Elevating CXCR7 Improves Angiogenic Function of EPCs via Akt/GSK-3β/Fyn-Mediated Nrf2 Activation in Diabetic Limb Ischemia

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Rationale: Endothelial progenitor cells (EPCs) respond to stromal cell–derived factor 1 (SDF-1) through chemokine receptors CXCR7 and CXCR4. Whether SDF-1 receptors involve in diabetes mellitus–induced EPCs dysfunction remains unknown.

Objective: To determine the role of SDF-1 receptors in diabetic EPCs dysfunction.

Methods and Results: CXCR7 expression, but not CXCR4 was reduced in EPCs from db/db mice, which coincided with impaired tube formation. Knockdown of CXCR7 impaired tube formation of EPCs from normal mice, whereas upregulation of CXCR7 rescued angiogenic function of EPCs from db/db mice. In normal EPCs treated with oxidized low-density lipoprotein or high glucose also reduced CXCR7 expression, impaired tube formation, and increased oxidative stress and apoptosis. The damaging effects of oxidized low-density lipoprotein or high glucose were markedly reduced by SDF-1 pretreatment in EPCs transduced with CXCR7 lentivirus but in EPCs transduced with control lentivirus. Most importantly, EPCs transduced with CXCR7 lentivirus were superior to EPCs transduced with control lentivirus for therapy of ischemic limbs in db/db mice. Mechanistic studies demonstrated that oxidized low-density lipoprotein or high glucose inhibited protein kinase B and glycogen synthase kinase-3β phosphorylation, nuclear export of Fyn and nuclear localization of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), blunting Nrf2 downstream target genes heme oxygenase-1, NAD(P)H dehydrogenase (quinone 1) and catalase, and inducing an increase in EPC oxidative stress. This destructive cascade was blocked by SDF-1 treatment in EPCs transduced with CXCR7 lentivirus. Furthermore, inhibition of phosphatidylinositol 3-kinase/protein kinase B prevented SDF-1/CXCR7-mediated Nrf2 activation and blocked angiogenic repair. Moreover, Nrf2 knockdown almost completely abolished the protective effects of SDF-1/CXCR7 on EPC function in vitro and in vivo.

Conclusions: Elevated expression of CXCR7 enhances EPC resistance to diabetes mellitus–induced oxidative damage and improves therapeutic efficacy of EPCs in treating diabetic limb ischemia. The benefits of CXCR7 are mediated predominantly by a protein kinase B/glycogen synthase kinase-3β/Fyn pathway via increased activity of Nrf2. (Circ Res. 2017;120:e7-e23. DOI: 10.1161/CIRCRESAHA.117.310619.)

Key Words: angiogenesis ■ chemokine CXCL12 ■ CXC chemokine receptor type 7 ■ endothelial progenitor cells ■ hindlimb ischemia

Diabetes mellitus is a common, chronic, metabolic disease producing great social and economic burden. Microvascular and macrovascular complications associated with type 2 diabetes mellitus are leading causes of morbidity and mortality for diabetic patients. Vascular complications in diabetes mellitus are associated with dysregulation of vascular remodeling and vascular growth, decreased responsiveness to ischemic/hypoxic stimuli, impaired or abnormal

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EPCs respond to stromal cell–derived factor 1 through receptors CXCR7 and CXCR4. The reduced CXCR7 but not CXCR4 expression leads to impaired angiogenic function of EPCs under diabetic conditions, whereas elevating CXCR7 expression enhances EPCs resistance to diabetes mellitus–induced oxidative damage and improves therapeutic efficacy of EPCs. The benefits of CXCR7 are mediated by a protein kinase B/glycogen synthase kinase 3β/tyrosine kinase Fyn pathway via nuclear factor (erythroid-derived 2)-like 2 activation. Our study demonstrates for the first time that reduced CXCR7 expression contributes to diabetes mellitus–induced EPCs dysfunction and provides a novel therapeutic target for enhancing endothelial repair capacity in diabetes mellitus.

Nonstandard Abbreviations and Acronyms

- EPCs: endothelial progenitor cells
- CXCR4: CXC chemokine receptor type 4
- CXCR7: CXC chemokine receptor type 7
- CXCR7-EPCs: EPCs transduced with CXCR7 lentivirus
- GSK-3β: glycogen synthase kinase 3β
- HO-1: heme oxygenase-1
- MNCs: mononuclear cells
- NQO-1: NAD(P)H dehydrogenase (quinone 1)
- Nrf2: nuclear factor (erythroid-derived 2)-like 2
- Null-EPCs: EPCs transduced with control lentivirus
- ox-LDL: oxidized low-density lipoprotein
- ROS: reactive oxygen species
- SDF-1: stromal cell–derived factor 1, also named CXCL12
- WT: wild-type
- Akt: protein kinase B

EPC-induced neovascularization is a highly coordinated, temporally regulated, and complex set of events including mobilization, migration, and homing.9,10 Chemokines play an essential role in each of these steps. Stromal cell–derived factor 1 (SDF-1, also known as CXCL12) is a key chemokine in regulating hematopoietic stem cell trafficking between the bone marrow and peripheral circulation and attracts EPCs to areas of ischemia. For some time, chemokine receptor type 4 (CXCR4) was thought to be the only SDF-1 receptor, and numerous studies demonstrated a role of the SDF-1/CXCR4 axis in mobilizing stem and progenitor cells from bone marrow, homing to target tissue, chemotaxis, adhesion, survival, and angiogenesis.11,12 In 2005, a second SDF-1 receptor was identified, CXCR7 that binds SDF-1 with 10-fold higher affinity than CXCR4.13 EPCs express both CXCR4 and CXCR714,15 and recently CXCR7 is shown to influence EPCs clinical status, that is, EPCs of hypertensive patients express low levels of CXCR7 and this contributes to impaired angiogenesis and re-endothelialization.16

In diabetes mellitus, the number of EPCs is reduced and EPC function is attenuated.17 Diabetes mellitus may alter expression and function of SDF-1 receptors in EPCs, but this remains untested. Our previous studies demonstrated that CXCR7 has a critical role regulating adhesion and survival of EPCs from rat bone marrow and human cord blood.14,15 Extrapolating from those studies, we hypothesized that elevating CXCR7 expression would improve angiogenic function of EPCs in type 2 diabetes mellitus. The aims of the present study were to determine the level of SDF-1 receptors in diabetic EPCs and determine whether overexpression of CXCR7 improved angiogenic function of EPCs in diabetes mellitus and to reveal mechanisms connecting EPC function and CXCR7 expression.

Methods

Animals

FVB wild-type (WT) and db/db (FVB background) male mice of 10 to 12 weeks were used in this study. All animal procedures were approved by the Animal Policy and Welfare Committee of Wenzhou Medical
University and the Institutional Animal Care and Use Committee of the University of Louisville, which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 1996).

Isolation and Culture of Bone Marrow–Derived EPC

EPCs were isolated from the bone marrow of WT (WT-EPCs) and db/db (db/db-EPCs) mice and cultured afterward according to our established methods with minor modifications. Briefly, bone marrow mononuclear cells (MNCs) were isolated from the femurs and tibiae of mice by density gradient centrifugation with histopaque-1083 (Sigma-Aldrich, St. Louis, MO). After 2 washing steps, MNCs were plated on vitronectin-coated culture dishes (Sigma-Aldrich) and maintained in endothelial growth factor–supplemented media (EGM-2 bullet kit; Lonza, Basel, Switzerland) with 10% FBS. Cells were cultured at 37°C with 5% CO2 in a humidified atmosphere. EGM-2 medium was replaced after the first 24 hours and every 3 days thereafter. Cell colonies that appeared after 7 days of culture were defined as early EPC, and cell colonies that appeared after 14 days of culture were defined as late EPC as previously reported.

Characterization of Bone Marrow–Derived MNCs

After 7 or 14 days in endothelial-specific media and the removal of nonadherent MNCs, the remaining cells were characterized by immunofluorescent staining and flow cytometry analysis. For immunofluorescent staining, cells were seeded on vitronectin-coated 8-well μ-slide (ibidi, Martinsried, Germany) 1 day before and then the cells were incubated with 5 μg/mL acetylated Dil lipoprotein from human plasma (Dil-Ac-LDL; Thermo Fisher Scientific, Waltham, MA) at 37°C for 4 hours followed by 3 washes with Dulbecco PBS (DPBS) and incubated with 10 μg/mL fluorescein isothiocyanate–labeled ulex europaeus lectin-1 (Sigma-Aldrich) for 1 hour at room temperature. After incubation, cells were rinsed with DPBS for 3× and were visualized via a confocal microscopy. For flow cytometry analysis, cells were incubated with 5% BSA (Sigma) for 15 minutes for blockade of nonspecific binding and then stained with antismouse CD34-phycocerythrin (PE), CD31-PE, CD14-PE, CD144-PE, Scal-1-fluorescein isothiocyanate, c-kit- fluorescein isothiocyanate (BD Biosciences, San Jose, CA), and VEGFR2-APC (vascular endothelial growth factor receptor 2-allophycocyanin), CD45-APC (Biolegend, San Diego, CA) at room temperature for 1 hour, respectively. The same fluorescein-labeled isotype IgG served as control to define the negative populations for each stain. Cells were analyzed with a BD FACSAria cell sorter (BD Biosciences), and data were analyzed using FlowJo software version 8.8.4 (TreeStar, Inc, Ashland, OR).

