Age-Related Decline in Reendothelialization Capacity of Human Endothelial Progenitor Cells Is Restored by Shear Stress

Wen Hao Xia, Zhen Yang, Shi Yue Xu, Long Chen, Xiao Yu Zhang, Jing Li, Xing Liu, Yan Xia Qiu, Xin Tao Shuai, Jun Tao

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Abstract—Aging is associated with dysfunction of endothelial progenitor cells (EPCs), and shear stress has a beneficial impact on EPC function; however, the effects of aging and shear stress on the endothelial repair capacity of EPCs after arterial injury have not been reported. Here we investigated the influence of aging and shear stress on the reendothelialization capacity of human EPCs and the related molecular mechanism. Compared with EPCs isolated from young subjects, EPCs from the elderly displayed an impaired migration and adhesion in vitro and demonstrated a significantly reduced reendothelialization capacity in vivo after transplantation into nude mice with carotid artery denudation injury. Shear stress pretreatment enhances the migration, adhesion, and reendothelialization capacity in both young and elderly EPCs; however, it was to a greater extent in EPCs from the elderly. Although basal CXC chemokine receptor 4 (CXCR4) expression was similar in EPCs from the 2 age groups, the stromal cell derived factor 1-induced CXCR4 and Janus kinase 2 phosphorylations were much lower in the elderly than in young EPCs. Shear stress treatment upregulated CXCR4 expression and phosphorylation and, importantly, restored the stromal cell-derived factor 1/CXCR4-dependent Janus kinase 2 phosphorylation in the elderly EPCs. Furthermore, short hairpin RNA-mediated knockdown of CXCR4 expression or pretreatment with Janus kinase 2 inhibitor diminished the enhancement in the migration, adhesion, and reendothelialization capacity of the elderly EPCs from shear stress treatments. Thus, our study demonstrates that upregulation of the CXCR4/Janus kinase 2 pathway by shear stress contributes to the enhanced reendothelialization capacity of EPCs from elderly men. (Hypertension. 2012;59:1225-1231.)

Online Data Supplement

Key Words: aging ■ endothelial progenitor cells ■ CXCR4 ■ shear stress ■ endothelial repair

Aging is a well-recognized risk factor for cardiovascular disease.1,2 The impact of aging, a traditional detrimental factor, for the increased development of cardiovascular disease is initiated by abnormalities in structure and function of the vascular endothelium.3–5 Thus, it is of particular importance to maintain the integrity of the vascular endothelium after arterial injury with aging.

Accelerated reendothelialization is an important therapeutic means for repair of injured artery. Accumulating evidence indicates that circulating endothelial progenitor cells (EPCs) provide an endogenous repair mechanism to counteract ongoing risk factor–induced endothelial injury and to replace dysfunctional endothelium,6–10 thus suggesting an important role of circulating EPCs for restoration of the integrity of the vascular endothelium with aging. Previous studies showed that aging leads to a reduction in the number of circulating EPCs, and aging is associated with dysfunctional EPCs in both healthy persons and patients with cardiovascular disease,11–16 which is, at least in part, responsible for the development of age-related endothelial injury in humans.17–19 However, the mechanism underlying age-related EPC dysfunction is not fully understood. It is, therefore, essential to search for a novel approach to improve the functional potential of EPCs in elderly individuals, with the aim of enhancing EPC-based endothelial repair and reducing the occurrence of cardiovascular disease.

The pharmacological therapy and the gene transfer intervention are the most popular methods to enhance the EPC function for their therapeutic potentials. However, less is focused on the nonpharmacologic interventions to regulate...
the function of EPCs. It is generally accepted that shear stress as a nonpharmacologic intervention measure contributes to the maintenance of homeostasis in the vascular endothelium. Accumulating evidence indicates that the beneficial effect of shear stress on the vascular endothelium is, at least in part, related to shear stress-mediated upregulation of EPC function. However, the molecular mechanisms underlying the favorable effect of shear stress on EPCs need to be further investigated.

CXC chemokine receptor 4 (CXCR4) is a 7-transmembrane G protein–coupled receptor, and the ligand for CXCR4 is chemokine stromal cell derived factor 1 (SDF-1). There is increasing evidence to show that CXCR4 is a key regulator of homing and retention of EPCs at the sites of injured artery, suggesting that an impaired CXCR4 signaling may lead to a decrease in endothelial repair capacity of EPCs. Our recent study indicated that impairment of CXCR4-mediated Janus kinase 2 (JAK-2) phosphorylation is involved in the aged-related reduction in endothelialization capacity of EPCs; however, the role of CXCR4 phosphorylation in the regulation of CXCR4/JAK-2 signaling is unknown. It is also not clear whether shear stress can enhance reendothelialization capacity of EPCs derived from the elderly. Based on the previous observations, we hypothesized that the impaired reendothelialization capacity of EPCs derived from elderly persons is related to the decreased CXCR4 phosphorylation, and shear stress can be used to promote reendothelialization capacity of EPCs from elderly persons, which correlates to increasing CXCR4 signaling. To test these hypotheses, in vivo reendothelialization capacity and CXCR4 signaling of EPCs were examined in both elderly and young subjects. Then, the EPCs from the elderly were exposed to an environment of shear stress produced by the biomimic device. Under physiological conditions of shear stress, the human EPCs were tested in vitro for their ability to affect the CXCR4 signaling, as well as migration and adhesion function. In addition, we also evaluated the effect of transplantation of shear stress-treated EPCs from elderly persons on in vivo reendothelialization capacity in a nude mouse model of carotid injury. The present study may add valuable information to our understanding of age-related endothelial injury and provide a novel therapeutic strategy to counteract the decline in EPC function in humans with aging.

Materials and Methods

Subjects Characteristics
Elderly (age, 68.4±2.5 years; n=10) and young (age, 27.3±3.0 years; n=10) healthy male subjects without clinical evidence of cardiovascular risk factors and significant medical history were enrolled into the study. Peripheral venous blood samples were obtained for EPC isolation. The study protocol was approved by the ethics committee of our hospital, and written informed consent was obtained from all of the study subjects. Detailed methods for the present study are provided in the online-only Data Supplement Materials and Methods section.

