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SDF-1/CXCR4 axis promotes directional migration of colorectal cancer cells through upregulation of integrin αvβ6

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Colorectal cancer (CRC) displays a predilection for metastasis to liver. Although stromal cell-derived factor-1 (SDF-1)/CXCR4 plays an important role in the liver metastasis, the molecular mechanism still remains obscure. We previously reported that integrin αvβ6 was implicated in the progression of CRC. However, no data are currently available on the cross talk between CXCR4 and αvβ6. In the present study, we first demonstrated the cross talk between CXCR4 and αvβ6 and their role in liver metastasis of CRC. We analyzed 159 human CRC samples and found that expression of CXCR4 and αvβ6 was significantly associated with liver metastasis, and interestingly expression of αvβ6 significantly correlated with expression of CXCR4. Both CXCR4 and αvβ6 were highly expressed in metastatic CRC cell lines HT-29 and WiDr, whereas both of them were exiguous in non-metastatic cell line Caco-2. Furthermore, inhibition of αvβ6 significantly decreased SDF-1α-induced cell migration *in vitro*. SDF-1/CXCR4 could upregulate αvβ6 expression through phosphorylation of ERK and activation of Ets-1 transcription factor. In conclusion, we demonstrate that SDF-1/CXCR4 induces directional migration and liver metastasis of CRC cells by upregulating αvβ6 through ERK/Ets-1 pathway. These data support combined inhibition of CXCR4 and αvβ6 to prevent development of liver metastasis of CRC.

Introduction

Colorectal cancer (CRC) is ranked as the second most common cause of cancer-related death, which has been mainly attributed to distant metastasis (1,2). A salient feature of CRC metastasis is the ability to metastasize to specific sites, including liver, lung and bone marrow. Among these sites, liver is the most preferred target organ (3). Therefore, it is urgent to explore the mechanisms for liver-specific metastasis of CRC, which will facilitate the identification of novel therapeutic targets. The process of cancer metastasis consists of a series of sequential and interrelated steps, including invasion, detachment from the primary sites, intravasation, survival in the circulation and translocation to microvessels of target organs, extravasation and colonization. A keystone in the process is cell migration. However, we know very little about the molecular mechanisms that regulate cancer cell migration, especially the directional migration to specific sites (1,4,5). Chemokine receptors are a superfamily of G-protein-coupled cell surface receptors (6). Interaction between such receptors and their respective chemokines can induce directional migration of cells toward a gradient of chemokines (namely chemotaxis). This process was initially found in the homing of leukocytes to sites of inflammation (7,8). Considering many similarities shared by cancer cell metastasis and leukocyte trafficking, chemokines and their receptors may play a crucial role in organ-specific cancer metastasis (5). CXCR4 is a commonly expressed chemokine receptor in CRC. Previous studies reported that CXCR4 together with its unique ligand stromal cell-derived factor-1 (SDF-1 or CXCL12) played a significant role in the progression and metastasis of CRC, breast cancer, prostate cancer, lung cancer, ovarian cancer and laryngeal carcinoma (9–16). Intriguingly, SDF-1 is highly expressed in liver, lung, bone marrow and lymph nodes, which just represent the most common sites for CRC metastasis (17). However, several studies indicate that CXCR4 itself does not directly contribute to cell adhesion and migration, but it is essential to transmit SDF-1-induced signals, which may further modify other receptors to promote cancer cell adhesion and migration (18,19). Integrins are heterodimeric receptors comprising α and β subunits, which function as major cell adhesion molecules mediating cell motility, adhesion, polarity, proliferation and survival (20). Integrin αvβ6 is the only heterodimer that β6 subunit can form and is expressed exclusively in epithelial cells. It is absent or lowly expressed in healthy adult epithelia, but it is highly expressed during embryogenesis, tissue repair and carcinogenesis (21). We previously reported the definite role of αvβ6 in cancer cell migration, proliferation, invasion, apoptosis, metastasis and chemo resistance (22–28). Recently, we have verified that αvβ6 can promote colon cancer cell migration in a mechanism of integrin trafficking (29). Thus, we hypothesize that SDF-1/CXCR4 axis may facilitate liver metastasis of CRC via integrin αvβ6. In the present study, we investigated the prognostic significance of CXCR4 and αvβ6 in liver metastasis of CRC and the potential role and underlying mechanism of αvβ6 in SDF-1/CXCR4-mediated CRC cell migration *in vitro*.

Abbreviations: BSA, bovine serum albumin; CRC, colorectal cancer; EMSA, electrophoretic mobility shift assay; mRNA, messenger RNA; PBS, phosphate-buffered saline; SDF-1, stromal cell-derived factor-1; siRNA, small interfering RNA.

Materials and methods

Antibodies and reagents

Antibodies anti-CXCR4 (ab2074) and phycoerythrin-conjugated anti-CXCR4 (clone 12G5) were obtained from Abcam and BioLegend, respectively. Monoclonal antibodies against integrin αvβ6, including 10D5 and E7P6, were purchased from Chemicon, and clone 442.5C4 from Calbiochem. Antibodies against αv (P2W7), β6 (C-19) were obtained from Santa Cruz, antibodies against ERK1/2 and phospho-ERK1/2 were from Cell Signaling Technology and antibodies against Ets-1 (Ab38) and Ets-1 (phospho-Thr38) were from Assay Biotechnology. Recombinant human SDF-1α was purchased from PeproTech. Specific CXCR4 antagonist (AMD3100) was purchased from Santa Cruz and MEK inhibitor U0126 from Cell Signaling Technology.

Patient management and follow-up

A total of 159 CRC patients who underwent surgery by the same surgical team at the Department of General Surgery of Qilu Hospital (Shandong University, China) were selected consecutively from 2005 to 2008. Twenty-one of these patients had synchronous CRC and liver metastasis at diagnosis, and they underwent both colorectal and hepatic curative surgeries. The histologic sections were reviewed by two expert pathologists to verify the histologic diagnosis. Patients were regularly followed up with detailed evaluation of symptoms, physical examination, laboratory studies, ultrasound scan and computed tomography until June 2012. Written informed consents were obtained from all patients and the study protocol was approved by the Institutional Ethics Committee of Shandong University.

Tissue microarray and immunohistochemistry

Tissue cores were obtained from formalin-fixed paraffin-embedded tissue blocks and were transferred into a recipient master using a Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI). Representative areas of the tumor were selected based on hematoxylin–eosin staining. Five micrometer sections of tissue microarray blocks were subjected to routine deparaffinization and rehydration and boiled in sodium citrate buffer solution (pH 6.0) at 95°C for 15 min. The sections were incubated in 3% hydrogen peroxide for 10 min and blocked with 10% normal goat serum for 30 min. Tissue microarray sections were incubated overnight at 4°C with rabbit anti-human CXCR4 (ab2074) or

mouse anti-human αvβ6 (clone 442.5C4) primary antibodies and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG polymer (Polymer Detection System; GBI, WA). CXCR4 or αvβ6 antigen were stained for 5 min with 3,3-diaminobenzidine. Normal rabbit or mouse IgG was substituted for primary antibody as the negative control. Staining for CXCR4 and αvβ6 was evaluated according to the modified methods described previously (30). In brief, the intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium) or 3 (strong), and the extent of staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). The product of intensity and extent scores was used as the final immunohistochemistry score ranging from 0 to 12. Tumors with a final score of 0–3 were defined as negative expression, and >3 were defined as positive.

Cell culture and interference

The human colon cancer cell lines HT-29, WiDr and Caco-2 (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. To inhibit expression of CXCR4, β6 and Ets-1, cells were transiently transfected with specific small interfering RNA (siRNA; GenePharma) or negative control in Opti-MEM Medium (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen). Experiments were performed 24 h after transfection. To overexpress CXCR4, Caco-2 cells were transfected with human CXCR4-expressed pcDNA3.1(+) plasmid (pcDNA-CXCR4) or mock vector. The pcDNA3.1(+) vector containing green fluorescence protein (pcDNA-GFP) was used as a control to demonstrate transfection efficiency. In some cases, cells were pretreated with AMD3100 (500 ng/ml) to inhibit function of CXCR4, 10D5 (100 μg/ml) to inhibit function of αvβ6 and U0126 (20 μmol/l) to inhibit activation of ERK.