Lentiviral Vector Construction, Virus Production, and Infection

To upregulate the CXCR7 expression in EPCs, recombinant lentiviruses encoding CXCR7 (pLVX-CXCR7-EGFP-3FLAG-Puro) was constructed by cloning the CXCR7 gene into the pLVX-EGFP-3FLAG-Puro vector (Shanghai Sunbio Medical Biotechnology, Shanghai, China) via EcoRI and BamHI sites. The CXCR7 cDNA was amplified using the following primers: forward: 5′-CGGAGCCAAAGAATTCCGGCAG-3′ reverse: 5′-CGGAGGCCCAAACTACTAGCCTCGG-3′. Viral supernatants were produced in human embryonic kidney 293 cells constitutively expressing the simian virus 40 (SV40) large T antigen after cotransfection of CXCR7 recombinant vector (pLVX-CXCR7-EGFP-3FLAG-Puro) with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The supernatant was harvested at 72 hours post-transfection, filtered through Millex-HV 0.45-μm polycarbonate filter (Millipore, Billerica, MA), and stored at −80°C until use.

Early EPCs or late EPCs (passages 3–4) were infected with the purified lentivirus carrying recombinant CXCR7 (CXCR7-EPCs) or control vector (Null-EPCs) overnight at multiplicity of infection of 25 with 2.5 μg/mL polybrene (Santa Cruz, Dallas, TX), and the medium was replaced with fresh growth medium 24 hours after infection. After transfection for 72 hours, the infection efficiency was determined by flow cytometry analysis of green fluorescent protein (GFP) expression. The levels of CXCR7 expression were detected by Western blot and flow cytometry assay.

To knockdown CXCR7 expression in early EPCs from WT mice, specific siRNAs against mouse CXCR7, alone with Silencer Select Negative Control (Thermo Fisher) were transfected into EPCs using Lipofectamine 2000 (Thermo Fisher). After transfection for 48 hours, the expression of CXCR7 was determined by Western blot.

To knockdown nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression in late EPCs from WT mice, EPCs were infected with the lentiviruses containing shRNA against Nrf2 or nonsense shRNA constructed by Shanghai Sunbio Medical Biotechnology (Shanghai, China). We selected 3 target sequences for Nrf2 and nonsense sequence as follows:

- Nrf2-shRNA 1: 5′-CTTGAAGTCTTCGACGTATGTTA-3′
- Nrf2-shRNA 2: 5′-GCTTTAATCTCCGAGTAA-3′
- Nrf2-shRNA 3: 5′-GACCTCTTATGACTAAATCC-3′

Nonsense shRNA: 5′-TTTCTCGAAGCCTGTCAGTTT-3′. The transfection was performed after the procedure described above. After transfection for 48 hours, the expression of Nrf2 was determined by Western blot. Then CXCR7-EPCs or Null-EPCs were infected with the lentiviruses containing shRNA against Nrf2 with the best knockdown efficiency or nonsense shRNA after the same protocol described above.

Apoptosis Assay

EPCs transduced with control lentivirus or lentivirus encoding CXCR7, Nrf2 shRNA, or nontarget shRNA were seeded on 12-well plates (1×10⁵ cells per well) and maintained under basal culture conditions or with oxidized low-density lipoprotein (ox-LDL, 50 μg/mL; Athens Research & Technology, Inc., Athens, GA), or high glucose (HG, 25 mmol/L; Sigma Chemical Co., St. Louis, MO) along with a same dose of mannitol (Sigma Chemical Co.) as osmotic control for 24 hours in the presence or absence of SDF-1 (100 ng/mL). Nonadherent cells were removed by washing with PBS. Subsequently, adherent cells were released with 0.25% trypsin without EDTA. EPCs were collected by centrifugation and stained with APC-conjugated Annexin V Apoptosis Detection Kit with propidium iodide, according to the manufacturer’s instructions (Biolegend). The apoptotic EPCs were detected by a flow cytometry. Early apoptotic cells were defined as AnnexinV+/propidium iodide+.

Angiogenesis Assay In Vitro

The in vitro angiogenic capability of EPCs was determined by a transwell migration assay. Briefly, 48-well plates were coated with growth factor–reduced matrix gel (150 μL/well; BD Biosciences). EPCs transduced with control lentivirus or lentivirus encoding CXCR7, Nrf2 shRNA, or nontarget shRNA were plated (5×10⁴ cells per well) in 200-μL basal culture medium or medium containing ox-LDL or HG in the presence or absence of SDF-1 and incubated at 37°C with 5% CO₂ for 12 hours to form tubes. Images of tubes in each well were taken using an inverted microscope (Nikon Eclipse E600; Nikon, Kanagawa, Japan). The tube lengths were calculated by Image J software.

Transendothelial Migration Assay

Transendothelial migration (TEM) is one of most important abilities for EPCs participating in angiogenesis. We evaluated TEM abilities of EPCs by a transwell assay according to our previous report. Human umbilical vein endothelial cells (1×10⁵ cells per well) were cultured in the upper chamber of a 24-transwell insert (8.0-μm pores; Falcon 353097; BD, Bedford, MA). Before each experiment, monolayer confluency was confirmed by inverted fluorescence microscopy. EPCs transduced with control lentivirus or lentivirus encoding CXCR7, Nrf2 shRNA, or nontarget shRNA were cultured in the presence or absence of ox-LDL for 12 hours or HG for 24 hours. Then EPCs were harvested and resuspended in basal culture medium (EBM-2, 0.5% BSA). EPC suspension (0.2 mL) was added to the upper chamber of the transwell, and 0.6 mL EBM-2 medium was added to the lower chamber after 24 hours. The tube formation by EPCs was visualized at high power magnification by an inverted microscope (Nikon, Japan). The tube lengths were quantitated by Image J software.
supplemented with either PBS or SDF-1 (100 ng/mL) was added to the lower chamber of the transwell. Cells were cultured for 12 hours at 37°C, and EPCs traversing from the upper to the lower chamber of the transwell was quantified by independent investigators blinded to treatment.

**Quantitative Determination of Oxidative Stress**

To detect the reactive oxygen species (ROS) level of EPCs with ox-LDL or HG treatment, dithiothreitol (Molecular Probes, Eugene, OR) probe was used to stain EPCs. Dithiothreitol is cell permeable and able to react with superoxide to form ethidium, which in turn intercalates with DNA and produces nuclear fluorescence. EPCs were seeded on 24-well plates and treated with ox-LDL or HG in presence or absence SDF-1 for 6 hours and then incubated with 5 μmol/L dithiothreitol in PBS for 30 minutes at 37°C. Nuclear dihydroethidium positive staining indicates superoxide generation in cells. The fluorescence intensity was detected by a microplate reader (SpectraMax M3; Molecular Devices, Sunnyvale, CA) under specific wavelength conditions (excitation, 518 nm; fluorescence, 605 nm). Meanwhile, the fluorescence images of random EPCs were captured at ×20 magnification (XI 71 Olympus, Tokyo, Japan).

**Western Blot Assay**

Western blot was performed as previous study. For cellular protein extraction, EPCs were rinsed twice with PBS and then suspended in ice-cold RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX) and incubated for 15 to 30 minutes on ice. Nuclear proteins from EPCs were extracted by a nuclear extraction kit (Abcam, Cambridge, MA). Proteins were collected by centrifugation, 12,000 g for 15 minutes at 4°C. The protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA). The total and nuclear proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in tris-buffered saline with 5% nonfat milk and 0.5% BSA for 1 hour and then incubated with the primary antibody overnight at 4°C, followed by incubation with the secondary antibodies for 1 hour at room temperature after standard washing procedures. The primary antibodies against Nrf2 (1:1000), histone H3 (1:1000), heme oxygenase-1 (HO-1, 1:1000), NAD(P)H dehydrogenase quinone 1 (NQO-1, 1:1000), catalase (1:5000), GAPDH (1:5000), and β-actin (1:3000) were purchased from Santa Cruz Biotechnology; protein kinase B (Akt) and p-Akt (Ser473; 1:2000), glycogen synthase kinase (GSK)-3β (1:1000), and β-actin (1:3000) were purchased from Cell Signaling Technology (Danvers, MA). Proteins were collected by centrifugation, 12,000 g for 15 minutes at 4°C. The protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA). The comparative cycle time (Ct) method was used to determine fold differences between samples, and the amount of target genes was normalized to β-actin as an endogenous reference (2−∆∆Ct).

