Impaired CXCR4 Signaling Contributes to the Reduced Neovascularization Capacity of Endothelial Progenitor Cells From Patients With Coronary Artery Disease

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Abstract—Transplantation of bone marrow cells as well as circulating endothelial progenitor cells (EPC) enhances neovascularization after ischemia. The chemokine receptor CXCR4 is essential for migration and homing of hematopoietic stem cells. Therefore, we investigated the role of CXCR4 and its downstream signaling cascade for the angiogenic capacity of cultured human EPC. Ex vivo, differentiated EPC derived from peripheral blood abundantly expressed CXCR4. Incubation of EPC from healthy volunteers with neutralizing antibodies against CXCR4 profoundly inhibited vascular endothelial growth factor- and stromal-derived factor-1–induced migration as well as EPC-induced angiogenesis in an ex vivo assay. Preincubation of transplanted EPC with CXCR4 antibody reduced EPC incorporation and impaired blood-flow recovery in ischemic hindlimbs of nude mice (57±4% of normal perfusion versus untreated EPC: 80±11%, P<0.001). Bone marrow mononuclear cells (BM-MNC) or EPC of heterozygous CXCR4+/− mice displayed reduced CXCR4 expression and disclosed impaired in vivo capacity to enhance recovery of ischemic blood flow in nude mice (blood flow 27±11% versus 66±25% using wild-type cells, P<0.01). Importantly, impaired blood flow in ischemic CXCR4+/− mice was rescued by injection of wild-type BM-MNC. Next, we investigated the role of CXCR4 for functional capacities of EPC from patients with coronary artery disease (CAD). Surface expression of CXCR4 was similar in EPC from patients with CAD compared with healthy controls. However, basal Janus kinase (JAK)-2 phosphorylation was significantly reduced and less responsive to stromal-derived factor-1 in EPC from patients with CAD compared with healthy volunteers, indicating that CXCR4-mediated JAK-2 signaling is dysregulated in EPC from patients with CAD. The CXCR4 receptor signaling profoundly modulates the angiogenic activity and homing capacity of cultured human EPC. Disturbance of CXCR4 signaling, as demonstrated by reduced JAK-2 phosphorylation, may contribute to functional impairment of EPC from patients with CAD. Stimulating CXCR4 signaling might improve functional properties of EPC and may rescue impaired neovascularization capacity of EPC derived from patients with CAD. (Circ Res. 2005;97:1142-1151.)

Key Words: coronary artery disease ■ endothelium ■ angiogenesis ■ EPC

Circulating endothelial progenitor cells (EPC) play a crucial role in postnatal neovascularization.1–3 Increasing evidence suggests that transplantation of culture-expanded progenitor cells or bone marrow–derived progenitor cells successfully promotes therapeutic neovascularization in both ischemic hindlimbs as well as acute myocardial infarction models.4–7 Moreover, recent clinical pilot studies suggest that not only restoration of blood flow in peripheral artery disease but also functional regeneration and left ventricular remodeling can be enhanced after autologous transplantation of bone marrow–derived cells or cultured EPC in patients with coronary atherosclerosis.8–11 Further evidence indicates that not only the cell number but also functional properties of transplanted cells determine the therapeutic success in autologous stem cell transplantation.12,13

The chemokine stromal-derived factor (SDF)-1 and its unique receptor CXCR4 are essential for normal cardiovascular development but also play a critical role in postnatal vasculogenesis.14–16 The CXCR4 receptor, which is highly expressed on both endothelial and hematopoietic progenitor cells,17,18 has been shown to be essentially involved in mobilization and homing of hematopoietic stem cells,19,20 and CXCR4-dependent migration toward stromal cell-derived factor-1 (SDF-1) correlated with stem cell function.18
Very recent studies have highlighted the crucial role of endogenously upregulated SDF-1 to mediate recruitment and homing of progenitor cells to ischemic tissues.\textsuperscript{21,22} In addition, locally delivered SDF-1 augments vasculogenesis and subsequently contributes to ischemic neovascularization in vivo by augmenting EPC recruitment to ischemic sites.\textsuperscript{23}

Given the close relation between hematopoietic progenitor cells and EPC, we investigated the role of CXCR4 and its downstream signaling cascade for the neovascularization capacity of cultured human EPC as well as progenitor cells derived from mice. Because EPC or bone marrow mononuclear cells (BM-MNC) from patients with coronary artery disease (CAD) or diabetes have been shown to be functionally impaired\textsuperscript{24–26} compared with EPC derived from healthy donors, we also assessed whether the CXCR4 receptor expression or the dysregulation of the CXCR4 signaling cascade might contribute to the limited functional neovascularization capacity in EPC from patients with CAD.