Quantitative real-time PCR

Total RNA was extracted from cells by Trizol (Invitrogen). The reverse transcription reaction was performed using RevertAid First Strand cDNA Synthesis Kit (Fermentas), according to the manufacturer's instructions. cDNA obtained from reverse transcription reaction was analyzed by a real-time PCR thermocycler (IQ5 Real-Time PCR cycler; Bio-Rad, CA) with SsoFast EvaGreen Supermix (Bio-Rad Laboratories). Quantitative values were obtained by the threshold cycle (CT) value. Relative mean fold change in expression ratios was calculated by the $2^{-\Delta\Delta CT}$ method. The primers for human β6 integrin were as follows: 5′-TTCCTAATGACGGGCTCTGT-3′ (forward) and 5′-TTGGGTTACAGCGAAGATCA-3′ (reverse). The housekeeping gene *GAPDH* was served as internal control.

Western blot analysis

Harvested cells were lysated and equal amounts of protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature with 5% non-fat milk. Then membranes were incubated overnight at 4°C with the primary antibodies anti-CXCR4, anti-β6, anti-αv, anti-β1, anti-β3, anti-β5, anti-ERK1/2, anti-phospho-ERK1/2, anti-Ets-1 and anti-Ets-1 (phospho-Thr38), then with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were visualized by chemiluminescence (Millipore).

Flow cytometry

Harvested cells were washed by phosphate-buffered saline (PBS), blocked by goat serum at 4°C for 30 min and then incubated with phycoerythrin-conjugated anti-CXCR4 monoclonal antibody (clone 12G5) or unconjugated anti-αvβ6 monoclonal antibody (E7P6) for 20 min at 4°C and washed twice with PBS. For αvβ6 analysis, cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 20 min at 4°C. Then cell concentration was adjusted to 1×10^6 cells/ml prior to flow cytometry analysis (FACSCalibur; BD).

Confocal microscopy

Cells were fixed with 4% paraformaldehyde for 20 min, blocked by goat serum for 1 h and incubated with primary antibodies anti-CXCR4 or anti-αvβ6 overnight at 4°C. For phospho-Ets-1 analysis, cells were incubated with primary antibodies anti-Ets-1 (phospho-Thr38) in PBS with 0.1% Triton X-100. Then cells were washed with PBS before incubation with secondary antibodies (1:500 dilution; Jackson Laboratories) for 30 min at 37°C. Coverslips were finally sealed by a drop of Prolong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen). Images were acquired by laser scanning confocal microscopy (LSM710; Carl Zeiss, Germany) and analyzed by Image-Pro Plus 6.0.

Electrophoretic mobility shift assay

DNA-binding activity of Ets-1 was detected by electrophoretic mobility shift assay (EMSA) using LightShift Chemiluminescent EMSA kit (Thermo Scientific). Briefly, nuclear proteins were extracted from cells by nuclear extraction kit (Active Motif, CA). Single-stranded oligonucleotides containing binding sites of Ets-1 (5′-GATCTCGAGCCGGAAGTTCGA-3′) were synthesized and biotin labeled (BioSune, Shanghai, China) and then annealed to double-strand probes. Five micrograms of nuclear protein was incubated with 20 fmol biotin-labeled probes for 20 min at room temperature in binding buffer consisting of 50 mM KCl, 10 mM ethylenediaminetetraacetic acid, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng poly (dI•dC) and 0.05% NP-40. The specificity of binding was verified by competitive reactions in which a 200-fold molar excess (4 pmol) of unlabeled probes was added. After electrophoresis in 6.5% polyacrylamide gel electrophoresis, protein-probe mixture was transferred to nylon membrane and then cross-linked with a UV lamp for 10 min. Then the membrane was blocked for 15 min at room temperature, incubated with stabilized streptavidin–horseradish peroxidase conjugate for 15 min, washed four times and incubated with substrate equilibration buffer for 5 min. Then the membrane was incubated with chemiluminescent substrate for 5 min and analyzed by ImageQuant LAS 4000 mini (GE Healthcare).

Migration assay

Migration assays were performed using modified Transwell chambers (8 μm pore size; Corning) with the membrane precoated by 10 μg/ml fibronectin or bovine serum albumin (BSA). Cells were pretreated with AMD3100, 10D5, isotype IgG, siRNA, U0126 or pcDNA-CXCR4. 1×10^5 cells per well in 150 μl serum-free medium were seeded into the upper chamber. Serum-free medium containing 200 ng/ml SDF-1α was used as a chemoattractant in the lower chamber. After culture for 24 h at 37°C, the upper surface of the Transwell membrane was wiped gently with a cotton swab to remove non-migrating cells. The membranes were fixed with methanol and stained with 0.1% crystal violet. A minimum of five high-power fields per membrane were counted under a microscope. The number of migrating cells in each experiment was adjusted by the cell viability assay to correct for proliferation effects of the SDF-1α treatment (corrected migrating cell number = counted migrating cell number/percentage of viable cells) (31).

Statistical analysis

Associations between liver metastasis and immunohistochemical staining of CXCR4 and αvβ6 were analyzed by chi-square test. The statistical significance was determined by Mann–Whitney test or one-way analysis of variance. $P < 0.05$ was considered statistically significant. Data were analyzed with SPSS 16.0 software and presented as mean ± SD.

Results

Expression of CXCR4 and αvβ6 was associated with liver metastasis in CRC patients

Immunohistochemistry analysis of 159 primary CRC samples demonstrated that CXCR4 was positive in 107 tumors (67.3%) and CXCR4 staining was observed predominantly in the cytoplasm of CRC cells (Figure 1A). Integrin αvβ6 was positive in 73 tumors (45.9%) and its staining was detected both in the cytoplasm and membrane of tumor cells (Figure 1B). However, there were no or equivocal staining of CXCR4 and αvβ6 in normal colon tissues (data not shown). Until June 2012, after a median follow-up of 39 months, liver metastases were detected in 57 patients. In 57 patients with liver metastasis, 49 cases (86%) showed positive CXCR4 staining in primary cancer and 34 cases (59.6%) showed positive αvβ6 staining. In 102 patients without liver metastasis, positive expressions of CXCR4 and αvβ6 were detected in 58 (56.9%) and 39 (38.2%) cases. There were significant correlations between CXCR4 ($P < 0.001$) or αvβ6 ($P = 0.009$) expression and liver metastasis of CRC. Twenty-one patients, with synchronous CRC and liver metastasis at diagnosis, underwent both colorectal and hepatic curative surgeries. In these liver metastatic samples, 19 cases (90.5%) showed positive CXCR4 staining (Figure 1C), and 16 (76.2%) cases showed positive αvβ6 staining (Figure 1D). In contrast, none to equivocal staining of CXCR4 and αvβ6 were observed in normal liver specimens (data not shown).

Correlation between expression of CXCR4 and αvβ6

To explore the relationship between CXCR4 expression and αvβ6 expression, 159 primary CRC samples were divided into two groups

