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The role of CXCR7 on the adhesion, proliferation and angiogenesis of endothelial progenitor cells

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Abstract

Previous studies confirmed that stromal cell-derived factor 1 (SDF-1) was a principal regulator of retention, migration and mobilization of haematopoietic stem cells and endothelial progenitor cells (EPCs) during steady-state homeostasis and injury. CXC chemokine receptor 4 (CXCR4) has been considered as the unique receptor of SDF-1 and as the only mediator of SDF-1-induced biological effects for many years. However, recent studies found that SDF-1 could bind to not only CXCR4 but also CXC chemokine receptor 7 (CXCR7). The evidence that SDF-1 binds to the CXCR7 raises a concern how to distinguish the potential contribution of the SDF-1/CXCR7 pathway from SDF-1/CXCR4 pathway in all the processes that were previously attributed to SDF-1/CXCR4. In this study, the role of CXCR7 in EPCs was investigated *in vitro*. RT-PCR, Western blot and flow cytometry assay demonstrate that both CXCR4 and CXCR7 were expressed highly in EPCs. The adhesion of EPCs induced by SDF-1 was inhibited by blocking either CXCR4 or CXCR7 with their antibodies or antagonists. SDF-1 regulated the migration of EPCs *via* CXCR4 but not CXCR7. However, the transendothelial migration of EPCs was inhibited by either blocking of CXCR4 or CXCR7. Both CXCR7 and CXCR4 are essential for the tube formation of EPCs induced by SDF-1. These results suggested that both CXCR7 and CXCR4 are important for EPCs in response to SDF-1, indicating that CXCR7 may be another potential target molecule for angiogenesis-dependent diseases.

Keywords: CXC chemokine receptor 7 • stromal cell-derived factor 1 • endothelial progenitor cells • CXC chemokine receptor 4 • angiogenesis

Introduction

Endothelial progenitor cells (EPCs) are a circulating, bone marrow derived cell population that can differentiate into mature endothelial cells [1]. Emerging evidence shows that EPCs play a crucial role in neovascularization of ischemic tissue [2, 3] and tumorigenesis [4]. The processes of EPCs participating in neovascularization are regulated by a variety of growth factors, cytokines and chemokines, which may affect the mobilization, homing, proliferation and differentiation of EPCs [3, 5, 6]. However, the exact mechanisms driving EPC mobilization and homing to neovascularization sites are largely unknown. Stromal cell-derived factor 1

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(SDF-1) has been confirmed as a principal regulator of retention, migration and mobilization of EPCs during steady-state homeostasis and injury [7, 8]. Reportedly the expression of SDF-1 could be up-regulated in the neovascularization sites, suggesting that SDF-1 gradient is required for facilitating mobilization of EPCs into peripheral blood and homing to neovascularization sites. For many years, CXC chemokine receptor 4 (CXCR4) has been considered as the unique receptor of SDF-1 and as the only mediator of SDF-1-induced biological effects [9, 10]. However, recent studies reported that SDF-1 was also a ligand of a novel chemokine receptor, CXC chemokine receptor 7 (CXCR7) [11, 12]. CXCR7 is widely expressed on haematopoietic system, heart, vascular endothelial cells, bone, kidney and brain [12–16]. CXCR7 has a significant higher binding affinity for SDF-1 than CXCR4 [12].

The functions of CXCR7 and its molecular interactions in the cells after binding with SDF-1 remain poorly defined. Some studies revealed that CXCR7 functions as a signalling receptor, promoting cell proliferation, adhesion, chemotaxis and activation of downstream signalling molecules such as extracellular signal-regulated kinase (ERK)1/2 and/or Akt [11, 17, 18]. However, these effects of CXCR7 are not observed in other conditions. For instance, during zebrafish development, CXCR7 seems to act as a decoy receptor to scavenge or sequester SDF-1, thereby forming SDF-1 gradient which is critical in guiding proper primordial germ cell migration [19]. Levoye *et al.* described that CXCR7 *per se* does not trigger Gαi protein-dependent signalling, but it can modulate SDF-1-mediated G protein signalling through heterodimerizing with CXCR4 [20]. Collectively, the functions of CXCR7 are very complex. However, most of studies on CXCR7 have focused on cancer biology, and the role of CXCR7 in EPCs remains largely unclear.

It was confirmed that CXCR7 plays a critical role in foetal endothelial biology, cardiac development and B-cell localization by characterizing CXCR7-deficient mice [21]. The expression of CXCR7 is elevated in endothelial cells associated with tumours [22]. Miao *et al.* further confirmed a critical role for CXCR7 in tumour vascular formation and angiogenesis [23]. All these previous experimental results support the possible involvement of CXCR7 in SDF-1-mediated angiogenesis. Mazzinghi *et al.* described that CXCR4 and CXCR7 played essential, but differential roles in the therapeutic homing of human renal progenitor cells: CXCR7 involves in the renal progenitor cell survival and adhesion to endothelium whereas CXCR4 involves in the chemotaxis [24]. This study clearly indicates that CXCR7 plays an important role in the renal progenitor survival. However, to date whether CXCR7 also plays certain role in other progenitor or stem cell physiology such as EPCs were unexplored.

In the present study, therefore, we aimed to identify the role of CXCR7 in EPCs. We found that both receptors of SDF-1, CXCR4 and CXCR7, were highly expressed in EPCs, and SDF-1 regulated the behaviours of EPCs through both receptors with distinct effects. SDF-1 regulated the adhesion, transendothelial migration, proliferation and tube formation of EPCs through both of CXCR4 and CXCR7, and the chemotaxis of EPCs through CXCR4 alone, whereas SDF-1 regulated the survival of EPCs *via* CXCR7 but not CXCR4.

Materials and methods

EPCs isolation and characterization

Mononuclear cells (MNCs) were isolated from rat bone marrow by density gradient centrifugation with percoll-1083 (Sigma, St. Louis, MO, USA), plated on 6-well plates coated with fibronectin (Sigma), and cultured in endothelial cell basal medium-2 (EBM-2, Lonza, Basel, Switzerland) supplemented with 10% foetal bovine serum (FBS, Hyclone, Logan, UT, USA) and EGM-2 SingleQuots (Lonza). After 4 days' culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), and then new medium was applied. Cell colonies appeared at day 7 after the isolation were defined as EPCs and were maintained in EBM-2 supplemented with 20% FBS. Isolated EPCs were used for *in vitro* studies within passages 2 to 3. At day 7, EPCs were characterized by acetylated low-density lipoprotein uptake and lectin binding. Cells were first incubated with Dil-acetylated low-density lipoprotein (Dil-acLDL, final concentration 10 μg/ml, Biomedical Technologies, Segrate, Milan, Italy) at 37°C for 4 hrs and then fixed with 3% paraformaldehyde for 10 min. After washing with PBS twice, the cells reacted with ulex europaeus agglutinin-1 (UEA-1, final concentration 10 μg/ml; Sigma) for 1 hr. After staining, samples were viewed with a confocal microscope (Leica, Wetzlar, Germany). Cells with double positive stainings were identified as differentiating EPCs [25]. Immunofluorescent staining was performed on EPCs to detect the expression of CD133 and vascular endothelial growth factor receptor 2 (VEGFR-2) with goat polyclonal anti-CD133 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal antibody against VEGFR-2 (Santa Cruz Biotechnology), respectively.