**Materials and Methods**

**Study Population and Patient Characteristics**

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy human volunteers and patients with stable CAD documented by angiographic evidence of coronary lesions. Patients with signs of acute myocardial ischemia documented by classic symptoms of chest pain, electrocardiogram alterations, and elevation of creatine kinase or troponin T within the past 3 months and patients with severely impaired left-ventricular function (ejection fraction, $<25\%$) were excluded. Further exclusion criteria were the presence of active or chronic infection, surgical procedures, trauma within the last 3 months, or evidence of malignant diseases. All women included were in postmenopause and did not take hormone-replacement therapy. The ethics review board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

**Human and Murine EPC Culture**

PBMCs were isolated from blood of healthy volunteers and patients with CAD as described previously.\textsuperscript{27} EPC were isolated from the

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**Figure 1.** CXCR4 mediates migratory and angiogenic activity of EPC. A, Quantification of basal migration and VEGF-induced and SDF-1–induced migration of cultured EPC from healthy volunteers with or without preincubation of anti-human CXCR4 antibody. *P*<0.05 vs EPC without anti-CXCR4. Isotype-identical antibodies served as negative control (N=3). Quantification (B) and representative photomicrographs (C) of tube formation in an ex vivo angiogenesis assay. Angiogenesis was quantified by measuring tube length in millimeters with a computer-assisted microscope. Data are mean±SD (N=3). \textsuperscript{*}P<0.01, EPC+CXCR4 antibody incubation vs EPC without anti-CXCR4.
BM-MNC were isolated as described previously after Ficoll density centrifugation of bone marrow aspirates. Cell Migration Assay

A total of 2×10⁴ EPC per group were isolated, resuspended in 250 μL of endothelial cell basal medium (EBM), and seeded at day 4 in the upper chamber of a modified Boyden chamber (Costar Transwell assay, 6.5 mm, 5-μm pore size; Corning, NY). The chamber was placed in a 24-well culture dish containing 500 μL of EBM supplemented with PBS, 50 ng/mL vascular endothelial growth factor (VEGF), or 100 ng/mL SDF-1. After 24 hours of incubation at 37°C, transmigrated cells were counted by independent investigators blinded to treatment.

Angiogenesis Assay

The human angiogenesis assay was performed according to the instructions of the manufacturer (Cell Systems/Clonetics), as described previously. CXCR4 Mediates Migratory and Angiogenic Activity of EPC

EPC were cultivated out of PBMCs on fibronectin-coated dishes. After 4 days of cultivation in endothelial differentiation–inducing medium, EPC express von Willebrand factor, vascular endothelial cadherin, vascular endothelial growth factor receptor 2 (VEGFR2), CD105, and CD146. Characterized by fluorescence-activated cell sorting (FACS) analysis, as described previously. CXCR4 receptor expression was determined by anti-human allophycocyanin (APC)-labeled antibody (BD Pharmingen) and an antibody against epitope 6H8, which recognizes an epitope located between residues 22 and 25 of the N-terminal extracellular domain of the CXCR4 receptor (kindly provided by Dr. Arenzana-Seisdesdos, Pasteur Institute, Paris). Isotype-identical directly conjugated antibodies served as a negative control. Labeled cells were fixed with 2% formaldehyde and analyzed by quantitative flow cytometry using FACStar flow cytometer (Becton Dickinson) and Cell Quest Software counting 10 000 events per sample. Cell proliferation was assessed using a 5-bromodeoxyuridine assay (BD-Pharmingen).

Statistical Analysis

Continuous variables were compared by means of Student t test or Mann–Whitney U Test. Multiple comparisons were performed by Kruskal–Wallis test or ANOVA with Bonferroni’s correction using SPSS version 11.0. A probability value of <0.05 was considered significant.