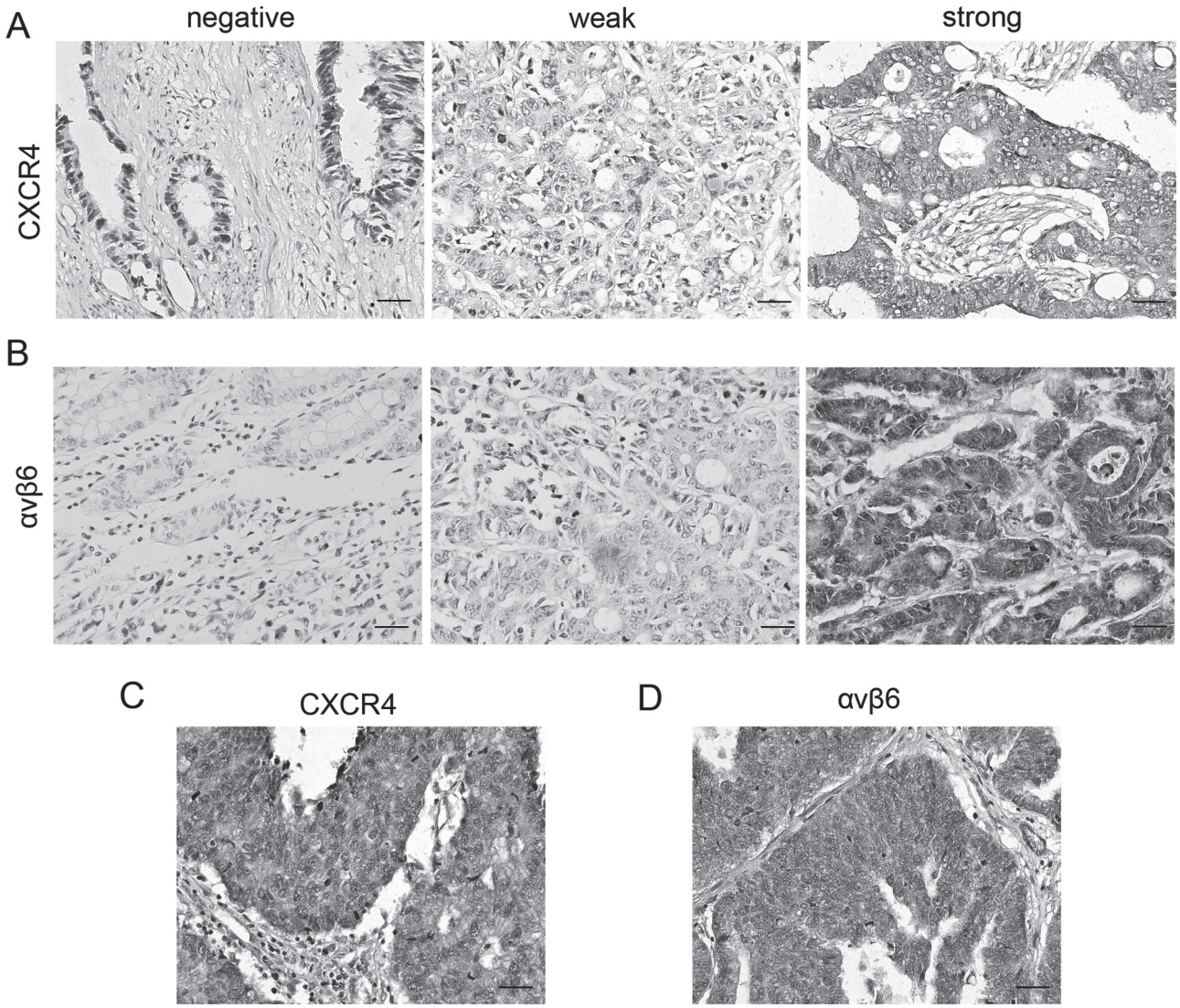

Fig. 1. Immunohistochemical expression of CXCR4 and αvβ6 in CRC and liver metastasis. (**A**) Immunostaining of CXCR4 in representative CRC tissues (×40). (**B**) Immunostaining of αvβ6 in representative CRC tissues (×40). (**C**) Immunostaining of CXCR4 in representative liver metastasis (×40). (**D**) Immunostaining of αvβ6 in representative liver metastasis (×40). Bar = 20 μm.

according to CXCR4 expression, and immunohistochemistry scores of αvβ6 in two groups were compared by Mann–Whitney test. Box-and-whisker graph demonstrated that CXCR4-positive group displayed significantly higher αvβ6 expression than CXCR4-negative group ($P < 0.001$; Figure 2).

Expression profiles of CXCR4 and αvβ6 in CRC cell lines

To investigate the cross talk between CXCR4 and αvβ6 as well as their potential role in distant metastasis, we first examined the expression patterns of CXCR4 and αvβ6 in CRC cell lines with different metastatic potential. CRC cell lines HT-29 and WiDr have been verified by *in vivo* and *in vitro* assays to be highly metastatic (especially highly metastatic to liver), whereas CRC cell line Caco-2 has been shown to be non-metastatic (32–35). Interestingly, reverse transcription–PCR in the present study demonstrated that relative messenger RNA (mRNA) levels of CXCR4 and β6 subunit (in affiliation only with the αv subunit) were high in metastatic cell lines HT-29 and WiDr, whereas non-metastatic cell line Caco-2 exhibited very low levels of both CXCR4 and β6 (Figure 3A). Similar results were observed in their protein expression by western blot (Figure 3B). With respect to cell surface presentation, flow cytometry showed that high membrane

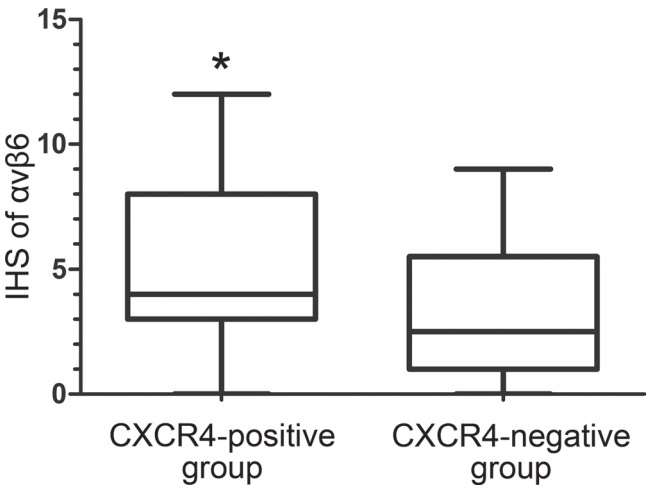

Fig. 2. Box-and-whisker graph demonstrated that IHS of αvβ6 in CXCR4-positive group were significantly higher than CXCR4-negative groups. *$P < 0.05$ versus CXCR4-negative group. IHS, immunohistochemistry scores.

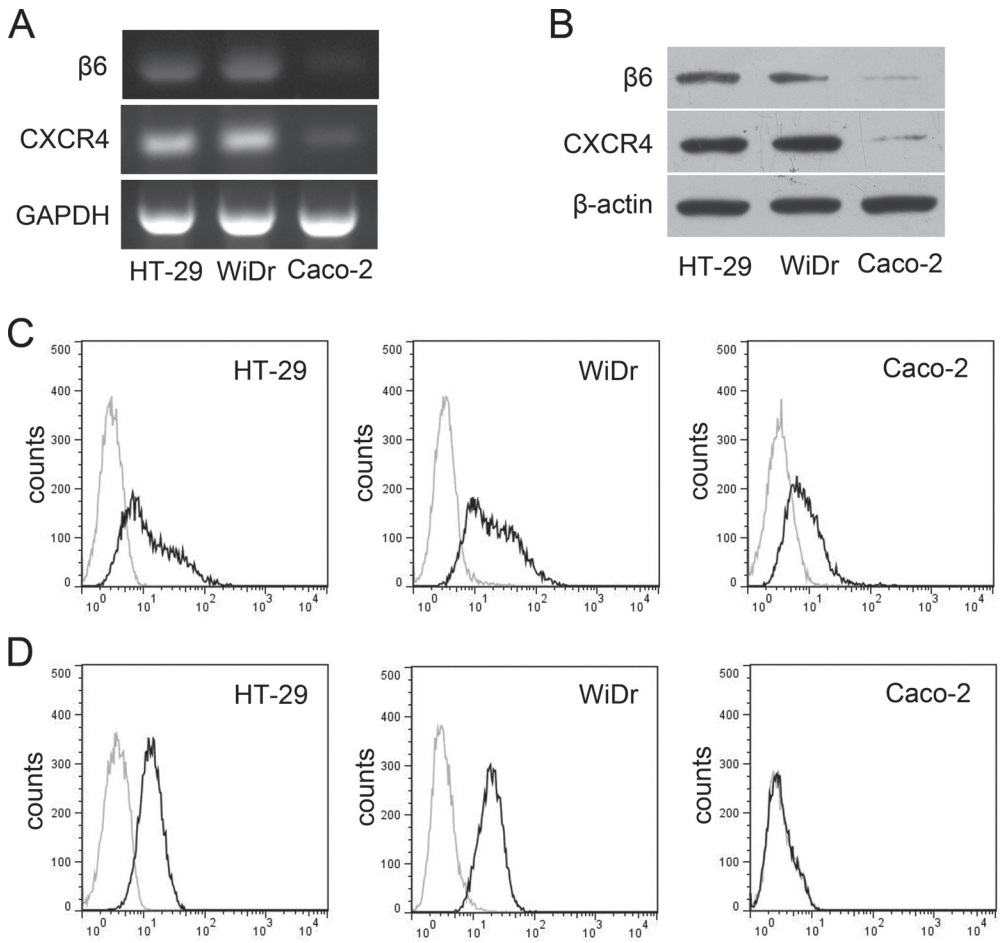

Fig. 3. Expression profiles of CXCR4 and αvβ6 in CRC cell lines HT-29, WiDr and Caco-2. (**A**) Reverse transcription–PCR analysis of mRNA levels of CXCR4 and β6 in metastatic cell lines HT-29 and WiDr and non-metastatic cell line Caco-2. (**B**) Western blot analysis of protein expression of CXCR4 and β6 in HT-29, WiDr and Caco-2. (**C** and **D**) Flow cytometry analysis of surface expression of CXCR4 (C) and αvβ6 (D) in HT-29, WiDr and Caco-2. The gray histograms represent isotype IgG controls, and the black histograms represent cells labelled with anti-CXCR4 antibody (**C**) or anti-αvβ6 antibody (**D**).

levels of both CXCR4 and integrin αvβ6 were detected in HT-29 and WiDr cells, whereas lower levels of CXCR4 and hardly any αvβ6 in Caco-2 (Figure 3C and D).