RT-PCR analysis of CXCR7 and CXCR4

Total RNA from EPCs was isolated using Trizol (Invitrogen, Carlsbad, CA, USA), and 1 μg of RNA was reverse-transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas International Inc., Burlington, Ontario, Canada). RT-PCR was performed with 1 μl of cDNA using 2$\times$ PCR Master Mix (Fermentas International Inc.) for 35 cycles (30 sec., 95°C; 30 sec., 52°C; 45 sec., 72°C). Primers: CXCR4 (sense), 5′-AAAATCTTCCTGCCCACC-3′ and (anti-sense) 5′-ATCCAGACGCCAACATAG-3′; CXCR7 (sense), 5′-CTGCGTCCAACAATGAGA-3′ and (anti-sense), 5′-AACAAGTAAACCCGTCCC-3′. GAPDH (sense), 5′-GAAGGTCGGAGT-CAACGG-3′ and (anti-sense) 5′-TCAAAGGTGGAGGAGTGG-3′.

Western blot analysis of CXCR7 and CXCR4

The expression of CXCR7 and CXCR4 on EPCs were detected by Western blot assay with human umbilical vein endothelial cells (HUVECs) as positive control. EPCs and HUVECs were washed with PBS and lysed in RIPA solution. Protein concentrations were determined for cell lysates clarified by centrifugation at 12,000 rpm for 10 min. Total lysate proteins (40 μg) were resuspended in loading buffer and loaded on a 10% SDS-PAGE. The gel was transferred onto a polyvinylidene difluoride membrane. For detection of CXCR7 and CXCR4, the membranes were incubated overnight with rabbit polyclonal antibody against CXCR4 (1:400; Abcam, Cambridge, MA, USA) and RDC1/CXCR7 (1:400; Abcam). Then, the membranes were washed with Tris-buffered saline with Tween 20 for three times and incubated with peroxidase conjugated goat anti-rabbit IgG (1:2000; Abcam) for 1 hr and detected by chromomeric substrate-3, 3′-diaminobenzidine.

Flow cytometry analysis for CXCR4 and CXCR7 surface expression on EPCs

Cell-surface expression of CXCR7 and CXCR4 was quantified by flow cytometric analysis. Cultured EPCs were suspended in PBS supplemented with 0.3% bovine serum albumin (BSA) and 0.1% sodium azide, and subsequently incubated for 30 min. at 4°C with rabbit anti-CXCR4 polyclonal antibody (1:100; Abcam), rabbit Anti-CXCR7 polyclonal antibody (1:100; Abcam) and rabbit IgG isotype antibody, respectively, and for another 30 min. with fluorescein isothiocyanate (FITC)-labelled goat polyclonal secondary antibody against rabbit IgG (1:200; Abcam). Flow cytometric analysis was performed with a FACScan (BD FACSCalibur, San Jose, CA, USA).

Adhesion assay

To investigate EPC adhering to extracellular matrix, dishes were coated with fibronectin (50 μg/ml; Sigma) or collagen I (100 μg/ml; BD Biosciences, San Jose, CA, USA), SDF-1 (10 ng/ml; Sigma) was immobilized on fibronectin or collagen for 1 hr. EPCs were pre-treated either with anti-CXCR4, anti-CXCR7 antibody, IgG isotype control (10 μg/ml each; Abcam), CXCR4 antagonist AMD3100 (10 μg/ml; Sigma) [26], CXCR7 antagonist CCX733 (1 μM, ChemoCentryx, Inc., Mt. View, CA, USA) [26], anti-CXCR4 plus anti-CXCR7 antibody or CXCR4 antagonist plus CXCR7 antagonist, respectively, for 30 min. at 37°C, and then added into dishes for 30 min. at 37°C after washing. Non-adherent cells were washed off thoroughly. Adherent cells were counted by inverted-phase contrast microscope on multiple 10× fields.

HUVECs (5×10^4 cells per well) were allowed to adhere to 24-well plastic tissue culture plates overnight. The monolayer was treated with medium containing 10 ng/ml SDF-1 for 5 hrs. EPCs labelled with calcein acetoxymethyl ester (AM) (Invitrogen) were pre-treated either with anti-CXCR4 and anti-CXCR7 antibodies or IgG isotype control (10 μg/ml each) as mentioned above in detail, CXCR4 antagonist AMD3100 (10 μg/ml; Sigma), CXCR7 antagonist CCX733 (1 μM, ChemoCentryx, Inc.), anti-CXCR4 plus anti-CXCR7 antibody or CXCR4 antagonist plus CXCR7 antagonist, respectively, for 30 min. at 37°C, and then added to the HUVEC monolayers for 30 min. at 37°C after washing. Non-adherent cells were washed off thoroughly. Adherent cells were quantified by inverted-phase contrast microscope (Olympus TH4–200, Tokyo, Japan) on multiple 10× fields.

Migration assay

Cells migration assay was performed in 24-transwell culture plates containing microporous (8.0 μM) membranes (Corning/Fisher Scientific, Schwerte, Germany). Briefly, EPCs suspended in EBM-2 medium supplemented with 0.5% BSA (Sigma) were added to the top chamber. SDF-1 was added to the low chamber at concentrations of 0, 1, 10 and 100 ng/ml in EBM-2 medium supplemented with 1% FBS. In some experiments, EPCs were added to the top chamber in presence of CXCR4 or CXCR7 antibody or antagonist, respectively. In these experiments, SDF-1 was used at a concentration of 10 ng/ml in EBM-2 medium supplemented with 1% FBS. After incubation for 8 hrs, non-migratory cells were removed by cotton-tipped swabs, and then the migratory cells were stained with 0.1% crystal violet solution and counted manually in random high magnification fields (×10) in each well.

