

Physical exercise attenuates age-associated reduction in endothelium-reparative capacity of endothelial progenitor cells by increasing CXCR4/JAK-2 signaling in healthy men

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Summary

Endothelial progenitor cells (EPCs) play an important role in repairing endothelial injury. Aging is associated with EPC dysfunction. Physical exercise has a beneficial impact on EPC activity. However, whether physical exercise can enhance the endothelial repair capacity of EPCs in healthy men with aging is not clear. Here, we investigated the effects of physical exercise on reendothelialization capacity and CXCR4 chemokine receptor four (CXCR4) signaling in human EPCs. Before and after 12-week exercise, EPCs were isolated from elderly and young men. *In vitro* function and *in vivo* reendothelialization capacity of EPCs in a mouse model of carotid artery injury were measured. The expression of CXCR4 and its downstream signaling target Janus kinase-2 (JAK-2) were determined. Before exercise, *in vitro* function and *in vivo* reendothelialization capacity of EPCs were significantly reduced in elderly men compared with young men. After exercise intervention, *in vitro* function and *in vivo* reendothelialization capacity of EPCs from elderly men were markedly enhanced. Physical exercise increased a higher CXCR4 protein expression and higher JAK-2 phosphorylation levels of EPCs. The augmentation in reendothelialization capacity of EPCs was closely correlated with the upregulation of CXCR4/JAK-2 signaling and improvement of endothelial function. This study demonstrates for the first time that physical exercise attenuates age-associated reduction in endothelium-reparative capacity of EPCs by increasing CXCR4/JAK-2 signaling. Our findings provide insight into the novel mechanisms of physical exercise as a lifestyle intervention strategy to promote vascular health in aging population.

Key words: aging; endothelial progenitor cells; exercise; CXCR4; endothelium; endothelial repair.

Introduction

Aging is a powerful independent risk factor for increased cardiovascular vascular disease (CVD) (Lakatta, 1993; Sniderman & Furberg, 2008). Loss in the endothelial homeostasis with aging is clearly a contributor to the

initiation and development of CVD (Taddei *et al.*, 1995; Tao *et al.*, 2004; Deanfield *et al.*, 2007; Rodríguez-Mañas *et al.*, 2009). Therefore, maintenance of the normal endothelial integrity becomes an important therapeutic strategy aimed at reducing the age-related high incidence of CVD.

Accumulating evidence indicates that endothelial progenitor cells (EPCs) contribute substantially to the preservation of a structurally and functionally intact endothelium (Hill *et al.*, 2003; Werner *et al.*, 2005; Moreno *et al.*, 2009). However, in the presence of CVD risk factors including aging, the fall in function and number of circulating EPCs has been reported (Vasa *et al.*, 2001; Tao *et al.*, 2006; Thijssen *et al.*, 2006; Müller-Ehmsen *et al.*, 2008; Umemura *et al.*, 2008), thus leading to impaired reendothelialization capacity of EPCs. Therefore, upregulation of functional potential of EPCs with subsequently enhanced endothelial repair in elderly individuals has important clinical implication in humans with aging.

Physical exercise is an important lifestyle intervention means to halt development of CVD with aging. In addition to favorably modifying traditional risk factors, physical exercise has been shown to prevent and restore age-associated loss in endothelial function in humans (DeSouza *et al.*, 2000; Werner *et al.*, 2009). Recent data also suggest that the beneficial effect of exercise on endothelial protection is, at least in part, related to the exercise-induced upregulation of EPC function and number (Laufs *et al.*, 2004; Yang *et al.*, 2007; Van Craenenbroeck *et al.*, 2010; Witkowski *et al.*, 2010). However, it is unclear whether physical exercise can improve *in vivo* reendothelialization capacity of EPCs from elderly men. Moreover, the mechanisms underlying the effect of exercise on endothelium-reparative capacity of EPCs remain unknown in humans with aging.

CXCR4 chemokine receptor four (CXCR4) is a seven-transmembrane G-protein-coupled receptor, and the ligand for CXCR4 is chemokine stromal cell-derived factor-1 (SDF-1). There is increasing evidence that CXCR4 is a key regulator of homing and retention of EPCs at the sites of injured artery, suggesting that an enhanced CXCR4 expression may facilitate the endothelial repair capacity of EPCs (Hristov *et al.*, 2007a; Sainz & Sata, 2007; Chen *et al.*, 2010). However, the influences of physical exercise on endothelialization capacity and CXCR4 signaling of human EPCs are not clear in persons with aging.