Integrin αvβ6 was essential for SDF-1α/CXCR4-induced CRC cell migration

SDF-1/CXCR4 axis has been proved to direct the migration of various cancer cells (17). To further explore whether αvβ6 was required for SDF-1/CXCR4-mediated migration of CRC cells, we examined HT-29 cells migration on fibronectin, a major ligand of αvβ6, and the BSA served as control. SDF-1α treatment dramatically increased cell migration on fibronectin, whereas it had no effect on the migration on BSA. Then specific αvβ6 blocking antibody 10D5 and specific β6 siRNA were used. Compared with the control, β6 expression in target cells was effectively repressed after 24 h transfection of β6 siRNA (Supplementary Figure 1A, available at *Carcinogenesis* Online). Inhibition of αvβ6 by 10D5 or siRNA significantly decreased SDF-1α-induced cell migration on fibronectin, comparable with the inhibitive effect of CXCR4-specific inhibitor AMD3100 (Figure 4A and B). These data suggested that SDF-1α might promote CRC cell migration via integrin αvβ6.

SDF-1α/CXCR4 axis induced upregulation of integrin αvβ6

Previous studies showed that SDF-1α could increase expression of integrin αvβ3 (36,37). Our aforementioned results suggested a correlation between expression of CXCR4 and αvβ6 in CRC tissues. We, therefore, investigated the effect of SDF-1α/CXCR4 on αvβ6 expression. Real-time PCR analysis demonstrated that SDF-1α dose dependently increased mRNA levels of β6 integrin in HT-29 and WiDr cells with maximum effect at a concentration of 200 ng/ml but failed to alter the mRNA levels of αv subunit (Figure 4C). This result was confirmed by western blot (Figure 4D). Then CXCR4 inhibitor AMD3100 and CXCR4 siRNA were applied in the following experiment. Transient transfection of specific CXCR4 siRNA significantly reduced protein expression of CXCR4 in HT-29 and WiDr cells (Supplementary Figure 1B, available at *Carcinogenesis* Online). Pretreatment of HT-29 and WiDr cells with AMD3100 or CXCR4 siRNA markedly attenuated SDF-1α-induced upregulation of β6 (Figure 4E and F). Integrin αvβ6 is the only heterodimer that β6 can form and upregulation of β6 can increase surface expression of integrin αvβ6 despite no change of αv expression (38). Flow cytometry showed that SDF-1α upregulated the cell surface αvβ6 integrins in both HT-29 and WiDr cells, which was inhibited by AMD3100 or CXCR4 siRNA (Figure 4G and H). In addition, the effect of SDF-1α on other forms of β integrins was also examined. Real-time PCR and western blot analysis revealed that SDF-1α could also induce upregulation of β1 integrin, whereas it had no effect on the expression of β3 and β5 integrins even at a higher concentration of 500 ng/ml (Supplementary Figure 2A–D, available at *Carcinogenesis* Online), which was consistent with a previous study using other CRC cell lines (39). In the present study, we mainly focused on the role of integrin αvβ6 in SDF-1/CXCR4-induced metastasis, and the role of β1 integrin in metastasis of CRC will be investigated in further study.

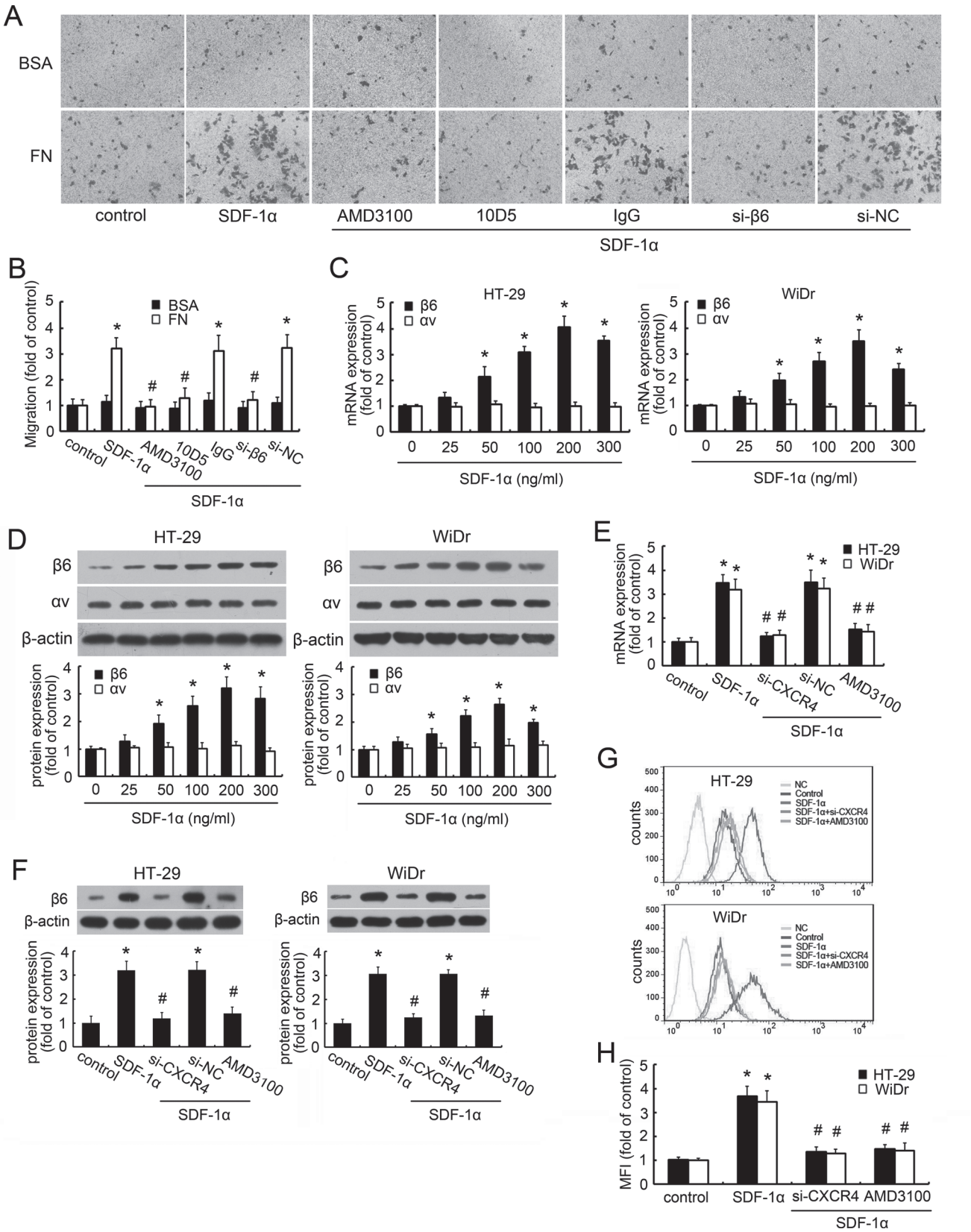

Fig. 4. SDF-1α/CXCR4-induced CRC cell migration by upregulating integrin αvβ6. (**A** and **B**) HT-29 cells were pretreated with CXCR4 inhibitor AMD3100 or αvβ6 function blocking antibody 10D5 for 30 min or transfected with αvβ6 siRNA for 24 h. Cell migration toward FN or BSA was measured with Transwell in the presence or absence of SDF-1α (200 ng/ml). (**C** and **D**) HT-29 and WiDr cells were incubated with SDF-1α at different concentrations for 24 h, and expression of αv or β6 subunit was analyzed by real-time PCR (C) and western blot (D). (**E** and **F**) HT-29 and WiDr cells were pretreated with CXCR4 siRNA, control siRNA or AMD3100 followed by stimulation with SDF-1α (200 ng/ml) for 24 h. Then expression of β6 subunit was determined by real-time PCR (E) and western blot (F). (**G** and **H**) Surface expression of αvβ6 receptors was determined by flow cytometry, and quantification of MFI was shown (H). Shown are mean ± SD of three independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus SDF-1α. FN, fibronectin; MFI, mean fluorescence intensity.