Transendothelial migration assay

The transendothelial migration assay was performed in 24-transwell culture plates containing microporous (8.0 μM) membranes. Briefly, HUVECs (5×104 cells per well) were seeded onto transwell culture plates for 24 hrs to form a confluent monolayer. EPCs were labelled with calcein AM and added to the top chamber in presence of CXCR4 or CXCR7 antibody or antagonist, respectively. Transmigration toward a 10 ng/ml SDF-1 gradient was quantified by fluorescence microscopy (Olympus TH4–200) on multiple 10× fields.

Cell proliferation

Cell proliferation was assayed by methyl thiazolyl tetrazolium (MTT; Sigma). Cells (5×10^4 cells per well) were treated for 8 hrs with SDF-1 (0, 1, 10 and 100 ng/ml). In parallel, cells were cultured with SDF-1 (10 ng/ml) in presence of CXCR4 or CXCR7 antibody or antagonist, respectively for 8 hrs. Cells were then incubated with 0.5 mg/ml MTT for 4 hrs at 37°C. After 4 hrs of incubation at 37°C, the medium was aspirated and the formazan reaction products were dissolved in 150 μl DMSO solution. The optical density (OD) of the formazan solution was measured with a microplate reader at 570 nm.

Assay for cell apoptosis

EPC apoptosis induced by serum starvation was detected by annexin V-FITC staining (KeyGenBiotech., Nanjing, China) to determine whether CXCR4/SDF-1 or CXCR7/SDF-1 exerted a survival effect on EPCs. Briefly, EPCs were cultured with EBM-2 basic medium with SDF-1 (10 ng/ml) in the presence or absence of CXCR4 or CXCR7 antibody or antagonist, respectively, for 48 hrs. After treated for 48 hrs, EPCs were collected and washed for three times. Annexin V-FITC and propidium iodide (PI) were added to the washed cells (10^6 cells/ml in FACS buffer) for 15 min. at room temperature in the dark. FACS buffer was added, and cells were analysed immediately by flow cytometry.

Tube formation assay

EPC tube formation was assessed in matrigel (BD Biosciences) as described previously [27]. EPCs (2×10^4 cells) suspended in EBM-2 medium containing 0.5% FBS were plated on a 24-well dish that has been coated of matrigel and incubated with SDF-1 (10 ng/ml) with and without CXCR4 or CXCR7 antibody or antagonist, respectively. After cultured at 37°C for 72 hrs, the tube-like structures were record under a light microscope (Olympus TH4–200) equipped with a digital camera (Olympus DP25) in a blind manner. The length of tube-like structures in the images was measured using ImageJ (http://rsbweb.nih.gov/ij/). At least six fields were examined per well and experiments were repeated with three independent EPCs cultures. A relative tube length was calculated as follows: total length of the tubes per field with SDF-1 in the presence or absence of CXCR4 or CXCR7 antibody or antagonist, respectively, was divided by the total length of the tubes without SDF-1 (control) in each experiment.

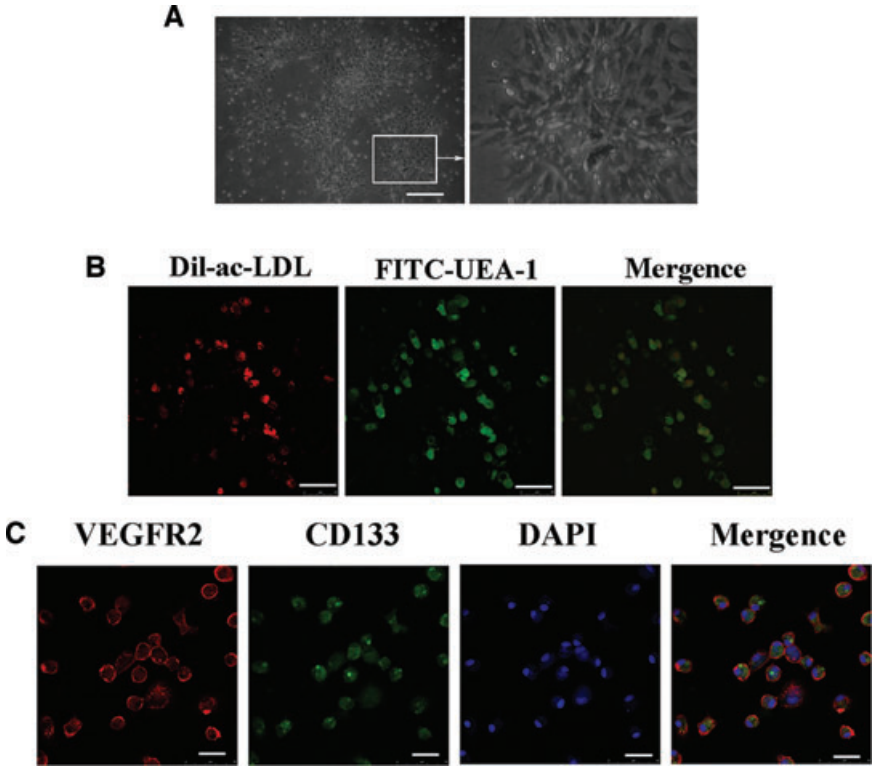

Fig. 1 Characterization of isolated EPCs. (**A**) Bone marrow derived MNCs appear a spindle-shape and cluster arrangement after 7-day culture under endothelial-specific conditions; the scale bars represent 200 μm. (**B**) DiI-acLDL and FITC-UEA-1 uptake assay show that the cells are both DiI-acLDL/FITC-UEA-1^{+}, which indicates that the isolated MNCs are EPCs, the scale bars represent 50 μm. (**C**) The cultured MNCs were further characterized by immunofluorescent staining using EPCs specific markers CD133/VEGFR-2 and nuclear maker 4′,6-diamidino-2-phenylindole (DAPI); the scale bars represent 50 μm.

Statistical analysis

All data are represented as mean ± S.D., obtained from at least three independent experiments. Statistical analysis was performed with Origin 7.5 (OriginLab data analysis and graphing software) with one-way ANOVA and Student's t-test analysis. A *P*-value less than 0.05 was considered statistically significant.

Results

Characterization of EPCs

Bone marrow derived MNCs cultured under endothelial-specific conditions developed a spindle-shaped appearance and typical cell clusters at day 7 after the isolation (Fig. 1A). The appearance and organization of these cell clusters resembled the characteristic blood island-like cell clusters. The outgrowth cells were positive for DiI-acLDL uptake and lectin binding. Most of the attached cells were positive for both stains (Fig. 1B). The double positive cells were recognized as differentiating EPCs. Immunofluorescent staining assay demonstrated that most of the cells were positive for CD133 and VEGFR2 (Fig. 1C), confirming the double positive cells as EPCs.