In this study, we hypothesized that physical exercise may attenuate age-related decline in reendothelialization capacity of EPCs and CXCR4 signaling is related to exercise-enhanced endothelial repair. To test these assumptions, *in vivo* reendothelialization capacity in a nude mouse carotid artery injury model as well as *in vitro* migration and adhesion functions of EPCs derived from elderly and young healthy men was examined. Then, we evaluated the effect of the transplantation of exercise-stimulated EPCs from elderly men after 12 weeks physical exercise on *in vivo* reendothelialization capacity in nude mice. The change of CXCR4 signaling in EPCs was determined. Endothelium-dependent vasodilation in the brachial artery was also assessed by high-resolution ultrasound. This study may provide novel evidence for further exploration of the crucial role of physical exercise as an effective lifestyle intervention measure to maintain the vascular homeostasis in aging population.

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Accepted for publication 17 October 2011



Results

Subject characteristics

Forty-seven individuals (22 elderly and 25 young men) were included into this study. Besides age, the two groups did not differ significantly with regard to other parameters before and after 12-week exercise (Table 1).

In vitro activities and *in vivo* reendothelialization capacity of EPCs from elderly men are impaired

The percentage of CD34+/KDR+ or KDR+/CD133+ cells was significantly lower in the peripheral blood mononuclear cells (PBMCs) from the elderly than those from young subjects ($P < 0.05$) (Fig. 1A,B). 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 (0.32 ± 0.13 vs. 0.81 ± 0.22 ; $P < 0.01$) in EPCs from the elderly (Fig. 1C). We evaluated the adhesion activity of EPC by counting the adherent cells of CM-Dil-labeled EPCs onto TNF- α -prestimulated monolayer human umbilical vein endothelial cells (HUVECs); the numbers of adherent EPCs from elderly were fewer than those from young subjects ($P < 0.01$) and fold change of TNF- α -induced increase of adhesion was lesser (0.73 ± 0.24 vs. 1.33 ± 0.43 ; $P < 0.01$) in EPCs from the elderly (Fig. 1D). The reendothelialization capacity of cultured EPCs was observed in a carotid artery denudation injury model in nude mice. Transplantation of EPCs from young people, but not EPCs from the elderly, significantly accelerated reendothelialization of the injured arteries (elderly vs. young: $15 \pm 4\%$ vs. $36 \pm 5\%$; $P < 0.001$) (Fig. 2A,B). 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 ($P < 0.001$) (Fig. 2C). Under the fluorescent microscope, the number of incorporated transplanted-labeled EPCs was lower in the

injured carotid artery (Fig. 2D). These results confirmed that EPCs from the elderly have an impaired reendothelialization capacity that is related to a decreased EPC incorporation in the injured vessels.

CXCR4/JAK-2 signaling disturbance contributes to the diminished reendothelialization capacity of EPCs from elderly men

The CXCR4 has been shown to be involved in the homing of EPCs (Hristov *et al.*, 2007a; Sainz & Sata, 2007; Chen *et al.*, 2010). Our data showed the surface CXCR4 protein expression did not have difference between young and elderly men ($p = NS$) (Fig. 3A). It is well known that Janus kinase-2 (JAK-2) is one of the downstream targets of CXCR4 signaling, so we investigated whether CXCR4-mediated JAK-2 signaling is deregulated in EPCs from elderly men. We found that the basal (young: 1 ± 0.15 vs. elderly: 0.43 ± 0.24 ratio of p-JAK-2/JAK-2; $P < 0.05$) and SDF-1-induced JAK-2 phosphorylation (p-JAK-2) (young: 1.48 ± 0.29 vs. elderly: 0.55 ± 0.19 ratio of p-JAK-2/JAK-2; $P < 0.001$) were significantly decreased in EPCs from elderly men, and the fold change of SDF-1-induced increase of JAK-2 phosphorylation was lesser (0.26 ± 0.09 vs. 0.51 ± 0.12 ; $P < 0.01$) in EPCs from the elderly (Fig. 3B).

To further investigate the relationship between dysregulation of CXCR4/JAK2 signaling pathway and impaired reendothelialization capacity of EPCs from elderly men, CXCR4 gene knockdown (Fig. 4A,B) and JAK-2 inhibitor AG-490 were used to block the CXCR4/JAK-2 signaling of EPCs from young men, respectively. Our data showed CXCR4 gene knockdown or pretreatment with AG490 profoundly reduced *in vitro* function of EPCs from young men, meanwhile combined JAK-2 and CXCR4 blockade did not result in an additive effect (Fig. 4C,D). Consistent with the results of *in vitro* function assays, CXCR4 gene knockdown or pretreatment with AG490 can also attenuate *in vivo* reendothelialization capacity of EPCs from young men, respectively (Fig. 4E,F).