Results

CXCR4 Mediates Migratory and Angiogenic Activity of EPC

EPC were cultivated out of PBMCs on fibronectin-coated dishes. After 4 days of cultivation in endothelial differentiation–inducing medium, EPC express von Willebrand factor, vascular endothelial cadherin, vascular endothelial growth factor receptor 2 (VEGFR2), CD105, and CD146.
istics of cultured human EPC have been described previously.30 The critical role for CXCR4 in angiogenic activities of EPC from healthy donors was investigated by preincubation of EPC with neutralizing antibodies against CXCR4. Blocking CXCR4 not only inhibited basal migration but also VEGF- and SDF-1–induced migration (Figure 1A). Isotype control antibodies were ineffective in blocking migratory activity (Figure 1A). In addition, EPC-induced angiogenesis in an ex vivo assay was significantly reduced by prior treatment of EPC with anti-CXCR4 antibody treatment (Figure 1B and 1C). The CXCR4 antibody treatment did not influence proliferation of EPC as assessed by 5-bromodeoxyuridine incorporation (0.42 ± 0.5% versus 0.56 ± 0.8%, *P < 0.05 vs EPC).

CXCR4 Contributes to In Vivo Incorporation of Human EPC and Therapeutic Neovascularization

Transplantation of human EPC from healthy donors accelerated therapeutic neovascularization and restored blood flow of ischemic hindlimbs in nude mice (Figure 2). Pretreatment of EPC with anti-CXCR4 antibody resulted in significantly reduced recovery of hindlimb blood flow (Figure 2), as well as reduced capillary density (Figure 3). Importantly, incorporation of EPC into ischemic tissues was significantly lower after preincubation of EPC with the anti-CXCR4 antibody (Figure 4).

Impaired Neovascularization Capacity of CXCR4+/− Heterozygous Mice

Having demonstrated that the CXCR4 receptor plays a crucial role in neovascularization, we attempted to characterize the phenotype of mice with genetic ablation of CXCR4. Because CXCR4−/− mice are embryonically lethal, we used CXCR4+/− mice. As expected, CXCR4 surface expression of BM-MNC from CXCR4+/− mice was significantly lower compared with BM-MNC from wild-type BL6/J mice (Figure 5A). We next investigated the functional in vivo capacity of cells derived from CXCR4+/− mice after transplantation to the ischemic hindlimb model in nude mice. Intravenous infusion of BM-MNC derived from CXCR4+/− mice into ischemic nude mice resulted in significantly impaired recovery of ischemic blood flow compared with the infusion of wild-type BM-MNC (Figure 5B). These data were confirmed by using ex vivo cultivated EPC from spleen. EPC from CXCR4+/− mice were significantly impaired to restore blood flow in ischemic nude mice compared with wild-type EPC (blood flow ratio: 43 ± 10% versus 64 ± 9%, *P < 0.05, N = 3). More importantly, however, transplantation of wild-type BM-MNC into CXCR4+/− mice significantly improved blood flow to ischemic hindlimbs (Figure 5D), clearly supporting the functional relevance of CXCR4 for the neovascularization capacity of progenitor cells.

Impaired Functional Capacity of EPC From Patients With CAD

The functional activity of EPC derived from patients with CAD is known to be limited. After having shown that CXCR4 blockade on EPC as well as reduced CXCR4 expression levels in bone marrow cells from CXCR4+/− mice

Figure 3. Capillary density. A through C, Representative histological examples of capillary staining (anti-CD31, nuclear colabeling with TO-PRO-3 iodide) in control mice, hindlimb muscles after EPC therapy, and EPC with anti-CXCR4 preincubation. D, Quantification of capillary density (N = 3 to 4 per group). Data are mean ± SD. *P < 0.05 vs EPC.
disclosed a major role of CXCR4 for angiogenic activities, we investigated whether the dysfunctional capacities of EPC from patients with CAD might be linked to the CXCR4 receptor.