RT-PCR analysis revealed that the receptors of SDF-1, CXCR4 and CXCR7 were expressed highly in EPCs (Fig. 2A), which was also confirmed at the protein level by Western blot analysis (Fig. 2B) and flow cytometric analysis (Fig. 2C).

The effects of CXCR7 and CXR4 on EPC adhesion

The adhesion capacity of EPCs to the activated endothelial cells and the extracellular matrix are very important for EPC participating in angiogenesis [28]. We tested whether SDF-1 promotes EPC adhesion to extracellular matrix or endothelial cells using an *in vitro* cell adhesion assay. As shown in Figure 3A and B, both CXCR7 antibody and CXCR4 antibody significantly inhibit SDF-1-mediated EPC adhesion to collagen (Fig. 3A) and fibronectin (Fig. 3B). The inhibitory effect of either CXCR7 antibody or CXCR4 antibody was confirmed with either CXCR7 antagonist CCX733 or CXCR4 antagonist AMD3100 (Fig. 3A and B). Although both CXCR4 and CXCR7 antibodies or antagonists could inhibit the adhesion of EPCs to fibronectin, the inhibitory effect of CXCR7 antibody or antagonist is more predominant. There was no significant additive effect when both CXCR4 and CXCR7 were blocked either with their antibodies or antagonists.

However, pre-treatment of calcein AM-labelled EPCs with an anti-CXCR7 antibody or CCX733 blocked the adhesion of these labelled EPCs to HUVEC monolayer, but pre-treatment of these EPCs

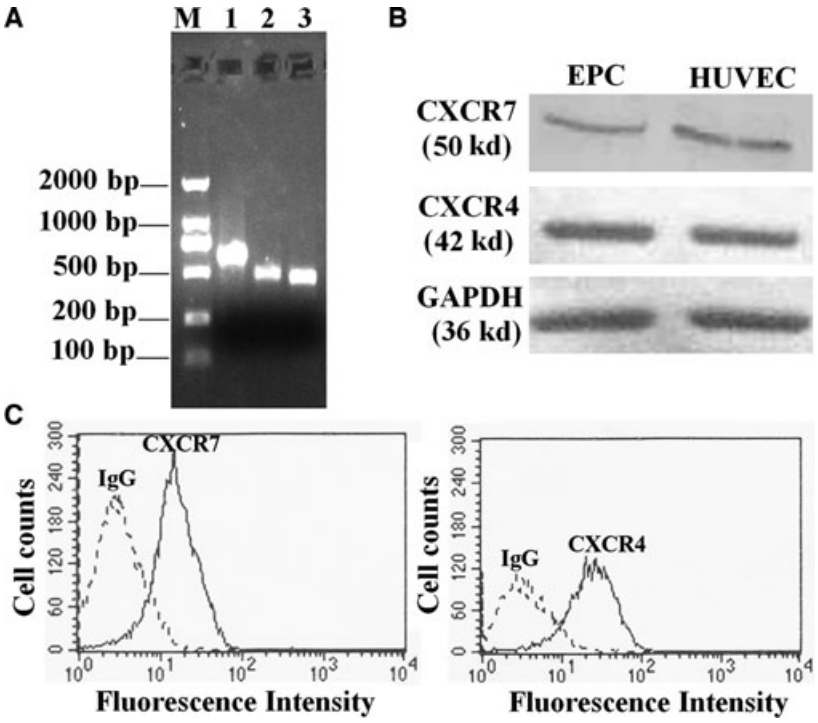

Fig. 2 The expression of CXCR7 and CXCR4 on EPCs. The expression of CXCR7 and CXCR4 on EPCs was detected by RT-PCR analysis for the mRNA (**A**) [M: Maker, DL2000; 1: GAPDH (618 bp); 2: CXCR7 (473 bp); 3: CXCR4 (446 bp)] and by Western blot analysis for the protein (**B**), respectively. GAPDH was used as loading control and HUVECs were used as CXCR7 and CXCR4 expression positive control. The cell surface expression of CXCR7 and CXCR4 was investigated by flow cytometry (**C**).

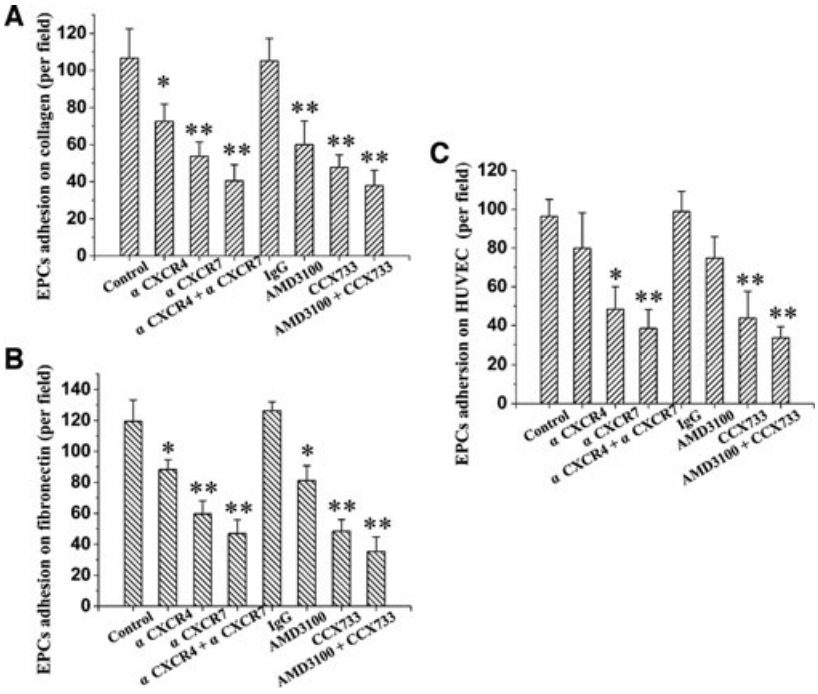

Fig. 3 The adhesion properties of EPCs to ECM components and HUVEC induced by SDF-1. EPCs pre-treated with anti-CXCR4 antibody (α CXCR4), anti-CXCR7 antibody (α CXCR7), IgG control, AMD3100 or CCX733, respectively, were added onto ECM components [collagen (**A**) and fibronectin (**B**)] or HUVEC monolayer bond with SDF-1 (**C**) for 30 min., and then the number of adherent cells was counted in multiple microscopic 10× fields. Data are given as mean ± S.D. (*$P < 0.05$; **$P < 0.01$ *versus* control).

with anti-CXCR4 antibody or AMD3100 had no such significant effect (Fig. 3C). It is worth mentioned that the inhibitory effect of blocking both CXCR7 and CXCR4 either with their antibodies or antagonists are similar to that of blocking CXCR7 alone (Fig. 3C). These results thus suggested that both CXCR4 and CXCR7 are essential for SDF-1-mediated EPCs adhesion to extracellular matrix, whereas SDF-1 mediates adhesion of EPCs to endothelial monolayer only *via* CXCR7.