Table 1 Clinical characteristics of included subjects

	Young group (n = 22)		Elderly group (n = 25)	
	Baseline	12 weeks	Baseline	12 weeks
Age (years)	26.3 \pm 3.15	26.3 \pm 3.15	67.8 \pm 3.38*	67.8 \pm 3.38
Weight (kg)	64.5 \pm 7.58	64.6 \pm 7.18	65.6 \pm 5.92	65.5 \pm 5.90
BMI (kg m ⁻²)	22.9 \pm 1.53	22.7 \pm 1.41	23.3 \pm 1.29	23.3 \pm 1.46
SBP (mmHg)	119.5 \pm 7.52	120.2 \pm 6.48	123.6 \pm 8.34	123.1 \pm 8.21
DBP (mmHg)	75.9 \pm 4.56	75.3 \pm 4.76	79.8 \pm 4.54	80.1 \pm 4.23
HR (beats min ⁻¹)	70.6 \pm 3.75	70.8 \pm 3.55	73.3 \pm 3.40	73.7 \pm 4.22
FPG (mm)	4.2 \pm 0.51	4.2 \pm 0.39	4.55 \pm 0.42	4.51 \pm 0.29
TC (mm)	5.1 \pm 0.70	5.2 \pm 0.35	5.0 \pm 0.68	4.8 \pm 0.56
TG (mm)	1.1 \pm 0.33	1.1 \pm 0.28	1.0 \pm 0.35	0.9 \pm 0.37
HDL (mm)	1.3 \pm 0.25	1.3 \pm 0.27	1.4 \pm 0.22	1.3 \pm 0.15
LDL (mm)	2.8 \pm 0.35	2.9 \pm 0.26	2.8 \pm 0.46	2.7 \pm 0.34
Cr (μ m)	71.4 \pm 5.70	71.1 \pm 5.53	71.7 \pm 4.65	72.6 \pm 5.50
BUN (mm)	4.5 \pm 0.75	4.5 \pm 0.73	4.4 \pm 0.73	4.3 \pm 0.54
ALT (U L ⁻¹)	21.1 \pm 8.10	23.2 \pm 7.79	24.4 \pm 6.48	25.7 \pm 7.06
AST (U L ⁻¹)	27.6 \pm 6.56	27.9 \pm 5.84	27.5 \pm 6.89	27.2 \pm 6.48

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Cr, creatinine; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase. Data are shown as mean \pm SD. * $P < 0.05$ vs. young group baseline.

Physical exercise augments *in vitro* activities and *in vivo* reendothelialization capacity of EPCs from elderly men

Physical exercise had positive effects on quantities and *in vitro* activities of EPCs from elderly men (Fig. 5). Similar to *in vitro* function, the *in vivo* reendothelialization capacity of EPCs from elderly men increased (before: $15 \pm 4\%$ vs. after: $36 \pm 9\%$; $P < 0.001$) after 12 weeks physical exercise (Fig. 6A,B). Moreover, our data showed that exercise upregulated surface CXCR4 protein expression and ratio of p-JAK-2/JAK-2 protein of EPCs ($P < 0.001$, Fig. 6C,D).

Physical exercise-enhanced reendothelialization capacity of EPCs from elderly men is correlated with CXCR4/JAK-2 signaling and endothelial function

Our data showed that flow-mediated dilation (FMD) was significantly decreased in elderly men compared with young men (young: $10.0 \pm 2.07\%$ vs. elderly: $6.39 \pm 1.35\%$; $P < 0.01$) (Fig. 6E). Physical exercise had a significant improvement of FMD in elderly men from $6.39 \pm 1.35\%$ to $7.97 \pm 1.21\%$ ($P < 0.05$, Fig. 6F). There is a close association between augmentation in reendothelialization capacity and upregulation of p-JAK-2/JAK-2 protein ratio of EPCs ($r = 0.63$, $P < 0.05$) in elderly exercise group. The augmentation in reendothelialization capacity of EPCs from elderly men after exercise is closely correlated with the upregulation of improvement of endothelial function ($r = 0.61$, $P < 0.05$).