Statistical Analysis
Statistical analyses were performed using SPSS 17.0 software (SPSS Inc). All of the data are reported as mean (SD) unless stated otherwise. Statistical significance was evaluated by means of a Student t test or ANOVA. A value of \( P<0.05 \) was considered statistically significant.

Results

Study Subjects
The baseline characteristics were within normal range and not different between elderly and young subjects except with regard to age (Table S1).

In Vitro Functions and In Vivo Reendothelialization Capacity of EPCs From Elderly Human Subjects Are Impaired
The percentage of CD34+/KDR− cells was significantly lower in the peripheral blood mononuclear cells from the elderly (\( P<0.05 \); Figures S1A and S2). We then grew EPCs by culturing peripheral blood mononuclear cells in specific medium for 7 days. Cultured EPCs were defined as cells dually positive for low-density lipoprotein from human plasma, acetylated, Dil complex uptake and fluorescein isothiocyanate–labeled BS-1 lectin binding or by flow cytometry analysis (Figure S3). In a modified Boyden chamber assay, the basal level of migration was lower (\( P<0.05 \)), and the fold change of SDF-1–induced increase of migration was lesser (0.33±0.11 versus 0.78±0.21; \( P<0.01 \)) in EPCs from the elderly than those from young subjects (Figure S1B). The EPC adhesion activity was evaluated by applying Dil-labeled EPCs onto tumor necrosis factor–prestimulated monolayer human umbilical vein endothelial cells, subsequently counting the adherent cells under the fluorescent microscope; the numbers of adherent EPCs from elderly were fewer than those from young subjects (\( P<0.01 \); Figure S1C). Consistently, the migration and adhesion activities of late EPCs from the elderly were significantly lower than those from young subjects (Figure S4). Collectively, these results suggest that aging is associated with a reduced number of circulating EPCs and impaired function of cultured EPCs.

Transplantation of EPCs from young people but not EPCs from the elderly markedly accelerated reendothelialization of the injured arteries (elderly versus young, 14±4% versus 36±5%; \( P<0.001 \); Figure 1A and 1B). Fluorescent microscope revealed that the transplanted EPCs were incorporated at sites of injury, however, less frequently with EPCs from the elderly than EPCs from young people (Figure 1C). Flow cytometry analyses of single-cell suspension made from the injured vessel fragments confirmed fewer Dil-labeled EPCs in mice transplanted with EPCs from the elderly (\( P<0.01 \); Figure 1D). Furthermore, transplantation of late EPCs from the elderly also displayed a lower degree of reendothelialization and less EPC incorporation than transplantation of late EPCs from young subjects (Figure S5). These results confirmed that EPCs from the elderly have a reduced reendothelialization capacity that is associated with a reduced EPC incorporation in the injured vessels.

CXCR4/JAK-2 Signaling Is Required for EPC Migration and Adhesion and EPC-Mediated Reendothelialization
To understand the molecular mechanism responsible for the impaired reendothelialization capacity of EPCs derived from the elderly, we examined the CXCR4 signaling in EPCs, because CXCR4 is key to homing and retention of EPCs at sites of arterial injury, therefore contributing to the reendothelialization. Surprisingly, Western blotting analyses
Aging endothelial progenitor cells (EPCs) display a reduced reendothelialization capacity. A and B, Quantification (A) and representative photographs (B) of reendothelialization areas of the injured carotid arteries in nude mice 3 days after carotid denudation surgery plus IV injection of PBS or EPCs (5 × 10^5 cells) cultured from young people or the elderly (n = 10 per group). C and D, Assessments of the transplanted EPCs incorporated in the injured vessels. C, a, light photograph of injured (stained with Evans blue) and contralateral uninjured carotid artery; b and c, electron microscope pictures of injured (b) and contralateral uninjured carotid arteries (c); d through f, cross-section and (g through i) en face view of the contralateral uninjured carotid arteries (d and g) and injured carotid arteries (e, f, h, and i) showing chloromethylbenzamido-Dil–labeled EPCs (red) attached to injured endothelium (green) in mice receiving young (e) or elderly EPCs (f). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue, d through f). Shown are representatives of 3 independent experiments. D, Fragments of injured carotid arteries were isolated, digested, and made into single cell suspension, and the Dil(+) EPCs were quantified by flow cytometry (n = 5 per group). Error bars represent SEM.

Figure 1. Aging endothelial progenitor cells (EPCs) display a reduced reendothelialization capacity. A and B, Quantification (A) and representative photographs (B) of reendothelialization areas of the injured carotid arteries in nude mice 3 days after carotid denudation surgery plus IV injection of PBS or EPCs (5 × 10^5 cells) cultured from young people or the elderly (n = 10 per group). C and D, Assessments of the transplanted EPCs incorporated in the injured vessels. C, a, light photograph of injured (stained with Evans blue) and contralateral uninjured carotid artery; b and c, electron microscope pictures of injured (b) and contralateral uninjured carotid arteries (c); d through f, cross-section and (g through i) en face view of the contralateral uninjured carotid arteries (d and g) and injured carotid arteries (e, f, h, and i) showing chloromethylbenzamido-Dil–labeled EPCs (red) attached to injured endothelium (green) in mice receiving young (e) or elderly EPCs (f). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue, d through f). Shown are representatives of 3 independent experiments. D, Fragments of injured carotid arteries were isolated, digested, and made into single cell suspension, and the Dil(+) EPCs were quantified by flow cytometry (n = 5 per group). Error bars represent SEM.

indicated that CXCR4 expression did not differ between EPCs from the young and the elderly (Figure 2A); however, the CXCR4 phosphorylation (at serine 339) was reduced in EPCs from elderly (Figure 2B). Because JAK-2 is a well-established downstream target of CXCR4 signaling, we investigated whether the functional status of JAK-2 is altered in EPCs from the elderly. Basal (P < 0.01) and the SDF-1–induced (P < 0.001) JAK-2 phosphorylations were significantly lower in EPCs from the elderly than EPCs from young subjects (Figure 2C). Importantly, both short hairpin RNA–mediated knockdown of CXCR4 or pretreatment of EPCs with JAK-2 inhibitor AG490 significantly attenuated EPC migration and adhesion activities and the EPC-mediated reendothelialization (Figure S6). Collectively, these results suggest that the CXCR4/JAK-2 signaling is essential for normal EPC functions, and the reduced reendothelialization capacity of EPCs from the elderly is at least partially related to the reduced CXCR4/JAK-2 signaling.