Basal migration was significantly lower in EPC derived from patients with CAD compared with healthy controls (Figure 6A). Despite similar relative increases in migratory responses to SDF-1 compared with basal migration, maximal migratory capacities in response to SDF-1 were significantly lower in EPC from patients with CAD compared with EPC from healthy volunteers. In fact, SDF-1–induced migratory capacity of EPC derived from patients with CAD was reduced to the level of CXCR4 antibody–treated EPC derived from healthy volunteers (Figures 6B and 1A). Similar findings were obtained when VEGF-induced migration was assessed (Figure 6C). In line with the reduced level of migratory capacity, the in vitro angiogenic activity was impaired in EPC derived from patients with CAD compared with EPC derived from healthy volunteers (Figure 6D). Finally, infusion of equal numbers of EPC derived from patients with CAD demonstrated reduced in vivo incorporation and impaired recovery of blood flow in the ischemic hindlimb model compared with EPC from healthy volunteers (Figure 6E). Neutralizing antibodies against CXCR4 slightly, but significantly, reduced the SDF-1–induced migratory capacity of EPC derived from patients with CAD, whereas the effects of CXCR4 blockade on VEGF-induced migration failed to achieve statistical significance. The in vitro angiogenic activity was significantly reduced from \(43 \pm 0.05\) to \(32 \pm 0.05\) by treating EPC derived from patients with CAD with neutralizing CXCR4 antibodies (Figure 6D). Finally, in vivo neovascularization capacity—as measured by blood flow recovery of the ischemic hindlimb—did not further deteriorate when EPC derived from patients with CAD were treated with neutralizing CXCR4 antibodies before infusion in the ischemic hindlimb model (Figure 6E). In line with these findings, histologic analysis of capillary density did not reveal any significant differences between patient-derived EPC with or without pretreatment with anti-CXCR4 antibody (patient EPC versus patient EPC + anti-CXCR4: \(32.2 \pm 10.2\) versus \(32.7 \pm 10.1\) capillaries/high power field, \(P = 1.0\)). Likewise, incorporation of EPC (EPC/high-power field: \(7.8 \pm 1.3\) versus \(5.7 \pm 2.3\), \(P = 0.47\)) was not further reduced after blocking CXCR4 in patient-derived EPC.

Figure 4. CXCR4 mediates incorporation of EPC to ischemic muscles. A, Representative microscopic photographs of incorporated EPC identified by double-fluorescent labeling (yellow/orange) in ischemic muscles. Transplanted human 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (Dil)–labeled EPC were identified by red fluorescence in histological sections retrieved from ischemic muscles. Vasculature was identified by green fluorescence (CD31 staining) in the same tissue sections. Density of incorporated EPC in tissue sections retrieved from ischemic muscles was significantly lower in the CXCR4 treatment group compared with untreated healthy EPC. B, Quantification of EPC incorporation into ischemic muscles as identified by Dil-ac-LDL/CD31 double-positive cells per high-power field, mean±SD. *\(P < 0.05\) vs EPC. C, High-power magnification of EPC incorporating into vessel structures.
these data indicate that the functional capacities of EPC from patients with CAD as well as the responsiveness toward SDF-1 and VEGF stimulation were significantly impaired. In addition, the effects of neutralizing antibodies against CXCR4 were less pronounced in EPC derived from patients with CAD compared with healthy volunteers.

CXCR4 Receptor Expression in EPC Derived From Patients With CAD

Based on these findings, we postulated that EPC from patients with CAD might bear dysfunctional properties linked to the CXCR4 receptor limiting the therapeutic potential. To address this issue, we first analyzed CXCR4 expression levels on cultured EPC.

Ex vivo, differentiated EPC derived from peripheral blood abundantly expressed CXCR4, as shown by FACS analysis after 4 days in culture. Importantly, surface expression of CXCR4 was similar in EPC from patients with CAD (N=14) and in EPC isolated from healthy volunteers (N=14) (Figure 7A).

Moreover, expression of the 6H8 epitope, which recognizes an epitope located between residues 22 and 25 of the N-terminal extracellular domain of the CXCR4 receptor essential for the function of CXCR4, was similar between EPC from patients with CAD versus healthy controls (data not shown). Thus, the surface expression of CXCR4 does not explain the profound reduction in neovascularization capacity of EPC derived from patients with CAD.

Dysregulation of Janus Kinase-2 Signaling Contributes to Impaired Neovascularization Capacity

Janus kinase (JAK)-2 is one of the downstream targets of CXCR4 signaling. Thus, we investigated whether CXCR4-mediated JAK-2 signaling is dysregulated in EPC from patients with CAD and may account for the impaired neovascularization capacity. Indeed, immunoblotting revealed that basal JAK-2 phosphorylation was significantly reduced in EPC from patients with CAD compared with healthy volunteers (Figure 7B). Moreover, SDF-1–induced increase in JAK-2 phosphorylation in healthy volunteers was blunted in patients with CAD (Figure 7B).

Finally, analysis of the CXCR4+/− mice, which were characterized by reduced CXCR4 expression levels, also
displayed reduced basal JAK-2 phosphorylation levels compared with wild-type littermates (Figure 7C).