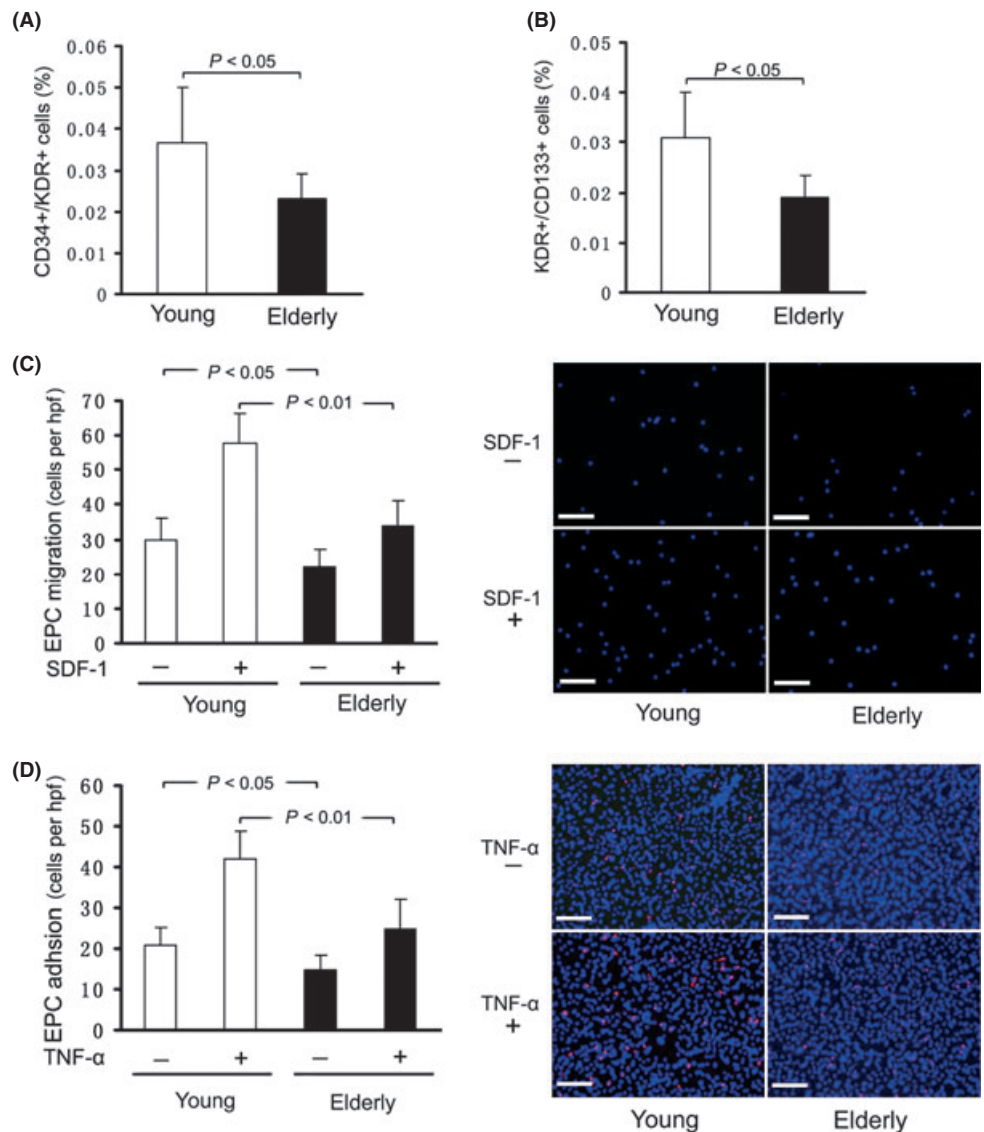


Fig. 1 Effects of aging on the number and *in vitro* function of endothelial progenitor cells (EPCs). The level of circulating CD34+/KDR+ (A) or KDR+/CD133+ (B) double positive cells from young and elderly men ($n = 6$ per group). Endothelial progenitor cells were obtained by culturing PBMCs in EBM-2 medium for 7 days. (B) A modified Boyden chamber assay was used to evaluate EPC migration. Endothelial progenitor cells (2×10^4 cells per well) were seeded into the upper chamber, and the lower chamber was filled with 100 ng mL^{-1} SDF-1 (+) or PBS (-); then, the chambers were incubated at 37°C for 24 h, and the numbers of the migrated cells were counted ($n = 6$ per group). (C) Endothelial progenitor cells adhesion was assessed by applying Dil-labeled EPCs onto the monolayer of human umbilical vein endothelial cells (HUVECs) that had been pretreated for 12 h with TNF- α (1 ng mL^{-1}) (+) or vehicle control (-); after incubation for 3 h, the nonadherent cells were gently removed, the remaining cells were fixed, and the Dil(+) EPCs were counted under fluorescent microscope ($n = 6$ per group). Error bars represent SEM. hpf, high power field.