Shear Stress Enhances the Function and Reendothelialization Capacity of EPCs From the Elderly

We then investigated the effect of in vitro shear stress on the functions of cultured EPCs. Exposure of EPCs to 15 dyne/cm^2 of shear stress for 12 or 24 hours (Figure S7A through S7C) or exposure of EPCs for 12 hours to 10, 15, or 25 dyne/cm^2 of shear stress (Figure S7D and S7E) significantly increased EPC migration toward SDF-1, adhesion to tumor necrosis factor-α–prestimulated HUVECs, and adhesion in flow on fibronectin (Figure S8) in both young and elderly EPCs (P < 0.05) and abrogated the differences between the 2 groups. In addition, the migration and adhesion activities of EPCs derived from HUVECs and late EPCs from the elderly were also enhanced by in vitro shear stress treatment (Figure S9). To determine the effect of shear stress on EPC-mediated reendothelialization in vivo, EPCs were exposed to 15 dyne/cm^2 of shear stress for 12 hours and then intravenously injected (separately) into nude mice 3 hours after surgical carotid artery denudation. Pretreatment with shear stress enhanced the reendothelialization capacity of both young and elderly EPCs, however, to a greater extent in elderly EPCs (Figure 3A and 3B). Furthermore, in vitro shear stress treatment also significantly enhanced the reendothelialization capacity of late EPCs from the elderly and endothelial-derived EPCs derived from HUVECs (Figure S10).

Shear Stress Upregulates CXCR4 Expression and Enhances SDF-1/CXCR4-Mediated JAK-2 Phosphorylation

We then investigated whether the enhancement of reendothelialization capacity of EPCs by shear stress treatment was related to CXCR4 signaling. Exposure of EPCs to 15 dyne/cm^2 of shear stress for 6, 12, or 24 hours or exposure of EPCs for 12 hours to 5, 10, 15, and 25 dyne/cm^2 of shear stress led to a significant increase in CXCR4 mRNA (Figure S11A and S11B), total (Figure S11C and S11D) and surface CXCR4 protein (Figure 3C and 3D), and phosphorylated surface CXCR4 protein (Figure 3E and 3F; P < 0.05) but did not alter CD31, kinase insert domain receptor, von Willebrand factor,
and CD14 expression in EPCs (Figure S12). Furthermore, our results indicated that CXCR4 protein expression in the late EPCs from the elderly (Figure S13A) and in EPCs derived from HUVECs (Figure S13B) was also increased after exposure to 15 dyne/cm² of shear stress for 12 hours.

There was no significant difference in basal JAK-2 phosphorylation between the shear stress-treated EPCs and EPCs cultured under static condition; however, the SDF-1-stimulated increase of JAK-2 phosphorylation was significantly greater in shear stress-treated EPCs than in EPCs cultured under static condition (P<0.01; Figure 4A), and this difference was diminished by CXCR4 knockdown or by pretreatment of the cells with JAK-2 inhibitor AG490 (P<0.01; Figure 4B), suggesting a dependency on the SDF-1/CXCR4 signaling. Interestingly, the combination of JAK-2 inhibition and CXCR4 knockdown did not produce an additive effect. Furthermore, shear stress also potentiated SDF-1-induced JAK-2 phosphorylation in late EPCs from the elderly (Figure S13C).

Shear Stress Enhances the Reendothelialization Capacity of EPCs From the Elderly via CXCR4/JAK-2 Signaling

Next, we investigated whether shear stress-induced upregulation of CXCR4/JAK-2 signaling contributes to the enhancement of EPC functions. Lentivirus short hairpin RNA-mediated CXCR4 knockdown or preincubation of EPCs with JAK-2 inhibitor AG490 significantly attenuated the in vitro migration and adhesion (P<0.05; Figure 4C and 4D) and in vivo reendothelialization capacity of the shear stress-treated EPCs from both the young and the elderly (P<0.01; Figure 4E and 4F) and abrogated the difference between the 2 groups of cells. In contrast, transduction of EPCs with scrambled short hairpin RNA lentiviral particles had no effect on these functional parameters. Furthermore, CXCR4 knockdown or AG490 treatment also attenuated the shear stress-mediated functions in endothelial-derived EPCs and late EPCs from the elderly (Figure S14). Collectively, these results suggest that shear stress enhances the reendothelialization capacity of EPCs from the elderly through the CXCR4/JAK-2 signaling pathway.

Discussion

The major findings of the present study are as follows: (1) in vitro migration and adhesion activities and in vivo reendothelialization capacity of EPCs in the elderly are significantly reduced; (2) the CXCR4 phosphorylation and CXCR4/JAK-2 signaling are impaired in EPCs from aging populations; and (3) shear stress ameliorates the functional defects of EPCs in the elderly and enhances their reendothelialization capacity by augmenting CXCR4/JAK-2 signaling. Collectively, our study demonstrates for the first time that the functional state of CXCR4 and CXCR4/JAK-2 signaling is critical to the function of EPCs from aging populations and that ex vivo treatment of EPCs with shear stress can be used as an effective approach to enhance the reendothelialization capacity of EPCs.

It has been known that advancing age is associated with a reduction in the number and function of EPCs in humans.11–13,15–17,19,29 Here we found that EPC migration toward SDF-1 and adhesion to the tumor necrosis factor-α–activated endothelial cells are specifically impaired. Furthermore, EPCs from the elderly exhibited a significantly reduced reendothelialization capacity in the injured carotid artery compared with EPCs from the young subjects, which suggests a reduction of endogenous repair capacity in the vasculature with aging.