ERK pathway was involved in SDF-1α/CXCR4-induced αvβ6 upregulation

We then investigated the mechanism of αvβ6 upregulation induced by SDF-1α/CXCR4. Western blot analysis demonstrated that stimulation of cells with 200 ng/ml of SDF-1α increased the phosphorylation of ERK 1/2 with a peak at 60 min (Figure 5A and B). Inhibition of CXCR4 by AMD3100 or CXCR4 siRNA significantly reduced SDF-1α-induced ERK 1/2 phosphorylation (Figure 5C and D). Addition of U0126 (specific ERK inhibitor) effectively reverted SDF-1α-induced increase of β6, which was associated with decreased phosphorylation of ERK1/2 (Figure 5E–G). Moreover, SDF-1α-induced cell migration on fibronectin was also greatly reduced by U0126 (Figure 5H and I).

SDF-1α/CXCR4 induced αvβ6 upregulation through activation of Ets-1 transcription factor

Integrin αvβ6 can be transcriptionally activated by Ets-1 transcription factor during the epithelial–mesenchymal transition process (40). To explore whether Ets-1 was involved in SDF-1α-induced αvβ6 upregulation, Ets-1 expression, activation (phosphorylation) and DNA-binding activity were detected. SDF-1α induced a slight increase in total Ets-1 expression and a significant increase in phosphorylation of Ets-1 on the threonine 38 residue (Thr38), which was inhibited by AMD3100 or CXCR4 siRNA (Figure 6A–C). Given that ERK1/2 was phosphorylated by SDF-1α, we examined its involvement in SDF-1α-induced activation of Ets-1. Inhibition of ERK activation with U0126 abolished SDF-1α-induced increase in Ets-1 expression and phosphorylation on Thr38 (Figure 6D–F). Immunofluorescence analysis showed that SDF-1α significantly increased nuclear levels of Thr38-phosphorylated Ets-1 (pThr38-Ets-1), and the elevated phosphorylation was reduced by AMD3100 or U0126 (Figure 6G). This increase in phosphorylation status is considered as a trigger for elevation of Ets-1 DNA-binding activity (41). EMSA results demonstrated that stimulation of HT-29 with SDF-1α significantly increased Ets-1 DNA-binding activity and the effect could be abolished by pretreatment of cells with

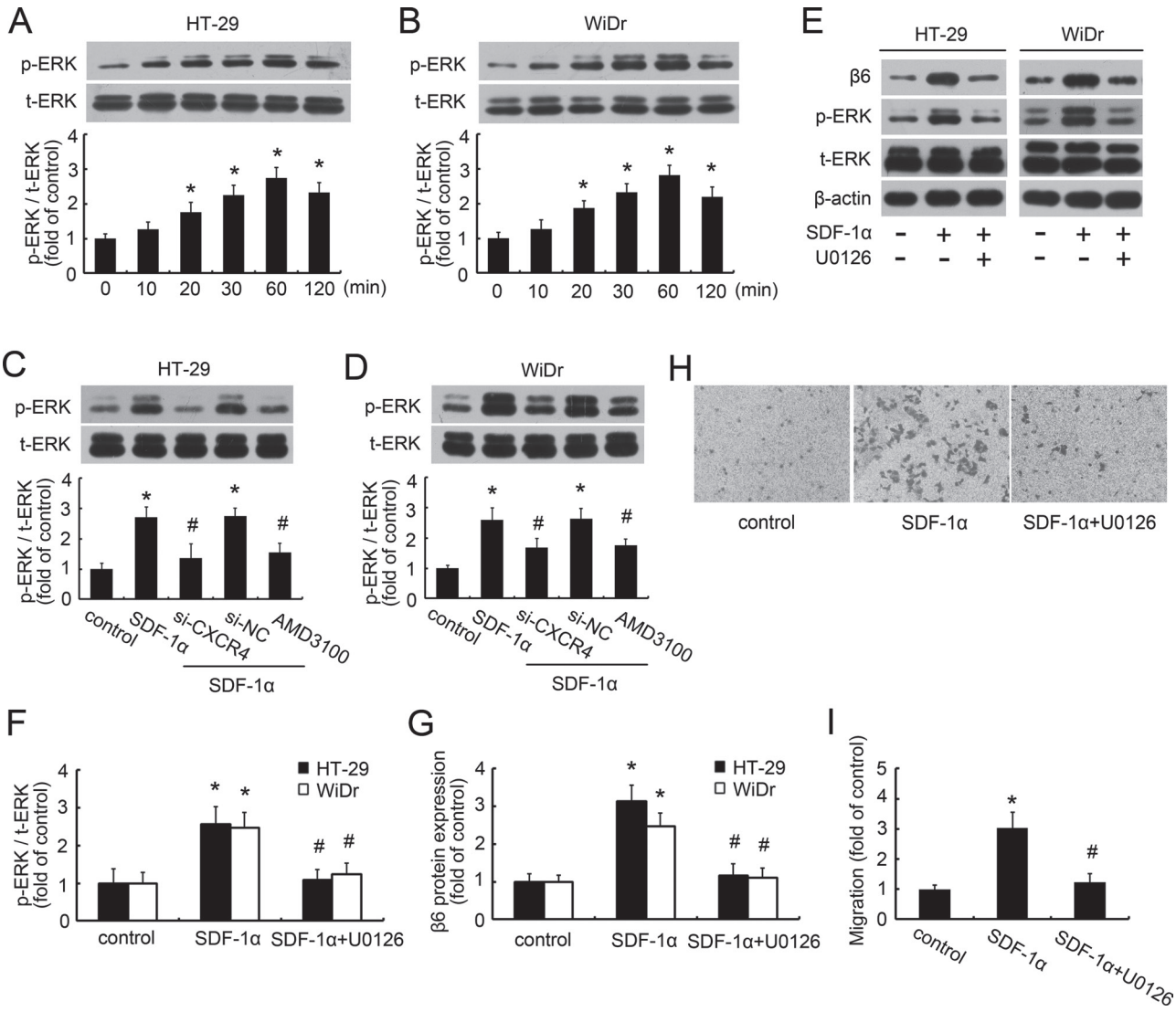

Fig. 5. ERK pathway was involved in SDF-1α/CXCR4-mediated upregulation of αvβ6. (**A** and **B**) HT-29 (A) and WiDr (B) cells were incubated with SDF-1α (200 ng/ml) for indicated time intervals, and expression of total ERK (t-ERK) and phospho-ERK (p-ERK) was determined by western blot. (**C** and **D**) HT-29 (C) and WiDr (D) cells were pretreated with CXCR4 siRNA, control siRNA or AMD3100 followed by stimulation with SDF-1α (200 ng/ml) for 60 min, and expression of t-ERK and p-ERK was determined by western blot. (**E–G**) HT-29 and WiDr cells were pretreated with ERK-specific inhibitor U0126 for 30 min followed by stimulation with SDF-1α (200 ng/ml) for 24 h, and expression of t-ERK, p-ERK and β6 subunit was detected by western blot. Quantification of ERK phosphorylation (F) and β6 expression (G) were shown. (**H** and **I**) HT-29 cells were pretreated with U0126 for 30 min, and cell migration toward fibronectin was measured with Transwell in the presence of SDF-1α (200 ng/ml). Quantification of cell migration (I) was shown. Shown are mean ± SD of three independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus SDF-1α.