Discussion

This study demonstrates that physical exercise increases *in vivo* reendothelialization capacity of EPCs from elderly men and improves age-related decline in endothelial function. Physical exercise facilitates CXCR4 protein expression and the phosphorylation of JAK-2 in EPCs. The increase in reendothelialization capacity of EPCs from elderly men after exercise is closely correlated with the upregulation of CXCR4/JAK-2 signaling and improvement of endothelial dysfunction. Our findings for the first time provide novel evidence that physical exercise contributes to the amelioration of EPCs-mediated endothelial repair capacity by augmenting CXCR4/JAK-2 signaling in humans with aging.

Previous studies demonstrated that the capacity of EPCs homing to the sites of endothelial damage and creating a cellular patch is necessary for the endothelial repair following arterial injury (Werner *et al.*, 2003; Hristov *et al.*, 2007b), indicating a particularly significance of EPCs for the restoration of endothelial integrity. In this study, we found that downregulation of CXCR4 expression in EPCs from young men by small interfering RNA knockdown not only resulted in impaired SDF-1-induced

migration and TNF- α -stimulated adhesion to HUVECs but was also accompanied by a reduced capacity of EPCs homing to the sites of vascular injury and diminished endothelial repair following arterial damage, suggesting that CXCR4 is crucial for therapeutic integrity of EPCs homing to the local vascular bed with subsequently enhanced reendothelialization.

Endothelial dysfunction is the hallmark of vascular damage with advancing age. Deficiency in function and number of EPCs is associated with endothelial dysfunction (Scheubel *et al.*, 2003; Heiss *et al.*, 2005; Tao *et al.*, 2006; Hoetzer *et al.*, 2007). The data reported here showed that endothelial function measured by flow-mediated vasodilation in the brachial artery as well as *in vitro* SDF-1-induced migration and TNF- α -stimulated adhesion to HUVECs was markedly impaired in elderly men compared with young men, similar with prior reports (Taddei *et al.*, 1995; Tao *et al.*, 2004; Heiss *et al.*, 2005; Hoetzer *et al.*, 2007). Moreover, reendothelialization capacity of *in vivo* EPCs derived from elderly men was significantly reduced in a nude mouse carotid artery injury model. Loss in reendothelialization capacity of EPCs was closely correlated with the injury of endothelial function, suggesting that the