**Histological Assessment**

To examine the homing and incorporation of EPCs in the ischemic muscle, ischemic gastrocnemius muscle and soleus muscle were embedded in optimal cutting temperature medium for frozen section. The GFP-positive EPCs were counted in randomly selected fields for a total of 20 different fields (×40 magnification) per section and 3 sections per animal. The EPC incorporation was expressed as the percentage of GFP-positive capillaries.

**Statistical Analysis**

All data are presented as mean±SD. Statistical analysis was performed using Origin data analysis and graphing software with 1-way or 2-way ANOVA, followed by post hoc multiple comparisons with the Scheffe test. Statistical significance was considered as P<0.05.

**Results**

**Characterization of Bone Marrow–Derived EPCs**

MNCs isolated from mouse bone marrow were cultured in EGM-2 medium on vitronectin-coated culture dishes. Three days after plating cell colonies appeared with centrally rounded cells and spindly peripheral cells (Online Figure IA, left). By 7 days, spindle-shaped adherent early EPCs were observed (Online Figure IA, middle). By 14 to 21 days of culture, endothelium-like cells with cobblestone-like morphology late EPCs were nearly confluent (Online Figure IA, right). By 14 to 21 days of culture, endothelium-like cells with cobblestone-like morphology late EPCs were nearly confluent (Online Figure IA, right). Both early and late EPCs are positive for DiI-acLDL and UEA-1 (Online Figure IB and IC). Flow cytometry demonstrated that many early EPCs were positive for CD34, CD31, CD14, CD45, Scale-1, c-KIT, and VEGFR2, but mostly negative for CD144 (Online Figure ID with quantitation). The cell surface profile of late EPCs was positive for CD34, CD31,
Figure 1. Diabetes attenuates CXC chemokine receptor type 7 (CXCR7) expression and impairs the angiogenic function of endothelial progenitor cells (EPCs). A–C, Early EPCs were isolated from bone marrow of wild-type (WT-EPCs) and db/db (db/db-EPCs) mice at 10 to 12 wk and assayed within 7 d of isolation. Expression of CXCR7 and CXCR4 was detected by Western blot (A) and flow cytometry (B). Angiogenic function of EPCs was evaluated by tube formation assay (C). D, Early WT-EPCs were transfected with specific siRNA against mouse CXCR7 (CXCR7-siRNA) or Silencer Select Negative Control (Ctrl-siRNA), and the transfection reagent (Lipofectamine (Continued))
VEGFR2, CD144, c-Kit, and Scal-1, but low or negative for both CD14 and CD45 (Online Figure IE with quantitation). These characteristics were consistent with previous descriptions of EPCs.18,20,21

**Diabetes Mellitus Attenuates CXCR7 Expression and Impairs Angiogenic Function of EPCs**

To determine how EPCs were affected by prolonged in vivo diabetes mellitus, early EPCs were isolated from bone marrow of WT and *db/db* mice and assayed for expression of SDF-1 receptors and for tube formation. Total and cell-surface expression of CXCR7 were significantly decreased in EPCs from *db/db* mice compared with those from WT mice, whereas no significant differences were seen for CXCR4 expression (Figure 1A and 1B). Tube formation by EPCs from *db/db* mice was also functionally deficient as indicated by the significantly shorter length of the tubes *db/db* EPCs produced compared with WT-EPCs (Figure 1C).

To determine whether reduced CXCR7 expression in diabetic EPCs was a cause of angiogenic dysfunction, gain- and loss-of-function studies were performed by CXCR7-siRNA knockdown in early EPCs from WT mice, and CXCR7 overexpression in early EPCs from *db/db* mice. CXCR7 levels were significantly reduced by the siRNA in WT-EPCs (Online Figure II), and this was accompanied by impaired tube formation (Figure 1D). Conversely, increasing CXCR7 levels in *db/db* EPCs with CXCR7 lentivirus (Online Figure II) completely reversed impaired tube formation function of diabetic EPCs (Figure 1D). Most importantly, transplantation of CXCR7 recombinant lentiviral vector transduced *db/db* EPCs time dependently improved blood perfusion in HLI in *db/db* mice (Figure 2A), which was accompanied by promoted angiogenesis mirrored by increased capillary density and exogenous EPCs incorporation mirrored by GFP encoded by the transduced lentivirus vector and isolatecolocalization in both soleus muscle (Figure 2B) and gastrocnemius muscle (Figure 2C) measured 28 days after HLI, but without obvious effects on arteriogenesis mirrored by no apparent changes in α-smooth muscle actin–positive arteriole area in adductor muscle (Figure 2D). These results suggest that diabetic downregulation of CXCR7 plays a causative role in diabetes mellitus–induced EPCs angiogenic dysfunction.

**Upregulating CXCR7 Expression Improves Survival and Angiogenic Function of EPCs Treated With ox-LDL and HG**

To overcome the shortage of number of limitation of early EPCs and facilitate gene manipulation, late EPCs from WT mice were used for the following mechanistic studies. Late EPCs from WT mice were transduced with CXCR7 recombinant lentiviral vector or control vector. At 72 hours after transduction, transfection efficiency was >70% as measured by GFP expression (Online Figure IIIA). CXCR7 expression was dramatically increased in CXCR7-EPCs compared with Null-EPCs and WT-EPCs as determined by flow cytometry (Online Figure IIIB) and Western blot assay (Online Figure IIIC).

To determine whether the effects of diabetes mellitus could be reproduced in vitro, late EPCs from WT mice were treated with ox-LDL or HG to mimic conditions of diabetic dyslipidemia or hyperglycemia as previous report.22 As found with *db/db* diabetes, both ox-LDL and HG dose dependently decreased the expression of CXCR7 but not that of CXCR4, except at a highest dose of HG (Online Figure IVA and IVC). Ox-LDL and HG also impaired tube formation by EPCs from WT mice (Online Figure IVB and IVD). These results show that both ox-LDL and HG treatment can recapture the effects of diabetes mellitus on CXCR7 expression and EPCs dysfunction.

To test the effects of CXCR7 manipulation on EPC function in vitro, EPC dysfunction was induced by 24-hour exposure late EPCs from WT mice to ox-LDL at 50 μg/mL or HG at 25 mmol/L, chosen on the basis of the dose–response study in Online Figure IV. Apoptosis, measured with Annexin V/propidium iodide staining (Figure 3A; Online Figure VA) was greatly increased in Null-EPCs by both ox-LDL and HG. This was slightly but not significantly reduced by SDF-1 pretreatment.

In CXCR7-EPCs, both ox-LDL and HG also induced apoptosis, but it was significantly less than in Null-EPCs. Most striking was the almost complete protection against apoptosis in CXCR7-EPCs by SDF-1 pretreatment.

Tube formation assays were used as one measure of in vitro angiogenic function (Figure 3B; Online Figure VB). Both ox-LDL and HG significantly impaired the tube formation abilities in Null-EPCs. As with apoptosis, the negative effect on tube formation was slightly but not significantly reduced by SDF-1 pretreatment in Null-EPCs. In CXCR7-EPCs, ox-LDL and HG had less inhibitory effect on tube formation and SDF-1 pretreatment provided much more effective protection against ox-LDL and HG inhibition than it did in Null-EPCs.

TEM is essential for EPC homing to the sites of blood vessel repair and formation and was assayed by transwell migration assay (Figure 3C; Online Figure VC). The assay showed no significant difference in basal TEM between Null-EPCs and CXCR7-EPC. When EPCs were treated with SDF-1, TEM of CXCR7-EPCs was much higher than that of Null-EPCs. Ox-LDL and HG pretreatment almost completely abolished SDF-1–stimulated increase of TEM in Null-EPCs, but in CXCR7-EPCs, the ability of SDF-1 to increase TEM was significantly preserved.

**Upregulating CXCR7 Expression Promotes EPCs-Mediated Angiogenesis and Blood Perfusion in HLI in db/db Type 2 Diabetes Mellitus**

To determine whether elevated CXCR7 improves late EPC-mediated neovascularization in type 2 diabetes mellitus in vivo, we transplanted CXCR7-EPCs or Null-EPCs into *db/db*
Both Null-EPCs and CXCR7-EPCs transplantation time dependently increased blood perfusion compared with mice without transplantation. Notably, CXCR7-EPCs were significantly more effective than Null-EPCs at all time points from days 7 to 28 (Online Figure VIA). The benefit of CXCR7-EPCs was also apparent in increased angiogenesis (Figure 2).