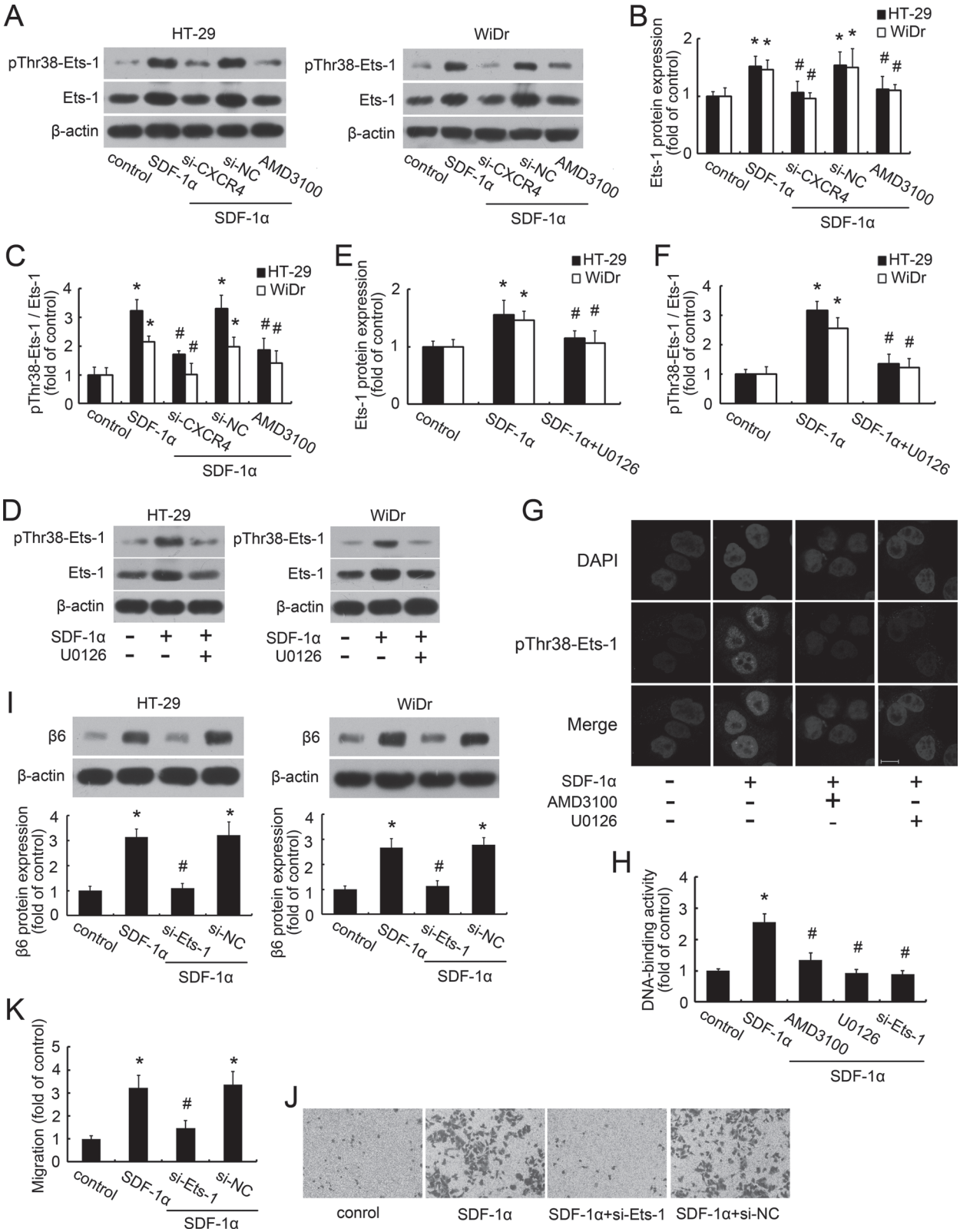

Fig. 6. SDF-1α/CXCR4-induced αvβ6 upregulation through activation of Ets-1 transcription factor. (**A** and **D**) HT-29 and WiDr cells were pretreated with CXCR4 siRNA, AMD3100 or U0126 followed by stimulation with SDF-1α (200 ng/ml) for 24 h, and expression of Ets-1 and phosphorylated Ets-1 on Thr38 (pThr38-Ets-1) was determined by western blot. Quantification of Ets-1 protein expression (**B** and **E**) and Ets-1 phosphorylation (**C** and **F**) were shown. (**G**) Nuclear pThr38-Ets-1 staining in HT-29 was detected by confocal microscopy. SDF-1α significantly increased nuclear staining of pThr38-Ets-1 ($p < 0.05$ versus control), and the enhanced staining was significantly reduced by AMD3100 or U0126 ($p < 0.05$ versus SDF-1). Bar = 10 μm. (**H**) Ets-1 DNA-binding assays were performed using nuclear extracts from control cells or SDF-1α-stimulated cells pretreated with AMD3100, U0126 or Ets-1 siRNA. (**I**) Effect of Ets-1 siRNA on SDF-1α-induced β6 upregulation was determined by western blot. (**J** and **K**) HT-29 cells were transfected with Ets-1 siRNA or control siRNA for 24 h, and cell migration toward fibronectin was measured with Transwell in the presence of SDF-1α (200 ng/ml). Shown are mean ± SD of three independent experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus SDF-1α.

AMD3100, U0126 or Ets-1 siRNA (Figure 6H). Successful knockdown of Ets-1 protein expression by specific siRNA was verified by western blot (Supplementary Figure 1C, available at *Carcinogenesis* Online). Furthermore, reducing Ets-1 activity with siRNA significantly blocked SDF-1α-induced αvβ6 upregulation (Figure 6I) and cell migration on fibronectin (Figure 6J and K). These data indicated that activation of Ets-1 was involved in SDF-1α/CXCR4-induced increase of αvβ6 expression and CRC cell migration.

Caco-2 was rendered migrating toward SDF-1α after overexpression of CXCR4 and upregulation of integrin αvβ6

Our aforementioned results have shown exiguous expression of both CXCR4 and αvβ6 in non-metastatic cell line Caco-2 (Figure 3). To further confirm the important role of αvβ6 upregulation in SDF-1α/CXCR4-induced cell migration, Caco-2 cells were transfected with human CXCR4-expressed plasmid (pcDNA-CXCR4) or mock vector (mock). Green fluorescence protein-expressed plasmid (pcDNA-GFP) was used as a control to demonstrate transfection efficiency, which was >80% in our experiment (Supplementary Figure 3A, available at *Carcinogenesis* Online). Western blot and flow cytometry demonstrated significant overexpression of CXCR4 in cells transfected with pcDNA-CXCR4 but not mock vector as compared with control (Supplementary Figure 3B and C, available at *Carcinogenesis* Online). Furthermore pcDNA-CXCR4 transfected Caco-2 cells were incubated with SDF-1α (200 ng/ml) for 24 h and, as a result, SDF-1α induced a significant increase in mRNA and protein levels of β6 integrin, which was evidently attenuated by CXCR4 inhibitor AMD3100 (Supplementary Figure 3D and E, available at *Carcinogenesis* Online). Flow cytometry also demonstrated similar results in respect of surface expression of integrin αvβ6 (Supplementary Figure 3F, available at *Carcinogenesis* Online). Migration assay on fibronectin was performed in non-transfected, mock vector or pcDNA-CXCR4 transfected Caco-2 cells. No statistical difference was observed between migration of SDF-1α-treated and untreated Caco-2 cells. On the contrary, SDF-1α dramatically increased the migration of pcDNA-CXCR4 but not mock vector transfected Caco-2 cells. Moreover, inhibition of αvβ6 by neutralizing antibody 10D5 significantly alleviated SDF-1α-induced migration of pcDNA-CXCR4 transfected Caco-2 cells (Supplementary Figure 3G and H, available at *Carcinogenesis* Online). These data suggested that Caco-2 cells were rendered migrating after overexpression of CXCR4, treatment with SDF-1α and subsequent upregulation of integrin αvβ6.