CXCR4 is crucial for EPC homing to the local vascular bed to mediate reendothelialization.26–28,30,31 Given the close association between CXCR4 signaling and EPCs targeted repair for endothelium, we hypothesized that disturbance in CXCR4 signaling is related to the impaired EPC function in the elderly. We show that SDF-1–induced phosphorylations of CXCR4 and JAK-2 are significantly lower in EPCs from the elderly than in EPCs from the young. Furthermore, CXCR4 knockdown and JAK-2 inhibition abrogate the in vitro function and in vivo reendothelialization capacity of EPCs. These data indicate that CXCR4/JAK-2 signaling is critical to the function of EPCs, and decreased phospho–JAK-2 level is, at least in part, related to the reduction in EPC-mediated endothelial repair capacity with aging. Therefore, a novel approach that enhances the CXCR4/JAK-2...
signaling may improve the endothelium-reparative potential of EPCs and restore the integrity and homeostasis of the vascular endothelium in the elderly.

We found that shear stress exerts beneficial effects on human EPCs for endothelial protection. Previous studies from our laboratory demonstrate that shear stress regulates the expression of endothelial NO synthase, Cu/Zn superoxide dismutase, and tissue plasminogen activator in human EPCs and that transplantation of shear stress-treated EPCs onto the prosthetic vascular grafts leads to the formation of a bioactive endothelial monolayer associated with significant inhibition of thrombosis formation. However, whether shear stress regulates CXCR4 signaling was unclear. In this study, we hypothesized that treatment of EPCs with shear stress may enhance CXCR4/JAK-2 signaling, contributing to EPC-mediated reendothelialization after arterial injury. We showed that shear stress upregulates CXCR4 expression and phosphorylation, as well as the responsiveness of EPCs to SDF-1/CXCR4-mediated JAK-2 phosphorylation. This augmentation in shear stress-induced CXCR4/JAK-2 signaling in the elderly EPCs parallels with the enhanced in vitro migration and adhesion activities and in vivo reendothelialization capacity and can be abrogated by CXCR4 knockdown or JAK-2 inhibition. These findings indicate that shear stress is an effective means to modify the biological phenotype of EPCs derived from the elderly and to facilitate EPC-mediated endothelial repair, adding a novel insight into the molecular mechanism of the shear stress on the regulation of EPC biological phenotype related to endothelial repair in humans.

The findings presented in this study have strong clinical implications. Aging results in imbalance of injury and repair in the endothelium and requires a higher efficiency of repair to maintain endothelial homeostasis. Our study clearly shows the dysfunctional properties of EPCs with aging, suggesting a negative impact of aging on the endothelial injury-and-repair balance. The identification of the CXCR4/JAK-2 signaling pathway downregulated with aging may provide molecular targets for the development of treatments to enhance the potential of endogenous EPCs and/or to increase the functional capacity of transplanted cells. The present study
provides evidence that shear stress other than direct stimulation of its natural ligand SDF-1 can be used to restore the CXCR4/JAK-2 molecular signaling pathway and enhance the endogenous endothelium-reparative capacity of EPCs, which constitutes an important cell-based therapeutic strategy to facilitate EPC function and improve repair capacity in the aging populations.

Perspectives

The present study demonstrates that increasing age leads to impaired reendothelialization capacity of EPCs that is at least partially related to the diminished CXCR4/JAK-2 signaling. Shear stress, therefore, may be a novel approach for EPC-based endothelial repair in the elderly by increasing CXCR4/JAK-2 signaling.

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Disclosures

None.

References


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Age-Related Decline in Reendothelialization Capacity of Human Endothelial Progenitor Cells Is Restored by Shear Stress

Short title: Age, EPCs, Shear Stress and Endothelial Repair

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Supplemental Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells (PBMNCs) and Flow Cytometry Analysis
PBMNCs were isolated by Ficoll density gradient centrifugation as described previously.\textsuperscript{1} Circulating EPCs were quantitatively analyzed by flow cytometry with PerCP-labeled anti-human CD45 (BD Pharmingen), phycoerythrin (PE)-labeled anti-human KDR (R&D Systems), and fluorescein isothiocyanate (FITC)-labeled anti-human CD34 (BD Pharmingen). We followed the gating strategy recommended by the International Society for Hematotherapy and Graft Engineering (ISHAGE),\textsuperscript{2} which is illustrated in the Online Supplemental Figure S2.

EPC Culture and Characterization
EPCs were cultured and characterized as detailed previously.\textsuperscript{1,3} Briefly, PBMNCs were cultured on fibronectin-coated 6-well plates in endothelial cell basal medium-2 (EBM-2) supplemented with endothelial growth medium-SingleQuots (Clonetics, San Diego, CA, USA). At day 4, nonadherent cells were removed, and adherent cells were maintained till day 7 and then used for EPC experiments. EPCs were defined as cells dually positive for Dil-acLDL (0.02mg/ml; Invitrogen, Carlsbad, CA, USA) uptake and FITC-labeled BS-1 lectin (0.01mg/ml; Sigma-Aldrich, St. Louis, MO, USA) binding as previously described (Supplemental Figure 3A).\textsuperscript{1} Endothelial marker proteins of cultured EPCs were also examined by flow cytometry analysis by using phycoerythrin (PE)-labeled monoclonal mouse anti-human antibodies recognizing CD31 (BD Pharmingen), von Willebrand factor (vWF) (BD Pharmingen) and kinase-insert domain receptor (KDR) (R&D system). Furthermore, expression of the monocytic lineage marker CD14 (BD Pharmingen) was analyzed. Overall, 82.33 ± 6.3% of the cells were positive for CD31, 94.37 ± 8.4% for vWF, 75.65 ± 5.9% for KDR and 71.38 ± 4.8% for CD14 (Supplemental Figure 3B). Based on the isolation and cultivation protocol, the adherent mononuclear cells were identified as EPCs, similar with previous studies.\textsuperscript{1,3,4}

Late EPC (LEPC) Culture and Characterization
Late EPCs were cultured and characterized by following the protocol described by other research labs.\textsuperscript{5,6} Briefly, PBMNCs were cultured on fibronectin-coated 6-well plates in EBM-2 supplemented with endothelial growth medium-SingleQuots (Clonetics, San Diego, CA, USA). After 4 days culture, nonadherent cells were removed by thoroughly washing with culture medium. Medium was changed daily for 7 days, and then every other day until the first passage. For all assays, late EPCs were used at passages 3 (about 28 days) (Supplemental Figure 3C). After 28 days culture, marker proteins of cultured EPCs were examined by flow cytometry analysis using phycoerythrin (PE)-labeled monoclonal mouse anti-human antibodies recognizing CD31 (BD Pharmingen), Tie-2 receptor (BD Pharmingen) and kinase-insert domain receptor (KDR) (R&D system). Overall, 94.53 ± 5.3% of the cells were positive for CD31, 87.56 ± 6.4% for Tie-2 receptor, 82.79 ± 6.9% for KDR (Supplemental Figure 3D). Based on the isolation and cultivation protocol, the adherent
mononuclear cells were identified as late EPCs, similar with previous studies.6,7