Figure 2. Upregulation of CXC chemokine receptor type 7 (CXCR7) expression rescues angiogenic function of db/db-endothelial progenitor cells (EPCs) in ischemic limb of db/db diabetic mice. A, Time course of blood perfusion shown in images and quantitative analysis after hindlimb ischemia surgery with or without EPC transplantation. Blood perfusion is the ratio of ischemic:nonischemic limb perfusion measured by a Pericam Perfusion Speckle Imager. B and C, Images and quantitation of immunofluorescent isolectin- and green fluorescent protein (GFP)-positive capillaries in transverse sections of soleus muscle (B) and gastrocnemius muscle (C) tissue from ischemic hind limbs. Capillary density was expressed as isolectin-positive capillaries per muscle fiber. The exogenous EPC incorporation (white arrows) was expressed as the percentage of GFP-positive capillaries. D, Representative images and quantitation of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA)–positive arteriole in the ischemic adductor muscle tissue. Arteriogenesis was expressed as \( \alpha \)-SMA–positive arteriole area per fiber normalized by PBS control. Data shown in graphs represent the mean\( \pm \)SD. \( n=8 \) mice per group. *\( P<0.05 \) vs PBS; \#\( P<0.05 \) vs lentivirus carrying control vector (Lv-Ctrl).

mice after HLI surgery. Both Null-EPCs and CXCR7-EPCs transplantation time dependently increased blood perfusion compared with mice without transplantation. Notably,
capillary density and exogenous EPCs incorporation measured 28 days after HLI (Online Figure VIB). These findings indicate that CXCR7 overexpression also promotes late EPCs homing and incorporating in endothelium in the ischemic tissue.

Upregulating CXCR7 Expression Attenuates ox-LDL- and HG-Induced Oxidative Stress in EPCs

Oxidative stress is a major factor responsible for the dysfunction of diabetic EPCs. Here, we show that ROS levels and oxidative damage produced by ox-LDL and HG are significantly reduced by elevated expression of CXCR7. Both ox-LDL and HG induced superoxide production in Null-EPCs, as measured by dihydroethidium staining, and superoxide was slightly reduced by SDF-1 pretreatment (Figure 4A; Online Figure VIIA). CXCR7 upregulation significantly attenuated ox-LDL- and HG-induced superoxide production in CXCR7-EPCs and almost completely blocked superoxide production after SDF-1 pretreatment (Figure 4A; Online Figure VIIA). Using 3-nitrotyrosine as an oxidative damage marker, we obtained similar results (Figure 4B; Online Figure VIIB): CXCR7 upregulation reduced 3-nitrotyrosine levels that were elevated by ox-LDL and HG exposure and CXCR7 completely blocked elevated 3-nitrotyrosine in the presence of SDF-1.

Upregulating CXCR7 Expression Activates Nrf2 via Akt/GSK-3β/Fyn Pathway in EPCs

Nrf2 plays a critical role in the cellular response to oxidative stress. Because increased CXCR7 expression decreased both ox-LDL- and HG-induced oxidative stress, we tested whether CXCR7 upregulation activate Nrf2 and its downstream target genes. There was no significant change in total Nrf2 protein and Nrf2 mRNA expression under any treatment condition in either Null-EPCs or CXCR7-EPCs (Figure 5A and 5B; Online Figure VIIIA). However when treated with ox-LDL or HG, nuclear Nrf2 levels significantly declined in Null-EPCs but not in CXCR7-EPCs. In fact, SDF-1 pretreatment significantly increased nuclear Nrf2 levels in CXCR7-EPCs compared with Null-EPCs (Figure 5A; Online Figure VIIIA), which was also supported by studies of Nrf2 nuclear immunofluorescent localization in Null-EPCs and CXCR7-EPCs (Figure 5C). These results indicate that CXCR7 upregulation increased Nrf2 levels in the nucleus but did not increase total Nrf2 expression. Most importantly, we found that protein and mRNA expression of antioxidant Nrf2 target genes HO-1, NQO-1, and catalase were significantly higher in CXCR7-EPCs than in Null-EPCs in the presence of ox-LDL or HG, especially with SDF-1 pretreatment (Figure 5A and 5B; Online Figure VIIIA). More importantly, CXCR7 upregulation-mediated Nrf2 nuclear translocation and downstream gene activation...
were also confirmed in early EPCs from \( \text{db/db} \) mice (Online Figure VIIIB). These changes in Nrf2 target gene expression are fully consistent with the changes found in Nrf2 nuclear localization produced by CXCR7 upregulation (Figure 5A and 5C; Online Figure VIIIA and VIIIB) and suggest that SDF-1/CXCR7 activates Nrf2 transcriptional function.

SDF-1/CXCR7 is thought to promote CD34+ hematopoietic stem/progenitor cell cycling and survival via Akt signaling, and SDF-1/CXCR7 also regulates cell migration by Akt signaling. Furthermore, the function of Nrf2 can be regulated by the Akt/GSK-3\( \beta \)/Fyn pathway in fibroblasts and PC12 cells by controlling Fyn-mediated export and degradation of nuclear Nrf2. We found that exposure of Null-EPCs and CXCR7-EPCs to ox-LDL or HG significantly decreased the phosphorylation of Akt and GSK-3\( \beta \) and increased the level of Fyn in the nucleus (Figure 6A; Online Figure IXA). In CXCR7-EPCs, SDF-1 pretreatment prevented ox-LDL– and HG-induced decreases in Akt and GSK-3\( \beta \) phosphorylation and nuclear accumulation of Fyn (Figure 6A; Online Figure IXA). These results suggest that upregulation of SDF-1/CXCR7 axis preserves EPCs function via regulating Akt/GSK-3\( \beta \)/Fyn pathway to activate Nrf2 function.

Knockdown of Nrf2 Impairs the Protective Effects of CXCR7 on EPCs

To confirm the pivotal role of Nrf2 in SDF-1/CXCR7 protection, we performed knockdown of Nrf2 gene expression with Nrf2-shRNA (Online Figure XA). The vector sh-Nrf2 2 had the best knockdown efficiency among the 3 Nrf2-shRNA vectors tested, and this vector was used to knockdown Nrf2 in subsequent studies: Nonsense shRNA did not alter Nrf2 expression and was used as control vector. Knockdown of Nrf2 expression blocked most of the protective effects we had seen with SDF-1 and CXCR7 (Figures 2 through 6; Online Figures V through IX). In SDF-1–treated...
Figure 5. Upregulating CXC chemokine receptor type 7 (CXCR7) activates nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling in endothelial progenitor cells (EPCs). Null-EPCs (EPCs transduced with control lentivirus) or CXCR7-EPCs were exposed to oxidized low-density lipoprotein (ox-LDL; 0 or 50 μg/mL) in the presence or absence of stromal cell–derived factor 1 (SDF-1; 100 ng/mL) for 12 h. A, Protein levels of Nrf2 and its downstream target genes heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase (quinone 1) (NQO-1), and catalase (CAT), and nuclear expression of Nrf2 (n-Nrf2) were detected by Western blot. B, The mRNA expression of Nrf2 and its downstream target genes HO-1, NQO-1, and CAT were determined by real-time polymerase chain reaction. C, Nrf2 nuclear translocation was determined in fixed cells by immunofluorescent staining. Three independent experiments were performed. Data shown in graphs represent the means±SD. *P<0.05 vs respective control in Null-EPCs or CXCR7-EPCs; #P<0.05 vs Null-EPC with the same treatment; &P<0.05 vs CXCR7-EPC with ox-LDL treatment.
Dai et al: CXCR7 and EPC Functions in Diabetes Mellitus

CXCR7-EPCs, Nrf2-shRNA eliminated the effect of elevated CXCR7 on nuclear accumulation of Nrf2 (Online Figure XB and XC), induction of antioxidant genes (Online Figure XB and XC), superoxide production (Figure 7B; Online Figure XIB) under ox-LDL or HG treatment conditions. The assay not affected by Nrf2 knockdown in SDF-1–treated CXCR7-EPCs was TEM ability (Figure 7C; Online Figure XIC), implying that Nrf2 is not important for TEM of EPCs. Knockdown of Nrf2 expression did not affect increased phosphorylation of Akt and GSK-3β or reduced nuclear accumulation of Fyn in SDF-1–treated CXCR7-EPCs (Online Figure XI A and XI B). This result indicates that SDF-1/CXCR7 effects on Nrf2 are downstream of Akt/GSK/Fyn. Nrf2 knockdown tested in vivo by transplantation into db/db mice effectively eliminated the ability of elevated CXCR7 expression in CXCR7-EPCs to augment the ability of EPC-mediated ischemic blood perfusion (Figure 8A) and angiogenesis (Figure 8B).

