al*., 2005). Prostate cancer cells have also been shown to migrate and invade through ECM components in response to SDF-1α–CXCR4 interactions, which were associated with enhanced expressions of mRNAs and active proteins of MMP-1, -2, -3, -9, -11 and MT1-MMP (MMP-14) in PC3 cells, as well as enhanced expressions of mRNAs and active proteins of MMP-1, -2 and -10 in LNCaP cells. However, MMP-13 expression was downregulated after SDF-1α–CXCR4 engagement in both PC3 and LNCaP cells (Singh *et al*., 2004). In contrast to this report, we found that SDF-1α induced MMP-13 expression and secretion in human BCC cells without significantly changing expression of MMP-3, -7 and -10 mRNAs, which were MMPs expressed in human BCC epithelium (Kerkelä and Saarialho-Kere, 2003). Although previous studies of gelatin zymography using 3-day culture fluids from 14 BCC tissues revealed high levels of activity corresponding to both MMP-2 and MMP-9 in all of the tumor specimens (Varani *et al*., 2000; Yucel *et al*., 2005), the experimental system of organ culture fluids may also reflect MMPs produced by reactive epithelium immediately adjacent to the tumor or especially by fibroblasts in the tumor-associated stroma (Yucel *et al*., 2005). This is also evident by the staining of MMP-9 in the normal epithelial cells adjacent to the tumor, but very little MMP-9 staining in the tumor epithelium itself (Varani *et al*., 2000). Gelatin zymography performed in this study revealed gelatinolytic bands at 48 kDa without obvious proteolytic bands at 72 and 92 kDa (Supplementary Figure 4), indicating that MMP-2 and -9 were not present in our BCC experimental system, which is consistent with previous studies (Poulsom *et al*., 1993; Dumas *et al*., 1999; Kerkelä and Saarialho-Kere, 2003). The discrepancies in the expression profiles of MMPs induced by SDF-1α in BCC cells and other cancer cells might contribute to the low metastatic potential of BCCs compared to other CXCR4-positive cancer cells. Our samples were concentrated 100-fold to exclude the presence of even weak MMP-2 activity. Nevertheless, the MMP-13 activity of BCC after SDF-1α treatment was readily detected in either 50- or 100-fold concentrates of the samples (Supplementary Figure 4).

A variety of growth factors stimulate the expression of MMP genes via signal-transduction pathways that converge to activate AP-1 complex of transcription factors. MAPK pathways ERK1/2, JNK and p38 induce the expression of AP-1 transcription factors (Ala-aho and Kähäri, 2005). We found SDF-1α-enhanced ERK1/2 phosphorylation without obvious changes of phosphorylation of Akt and other MAPK pathways (e.g., JNK and p38 MAPK pathways) in human BCC cells. Previous studies have revealed that SDF-1α treatment activates ERK1/2 in human lung cancer cells, astrocytes and glioblastoma cells (Bajetto *et al*., 2001; Kijima *et al*., 2002; Barbero *et al*., 2003; Phillips *et al*., 2003). The SDF-1α-directed BCC invasion was effectively inhibited by PD98059 treatment but not SB203580 treatment. However, SP600125 treatment also had an inhibitory effect on SDF-1α-induced BCC invasion, but it was statistically less than the effect of PD98059 treatment. This indicates that expression and activation of JNK might play some role in the invasiveness of human BCC cells and that this role is independent of SDF-1α-induced MMP-13 activation.

Functional characterization of the regulatory elements in human MMP-13 promoter has revealed that AP-1 site is functional and responsible for the inducibility by phorbol ester, transforming growth factor-β, and interleukin-1β in human fibroblasts (Ala-aho and Kähäri, 2005; Uría *et al*., 1997, 1998). The adjacent PEA-3 site does not seem to play a significant role in the transcriptional regulation of the human *MMP-13* gene (Pendás *et al*., 1997). In addition, the promoter region of human MMP-13 contains an OSE-2 that mediates the expression of osteoblastic specific genes (Ducy *et al*., 1997). The OSE-2 and PEA-3 sites did not seem to be involved in SDF-1α-mediated transcriptional regulation of MMP-13 gene as evidenced by the fact that transfection with the p56, p83 or p182 construct all resulted in a similar increase in luciferase activity compared to basal levels.

In conclusion, we present here a novel mechanism of SDF-1α/CXCR4-directed invasion of BCC cells by upregulation of both MMP-13 mRNA and MMP-13 active protein. The identification of SDF-1α as a potential stimulatory factor of MMP-13 during BCC cell invasion may contribute to better knowledge of the mechanisms involved in the aggressive potential of human BCC. In addition, the identification of SDF-1α/CXCR4 interaction as an important factor in BCC invasiveness may lead to potential improvements in therapeutic approaches of aggressive BCC.