**Endothelial Derived Progenitor Cell (EPC) Culture**
Endothelial derived progenitor cells were isolated and cultured from HUVECs by following the protocol described by Ingram et al.8

**Shear Stress Assay**
EPCs were exposed to shear stress with a fluid shear stress loading device. Briefly, glass slides seeded with EPCs were placed on the stage of the parallel-plate flow chamber (length, 80mm; width, 30mm; height, 2mm) channel. After 1 day culture, hydrodynamic shear stress loaded on adherent seeded cells on parallel-plate flow chamber was carried out according to procedures described by Frangos et al.9 The seeded cells were exposed to 5, 10, 15 and 25dyn/cm² shear stress for 12 hours or 15dyn/cm² shear stress for 6, 12 and 24 hours, respectively. Shear stress was calculated using the equation T=6μ/βh², where T is the shear stress, Q is the flow rate, μ is the medium viscosity, b is channel width, and h is channel height. Control cells were kept under static condition. All experiments were performed at 37°C in a CO₂ incubator.

**EPC Migration *in Vitro***
EPC migration was determined using a modified Boyden chamber. Briefly, 2×10⁴ EPCs, resuspended in 250 µl EBM-2, were pipetted in the upper chamber of a modified Boyden chamber (Costar Transwell ® assay, 8 µm pore size, Corning, NY). The chamber was placed in a 24-well culture dish containing 500 µl EBM-2 supplemented with either PBS, or 100ng/ml SDF-1(Peprotech, Rocky Hill, NJ, USA). After 24 hours incubation at 37°C, transmigrated cells were counted by independent investigators blinded to treatment groups.

**EPC Adhesion to Endothelial Cells *in Vitro***
A monolayer of HUVECs was prepared 48 hours before the assay by plating 2×10⁵ cells in each well of a 4-well plate. HUVECs were pretreated with or without 1 ng/ml tumor necrosis factor-α (TNF-α, Peprotech) for 12 hours. Then 1×10⁵ CM-DiI (CellTracker™ CM-DiI, Invitrogen)-labeled EPCs were added to each well and incubated for 3 hours at 37°C. Nonattached cells were gently removed with PBS, and adherent EPCs were fixed with 4% paraformaldehyde and counted by independent investigators blinded to treatment groups.

**EPC Adhesion Assays in Flow on Fibronectin *in Vitro***
Laminar flow assays were as described.¹ Dishes were coated with fibronectin (10µg/mL). EPCs (5×10⁵/ml) were stimulated with or without 100 ng/ml SDF-1 for 10 minutes before the assays, then resuspended in assay buffer (HEPES-buffered Hank’s Balanced Salt Solution, 1 mmol/L Mg²⁺/Ca²⁺, 0.5% BSA), and perfused into the flow chamber (proprietary item; commercially obtained from RWTH Aachen University) at a shear rate of 1.5 dyn/cm² for 4 min at 37°C. After 4 minutes, firmly adherent cells were quantitated in multiple fields by independent investigators blinded to treatment randomly.

**RT-PCR and Western Blotting Analysis**
Total RNA was extracted with the mRNA abstraction kit (Takara Biotecnology, Dalian CO.,
LTD.). RT-PCR was carried out by the routine 2-step method. The primer CXCR4 A (sense) is 5'- TCTTCCCTGCCCACCACACTCTACTC-3’, and the primer CXCR4 B (antisense) is 5’-GTAGATGACATGGACTGGCTTGC-3’. The primers for RT-PCR in the telomerase reverse transcriptase (hTERT) gene were follows: sense, CACCTCACCTCACCCACGCAGAAA; antisense, CCAGAGGTGGCCGACGATTTTGT. EPC proteins were harvested by cell lysis buffer (Cell Signaling Technology, Beverly). Protein extracts were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes (Roche, Indianapolis). The following antibodies were used: rabbit anti-CXCR4 and anti-CXCR4 (phospho Serine 339) antibodies (1:1000; abCAM, Cambridge), rabbit anti-phospho-JAK-2 and anti-JAK-2 antibody (1:2000; Cell Signaling Technology), rabbit anti-actin antibody (1:2000; Cell Signaling Technology). Proteins were visualized with HRP-conjugated anti-rabbit IgG (1:3000; Cell Signaling Technology), followed by use of the ECL chemiluminescence system (Cell Signaling Technology). To detect SDF-1 stimulated phosphorylation of CXCR4 and JAK-2, EPCs were preincubated with 100ng/ml SDF-1 for 10 min before protein harvesting.

**CXCR4 Knockdown**

The Mission shRNA lentiviral transduction particles were used to knockdown CXCR4 expression of EPCs. Viral transduction was performed according to manufacturer’s instruction (GeneChem company, Ltd, Shanghai). The following oligomers were used: CXCR4-shRNA-1.5’TGGAGGGGATCAGTATACACA-3’;CXCR4-shRNA-2.5’-GTTTCTA CTCAGCTAACACA-3’. Briefly, after 7 days or 28 days culture, the lentiviral particles with shRNA targeting CXCR4 gene (CXCR4-shRNA) or non-targeting shRNA (Scrambled-shRNA) were added to the cells for 120 min in culture without serum. After transduction, cells were washed with PBS and incubated with EPC medium for 48 hours before subsequent experiments for assessment of CXCR4 expression and EPC functions.

**JAK-2 Inhibition**

JAK-2 activity was inhibited by incubation of EPCs with the selective JAK-2 inhibitor AG490 (10μM; Alexis, Plymouth Meeting, PA, USA) for 1 hours.