Discussion

Liver is the most common site for CRC metastasis and hepatic metastasis is considered as a critical factor determining the prognosis of CRC patients (2). Although SDF-1/CXCR4 axis has been implicated in liver metastasis (10), the fundamental mechanism still remains unclear. In the present study, we focused on the potential role of integrin αvβ6 in SDF-1/CXCR4-induced liver metastasis of CRC. By analyzing clinical CRC specimens, we demonstrated that expression of CXCR4 and αvβ6 was significantly associated with liver metastasis, and αvβ6 expression significantly correlated with CXCR4 expression. SDF-1/CXCR4 could promote CRC cell migration by upregulating αvβ6 through activation of ERK pathway and Ets-1 transcription factor *in vitro*. The process of metastasis can be divided into several migration steps including detachment from primary site, intravasation, transport in the circulation, arrest in a capillary bed and extravasation into the target organ. Although migration of CRC cells from primary site to liver is essential to establish metastasis, little is known about the molecular mechanisms that regulate cancer cell migration (4,5). Cancer cell migration and metastasis share many similarities with the homing of leukocytes to inflammation sites, and its migration pattern is not random but directional (5,8). The definition of directional migration consists of two principles. First, organ-specific metastasis requires a complex signaling mechanism to guide CRC cells to the liver, which can be liken to a 'navigation system'. As a major chemokine receptor on CRC cells, CXCR4 has been proved to induce chemotaxis of cells toward a gradient of SDF-1 (5,17). Furthermore, high levels of SDF-1 were detected in liver, lung and bone marrow, representing the most common target organs for CRC metastasis (17). This suggests that SDF-1/CXCR4 axis could function as a 'navigation system' for CRC cells, guiding cancer cell migration from their original site to final destination. Indeed, SDF-1/CXCR4 axis has been reported to play a significant role in liver metastasis of CRC (9–11). Second, the ability of migration or motility is also required. A keystone of cell migration is the formation and regulation of adhesion contacts, especially integrin contacts, which are dynamically disassembled at the rear and reassembled at the leading edge (29,42). However, previous studies demonstrated that CXCR4 itself did not directly contribute to adhesion of prostrate and renal carcinoma cells to extracellular matrix but served as signal transmitters to modulate integrin α5- and β3-mediated adhesion (18,19). Therefore, it is possible that SDF-1/CXCR4 axis may promote CRC cell migration through cross talk with other receptors, especially the adhesion receptors. Integrin αvβ6 is an important adhesion receptor on CRC cells and is involved in several aggressive processes, including cell adhesion and spreading on fibronectin, tumor growth, apoptosis, matrix metalloproteinase secretion and chemo resistance (22,23,26,28,40). In our experiment, we analyzed the expression of CXCR4 and αvβ6 in 159 human CRC specimens and found that both CXCR4 and αvβ6 were associated with liver metastasis. Interestingly, as a transmembrane receptor, CXCR4 staining in our experiment was mainly cytoplasmic, whereas some cases presented an additional membranous localization. Similar expression pattern has been described previously in various cancers (10,14,30,43). This phenomenon could be explained by translocation of CXCR4 from membrane to cytoplasm after binding to SDF-1 (30,44). Previous studies reported that the cytoplasmic localization of CXCR4 might represent a functional status, which could subsequently activate downstream signaling events and indicate progression to highly aggressive phenotype (30,44,45). In addition, although CXCR4 nuclear expression has been reported in some studies (11,46), this expression pattern was not observed in our series. The absence of CXCR4 nuclear expression in our present study could not result from differences in primary antibodies, because Spano *et al*. (46) detected its nuclear localization in non-small-cell lung cancer using the same antibody (ab2074). Therefore, the subcellular distribution of CXCR4 and its biological significance needs further investigation in the future. Furthermore, we detected the expression profiles of CXCR4 and αvβ6 in CRC cell lines with different metastatic potential. Previous studies have examined the metastatic potential of CRC cell lines by *in vivo* and *in vitro* methods and demonstrated that HT-29 and WiDr were highly metastatic and Caco-2 was non-metastatic (32–35). Intriguingly, both CXCR4 and αvβ6 were highly expressed in metastatic CRC cell lines HT-29 and WiDr, whereas both of them were exiguous in non-metastatic CRC cell line Caco-2. These data indicated important role of both CXCR4 and αvβ6 in liver metastasis of CRC. Integrin αvβ6 is the major fibronectin receptor in HT-29 cells. We recently verified that αvβ6 could mediate colon cancer cell migration on fibronectin by internalization and recycling of αvβ6 integrins (29), indicating direct contribution of αvβ6 to colon cancer cell migration. In our study, SDF-1α treatment could promote the migration of HT-29 cells on fibronectin and the enhanced migration was significantly attenuated by inhibition of CXCR4 or αvβ6. In contrast, cell migration on BSA was unaffected. Thus, it is plausible that SDF-1/CXCR4 axis may promote CRC cells metastasis via integrin αvβ6. We further investigated the potential mechanisms of αvβ6 in SDF-1/CXCR4-induced metastasis of CRC. Sun *et al*. (37) demonstrated that SDF-1α enhanced both the expression and activation of integrin αvβ3 in metastatic prostate cancer cells but did not affect non-metastatic prostate cancer cells. In addition, elevated surface expression of β1 and β3 integrin was found to be involved in SDF-1α-induced lung cancer migration (47). Our present study showed that expression of CXCR4 in CRC specimens was significantly correlated with that of αvβ6. Therefore, we further investigated the effect of SDF-1 on αvβ6

expression. As we expected, SDF-1α could increase the mRNA and protein levels of β6 subunit and surface expression of integrin αvβ6 in HT-29 and WiDr cells. Moreover, overexpression of human CXCR4 in Caco-2 cells, followed by treatment with SDF-1α, also significantly increased the expression of β6 integrin and surface presentation of integrin αvβ6, which eventually led to elevated migration of Caco-2 cells. CXCR4 overexpression-induced increase in Caco-2 cell migration on fibronectin could be alleviated by inhibition of integrin αvβ6. These results confirmed the important role of αvβ6 upregulation in SDF-1/CXCR4-induced CRC cell migration. On the other hand, inhibition of CXCR4 by AMD3100 or siRNA significantly reduced SDF-1α-induced upregulation of αvβ6. The inhibitive effect of AMD3100 and CXCR4 siRNA on SDF-1α-induced αvβ6 upregulation also suggested a crucial role of CXCR4 in the process. The binding of SDF-1 to CXCR4 initiates divergent signaling pathways resulting in a variety of responses. Among these pathways, MEK/ERK 1/2 pathway has been linked to SDF-1-induced gene transcription and expression (48). We previously showed that αvβ6 upregulated its own expression at high cell density and ERK 1/2 was involved in this process (38). Our present data demonstrated that SDF-1α/CXCR4 increased phosphorylation of ERK 1/2 and inhibition of ERK by U0126 attenuated SDF-1α-induced upregulation of β6 integrin. Bates *et al*. (40) reported that β6 integrin could be transcriptionally activated by Ets-1 transcription factor during the epithelial–mesenchymal transition process and identified the Ets-1-binding site in human β6 promoter. In our experiment, SDF-1α-CXCR4 could induce a slight increase in Ets-1 expression, significant increase in phosphorylation of Ets-1 on Thr38 and enhancement of Ets-1 DNA-binding activity, all of which were dependent on ERK 1/2 activation. Inhibition of Ets-1 by siRNA led to abrogation of SDF-1α-induced upregulation of β6 integrin. These findings are consistent with previous reports showing that ERK, once activated, can form a signaling complex with Ets-1 to facilitate its phosphorylation on Thr38, resulting in increased transcription activity (49,50). Moreover, Ets-1 can also bind its own promoter and upregulate its own expression when phosphorylated (50). Therefore, the following mechanisms may be involved in SDF-1/CXCR4-induced upregulation of αvβ6: (i) SDF-1/CXCR4 axis enhances phosphorylation of ERK, (ii) activated ERK forms a signaling complex with Ets-1 and subsequently triggers phosphorylation of Ets-1 on Thr38 and (iii) phosphorylation of Ets-1 leads to transcriptional activation of downstream targets including β6 integrin and Ets-1 itself. However, whether other mechanisms are involved needs further investigation, such as conformational changes, accelerated internalization and recycling of integrin αvβ6. In conclusion, we firstly report that integrin αvβ6 plays a critical role in SDF-1/CXCR4-induced liver metastasis of CRC, and expression of αvβ6 can be upregulated by SDF-1/CXCR4 through increased phosphorylation of ERK and subsequent activation of Ets-1. Our present study presents a novel mechanism for SDF-1/CXCR4-induced organ-specific metastasis and shed light on effective therapeutic approaches for liver metastasis of CRC.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References