Materials and methods

Cell origin and cell culture

The human BCC cell line was established from human BCC derived from the undifferentiated type of BCC tumor arising on a thermal traumatic scar (Yen *et al*., 1996; Jee *et al*., 2002, 2004). Passages 130–140 of this cell line were used here. The human HaCaT cell line is an immortalized human keratinocyte cell line, kindly provided by Dr NE Fusenig (German Cancer Research Center, Heidelberg, Germany). Human melanoma

cell lines (SK-MEL-2 and SK-MEL-28), the human epidermoid carcinoma cell line (A431) and the human cervical cancer cell line (HeLa) were purchased from American Type Culture Collection (Manassas, VA, USA). We obtained primary cultures of normal human keratinocytes and normal melanocytes from normal appearing skin of at least three different individuals.

Establishment of BCC/CXCR4 transfectants
Details are available as Supplementary Information.

Generation of DNA constructs encoding a siRNA against human CXCR4
Oligonucleotides against human *CXCR4* genes were generated and cloned into a pSilencer 3.1-H1 vector (Ambion, Austin, TX, USA), as described (Lapteva *et al*., 2005). We used the TransFast transfection reagent (Promega, Madison, WI, USA) to transfect the BCC cells with pSilencer 3.1-H1-siCXCR4 or pSilencer 3.1-H1-siCXCR4-mut. Twenty-four hours after transfection, cells were replated in Roswell Park Memorial Institute (RPMI) 1640 medium (GibcoBRL, Rockville, MD, USA) with 10% fetal calf serum and 0.2 μg/ml puromycin (Sigma, St Louis, MO, USA).

Antibodies and reagents
The anti-CXCR4 antibody (clones 12G5 and 44708) and anti-MMP-13 antibody (clone 87512) were purchased from R&D Systems (Minneapolis, MN, USA). The CXCR4-specific chemical inhibitor AMD 3100 (Hatse *et al*., 2002; De Clercq, 2003) was a gift from TaiGen Biotechnology Company (Taipei, Taiwan). Affinity-purified monoclonal mouse anti-phospho-Akt, anti-Akt, anti-JNK, anti-phospho-JNK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK1/2 and anti-phospho-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phospho-c-Jun and anti-c-Jun were from Cell Signaling Technology (Danvers, MA, USA). The mitogen-induced extracellular kinase (MEK) inhibitor (PD98059) and p38 MAPK inhibitor (SB203580) were obtained from Calbiochem (San Diego, CA, USA). The selective JNK inhibitor, SP600125, was purchased from Sigma.

Immunohistochemistry, RT–PCR and Western blot analysis of the cell lysate and supernatant
Details are available as Supplementary Information.

Nuclear and cytosolic protein extraction
For nuclear and cytosolic protein extraction, the protein extracts were prepared from SDF-1α-treated BCC cells using a modified procedure, as described previously (Dignam *et al*., 1983).

Chemoinvasion assay
Details of chemoinvasion assay are available as Supplementary Information.

Zymography
Conditioned media were collected, centrifuged and concentrated 50- or 100-fold with Amicon Centriprep (Amicon Inc., Beverly, MA, USA). Concentrated supernatants were mixed with sample buffer without reducing agent or heating. The sample was loaded into a gelatin (1 mg/ml) containing sodium dodecyl sulfate–polyacrylamide gel and underwent electrophoresis with constant voltage. The gelatinase activity of MMP-13 was assayed, as described previously (Forsyth *et al*., 2002).

siRNA treatment for MMP-13
Details are available as Supplementary Information.

Antisense c-jun oligonucleotide treatment
The c-*jun* antisense oligonucleotide used in this study was c-*jun* antisense, 5′-CGTTTCCATCTTTGCAGT-3′. The c-*jun* sense oligonucleotide, 5′-ACTGCAAAGATGGAAACG-3′, served as control (Zhang *et al*., 2002). For transfection, BCC cells were plated on six-well plates at a density of 4×10^5 cells/well 1 day before transfection. A total of 0.3 μM oligonucleotides were mixed with 8 μl of TransFast transfection reagent (Promega) in RPMI-1640 medium for 24 h. Transfection efficiency was normalized by co-transfection of 0.1 μg of a green fluorescent protein (GFP)-encoding plasmid (pEGFP) (Clontech, Mountain View, CA, USA). Transfected cells were checked visually for GFP expression by inverted fluorescence microscopy (Nikon, Yokohama, Kanagawa, Japan).

MMP-13 promoter assay
We generated different promoter constructs of human MMP-13 genes according to previous reports with some modifications (Pendás *et al*., 1997; Uría *et al*., 1998; Jiménez *et al*., 1999). The details are available as Supplementary Information.

Statistical methods
The two-tailed Student's *t*-test was used for simple comparison of two values where appropriate. All data were expressed as mean $\pm$ s.d. from at least three independent experiments. A χ^2 test was used to compare the pathological characteristics of tumors with high and low CXCR4 expression. All statistical tests were two-sided. A *P*-value of less than 0.05 was considered statistically significant for all tests. All analyses were performed with the use of SAS software (version 8.02, SAS Institute, Cary, NC, USA).

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