**Mouse Model of Carotid Artery Denudation Injury and Reendothelialization Assay**

Male NRMInu/nu athymic nude mice (SLAC laboratory animal center, Shanghai), 8 to 10 weeks of age, were anesthetized with ketamine (100 mg/kg IP) and xylazine (5 mg/kg IP). Surgery was carried out using a dissecting microscope. The left carotid artery was exposed via a midline incision on the ventral side of the neck. The bifurcation of the carotid artery was located, and two ligatures were placed around the external carotid artery, which was then tied off with the distal ligature. An incision hole was made between the two ligatures to introduce the denudation device. The curved flexible wire (0.35-mm diameter) was introduced into the common carotid artery, and passed three times in order to denude endothelium. The wire was then removed, and the external carotid artery was tied off proximal to the incision hole with the proximal ligature.

To assess the reendothelialization capacity of the cultured EPCs, EPCs or shear stress treated EPCs (15 dyn/cm² treatment for 12 hours) (5×10⁵ cells) were resuspended in 100μl of prewarmed PBS (37°C) and transplanted 3 hours after carotid artery injury via tail vein
injection with a 27-gauge needle. The same volume of PBS was injected into placebo mice. Three days after carotid artery injury, endothelial regeneration was evaluated by staining denuded areas with 50μl of solution containing 5% Evans blue dye via tail vein injection.

To assess EPC incorporation, EPCs were labeled with CM-DiI (CellTracker™ CM-DiI, Invitrogen) and injected into the tail vein of nude mice after carotid injury. After 24 hours the animals were euthanized, blood was immediately removed and the injured sections of the carotid arteries from two groups were dissected. The carotid arteries were then opened and incubated with 0.2% collagenase for 30 min at 37 °C, and then flushed with precooled washing buffer (10 mM HEPES, 0.1% BSA in HBSS). The cell suspension was filtered through a 100 μm mesh and centrifuged at 1200 rpm for 5 min. The cells were resuspended in 2 ml of FACS buffer and analysed by the BD FACSCanto II system. Alternatively, the injured sections were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned. The CM-DiI-labeled EPCs incorporated in the injured vessels were quantitatively analyzed under a fluorescent microscope (Olympus BX51).

All experimental protocols complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH) and the Animal Care and Use Committees of Sun Yat-Sen University.
Supplemental References


Supplemental Table

**Supplemental Table S1. Baseline Clinical Characteristics of Study Subjects**

<table>
<thead>
<tr>
<th>subjects</th>
<th>Young (n=10)</th>
<th>Elderly (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>27.3±3.0</td>
<td>68.4±2.5*</td>
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<tr>
<td><strong>Weight (kg)</strong></td>
<td>64.8±5.8</td>
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<td><strong>Body mass index (kg/m²)</strong></td>
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<td><strong>Systolic blood pressure (mmHg)</strong></td>
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<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
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</tr>
<tr>
<td><strong>Heart rate (beats/minute)</strong></td>
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<td>79.2±4.6</td>
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<tr>
<td><strong>Fasting plasma glucose (mmol/L)</strong></td>
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<td>4.8±0.5</td>
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<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
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<tr>
<td><strong>Triglyceride (mmol/L)</strong></td>
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<td>1.0±0.7</td>
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<tr>
<td><strong>HDL cholesterol (mmol/L)</strong></td>
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<td>1.0±0.4</td>
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<tr>
<td><strong>LDL cholesterol (mmol/L)</strong></td>
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<td>3.3±1.3</td>
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<tr>
<td><strong>Creatinine (µmol/L)</strong></td>
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<td><strong>Blood urea nitrogen (mmol/L)</strong></td>
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<td><strong>Alanine aminotransferase (U/L)</strong></td>
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<td><strong>Aspartate aminotransferase (U/L)</strong></td>
<td>23.6±15.7</td>
<td>18.8±9.2</td>
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</table>

Data are shown as mean ± SEM. *P<0.001 vs. young group baseline. HDL, high-density lipoprotein; LDL, low-density lipoprotein.
**Supplemental Figures and Figure Legends**

**Supplementary Figure S1**

![Figure S1](image)

**Figure S1.** Effect of aging on the number of circulating EPCs and the functions of cultured EPCs. (A) Shown are representative flow cytometry analyses of PBMNCs that were double positive for KDR and CD34 (i.e., circulating EPCs) (*right panel*) and quantification of the percentage of CD34+KDR+ cells in PBMNCs (*left panel*) (n=10 per group). Detailed gate strategies are illustrated in Online Supplementary Figure S2. (B-C) EPCs were obtained by culturing PBMNCs in EBM-2 medium for 7 d. (B) EPC migration was evaluated with a modified Boyden chamber assay. (n=10 per group). (C) EPC adhesion was assessed by applying Dil-labeled EPCs onto the monolayer of HUVECs that had been pre-treated for 12 h with TNF-α (1 ng/mL) (+) or vehicle control (−). (n=10 per group). Error bars represent SEM. hpf = high power field.
**Supplementary Figure S2**

![Flow Cytometry Plots](image)

**Figure S2.** Flow cytometry quantification of EPCs following the ISHAGE (International Society for Hematotherapy and Graft Engineering) gating strategy. Plot R1, contain all CD45+ events. Plot R2, include CD34+ events of the events in gate R1. Plot 3, is obtained by plotting the events that fulfills the criteria of gates R1 and R2. Cell forming a cluster of blasts with characteristic low SSC and low CD45 fluorescence (SSC<sub>low</sub> and CD45<sub>low</sub> cells) are then gated on this plot to produce a third region (R3 Plot 3). Plot 4, the events fulfilling the criteria of all three gates (R1, R2 and R3) are then displayed on a forward light scatter (FSC) vs. SSC dot plot to confirm that the selected blasts fall into the lymphocyte region (R5). Plot 5, the events fulfilling the criteria of all three gates (R1, R2, R3 and R5) are then displayed CD45<sup>dim</sup>/CD34<sup>+</sup> (R6). The lymphocyte region (plot 6) was adjusted from a SSC vs. FSC plot gated on lymphocytes from R4 (plot 1), employing only small lymphocytes (FSC<sub>low</sub>, R5 in plot 6). CD45<sup>dim</sup>/CD34<sup>+</sup>/KDR<sup>+</sup> endothelial progenitor cells are then deducted from the upper right quadrant of plot 7, defining CD34<sup>+</sup> KDR<sup>-</sup> cells, when falling into all three regions (R2, R3 and R5).
**Supplementary Figure S3**