Thus, the impaired activation of the downstream signaling target of the CXCR4 receptor, JAK-2, may contribute to the functional impairment of EPC from patients with CAD. To assess the overall responsiveness of JAK-2, we used interleukin (IL)-3, which stimulates JAK-2 independent of CXCR4. In contrast to the blunted SDF-1–induced JAK-2 activation, incubation with IL-3 increased JAK-2 phosphorylation in EPC derived from patients with CAD (2.8-fold increase) and in EPC derived from healthy controls (1.4-fold increase) (Figure 7D).

To determine whether CXCR4-mediated angiogenic activities are dependent on JAK-2, we incubated EPC from healthy volunteers with the JAK-2 inhibitor AG490 or in combination with the anti-CXCR4 antibody. Indeed, preincubation of EPC with AG490 profoundly reduced migration, but combined JAK-2 and CXCR4 blockade did not result in an additive effect (Figure 8A).

Next, we confirmed the importance of JAK-2 for the invasion capacity of BM-MNC from wild-type mice (Figure 8B). Inhibition of JAK-2 by using AG490 yielded a comparable reduction of the invasion capacity compared with the effects of the anti-CXCR4 antibody. In accordance with the data obtained in EPC, coin incubation with the JAK-2 inhibitor and the anti-CXCR4 antibody did not result in an additive inhibition of the invasion capacity compared with AG490 alone, underscoring the relevance of JAK-2 activation for angiogenic properties.

**Discussion**

The results of the present study extend previously published data indicating that the CXCR4 receptor importantly modu-
lates the migratory and angiogenic capacities of cultured human EPC. In line with recent studies indicating that progenitor cell trafficking is regulated by SDF-1,21–23 our data underscore the critical role of CXCR4 for homing of transplanted human EPC into ischemic tissues.

CXCR4 blockade not only resulted in impaired migratory activity toward SDF-1 as well as VEGF but was also associated with an impaired incorporation of EPC into sites of ischemia-induced neovascularization and disturbed restoration of blood flow to ischemic limbs, suggesting that CXCR4 is important for therapeutic integration of EPC into the vascular bed. The role of CXCR4 was supported by experiments using BM-MNC or EPC derived from spleen from heterozygous CXCR4<sup>+/−</sup> mice (N=3 per group); quantification expressed as pJAK/JAK ratio. *P<0.05. D, Representative immunoblot showing JAK-2 phosphorylation levels in EPC from healthy donors vs patients with CAD after stimulation with IL-3.

Figure 7. CXCR4 expression and JAK-2 phosphorylation of cultured EPC. A, Quantification of CXCR4 expression by FACS analysis of EPC from patients (N=14) and healthy donors (N=14). Cell surface expression presented as geometric mean±SD did not differ significantly between both groups (P=0.8). B, Representative JAK-2 protein phosphorylation by immunoblotting in cultured EPC from patients compared with healthy donors with or without stimulation by SDF-1. *P=0.02, healthy vs healthy+SDF; **P=0.03, patient+SDF vs healthy donor+SDF; N=6 per group. Equal loading was confirmed by JAK-2 protein expression and quantification expressed as pJAK/JAK ratio. C, Basal JAK-2 phosphorylation levels in BM-MNC from wild-type and CXCR4<sup>−/−</sup> mice (N=3 per group); quantification expressed as pJAK/JAK ratio. *P<0.05. D, Representative immunoblot showing JAK-2 phosphorylation levels in EPC from healthy donors vs patients with CAD after stimulation with IL-3.

Figure 8. JAK-2 activation is required for CXCR4-dependent migration or invasion. A, Migration of human EPC, preincubated with the JAK-2 inhibitor AG490 or in combination with anti-CXCR4. *P<0.05 vs control (N=4). B, Invasion of BM-MNC from wild-type mice, preincubated with the JAK-2 inhibitor AG490, anti-CXCR4, or the combination. *P<0.05 vs control, N=5.
MNC, clearly documenting the in vivo relevance of CXCR4 for the neovascularization capacity of progenitor cells and further supporting the data obtained by CXCR4-blocking antibodies.

EPC or BM-MNC derived from patients with CAD have been shown to be functionally impaired compared with progenitor cells from healthy donors. We confirmed that migratory responses to SDF-1 and VEGF as well as angiogenic capacities of EPC derived from patients with CAD were significantly lower compared with EPC from healthy volunteers. In addition, the effects of neutralizing CXCR4 antibodies profoundly reduced migratory and angiogenic activities of EPC derived from healthy donors, whereas the effects of CXCR4 blocking antibodies, in general, were less pronounced in EPC from patients with CAD. These data suggested that EPC derived from patients with CAD might bear dysfunctional properties linked to the CXCR4 receptor, limiting their therapeutic potential to improve neovascularization.