1. Steinert,G. *et al.* (2012) Biology and significance of circulating and disseminated tumour cells in colorectal cancer. *Langenbecks. Arch. Surg*., **397**, 535–542.
2. Molleví,D.G. *et al.* (2007) Mutations in TP53 are a prognostic factor in colorectal hepatic metastases undergoing surgical resection. *Carcinogenesis*, **28**, 1241–1246.
3. Nguyen,D.X. *et al.* (2009) Metastasis: from dissemination to organ-specific colonization. *Nat. Rev. Cancer*, **9**, 274–284.
4. Fidler,I.J. (2003) The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat. Rev. Cancer*, **3**, 453–458.
5. Koizumi,K. *et al.* (2007) Chemokine receptors in cancer metastasis and cancer cell-derived chemokines in host immune response. *Cancer Sci*., **98**, 1652–1658.
6. Lira,S.A. *et al.* (2012) The biology of chemokines and their receptors. *Immunol. Res*., **54**, 111–120.
7. Yoshie,O. *et al.* (1997) Novel lymphocyte-specific CC chemokines and their receptors. *J. Leukoc. Biol*., **62**, 634–644.
8. Zlotnik,A. *et al.* (2000) Chemokines: a new classification system and their role in immunity. *Immunity*, **12**, 121–127.
9. Kim,J. *et al.* (2005) Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. *J. Clin. Oncol*., **23**, 2744–2753.
10. Ottaiano,A. *et al.* (2006) Overexpression of both CXC chemokine receptor 4 and vascular endothelial growth factor proteins predicts early distant relapse in stage II-III colorectal cancer patients. *Clin. Cancer Res*., **12**, 2795–2803.
11. Yoshitake,N. *et al.* (2008) Expression of SDF-1 alpha and nuclear CXCR4 predicts lymph node metastasis in colorectal cancer. *Br. J. Cancer*, **98**, 1682–1689.
12. Müller,A. *et al.* (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature*, **410**, 50–56.
13. Taichman,R.S. *et al.* (2002) Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res*., **62**, 1832–1837.
14. Su,L. *et al.* (2005) Differential expression of CXCR4 is associated with the metastatic potential of human non-small cell lung cancer cells. *Clin. Cancer Res*., **11**, 8273–8280.
15. Kajiyama,H. *et al.* (2008) Involvement of SDF-1alpha/CXCR4 axis in the enhanced peritoneal metastasis of epithelial ovarian carcinoma. *Int. J. Cancer*, **122**, 91–99.
16. Tan,C.T. *et al.* (2008) CXCL12/CXCR4 promotes laryngeal and hypopharyngeal squamous cell carcinoma metastasis through MMP-13-dependent invasion via the ERK1/2/AP-1 pathway. *Carcinogenesis*, **29**, 1519–1527.
17. Domanska,U.M. *et al.* (2013) A review on CXCR4/CXCL12 axis in oncology: no place to hide. *Eur. J. Cancer*, **49**, 219–230.
18. Engl,T. *et al.* (2006) CXCR4 chemokine receptor mediates prostate tumor cell adhesion through alpha5 and beta3 integrins. *Neoplasia*, **8**, 290–301.
19. Jones,J. *et al.* (2007) CXCR4 chemokine receptor engagement modifies integrin dependent adhesion of renal carcinoma cells. *Exp. Cell Res*., **313**, 4051–4065.
20. Hynes,R.O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell*, **110**, 673–687.
21. Bandyopadhyay,A. *et al.* (2009) Defining the role of integrin alphavbeta6 in cancer. *Curr. Drug Targets*, **10**, 645–652.
22. Niu,J. *et al.* (1998) Integrin-mediated signalling of gelatinase B secretion in colon cancer cells. *Biochem. Biophys. Res. Commun*., **249**, 287–291.
23. Yang,G.Y. *et al.* (2008) Integrin alpha v beta 6 mediates the potential for colon cancer cells to colonize in and metastasize to the liver. *Cancer Sci*., **99**, 879–887.
24. Zhang,Z.Y. *et al.* (2008) Integrin alphanvbeta6 acts as a prognostic indicator in gastric carcinoma. *Clin. Oncol. (R. Coll. Radiol)*., **20**, 61–66.
25. Zhao-Yang,Z. *et al.* (2008) Signaling and regulatory mechanisms of integrin alphavbeta6 on the apoptosis of colon cancer cells. *Cancer Lett*., **266**, 209–215.
26. Peng,C. *et al.* (2009) Norcantharidin induces HT-29 colon cancer cell apoptosis through the alphavbeta6-extracellular signal-related kinase signaling pathway. *Cancer Sci*., **100**, 2302–2308.
27. Niu,W. *et al.* (2010) Effects of alphavbeta6 gene silencing by RNA interference in PANC-1 pancreatic carcinoma cells. *Anticancer Res*., **30**, 135–142.
28. Liu,S. *et al.* (2013) The β6-integrin-ERK/MAP kinase pathway contributes to chemo resistance in colon cancer. *Cancer Lett*., **328**, 325–334.

29. Wang,J. *et al.* (2011) PKC promotes the migration of colon cancer cells by regulating the internalization and recycling of integrin alphavbeta6. *Cancer Lett.*, **311**, 38–47.
30. Maréchal,R. *et al.* (2009) High expression of CXCR4 may predict poor survival in resected pancreatic adenocarcinoma. *Br. J. Cancer*, **100**, 1444–1451.
31. Tang,C.H. *et al.* (2008) Involvement of matrix metalloproteinase-9 in stromal cell-derived factor-1/CXCR4 pathway of lung cancer metastasis. *Carcinogenesis*, **29**, 35–43.
32. Zirvi,K.A. *et al.* (1993) Sensitivity of human colon tumor metastases to anticancer drugs in athymic (nude) mice. *Cancer Lett*., **72**, 39–44.
33. Sato,M. *et al.* (1997) Interaction between human cancer cells and cultured murine endothelial cells, and its relationship with metastatic potential. *Int. J. Oncol*., **10**, 1173–1178.
34. Hamada,K. *et al.* (2008) Liver metastasis models of colon cancer for evaluation of drug efficacy using NOD/Shi-scid IL2Rgammanull (NOG) mice. *Int. J. Oncol*., **32**, 153–159.
35. Tammali,R. *et al.* (2011) Inhibition of aldose reductase prevents colon cancer metastasis. *Carcinogenesis*, **32**, 1259–1267.
36. Lai,T.H. *et al.* (2009) Stromal cell-derived factor-1 increase alphavbeta3 integrin expression and invasion in human chondrosarcoma cells. *J. Cell. Physiol*., **218**, 334–342.
37. Sun,Y.X. *et al.* (2007) Expression and activation of alpha v beta 3 integrins by SDF-1/CXC12 increases the aggressiveness of prostate cancer cells. *Prostate*, **67**, 61–73.
38. Niu,J. *et al.* (2002) Integrin expression in colon cancer cells is regulated by the cytoplasmic domain of the beta6 integrin subunit. *Int. J. Cancer*, **99**, 529–537.
39. Huang,W.S. *et al.* (2012) Stromal cell-derived factor-1/CXC receptor 4 and β1 integrin interaction regulates urokinase-type plasminogen activator expression in human colorectal cancer cells. *J. Cell. Physiol*., **227**, 1114–1122.
40. Bates,R.C. *et al.* (2005) Transcriptional activation of integrin beta6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. *J. Clin. Invest*., **115**, 339–347.
41. Stamatovic,S.M. *et al.* (2006) CCL2 regulates angiogenesis via activation of Ets-1 transcription factor. *J. Immunol*., **177**, 2651–2661.
42. Ulrich,F. *et al.* (2009) Trafficking and cell migration. *Traffic*, **10**, 811–818.
43. Scala,S. *et al.* (2005) Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. *Clin. Cancer Res*., **11**, 1835–1841.
44. Burger,M. *et al.* (2003) Functional expression of CXCR4 (CD184) on small-cell lung cancer cells mediates migration, integrin activation, and adhesion to stromal cells. *Oncogene*, **22**, 8093–8101.
45. Engl,T. *et al.* (2006) Prostate tumor CXC-chemokine profile correlates with cell adhesion to endothelium and extracellular matrix. *Life Sci*., **78**, 1784–1793.
46. Spano,J.P. *et al.* (2004) Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome. *Ann. Oncol*., **15**, 613–617.
47. Huang,Y.C. *et al.* (2007) Stromal cell-derived factor-1 enhances motility and integrin up-regulation through CXCR4, ERK and NF-kappaB-dependent pathway in human lung cancer cells. *Biochem. Pharmacol*., **74**, 1702–1712.
48. Teicher,B.A. *et al.* (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin. Cancer Res*., **16**, 2927–2931.
49. Callaway,K.A. *et al.* (2006) Properties and regulation of a transiently assembled ERK2.Ets-1 signaling complex. *Biochemistry*, **45**, 13719–13733.
50. Milkiewicz,M. *et al.* (2008) Shear stress-induced Ets-1 modulates protease inhibitor expression in microvascular endothelial cells. *J. Cell. Physiol*., **217**, 502–510.

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