![Image](image.png)

**Figure S3.** Phenotypic characterization of early EPCs and late EPCs. (A) After 7 days culture, 95% cultured EPCs were dually positive for Dil-acLDL uptake and FITC-labeled BS-1 lectin binding. (B) Marker proteins of cultured EPCs were also examined by flow cytometry analysis using PE-labeled monoclonal mouse anti-human antibodies recognizing CD31, vWF, KDR and CD14. n=5 per group. (C) Sequential changes of cultured EPC. a, Peripheral blood mononuclear cells immediately after plating. b, One day after seeding. c, Three days after seeding. d, Seven days after plating. e, Ten days after plating. f, Two weeks after plating. g, Late EPC grew exponentially. h, Late EPC grown to confluence showing a cobblestone-like monolayer. Scale bar=100 μm. (D) Flow cytometry analysis of marker proteins CD31, KDR and Tie-2 of cultured late EPCs. n=5 per group.
Supplementary Figure S4

**Figure S4.** Effect of aging on the function of cultured late EPCs. Late EPCs were obtained by culturing PBMCs isolated from healthy young and elderly human subjects. (A) Late EPC migration was evaluated with a modified Boyden chamber assay. Late EPCs (2×10⁴ cells/well) were seeded into the upper chamber, and the lower chamber was filled with 100 ng/ml SDF-1 (+) or PBS (−); then the chambers were incubated at 37°C for 24 h, and the numbers of the migrated cells were counted. n=5 per group. (B) Late EPC adhesion was assessed by applying DiI-labeled EPCs onto the monolayer of HUVECs that had been pre-treated for 12 h with TNF-α (1 ng/ml) (+) or vehicle control (−); after incubation for 3 h, the non-adherent cells were gently removed, the remaining cells were fixed, and the DiI(+) EPCs were counted under fluorescent microscope. n=5 per group. Error bars represent SEM. hpf = high power field.
**Supplementary Figure S5**

**Figure S5.** Late EPCs from the elderly display a reduced reendothelialization capacity. (A) Quantification of reendothelialization areas of the injured carotid arteries in nude mice 3 days after carotid denudation surgery plus i.v. injection of PBS or late EPCs (5×10⁵ cells) from young or the elderly. (n=5 per group). (B-C) Assessments of the transplanted late EPCs incorporated in the injured vessels. Late EPCs were labeled with CM-DiI (red) and i.v. injected into nude mice 3 h after carotid injury. Twenty-four hours later, the recipient mice were perfused with FITC-Lectin (green) to stain vasculature and euthanized. (B) (a-b) Cross section and (c-d) en face view of the injured carotid arteries showing CM-DiI–labeled EPCs (red) attached to injured endothelium (green) in mice receiving young (a,c) or elderly EPCs (b,d). Nuclei were stained with DAPI (blue). Shown are representatives of 3 independent experiments. (C) Fragments of injured carotid arteries were isolated, digested, and made into single cell suspension, and the DiI(+) EPCs were quantified by flow cytometry (n=5 per group). Error bars represent SEM.
**Supplementary Figure S6**

**Figure S6.** CXCR4/JAK-2 signaling is critical to the function of EPCs from elderly. Representative photographs (*upper panels*) and quantitative analyses (*lower panels*) of CXCR4 (A) and p-JAK-2 (B) protein expression in the cultured EPCs. (*P*<0.001 vs. young EPCs with SDF-1, *P*<0.01 vs. young EPCs without SDF-1; n=10 per group). (C-D) Representative photographs (*upper panels*) and quantification analyses (*lower panels*) of CXCR4 mRNA (C) and protein (D) expression in EPCs 48 h after Mission CXCR4-shRNA or scrambled-shRNA lentiviral transduction. (*P*<0.01 vs. Non-transduced or Scrambled-shRNA transduced EPCs, n=5 per group). (E-F) Quantification analyses of EPC migration (E) and adhesion to HUVECs (F). (*P*<0.05 vs. young EPCs without any treatment or only with Scrambled-shRNA treatment; *P*<0.05 vs. elderly EPCs without any treatment or only with Scrambled-shRNA treatment.) (G-H) Quantification analyses (G) and representative photographs (H) showing reendothelialization areas (white) of carotid arteries 3 days after denudation injury plus *i.v.* injection of EPCs with different pre-treatments. (*P*<0.01 vs. young EPCs without shRNA-transduction/AG-490 incubation; *P*<0.01 vs. elderly EPCs without shRNA-transduction/AG-490 incubation; n=5 per group). Error bars represent SEM. SS = shear stress. hpf = high power field.
**Supplementary Figure S7**

**Figure S7.** Shear stress enhances *in vitro* fusions and *in vivo* reendothelialization capacity of EPCs. (A-C) EPCs were firstly exposed to 15 dyn/cm² shear stress for the indicated length of time. (A) Quantitative analyses of the migratory activity of EPCs (*P*<0.05 vs. static young EPCs without chemotactant SDF-1, *P*<0.05 vs. static elderly EPCs without SDF-1; n=10 per group). (B) Quantitative analyses and (C) representative photographs of Dil-labeled EPC adhesion to HUVECs. (*P*<0.05 vs. static young EPCs with vehicle control treatment, *P*<0.05 vs. static elderly EPCs with vehicle control treatment; n=10 per group). (D-E) EPCs were treated for 12 h with the indicated force of shear stress, and quantitatively analyzed for (D) migratory activity (*P*<0.05 vs. static young EPCs without SDF-1, *P*<0.05 vs. static elderly EPCs without SDF-1; n=10 per group) and (E) adhesion to HUVECs (*P*<0.05 vs. static young EPCs with vehicle control treatment; *P*<0.05 vs. static elderly EPCs with vehicle control treatment; n=10 per group). & P<0.05; n.s., not significant. Error bars represent SEM. SS = shear stress. hpf = high power field.
**Figure S8.** Shear stress enhances *in vitro* adhesion in flow to fibronectin of EPCs. (A-B) EPCs were firstly exposed to 15 dyn/cm² shear stress for the indicated length of time. (A) Quantitative analyses and (B) representative photographs of EPC adhesion in flow to fibronectin. ("P<0.05 vs. static young EPCs with vehicle control treatment, *P<0.05 vs. static elderly EPCs with vehicle control treatment; n=5 per group). (D-E) EPCs were treated for 12 h with the indicated force of shear stress, and quantitatively analyzed for adhesion in flow to fibronectin ("P<0.05 vs. static young EPCs with vehicle control treatment; *P<0.05 vs. static elderly EPCs with vehicle control treatment; n=5 per group). &P<0.05; n.s., not significant. Error bars represent SEM.Scar bars = 100μm. SS = shear stress.
**Supplementary Figure S9**