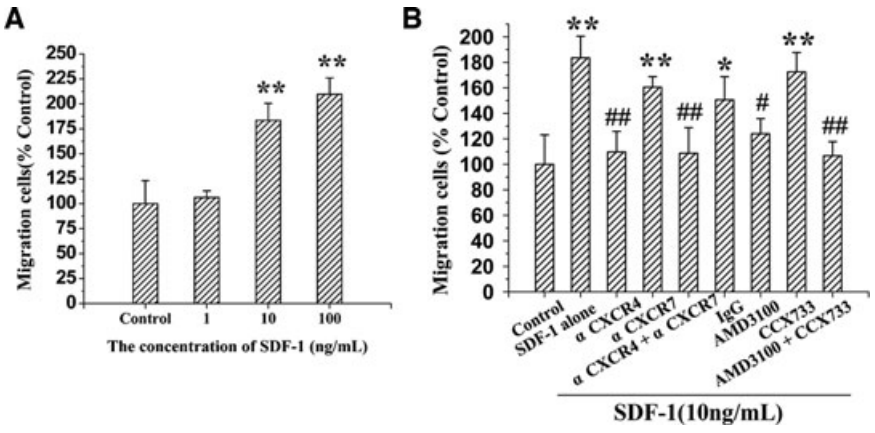

Fig. 4 Requirement of CXCR4 but not CXCR7 for SDF-1-induced migration of EPCs. EPC migration was assayed in 24-transwell culture plates containing microporous (8.0 μM) membranes. (**A**) Dose–response assay, for which EPCs suspended in EBM-2 medium supplemented with 0.5% BSA were added to the top chamber, and SDF-1 was added to the low chamber at a concentrations of 0, 1, 10 and 100 ng/ml in EBM-2 medium supplemented with 1% FBS. (**B**) Effect of CXCR7 or CXCR4 inhibition on the SDF-1-induced migration of EPCs, for which EPCs suspended in the above mentioned medium were added to the top chamber in presence of anti-CXCR4 antibody (α CXCR4), anti-CXCR7 antibody (α CXCR7), IgG control, AMD3100 or CCX733, and SDF-1 at a concentration of 10 ng/ml in EBM-2 medium supplemented with 1% FBS was added to the low chamber. Results are given as mean ± S.D. of three independent experiments (*$P < 0.05$; **$P < 0.01$, *versus* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *versus* SDF-1).

Requirement of CXCR4, but not CXCR7, for the migration of EPCs

The chemotactic activity of SDF-1 on EPCs was evaluated *in vitro*. SDF-1 induced the migration of EPCs in a dose-dependent manner, and 10–100 ng/ml SDF-1 can significantly induce the migration of EPCs (Fig. 4A). Pre-treatment with CXCR4 antibody or antagonist significantly inhibited SDF-1-induced EPC migration (109.7 ± 16.12% or 105.67 ± 14.11% *versus* 183.5 ± 17.01%, $P < 0.01$), whereas pre-treatment of EPCs with CXCR7 antibody or antagonist had no such effect on the chemotactic response (Fig. 4B). Blocking both CXCR7 and CXCR4 either with their antibodies or antagonists inhibited the migration of EPCs in a similar level to blocking CXCR4 alone (Fig. 4B). The results suggest that SDF-1-induced chemotactic response of EPCs was mainly mediated through CXCR4.

Essentiality of both CXCR4 and CXCR7 for the transendothelial migration of EPCs

The process of EPC mobilizing from bone marrow and engrafting to neo-angiogenesis sites requires transendothelial migration [27]. Thus, the contribution of CXCR4 and CXCR7 to SDF-1-induced transendothelial migration of EPCs *via* a HUVEC monolayer was also investigated. As shown in Figure 5, the exposure of EPCs to SDF-1 (10 ng/ml) in the bottom chamber significantly increased the transendothelial migration of EPCs (96.7 ± 8.6 *versus* 15.8 ± 4.2 cells/field; $P < 0.01$). The transendothelial migration response to SDF-1 was significantly suppressed by anti-CXCR4 antibody and AMD 3100 (54.7 ± 7.5 or 42.37 ± 8.87 *versus* 96.7 ± 8.6 cells/field, $P < 0.01$). Similarly anti-CXCR7 antibody and antagonist CCX733 also significantly suppressed the migration (41.6 ± 5.6 or 47.2 ± 10.82 *versus* 96.7 ± 8.6 cells/field, $P < 0.01$). When both of CXCR4 and CXCR7 were blocked either by their antibodies or antagonists, the number of transendothelial migration cells was significantly less than that of blocking either CXCR4 or CXCR7 alone (Fig. 5B). These findings suggest that both CXCR4 and CXCR7 are required for the transendothelial migration of EPCs, and the effects of CXCR4 and CXCR7 are additive.

The effects of CXCR4 and CXCR7 on the proliferation of EPCs

The proliferation of EPCs cultured in growth factor-deprived EBM-2 medium containing 1 ng/ml, 10 ng/ml or 100 ng/ml SDF-1 was measured by MTT. As shown in Figure 6A, 10–100 ng/ml SDF-1 significantly induced EPCs' proliferation. Because it is known that SDF-1 plays an important role in EPCs' proliferation through CXCR4 receptor [27], the next study was to investigate the role of CXCR7 in EPCs' proliferation. To this end, we treated SDF-1-stimulated EPCs with the antibodies and antagonists of CXCR4 or CXCR7 and then analysed their proliferation rates. MTT assay demonstrated that blocking either CXCR7 or CXCR4 could not significantly reduced the enhancement of EPC proliferation induced by SDF-1, whereas blocking both CXCR7 and CXCR4 significantly inhibited the EPC proliferation induced by SDF-1 (Fig. 6B). Thus SDF-1 enhanced EPCs proliferation is mediated by both of CXCR7 and CXCR4.