We, therefore, hypothesized that downregulation of the CXCR4 receptor surface expression might contribute to the reduced cell function in patients with CAD. However, cell surface expression of CXCR4 in cultured EPC from patients with CAD compared with healthy donors was similar.

Furthermore, our data revealed that the impaired functional capacity of EPC derived from patients with CAD was not confined only to SDF-1/CXCR4 interactions but also extended to basal and VEGF-stimulated cell functions. In particular, the finding that VEGF-mediated migration was also influenced by CXCR4 antibodies points toward a more general involvement of CXCR4 and its downstream signaling in homing mechanisms of EPC. Indeed, recent studies suggested that EPC derived from patients with CAD might bear dysfunctional properties linked to the CXCR4 receptor, limiting their therapeutic potential to improve neovascularization.

In summary, the CXCR4 receptor signaling profoundly regulates the angiogenic activity and homing capacity of cultured human EPC. Disturbance of CXCR4 signaling, as demonstrated by reduced JAK-2 phosphorylation, may contribute to functional impairment of EPC from patients with CAD, thus, providing a rationale for therapeutic intervention. Stimulating CXCR4 signaling might improve functional properties of EPC and may rescue impaired neovascularization capacity of EPC derived from patients with CAD undergoing cell therapy for ischemic diseases.

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Online supplement: Methods

Murine hindlimb ischemia model

The incorporation of EPC and contribution to neovascularization was investigated in a murine model of hind limb ischemia, using 8–10 wk old athymic NMRI nude mice (The Jackson Laboratory, Bar Harbor, Maine) weighing 18–22 g. Ischemia was induced by ligation of the proximal femoral artery including the superficial and the deep branch with 7-0 silk suture. All arterial side branches were obliterated using an electrical coagulator (Erbe, Tübingen, Germany). The overlying skin was closed using surgical staples. After 24 hours, mice received an intravenous injection of $5 \times 10^5$ EPC from healthy volunteers or patients with or without pre-incubation of neutralizing CXCR4 antibody. CXCR4 +/- mice and C57Bl6/J were purchased from Jackson Laboratories. Blood flow in ischemic and non-ischemic limbs was measured at 7 days and 2 weeks using a laser Doppler blood flow meter (Laser Doppler Perfusion Imager System, moorLDI™-Mark 2, Moor Instruments, Wilmington, Delaware).

Histological Analysis

Incorporation of EPC and tissue vascularization was determined in 7-µm frozen sections of the adductor and semimembranous muscles from the ischemic and the non-ischemic hindlimb. For cell tracking, human EPC were labeled with CM-DiI (Cell Tracker CM-DiI, Molecular Probes) prior to injection and identified by co-staining with FITC-labeled mouse monoclonal antibody against CD31 (Pharminingen). Human origin of EPC was additionally confirmed by co-staining with HLA-ABC-PE-labeled antibodies (Caltag). Nuclear co-labeling with TO-PRO-3 iodide (Molecular Probes) was performed. Incorporation of EPC into neovessels is expressed as number of EPC / high power field (40X). Capillary density is expressed as number of capillaries/high power field (40X). At least 3 randomly selected sections from N=4 muscles per group were analyzed.
**Invasion assay**

A modified Boyden chamber (Transwell, 8 μm pore size, BD Falcon) was filled with Matrigel (4 mg/ml; BD Biosciences). BM-MNC (5x10^4 cells) were placed in the upper compartment of the chamber in the presence or absence of anti-CXCR4 (10 ng/ml), AG490 (JAK-2 inhibitor, Calbiochem) (10 μM) or the combination. After 24 h at 37 °C, chambers were removed and the cells were manually counted in the lower part of the culture plate by two different investigators.

**Immunoblotting**

EPC derived from healthy volunteers or CAD patients were directly lysed in SDS sample-dye (62.5 mM Tris-HCl (pH 6.8, 2 % SDS, 10 % glycerol, 50 mM DTT, 0.2 % bromphenolblue). JAK-2 phosphorylation protein levels were determined by anti-phospho-JAK-2 antibody (Upstate, 1:500). Equal loading was confirmed by anti-JAK2 antibody (Upstate). Densitometric analysis was performed (Scion imaging program) to allow for semi-quantitative comparison of protein expression.