**Figure S9.** Effect of shear stress on *in vitro* functions of endothelial-derived EPCs (EEPCs) and late EPCs (LEPCs). EEPCs and LEPCs were firstly treated with 15 dyn/cm² shear stress for 12 h. (A) Quantitative analyses (left panel) and representative photographs (right panel) of the migratory activity of EEPCs and late EPCs toward SDF-1 (100 ng/ml). The migrated cells were stained with DAPI (blue); n=5 per group. (B) Quantitative analyses (left panel) and representative photographs (right panel) of Dil-labeled EPC adhesion to HUVECs that had been pre-treated with TNF-α (1 ng/ml) (+) or vehicle control (−) for 12 h. The cells were counterstained with DAPI (blue); n=5 per group. (C) Quantitative analyses (left panel) and representative photographs (right panel) of adhesion in flow to frbronectin of EEPCs and late EPCs that had been pre-treated with SDF-1 (100 ng/ml) (+) or vehicle control (−) for 10 min. n=5 per group. Error bars represent SEM. Scar bars= 100µm. SS = shear stress. hpf = high power field.
Supplementary Figure S10

Figure S10. Shear stress enhances reendothelialization capacity of endothelial-derived EPCs (EEPCs) and late EPCs (LEPCs). EEPCs and LEPCs were cultured from HUVECs and PBMCs of the elderly, respectively, treated with 15 dyn/cm² shear stress for 12 h, and i.v. injected (5×10⁵ cells/mouse) into nude mice 3 h after carotid-artery denudation injury; 3 days later, 50μl Evans blue dye (5%) was i.v. injected to stained the un-endothelialized areas (blue) in the injured vessels for the assessment of reendothelialization. (A) Quantitative analyses and (B) representative photographs of reendothelialization areas. n=5 per group. Error bars represent SEM. SS = shear stress.
Supplementary Figure S11

**Figure 11.** Shear stress upregulates CXCR4 expression in EPCs. Representative photograph (upper panels) and quantification (lower panels) of CXCR4 mRNA expression by RT-PCR (A), total (C) CXCR4 protein expression by Western blotting in EPCs treated with 15 dyn/cm² shear stress for the indicated length of time (*P<0.05 vs. static young EPCs; *P<0.05 vs. static elderly EPCs; n=10 per group). Representative photograph (upper panels) and quantification (lower panels) of CXCR4 mRNA (B), total (D) protein expression in EPCs treated for 12 h with the indicated force of shear stress. (*P<0.05 vs. static young EPCs; *P<0.05 vs. static elderly EPCs; n=10 per group). Error bars represent SEM. SS = shear stress. T-CXCR4 = total CXCR4.
**Supplementary Figure S12**

**Figure S12.** Effect of shear stress on EPC marker proteins expression. At day 7, the cells were exposed to 15 dyn/cm² shear stress (+) or control treatment (-) and 12 h later, analyzed for expression of CD31, KDR, vWF and CD14 by FACS (A, representative photographs; B, quantitative analyses). n=5 per group. SS=shear stress; PE=phycoerythrin. MFI=mean fluorescence intensity.
Figure S13. The reduced CXCR4/JAK-2 signaling in late EPCs of the elderly is upregulated by in vitro treatment with shear stress. (A) Representative photographs (right panels) and quantitative analyses (left panels) of CXCR4 protein expression in the cultured late EPCs with (+) or without (-) SS treatment (15 dyn/cm²) for 12 h. Representative photographs (right panels) and quantification analysis (left panels) of p-JAK-2 protein expression of EEPCs after 15 dyn/cm² SS treatment for 12 h. n=5 per group. (B) Representative photographs (right panels) and quantification analysis (left panels) of CXCR4 protein expression of EEPCs after 15 dyn/cm² SS treatment for 12 h. n=5 per group. (C) Representative photographs (right panels) and quantitative analyses (left panels) of p-JAK-2 protein expression in the cultured late EPCs with (+) or without (-) SS treatment (15 dyn/cm²) for 12 h. n=5 per group. Error bars represent SEM. SS=shear stress.
**Supplementary Figure S14**

**Figure S14.** Blockade of CXCR4/JAK-2 signaling attenuates shear-stress–mediated enhancement of *in vitro* functions and *in vivo* reendothelialization capacity of endothelial derived progenitor cells (EPCs) and Late EPCs from the elderly. (A-B) The cultured EPCs were infected with the Mission lentiviral CXCR4-shRNA or Scrambled-shRNA, and 48 h later, treated with 15 dyn/cm² SS for another 12 h; then the cells were incubated with AG-490 (+) or vehicle (-) for 1h and subjected to migration and adhesion assays. (A) Quantification analysis of EPC migration toward SDF-1 (*P<0.05 vs. SS-treated EPCs without shRNA-transduction/AG-490 incubation; **P<0.05 vs. SS-treated LEPCs without shRNA-transduction/AG-490 incubation; n=5 per group). (B) Quantification analysis of EPC adhesion to the TNF-α prestimulated HUVECs (*P<0.05 vs. SS-treated EPCs without shRNA-transduction/AG-490 incubation; **P<0.05 vs. SS-treated LEPCs without shRNA-transduction/AG-490 incubation; n=5 per group). (C) Quantification of reendothelialization areas of carotid arteries 3 days after denudation injury plus *i.v.* injection of EPCs or LEPCs pre-treated with CXCR4-shRNA, scrambled-shRNA or AG490. (*P<0.01 vs. SS-treated EPCs; **P<0.01 vs. SS-treated LEPCs; n=5 per group). Error bars represent SEM. SS=shear stress. hpf = high power field.