The effects of CXCR4 and CXCR7 on EPC survival

To examine the role of CXCR4 and CXCR7 in EPC survival, we determined serum deprivation-induced EPCs apoptosis by flow cytometry with FITC-conjugated annexin V and PI staining. As

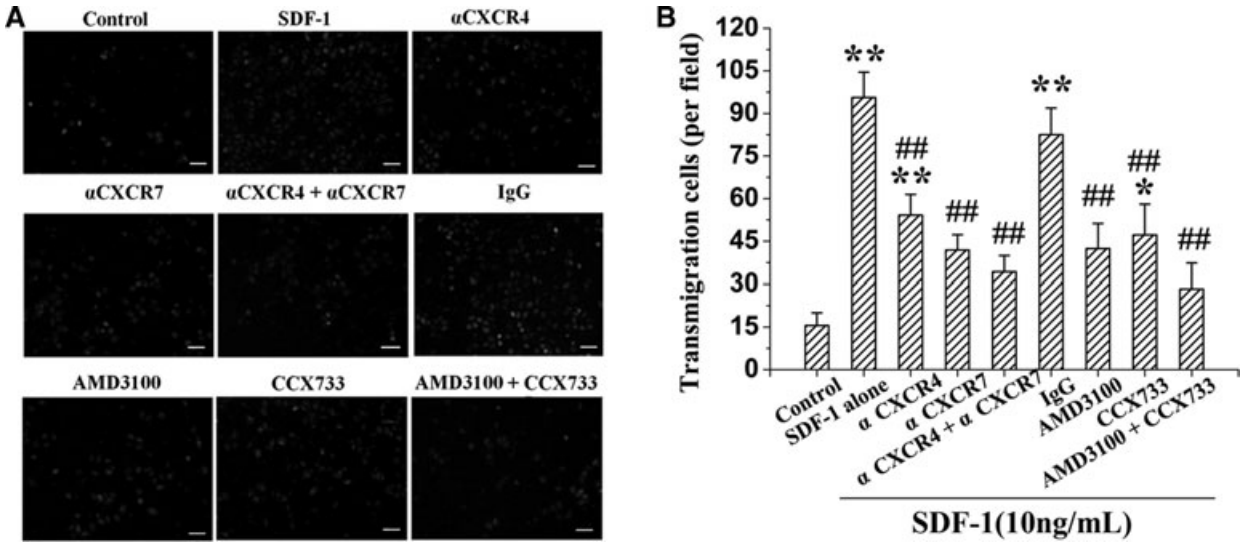

Fig. 5 Requirement of both CXCR7 and CXCR4 for the transendothelial migration of EPCs. (**A**) The transendothelial migration of EPCs toward 10 ng/ml SDF-1 was examined by fluorescence microscopy, and one representative image of three independent experiments is presented for each group. Scale bars represent 100 μM. (**B**) Quantitative analysis of the transmigration of EPCs as shown in (**A**), showing that the transendothelial migration of EPCs induced by SDF-1 (10 ng/ml) is suppressed by pre-treatment with anti-CXCR4 antibody (α CXCR4), anti-CXCR7 antibody (α CXCR7), IgG control, AMD3100 or CCX733, respectively. Results are expressed as the mean ± S.D. ($*P < 0.05$, $**P < 0.01$, *versus* control; $^{\#\#}P < 0.01$, *versus* SDF-1).

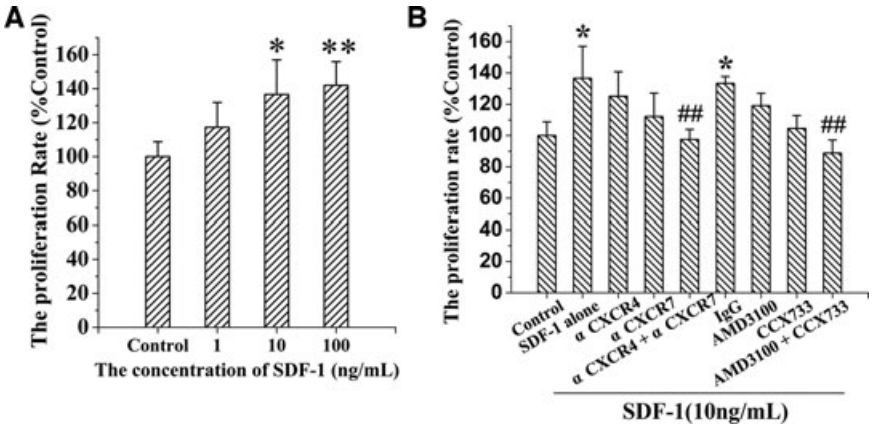

Fig. 6 Inhibition of SDF-1-induced proliferation of EPCs by blocking both CXCR4 and CXCR7. The proliferation of EPCs induced by SDF-1 was determined by MTT assay. (**A**) Dose–response of the proliferation of EPCs treated with SDF-1 (0, 1, 10 and 100 ng/ml) for 8 hrs. (**B**) Inhibitory effects on 10 ng/ml SDF-1-induced proliferation of EPCs with pre-treatment of either anti-CXCR4 antibody (α CXCR4), anti-CXCR7 antibody (α CXCR7), IgG control, AMD3100 or CCX733, respectively. Data are given as mean ± S.D. ($*P < 0.05$, $**P < 0.01$, *versus* control, $^{\#\#}P < 0.01$, *versus* SDF-1).

shown in Figure 7, serum deprivation induced about 19% of apoptotic cell death, but this apoptotic cell death can be significantly prevented with treatment of SDF-1 for 48 hrs (7.47 ± 0.71% *versus* 18.93 ± 2.58%, $P < 0.01$). The anti-apoptotic effect of SDF-1 was almost completely attenuated by CXCR7 antibody or antagonist CCX733 (16.05 ± 1.69% or 18.02 ± 1.95% *versus* 7.47 ± 0.71%, $P < 0.01$). However, blocking CXCR4 with anti-CXCR4 antibody or CXCR4 antagonist AMD3100 could not significantly diminish the anti-apoptotic effect of SDF-1 on the EPCs with serum deprivation. In addition, blocking both CXCR4 and CXCR7 has a similar effect to blocking CXCR7 alone in the protection of EPCs from apoptosis. Collectively, the results suggest that SDF-1 mediates EPC survival predominantly *via* CXCR7.