Discussion
Patients with type 1 or type 2 diabetes mellitus have decreased number and impaired angiogenic function of EPCs, which plays a causative role in the development and progression

Figure 6. Upregulating CXC chemokine receptor type 7 (CXCR7) activates nuclear factor (erythroid-derived 2)-like 2 (Nrf2) via protein kinase B (Akt)/glycogen synthase kinase (GSK)-3β/tyrosine kinase Fyn (Fyn) pathway. Null-EPCs (endothelial progenitor cells transduced with control lentivirus) or CXCR7-EPCs were pretreated with or without phosphatidylinositol 3-kinase inhibitor wortmannin for 30 min and then exposed to oxidized low-density lipoprotein (ox-LDL; 50 μg/mL) for 12 h in the presence or absence of stromal cell–derived factor 1 (SDF-1; 100 ng/mL). A, The phosphorylation of Akt and GSK-3β, and the nuclear translocation of Fyn (n-Fyn) were evaluated by Western blot. B and C, The expression of nuclear Nrf2 (n-Nrf2) and its downstream target genes (heme oxygenase-1 [HO-1], NAD(P)H dehydrogenase (quinone 1) [NQO-1], and catalase [CAT]) was evaluated by Western blot (B) and real-time polymerase chain reaction (C). (Continued)
of virtually all diabetic complications. Despite its critical importance, the mechanism for impaired EPC function in diabetes mellitus remains largely unknown. The present study provides 3 new lines of evidence implicating CXCR7 initiated pathways in diabetic EPC dysfunction: The first novel finding is the strong relationship between CXCR7 levels and diabetic EPC dysfunction: CXCR7 levels are decreased in diabetic EPCs, diabetic dysfunction is mimicked by CXCR7 knockdown, and EPC dysfunction is reversed by upregulation of CXCR7 levels. The second innovative finding is that the benefit of increased CXCR7 expression requires activation of Nrf2 antioxidant signaling. Our third novel finding is that the Akt/GSK-3β/Fyn pathway mediates CXCR7-induced Nrf2 activation in diabetic EPCs.

Emerging evidence indicates that CXCR7 is essential for regulation of multiple cellular functions. Our previous studies demonstrated that CXCR7 plays a critical role in the regulation of adhesion and survival of EPCs from both rat bone marrow and human cord blood. In the present study, we demonstrate that diabetes mellitus significantly decreased EPC cellular and cell surface levels of CXCR7 but produces no similar change in CXCR4 levels (Figure 1A and 1B). We addressed what factors in diabetes mellitus might cause downregulation of CXCR7 and dysfunction of EPCs. To test whether the decrease of CXCR7 expression in db/db EPCs could be caused by the dysmetabolic environment of diabetes mellitus, we exposed normal EPCs to elevated ox-LDL or HG to mimic dysmetabolic diabetic conditions, as previously reported. The results show that ox-LDL and HG both dose dependently decrease CXCR7 but not CXCR4 levels (Online Figure IVA and IVC) and indicate that CXCR7 is a strong candidate for impairment

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**Figure 6 Continued.** The results were normalized to the control group of Null-EPCs. D, The apoptosis of EPCs was analyzed by flow cytometry using Annexin V/propidium iodide (PI) staining. E, The angiogenic function of EPCs was determined by tube formation assay; the tube length was normalized to the control group of Null-EPCs. F, The transendothelial migration abilities of EPCs was analyzed by transwell assay. Three independent experiments were performed for each study. Data shown in graphs represent the means±SD. *P<0.05 vs respective control in Null-EPCs or CXCR7-EPCs; #P<0.05 vs Null-EPC with the same treatment; &P<0.05 vs CXCR7-EPC with ox-LDL treatment in the presence of SDF-1.
of EPC function under dysmetabolic diabetic conditions. However, the exact molecular mechanism by which the diabetic stressors HG and ox-LDL, as well as nondiabetic stressors such as hypertension,\textsuperscript{16} downregulate CXCR7 levels in EPCs remains unknown.

The most important, new finding of this study is that overexpression of CXCR7 rescued function of both diabetic EPCs and normal EPCs impaired by exposure to diabetic conditions in vitro or in vivo. Upregulation of CXCR7 levels restored angiogenic function of type 2 diabetic, \textit{db/db} EPCs (Figure 1D), and protected normal EPCs treated with ox-LDL or HG from excess apoptosis and angiogenic dysfunction (Figure 3A and 3B; Online Figure VA and VB). Moreover, upregulation of CXCR7 expression in normal or
EPCs promoted homing of EPCs to ischemic tissue, increased capillary density, and improved blood flow perfusion when EPCs were transplanted into a type 2 diabetic HLI model (Online Figure VI; Figure 2). It is well documented that elevated ox-LDL and hyperglycemic conditions can promote endothelial and smooth muscle dysfunction in

Figure 8. Knockdown of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) attenuates the beneficial effects of CXC chemokine receptor type 7 (CXCR7) upregulation on endothelial progenitor cell (EPC)-mediated angiogenesis in ischemic limb of db/db diabetic mice. The effects of Nrf2 knockdown on the beneficial effects of CXCR7 upregulation on EPC-mediated angiogenesis and blood perfusion were evaluated in the hindlimb ischemia (HLI) model in db/db diabetic mice as described in Figure 2 and Online Figure VI. A. Time course of blood perfusion after HLI surgery with or without EPCs transplantation shown in images and quantitative data analysis. Blood perfusion is the ratio of ischemic to nonischemic limb perfusion measured by PSI. B. Immunofluorescent staining and quantitation of isolectin-positive capillaries (white arrows) in transverse sections of gastrocnemius muscle tissue from ischemic hind limbs 28 days after HLI surgery. Capillary density was expressed as isolectin-positive capillaries per muscle fiber. n=8 mice per group. Data shown in graphs represent the means±SD. *P<0.05 vs PBS, #P<0.05 vs Null/sh-Ctrl-EPC group; &P<0.05 vs CXCR7/sh-Ctrl-EPC group. C. Schematic illustration of the protective effects of SDF-1/CXCR7 on EPCs under diabetic conditions. Diabetes mellitus decreases expression of CXCR7 in EPCs and induces oxidative stress, which impairs the survival and angiogenic function of EPCs. Under diabetic conditions upregulation of SDF-1/CXCR7 signaling improves EPC survival and function predominantly by Nrf2 activation mediated by increasing phosphorylation of protein kinase B and glycogen synthase kinase (GSK)-3β and inhibiting Fyn-mediated export and degradation of nuclear Nrf2. CAT indicates catalase; HO-1, heme oxygenase-1; NQO-1, NAD(P)H dehydrogenase (quinone 1); ROS, reactive oxygen species; and SDF-1, stromal cell–derived factor 1.
established arterial and arteriolar vessels during diabetes mellitus.\textsuperscript{33,34} Thus, in addition to a profound impact on capillary density and neovascularization in diabetic animals reported here, a potential contribution of changes in EPC CXCR7 expression to basal myogenic tone development and vasodilatory capacity in small diameter arterioles and its impact on blood flow in diabetes mellitus warrants further investigation. Nonetheless, considering that CXCR7 overexpression reverses diabetic EPC dysfunction and improves EPC survival these results strongly support the concept that the decline in CXCR7 levels is a significant, causative factor in development of diabetic EPC dysfunction, and further indicate that CXCR7 is a new therapeutic target for improving the ischemia-reparative capacity of EPCs in patients with diabetes mellitus. Intriguingly, EPCs from hypertensive patients have also been shown to have reduced CXCR7 levels, which coincides with impaired function,\textsuperscript{16} and like diabetic EPCs, their function can be rescued by overexpression of CXCR7.\textsuperscript{16} The analogous findings for CXCR7 in diabetic and hypertensive EPCs emphasize the importance of CXCR7 in maintaining adequate EPC function.

The second innovative finding of this study is that the benefit of increased CXCR7 expression requires activation of Nrf2 antioxidant signaling. Accumulating evidence demonstrates that oxidative stress in type 2 diabetes mellitus contributes to endothelial cell and EPC dysfunction.\textsuperscript{23,24,35} Intracellular ROS level is elevated in EPCs from diabetic mice or EPCs cultured under HG or high lipid conditions.\textsuperscript{24,35,36} Methods that reduced intracellular ROS levels restore function of diabetic EPCs.\textsuperscript{37,38} In the present study, we found that the ROS level of EPCs cultured with ox-LDL or HG was increased. Upregulating CXCR7 expression, especially when combined with SDF-1 pretreatment, almost completely inhibited the increase in ROS level of EPCs exposed to ox-LDL or HG (Figure 4; Online Figure VII). This indicates that upregulation of the SDF-1/CXCR7 axis improves EPC antioxidant capacity.