Essentiality of both CXCR4 and CXCR7 for SDF-1-induced tube formation by EPCs

EPCs in EBM-2 medium containing 1 ng/ml, 10 ng/ml or 100 ng/ml SDF-1 was planted on matrigel, and length of tube-like structures was measured. SDF-1 (10–100 ng/ml) significantly induced EPC tube formation (Fig. 8A). To evaluate the roles of CXCR4 and CXCR7 in SDF-1-induced angiogenesis, we treated SDF-1-stimulated EPCs with CXCR4 and CXCR7 antibodies or antagonists, respectively, and then measured tube length. As shown in Figure 8B, CXCR4 antibody or antagonist AMD3100 significantly decreased the length of SDF-1-induced tubes in comparison of the

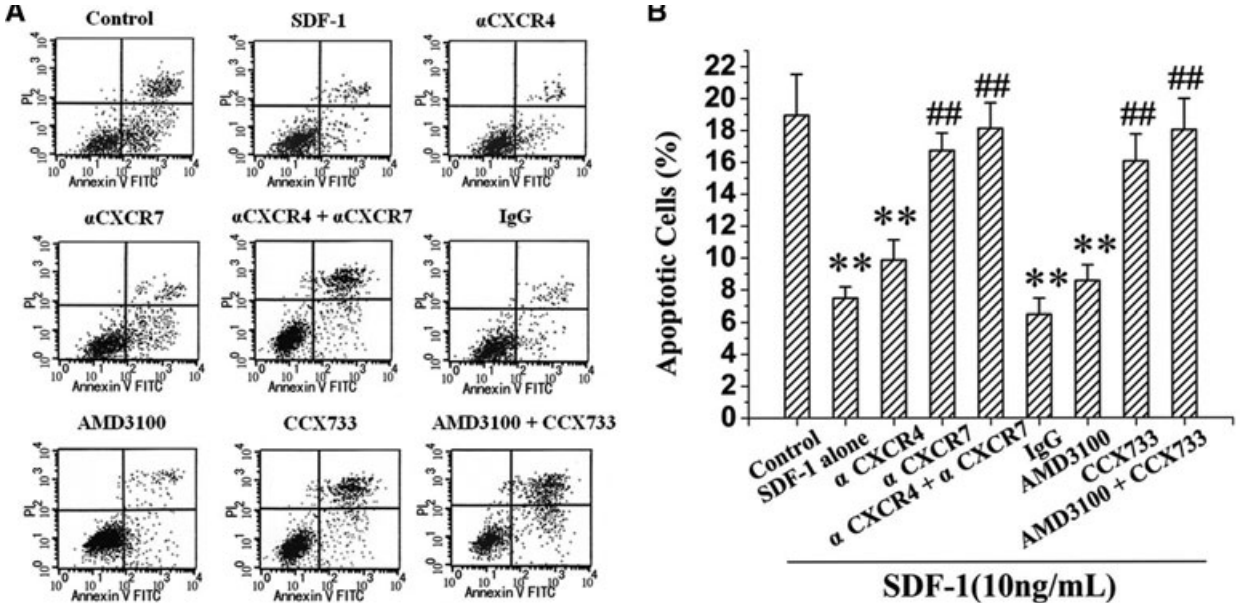

Fig. 7 SDF-1-promoted EPC survival predominantly *via* CXCR7. EPCs were cultured for 48 hrs in serum-free medium supplemented with SDF-1 (10 ng/ml) in the presence or absence of anti-CXCR4 antibody (α CXCR4), anti-CXCR7 antibody (α CXCR7), IgG control, AMD3100 or CCX733, respectively, and then the apoptotic cells were assessed by staining with FITC-conjugated annexin V and PI and evaluated by flow cytometry. (**A**) Representative flow cytometry gating data for annexin^{+} cells in EPCs with different treatments. (**B**) The apoptotic cells percentage was summarized and presented as mean ± S.D. ($^{**}P < 0.01$, *versus* control without SDF-1; $^{\#\#}P < 0.01$, *versus* SDF-1).

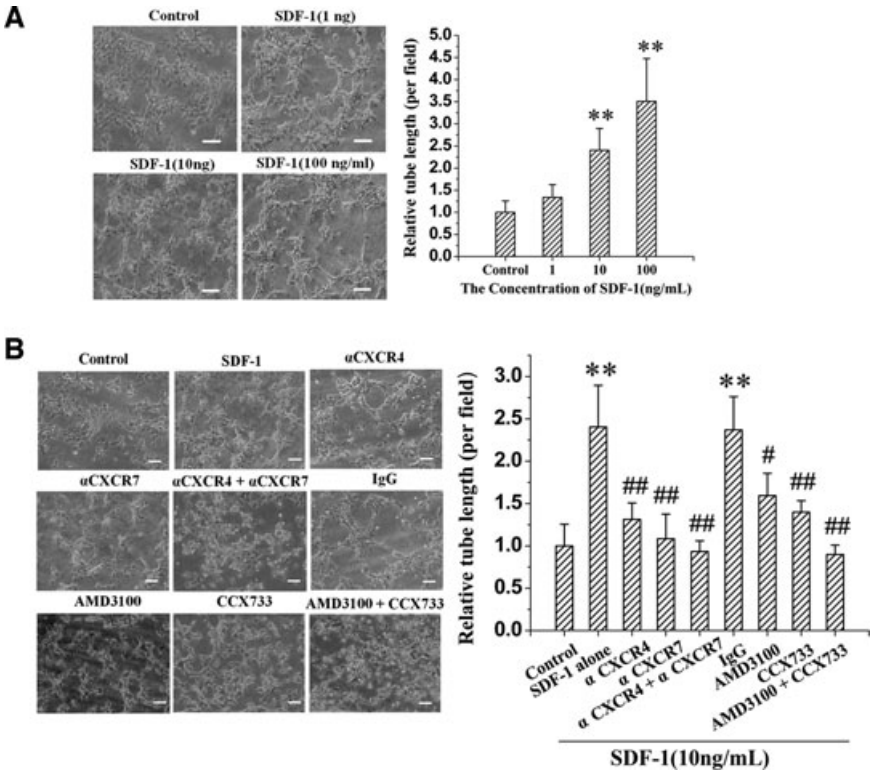

Fig. 8 The effects of CXCR7 and CXCR4 on SDF-1-induced angiogenesis *in vitro*. Angiogenesis was assayed by measuring the lengths of tube-like structures under a light microscope equipped with a digital camera in a blind manner. (**A**) EPCs were cultured on the matrigel with different concentrations of SDF-1 or without SDF-1 for 72 hrs. Representative images of tube-like structures from different groups (left panel) and quantitative data (right panel) are presented. (**B**) EPCs cultured on the matrigel with SDF-1 (10 ng/ml) were simultaneously treated with and without anti-CXCR4 antibody (α CXCR4), anti-CXCR7 antibody (α CXCR7), IgG control, AMD3100 or CCX733, respectively, for 72 hrs. The lengths of tube-like structures were measured as (**A**). Data are given as mean ± S.D. ($^{**}P < 0.01$, *versus* control without SDF-1; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, *versus* SDF-1). Scale bars represent 100 μM.

group without blocking CXCR4 (1.31 ± 0.19 or 1.59 ± 0.26 *versus* 2.4 ± 0.48, $P < 0.01$); Similarly CXCR7 antibody or antagonist CCX733 also significantly inhibited SDF-1-induced EPC tube formation (1.08 ± 0.29 or 1.40 ± 0.133 *versus* 2.4 ± 0.48, $P < 0.01$). These results suggest that SDF-1-induced tube formation from EPCs through both CXCR4 and CXCR7. When blocking both of CXCR4 and CXCR7 with their antibodies or antagonists, the effect of SDF-1 on tube formation was completely abolished.