We had previously seen that Nrf2 is a critical redox sensor and one of the master regulators of the antioxidant response.\textsuperscript{39} Nrf2 binds to regulatory antioxidant response elements and activates transcription of many antioxidant genes, such as HO-1, and NQO-1 that counteract ROS.\textsuperscript{40} In this study, we demonstrate for the first time that upregulating CXCR7 markedly increased nuclear accumulation of Nrf2 and increases the expression of downstream antioxidant target genes (HO-1, NQO-1, and catalase; Figure 5; Online Figure VIII). These results indicate that upregulating CXCR7 improves EPC antioxidant capacity by activating Nrf2 transcriptional function. The essential role Nrf2 plays in endothelial function has been widely appreciated. Florczyk et al\textsuperscript{41} reported that lack of Nrf2 attenuated survival, proliferation, and angiogenic function of endothelial cells in vitro and in vivo, whereas angiogenic factors increased the nuclear localization of Nrf2 and the expression of its target genes HO-1 and NQO-1, which stimulated tube formation of endothelial cells. Furthermore, knockdown of Nrf2 inhibits angiogenesis of rat cardiac microvascular endothelial cells under hypoxic conditions.\textsuperscript{42} Consistent with these findings in endothelial cells, our studies in EPCs revealed that lentivirus-mediated, shRNA knockdown of Nrf2 almost completely abrogated the ability of elevated CXCR7 levels to augment EPC survival and tube formation in vitro (Figure 7; Online Figure XI) and to enhance ischemic angiogenesis and blood perfusion in vivo (Figure 8). These findings prove that Nrf2 plays an essential role in the ability of CXCR7 upregulation to augment EPC function.

Our third important, new finding is that the Akt/GSK-3β/Fyn pathway mediates the activation of Nrf2 produced by elevated CXCR7 in diabetic EPCs. The mechanism of Nrf2 activation has been widely investigated.\textsuperscript{43,44} In PC12 cells, it was found that puerarin triggers Akt activation, which in turn inhibits GSK-3β activation resulting in more Nrf2 nuclear translocation. Moreover, it was demonstrated that CXCR7 activates phosphatidylinositol 3-kinase/protein kinase B independently of CXCR4.\textsuperscript{27,28} In this study, diabetes mellitus, ox-LDL, and HG all reduced Akt phosphorylation in EPCs. Using in vitro studies, we showed that decreased Akt phosphorylation produced by ox-LDL or HG exposure could be prevented by SDF-1 if CXCR7 expression was elevated in EPCs (Figure 6A; Online Figure IXA and IXB), showing that augmentation of the SDF-1/CXCR7 axis activates Akt in EPCs. However, the direct linkage between the SDF-1/ CXCR7 axis and phosphorylation of Akt in EPCs remains a key unanswered question.

Several recent studies showing that the Akt/GSK-3β/Fyn pathway plays a pivotal role in regulation of Nrf2 nuclear export and degradation.\textsuperscript{35,39,45,46} Based on those studies and Akt phosphorylation results, we hypothesized that SDF-1/CXCR7 signaling activates Nrf2 via the Akt/GSK-3β/Fyn pathway. Consistent with this hypothesis, we found that stimulating the SDF-1/CXCR7 axis not only increased phosphorylation of Akt but also inhibited GSK-3β activity that decreased nuclear accumulation of Fyn (Figure 6A; Online Figure IXA and IXB). It is known that Fyn can phosphorylate Nrf2 tyrosines, promoting Nrf2 nuclear export and degradation.\textsuperscript{45,47} Importantly, we found that a phosphatidylinositol 3-kinase/protein kinase–specific inhibitor (wortmannin) almost completely abolished SDF-1/CXCR7 activation of Nrf2 and expression of its downstream genes (Figure 6B and 6C; Online Figure IXC). This was accompanied by almost complete attenuation of the protective effects of SDF-1/CXCR7 activation on EPC survival and angiogenic function under ox-LDL and HG treatment conditions (Figure 6D and 6E; Online Figure IXD and IXE).

In conclusion, upregulation of signaling of the SDF-1/CXCR7 axis improves EPC survival and function under diabetic conditions. This is predominantly because of increased Nrf2 activation, mediated in EPCs by increasing Akt and GSK-3β phosphorylation and inhibiting Fyn-mediated Nrf2 nuclear export and degradation. By preserving Nrf2 nuclear localization, Nrf2 function is enhanced and antioxidant gene expression is increased, protecting EPCs from diabetes mellitus, ox-LDL, and HG-induced oxidative damage as illustrated in Figure 8C.

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Disclosures
None.

References


Elevating CXCR7 Improves Angiogenic Function of EPCs via Akt/GSK-3β/Fyn-Mediated Nrf2 Activation in Diabetic Limb Ischemia

Xiaozhen Dai, Xiaqing Yan, Jun Zeng, Jing Chen, Yuehui Wang, Jun Chen, Yan Li, Michelle T. Barati, Kupper A. Wintergerst, Kejian Pan, Matthew A. Nystoriak, Daniel J. Conklin, Gregg Rokosh, Paul N. Epstein, Xiaokun Li and Yi Tan

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Online Figure I

A
Day 3  Day 7  Day 14

B
FITC-UEA-1  Dil-ac-LDL  Merge
Early EPCs

C
FITC-UEA-1  Dil-ac-LDL  Merge
Late EPCs

D
Early EPCs
PE-isotype-IgG  PE-CD34  PE-CD31  PE-CD14  PE-CD144
APC-isotype-IgG  APC-VEGFR2  APC-CD45  FITC-isotype-IgG  FITC-Scal-1  FITC-c-Kit

E
Late EPCs
PE-isotype-IgG  PE-CD34  PE-CD31  PE-CD14  PE-CD144
APC-isotype-IgG  APC-VEGFR2  APC-CD45  FITC-isotype-IgG  FITC-Scal-1  FITC-c-Kit
**Online Figure I.** Phenotypic characterization of bone marrow derived EPCs in normal mice. **A,** Morphology of bone marrow mononuclear cell cultures at different time points after plating. Cell colonies that appeared after 7 and 14 days of culture were defined as early and late EPCs, respectively. **B, C,** Dil-acLDL and FITC-UEA-1 uptake assay showed that both early (B) and late (C) EPCs were Dil-acLDL and FITC-UEA-1 positive. **D, E,** Histograms for cell surface markers of early (D) and late (E) EPCs as determined by flow cytometry. Three independent experiments were performed.
Online Figure II. shRNA knockdown of CXCR7 in early EPCs from WT mice and recombinant CXCR7 lentiviral vector upregulation of CXCR7 in early EPCs from db/db mice. Early WT-EPCs were transfected with specific siRNA against mouse CXCR7 (CXCR7-siRNA) or Silencer Select Negative Control (Ctrl-siRNA), and the transfection reagent (Lipofectamine 2000, Lipo 2000) was used as a blank control; early db/db-EPCs were infected with purified lentivirus carrying recombinant CXCR7 (Lv-CXCR7) or control vector (Lv-Ctrl), and phosphate buffered saline (PBS) was used as vehicle control (Vehicle); the efficiency of CXCR7 knockdown or upregulation was determined by Western blot. Three independent experiments were performed. Data shown in graphs represents the Means ± SD. * p<0.05, vs WT-EPC with Lipo 2000 treatment; # P< 0.05, vs db/db-EPCs with vehicle treatment; & P<0.05, vs db/db-EPCs with Lv-Ctrl infection.
Online Figure III

A

Null-EPC

CXCR7-EPC

Black/white

GFP

\[ \text{GFP} \]

\[ \text{Null-EPC} \]

\[ \text{CXCR7-EPC} \]

\[ \text{\( \geq 75\% \)} \]

\[ \text{\( \geq 70\% \)} \]

B

Isotype-IgG

WT-EPC

Null-EPC

CXCR7-EPC

19.63%

21.06%

87.65%

C

WT-EPC

Null-EPC

CXCR7-EPC

CXCR7

GAPDH

CXCR4

GAPDH

\[ \text{CXCR7/GAPDH (folds of WT)} \]

\[ \text{CXCR4/GAPDH (folds of WT)} \]

\[ \text{\( \star \# \)} \]
Online Figure III. CXCR7 recombinant lentiviral vector transfection upregulation of CXCR7 expression in EPCs. EPCs from WT mice (WT-EPCs) were transfected CXCR7/GFP recombinant lentiviral vector (CXCR7-EPCs) or GFP control lentivirus vector (Null-EPCs). The transfection efficiency and CXCR7 expression were evaluated at 72 h after transfection. A, The transfection efficiency was determined by detect the expression of GFP. B, C, The expression of CXCR7 in EPCs was detected by flow cytometry (B) and Western blot (C). Three independent experiments were performed. Data shown in graphs represents the Mean ± SD. * p<0.01 vs WT-EPC; # p<0.01, vs Null-EPC.
Online Figure IV

A

ox-LDL (μg/mL)

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<td>GAPDH</td>
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Tube length (fold of Control)

B

ox-LDL (μg/mL)

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C

Glucose + Mannitol (mmol/L)

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CXCR7/GAPDH (fold of 5.5mM)

D

Glucose + Mannitol (mmol/L)

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Tube length (fold of Control)
Online Figure IV. ox-LDL and high glucose (HG) attenuate CXCR7 expression and impair the angiogenic function of EPCs. WT-EPCs were treated with different concentrations of ox-LDL for 12 h or glucose for 24 h. A, C, The expression of CXCR7 and CXCR4 in ox-LDL or glucose treated WT-EPCs was detected by Western blot and B, D, angiogenic function was evaluated by tube formation assay. The tube length or protein expression level was normalized to 0 μg/mL ox-LDL treatment group or 27.5 mmol/L mannitol treatment group. Three independent experiments were performed for each set of conditions. Data shown in graphs represents the Means ± SD. *p<0.05, **p<0.01, vs 0 μg/mL ox-LDL treatment group or 27.5 mmol/L mannitol osmotic control treatment group.
Online Figure V

A. Flow cytometry analysis showing apoptosis in EPCs under different conditions. The bar graph on the right shows the percentage of apoptotic cells (%). The statistical significance is indicated by **p < 0.01 and *p < 0.05.

B. Light microscopy images of EPC tube formation under different conditions. The bar graph on the right shows the relative tube length. The statistical significance is indicated by **p < 0.01 and *p < 0.05.

C. Immunofluorescence images of EPCs under different conditions. The bar graph on the right shows the number of EPCs per field. The statistical significance is indicated by **p < 0.01 and *p < 0.05.
Online Figure V. Upregulating CXCR7 expression protects EPCs from HG-induced apoptosis and angiogenic dysfunction. A, The apoptosis of EPCs was analyzed by flow cytometry analysis using Annexin V/propidium iodide (PI) staining after exposure to HG (25 mmol/L, 24 h). Apoptotic cells were defined as Annexin V+/PI− (Quadrant 4). B, The effects of CXCR7 upregulation on angiogenic function of EPCs under HG treatment condition were determined by tube formation assay. Tube length was normalized to the Null-EPC mannitol control group. C, Trans-endothelial migration of EPCs was analyzed by trans-well assay. Images are representatives of 3 independent experiments. Data shown in graphs represents the Mean ± SD. * P<0.05, vs respective mannitol osmotic control group in Null-EPCs or CXCR7-EPCs; # P< 0.05, vs Null-EPCs with the same treatment; & P<0.05, vs CXCR7-EPCs with HG treatment. CXCR7-EPCs or Null-EPCs: EPCs from WT mice were transduced with CXCR7 recombinant lentiviral vector or control vector.
Online Figure VI

A

PBS Null-EPC CXCR7-EPC

Blood perfusion

ischemia/non-ischemia(%) 0 20 40 60 80 100 120

BEF 0 3 7 14 21 28 day

B

GFP

Isolectin

Merge

PBS Null-EPC CXCR7-EPC

Capillary number (per fiber)

GFP positive capillary (%)

Blood perfusion

ischemia/non-ischemia(%) 0 20 40 60 80 100 120

BEF 0 3 7 14 21 28 day

PBS Null-EPC CXCR7-EPC

GFP positive capillary (%)

Blood perfusion

ischemia/non-ischemia(%) 0 20 40 60 80 100 120

BEF 0 3 7 14 21 28 day

PBS Null-EPC CXCR7-EPC

GFP positive capillary (%)

Blood perfusion

ischemia/non-ischemia(%) 0 20 40 60 80 100 120

BEF 0 3 7 14 21 28 day

PBS Null-EPC CXCR7-EPC
**Online Figure VI.** Improved EPC mediated limb blood perfusion and angiogenesis by transplanting CXCR7-EPCs in ischemic limb of *db/db* diabetic mice. **A**, Time course of blood perfusion shown in images and quantitative analysis after hind limb ischemia (HLI) surgery with or without EPC transplantation. Blood perfusion is the ratio of ischemic to non-ischemic limb perfusion measured by a Pericam Perfusion Speckle Imager (PSI). **B**, Images and quantitation of immunofluorescent isolectin- and/or GFP-positive capillaries in transverse sections of gastrocnemius muscle tissue from ischemic hind limbs. Capillary density was expressed as isolectin-positive capillaries per muscle fiber. The exogenous EPC incorporation (white arrows) was expressed as the percentage of GFP positive capillaries. Data shown in graphs represents the Means ± SD. n=8 mice per group. * p<0.05, vs PBS; # p<0.05, vs Null-EPCs.
Online Figure VII. Upregulating CXCR7 expression attenuates the superoxide level and oxidative damage in EPCs induced by HG. A, Fluorescent Images and quantitation of superoxide levels in EPCs treated with or without HG (25 mmol/L) in the presence or absence of SDF-1 (100 ng/mL) for 6 h. Superoxide was determined with the fluorescent indicator DHE, and the fluorescent intensity of DHE was measured by a fluorescent microplate reader. B, Levels of the oxidative damage marker 3-nitrotyrosine (3-NT) in EPCs treated with or without HG (25 mmol/L) in the presence or absence of SDF-1 (100 ng/mL) for 24 h was detected by Western blot. Three independent experiments were performed. Data shown in graphs represents the Mean ± SD. * P<0.05, vs respective mannitol osmotic control in Null-EPCs or CXCR7-EPCs; # P< 0.05, vs Null-EPCs with the same treatment; & P<0.05, vs CXCR7-EPCs with HG treatment.
Online Figure VIII

A

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<td>+</td>
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<td>+</td>
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B

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<tr>
<td>HO-1</td>
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<tr>
<td>NQO-1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CAT</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-Actin</td>
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Online Figure VIII. Nuclear Nrf2 signaling is reduced in EPCs by HG and activated by upregulating CXCR7. A, Null-EPCs or CXCR7-EPCs were exposed to HG (25 mmol/L) in the presence or absence of SDF-1 (100 ng/mL) for 24 h. Protein levels of Nrf2 and its downstream target genes HO-1, NQO-1 and CAT and nuclear levels of Nrf2 (n-Nrf2) were detected by Western blot. B, Early db/db-EPCs were infected with purified lentivirus carrying recombinant CXCR7 (Lv-CXCR7) or control vector (Lv-Ctrl), and phosphate buffered saline (PBS) was used as vehicle control (Vehicle); the efficiency of CXCR7 upregulation was confirmed in Online Figure II; and the protein levels of Nrf2 and its downstream target genes HO-1, NQO-1 and CAT were detected by Western blot. Three independent experiments were performed. Data shown in graphs represents the Means ± SD. * P<0.05, vs respective mannitol osmotic control in Null-EPCs or CXCR7-EPCs; # P<0.05, vs Null-EPC with the same treatment; & P<0.05, vs CXCR7-EPC with HG treatment for A. *p<0.05, vs db/db-EPC with Vehicle treatment, # P<0.05, vs db/db-EPCs with Lv-Ctrl infection for B. NQO-1: NAD(P)H dehydrogenase quinone 1; HO-1: Heme oxygenase-1; CAT: catalase.
Online Figure IX

A

<table>
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<th>CXCR7-EPC</th>
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<td>- + + +</td>
</tr>
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<td>SDF-1</td>
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<td>- - - +</td>
</tr>
<tr>
<td>p-Akt</td>
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</tr>
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<td>p-GSK-3β</td>
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B

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Online Figure IX Continued

C

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<th>CXCR7-EPC</th>
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<td>Wortmannin</td>
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<tr>
<td>(\alpha)-Actin</td>
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<td>+</td>
</tr>
<tr>
<td>NQO-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CAT</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>(n)-Nrf2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Histone 3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HO-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NQO-1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CAT</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>(\beta)-Actin</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

![Bar charts for expression of n-Nrf2, HO-1, NQO-1, and CAT](image)

- Mannitol
- Glucose
- Glucose+SDF-1
- Glucose+SDF-1+W

**Legend:**

- Mannitol
- Glucose
- Glucose+SDF-1
- Glucose+SDF-1+W

**Significance:**

* p < 0.05
& p < 0.01
# p < 0.001
Online Figure IX Continued

D

Mannitol  Glucose  Glucose+SDF-1  + Wortmannin

Null-EPC  CXCR7-EPC  Null-EPC  CXCR7-EPC

APC-Annexin-V

Apoptotic cells(%)  Null-EPC  CXCR7-EPC

*  *  &

E

Mannitol  Glucose  Glucose+SDF-1  + Wortmannin

Null-EPC  CXCR7-EPC  Null-EPC  CXCR7-EPC

Relative tube length

*  #  &

F

Mannitol  SDF-1  Glucose+SDF-1  + Wortmannin

Null-EPC  CXCR7-EPC  Null-EPC  CXCR7-EPC

Number of EPC (per field)