Discussion

In this study, we investigated the roles of chemokine receptor CXCR4 and CXCR7 in the adhesion, proliferation and angiogenesis of EPCs isolate from rat bone marrow using an *in vitro* model. The key findings are that: (1) both receptors of SDF-1, CXCR4 and CXCR7, were highly expressed on EPCs; (2) the adhesion, transendothelial migration, proliferation and tube formation of EPCs induced by SDF-1 was mediated by both CXCR4 and CXCR7; (3) SDF-1 regulated the migration of EPCs *via* CXCR4 but not CXCR7 and (4) CXCR7 but not CXCR4 was essential for SDF-1-mediated EPC survival under stress condition such as serum deprivation. These results indicate that both CXCR4 and CXCR7 are important for EPCs response to SDF-1.

Neoangiogenesis and neovascularization are not exclusively attributed to adjacent mature and terminally differentiated endothelial cells, but also to the bone marrow derived EPCs [29]. EPCs could mobilize from bone marrow to neovascularization sites to participate the neoangiogenesis. During this process, it has been shown that SDF-1 plays a major role in recruitment and retention of EPCs to the neoangiogenic niches for revascularization of ischemic tissue and tumour growth *via* CXCR4, which has been considered as the unique receptor of SDF-1 for many years [9, 10]. However, Burns *et al.* [11] characterized an alternate receptor of SDF-1, CXCR7, and found that CXCR7 involved in cell survival, cell adhesion and tumour development. This study raised several questions regarding the potential contributions of the SDF-1/CXCR7 axis to the effects that have been all attributed to SDF-1/CXCR4 interactions. Subsequent studies have unravelled a broad range of CXCR7 functions. First, during the development CXCR7 seems to act as a SDF-1 scavenger to shape the extracellular SDF-1 gradient that directs CXCR4-induced migration of primordial cells [19, 30]. Second, in the T lymphocytes CXCR7 its own does not mediate SDF-1-triggered integrin activation but is essential for CXCR4 to mediate SDF-1-dependent integrin activation [31]. In line with this, a recent study also reported that CXCR7 its own did not trigger SDF-1-mediated G protein signalling, but heterodimerized with CXCR4 to form a CXCR7/CXCR4 heterodimer that in turn can initiate SDF-1-mediated signals [20]. Third, CXCR7 also acted as an independent signalling receptor in some tumour cells [23, 32] and peripheral nerve system [17]. Collectively, more and more research on the cellular function of CXCR7 has provided new insights into understanding the puzzling picture.

The role of CXCR7 in EPC adhesion and migration

The first novel finding of the present study is that CXCR4 and CXCR7 have differential roles in EPC migration (Figs 4 and 5). Unlike classical chemokine receptors, CXCR7 does not mobilize Ca^{2+} from intracellular stores or extracellular sources after ligand binding [19, 31]. So it had been considered that CXCR7 could not directly induce cell migration. In the present study, we found that blocking of CXCR4 significantly inhibited the chemotaxis of EPCs induced by SDF-1, whereas blocking of CXCR7 did not inhibit the chemotactic migration of EPCs (Fig. 4B). Our result was consistent with the recent findings that SDF-1-induced migration of renal progenitor cells [24] and mesenchymal stem cells [33] was mediated only by CXCR4. However, a few more recent studies suggested that CXCR7 can regulate neuron [34, 35] and tumour cell [36] migration induced by SDF-1. These results indicate that the differential role of CXCR7 in cell movement may depend on cell or tissue specificity.

Zabel *et al.* [37] confirmed that CXCR7 played an essential role in the CXCL12/$CXCR4^+$ mediated transendothelial migration of $CXCR4^+CXCR7^+$ human tumour cells. In our study, we found that the transendothelial migration of EPCs toward SDF-1 gradient was significantly inhibited by either blocking of CXCR4 or CXCR7 and synergistically inhibited by blocking both CXCR4 and CXCR7 (Fig. 5), which are in line with the role of CXCR7 in human renal progenitor cells [24]. These results indicate that both CXCR4 and CXCR7 are necessary for the transendothelial migration of EPCs, but they might act *via* separate mechanisms. The transendothelial migration needs EPCs adhering to endothelial cells [38], and other group has confirmed that CXCR7 mediated the adhesion of human renal progenitor cells to endothelial cells [24], so we deduced that CXCR7 mediated the transendothelial migration of EPCs by mediating the adhesion of EPCs.

The role of CXCR7 in EPC angiogenesis

The second novel finding is that both CXCR4 and CXCR7 are essential for SDF-1-induced EPC angiogenesis. Previous evidence has indicated that the expression of CXCR7 was frequently up-regulated in tumour-associated endothelial cells and activated endothelial cells [23], and CXCR7 may promote tumour-associated angiogenesis [39]. However, it remains unclear whether CXCR7 plays any role in EPC differentiating into mature endothelial cells and forming new vessels. We investigated the role of CXCR7 in the angiogenic potential of EPCs by examining tube formation. We demonstrated that both CXCR7 and CXCR4 were required for the tube formation from EPCs in response to SDF-1 *in vitro* (Fig. 8). Our study is support of the most recent study that reported the requirement of CXCR7 and CXCR4 for the angiogenesis in the rheumatoid arthritis [26]. Therefore, CXCR7 may be a target molecule in new therapies for angiogenesis-involved diseases.

In summary, we have demonstrated for the first time that both CXCR7 and CXCR4 were highly expressed on EPCs. CXCR7 involved in EPC proliferation, survival and adhesion to extracellular

matrix or endothelium but not in the migration of EPCs. Both CXCR7 and CXCR4 are essential for the transmigration and tube formation of EPCs induced by SDF-1. These evidence indicated that CXCR7 plays a critical role in EPC homing and participating in angiogenesis, and CXCR7 may be another potential target molecule for angiogenesis-dependent diseases.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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