*  #  &

100μm
**Online Figure IX.** Upregulating CXCR7 facilitates Nrf2 and SDF-1 activation of Nrf2 via Akt/GSK-3β/Fyn pathway under HG treatment condition. **A, C,** Null-EPCs or CXCR7-EPCs were pretreated with or without PI3K inhibitor wortmannin for 30 min, and then exposed to HG (25 mmol/L) for 24 h in the presence or absence of SDF-1 (100 ng/mL). The phosphorylation of Akt and GSK-3β, the nuclear translocation of Fyn (A), and the expression of nuclear Nrf2 (n-Nrf2) and its downstream target genes HO-1, NQO-1 and CAT (C) were evaluated by Western blot. **D,** Apoptosis was analyzed by flow cytometry using Annexin V/PI staining. **E,** The angiogenic function of EPCs was determined by tube formation assay, the tube length was normalized to the control group of Null-EPCs. **F,** The trans-endothelial migration abilities of EPCs was analyzed by trans-well assay. **B,** Early *db/db*-EPCs were infected with purified lentivirus carrying recombinant CXCR7 (Lv-CXCR7) or control vector (Lv-Ctrl), and PBS was used as vehicle control (Vehicle); the efficiency of CXCR7 upregulation was confirmed in Online Figure II; and the phosphorylation of Akt and GSK-3β, and the nuclear translocation of Fyn (n-Fyn) were evaluated by Western blot. Three independent experiments were performed for each study. Data shown in graphs represents the Means ± SD. * P<0.05, vs respective mannitol osmatic control in Null-EPCs or CXCR7-EPCs; # P< 0.05, vs Null-EPC with the same treatment; & P<0.05, vs CXCR7-EPC with HG treatment for A, C, D, E & F. *p<0.05, vs *db/db*-EPC with Vehicle treatment, # P< 0.05, vs *db/db*-EPCs with Lv-Ctrl infection for B.
Online Figure X

A

WT | sh-Ctrl | sh-Nrf2 1 | sh-Nrf2 2 | sh-Nrf2 3

GAPDH

Nrf2

B

<table>
<thead>
<tr>
<th>ox-LDL</th>
<th>SDF-1</th>
<th>n-Nrf 2</th>
<th>Histone 3</th>
<th>HO-1</th>
<th>NQO-1</th>
<th>CAT</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null/ sh-Ctrl-EPC</td>
<td>CXCR7/ sh-Ctrl-EPC</td>
<td>CXCR7/ sh-Nrf2-EPC</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
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</table>

ox-LDL: - + - + - + +
SDF-1: - - + + - + +
n-Nrf 2: - - - - - - +
Histone 3: - - - - - - -
HO-1: - - - - - - -
NQO-1: - - - - - - -
CAT: - - - - - - -
β-Actin: - - - - - - -

C

<table>
<thead>
<tr>
<th>Glucose</th>
<th>SDF-1</th>
<th>n-Nrf 2</th>
<th>Histone 3</th>
<th>HO-1</th>
<th>NQO-1</th>
<th>CAT</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null/ sh-Ctrl-EPC</td>
<td>CXCR7/ sh-Ctrl-EPC</td>
<td>CXCR7/ sh-Nrf2-EPC</td>
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<td></td>
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</tbody>
</table>

Glucose: - + - + - + +
SDF-1: - - + + - + +
n-Nrf 2: - - - - - - +
Histone 3: - - - - - - -
HO-1: - - - - - - -
NQO-1: - - - - - - -
CAT: - - - - - - -
β-Actin: - - - - - - -
Online Figure X Continued

D

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<th>Control</th>
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<td><img src="ox-LDL_sdf_null_sh-Ctrl-EPC" alt="Image" /></td>
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<tr>
<td>CXCR7/sh-Nrf2-EPC</td>
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E

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<th>Glucose</th>
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<tbody>
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<td>Null/sh-Ctrl-EPC</td>
<td><img src="null_sh-Ctrl-EPC" alt="Image" /></td>
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<tr>
<td>CXCR7/sh-Ctrl-EPC</td>
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<tr>
<td>CXCR7/sh-Nrf2-EPC</td>
<td><img src="null_sh-Ctrl-EPC" alt="Image" /></td>
<td><img src="glucose_null_sh-Ctrl-EPC" alt="Image" /></td>
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</table>
Online Figure X. Lentivirus-mediated shRNA knockdown of Nrf2 significantly reduces the expression of Nrf2 and its downstream target genes (HO-1, NQO-1 and CAT) and decreases the nuclear accumulation of Nrf2 under basal, ox-LDL (50 μg/ml) or HG (25 mmol/L) treatment conditions. A, Protein expression of Nrf2 in WT-EPCs after transfecting with Nrf2 shRNAs lentivirus vectors for 72 h assayed by Western blot to verify the efficacy of Nrf2 shRNAs. B, C, The expression of nuclear Nrf2 (n-Nrf2) and its downstream target genes (HO-1, NQO-1 and CAT) in CXCR7-EPCs or Null-EPCs transfected with lentivirus vector encoding Nrf2 shRNA (CXCR7/sh-Nrf2-EPCs) or control shRNA (CXCR7/sh-Ctrl-EPCs or Null/sh-Ctrl-EPCs) were determined by Western blot and/or real time PCR under ox-LDL (B) or HG (C) treatment condition. The results were normalized to the Null/sh-Ctrl-EPCs control group. D, E, Fluorescent images showed the ROS level in EPCs treated with or without ox-LDL (D) or HG (E) in the presence or absence of SDF-1 (100 ng/mL) for 6 h (left), and the fluorescent intensity of DHE was measured with a fluorescent microplate reader (right). The images are representatives of 3 independent experiments. Data shown in graphs represents the Means ± SD. * P<0.05 vs respective control in Null/sh-Ctrl-EPCs, CXCR7/sh-Ctrl-EPCs or CXCR7/sh-Nrf2-EPCs; # P< 0.05 vs Null/sh-Ctrl-EPC with the same treatment; & P<0.05 vs CXCR7/sh-Ctrl-EPC with the same treatment.
Online Figure XI

A

Null/sh-Ctrl-EPC

CXCR7/sh-Ctrl-EPC

CXCR7/sh-Nrf2-EPC

Mannitol Glucose Glucose+SDF-1

APC-Annexin-V

B

Null/sh-Ctrl-EPC

CXCR7/sh-Ctrl-EPC

CXCR7/sh-Nrf2-EPC

Mannitol Glucose Glucose+SDF-1

Relative tube length

C

Null/sh-Ctrl-EPC

CXCR7/sh-Ctrl-EPC

CXCR7/sh-Nrf2-EPC

Mannitol Glucose Glucose+SDF-1

Number of EPC (per field)
Online Figure XI. Knockdown of Nrf2 in EPCs attenuates the protective effects of CXCR7 upregulation against HG (25 mmol/L) damage. CXCR7-EPCs or Null-EPCs were transfected with lentivirus vector encoding Nrf2 shRNA (CXCR7/sh-Nrf2-EPCs) or control shRNA (CXCR7/sh-Ctrl-EPCs or Null/sh-Ctrl-EPCs). Following shRNA transfection the protective effects of SDF-1/CXCR7 against HG impairment of EPC survival, angiogenesis and trans-endothelial migration were evaluated as described in Figure 6. A, Apoptosis was analyzed by flow cytometry. B, Angiogenic function was determined by tube formation assay. C, Trans-endothelial migration was analyzed by trans-well assay. Three independent experiments were performed for each study. Data shown in graphs represents the Means ± SD. * P<0.05 vs respective control in Null/sh-Ctrl-EPCs, CXCR7/sh-Ctrl-EPCs or CXCR7/sh-Nrf2-EPCs; # P< 0.05 vs Null/sh-Ctrl-EPC with the same treatment; & P<0.05 vs CXCR7/sh-Ctrl-EPC with the same treatment.
Online Figure XII

A

<table>
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B

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Online Figure XII. Knockdown of Nrf2 does not alter phosphorylation of Akt and GSK-3β and the nuclear accumulation of Fyn (n-Fyn) nor does it affect the ability of SDF-1/CXCR7 to protect these EPC responses under basal, ox-LDL (50μg/ml) or HG (25 mmol/L) treatment condition. The phosphorylation of Akt and GSK-3β and the nuclear accumulation of Fyn were detected by Western blot under ox-LDL (A) or HG (B) treatment condition. The blots are representatives of 3 independent experiments. Data shown in graphs represents the Means ± SD. * P<0.05 vs respective control in Null/sh-Ctrl-EPC, CXCR7/sh-Ctrl-EPC or CXCR7/sh-Nrf2-EPC; # P< 0.05 vs Null/sh-Ctrl-EPC with the same treatment.