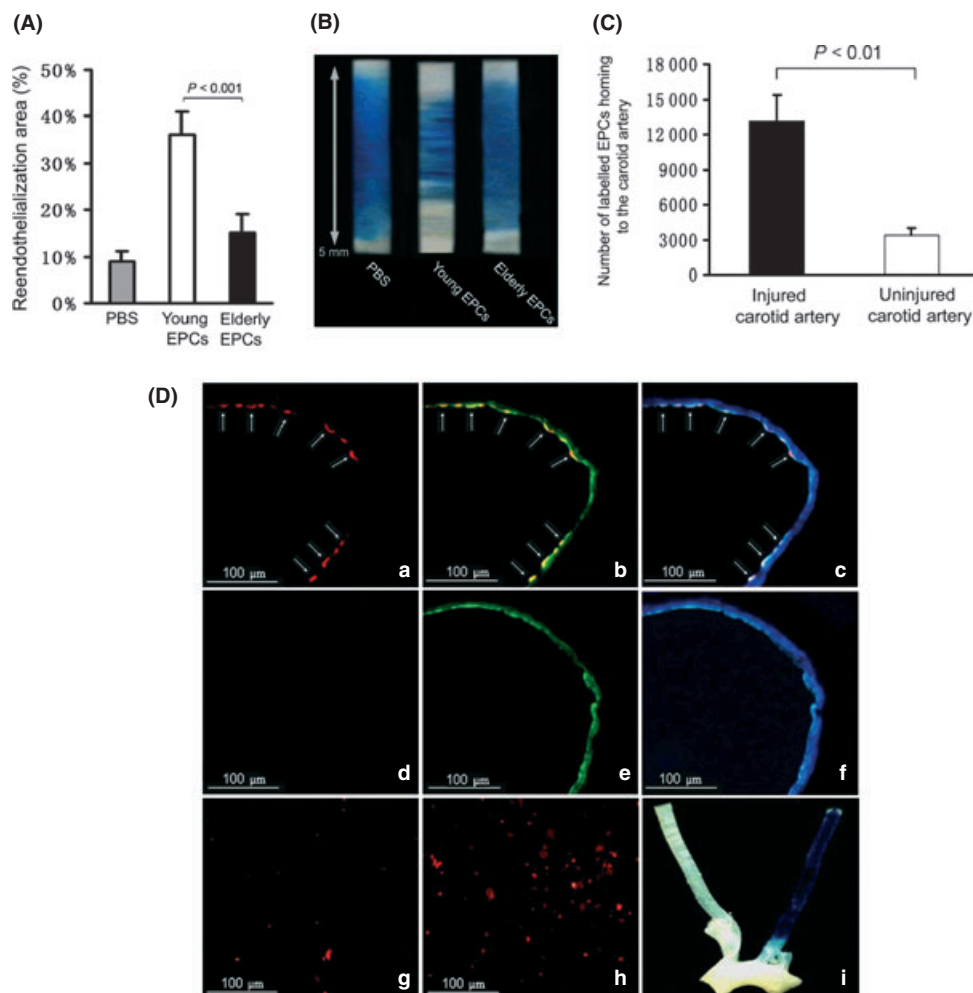


Fig. 2 Aging impairs *in vivo* reendothelialization capacity of endothelial progenitor cells (EPCs). Quantification analysis (A) and representative photographs (B) of reendothelialization area at day 3 after carotid injury in nude mice with PBS injection, transplantation of EPCs (5×10^5 cells) cultured from young or elderly men ($n = 6$ per group). (C) The number of DiI-labeled cultured EPCs (from young or elderly) homing to the injured carotid arteries quantified by flow cytometry analyses ($n = 6$ per group) (D) Assessments of the transplanted EPCs incorporated in the injured vessels. Fluorescent microscope: the cross-section (young: a-c; elderly: d-f) and the *en face* view (young: g; elderly: h) of carotid artery at day 3 after injury showing EPCs (CM-DiI-labeled, red) attached to injured endothelium (FITC-Lectin-stained, green) in mice receiving young or elderly EPCs. Nuclei were stained with DAPI (blue) ($n = 6$ per group). (i) Light photographs of injured and contralateral uninjured carotid artery (injury endothelium stained blue, Evans blue; $n = 6$ per group). Error bars represent SEM.

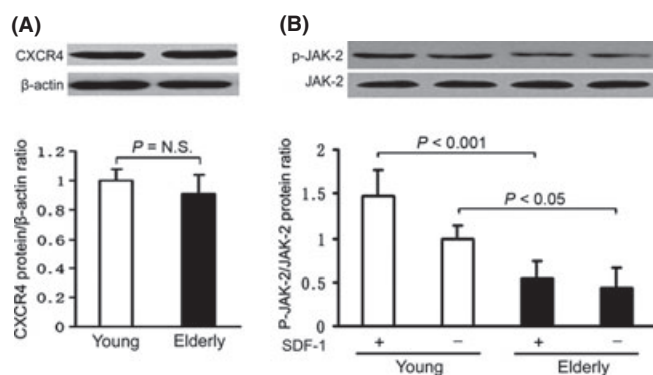


Fig. 3 Effect of aging on CXCR4/JAK-2 signaling of endothelial progenitor cells (EPCs). Representative photographs and quantification analysis of surface CXCR4 (A), p-JAK-2 and JAK-2 (B) protein expression of EPCs from young and elderly men ($n = 6$ per group).

age-associated fall in endogenous endothelium-reparative capacity for the vasculature contributes to development of endothelial dysfunction.

To further investigate the molecular mechanism of impaired EPCs-mediated endothelial repair, we hypothesized that abnormality in CXCR4 signaling resulted in reduced EPC function with aging. We found no

difference in the surface CXCR4 receptor expression in EPCs between elderly and young men. However, phosphorylation of JAK-2, a CXCR4 downstream signaling, was significantly reduced in EPCs derived from elderly men. Moreover, small interfering RNA knockdown of CXCR4 and JAK-2 inhibitor AG490 inhibited the *in vitro* function and *in vivo* reendothelialization capacity of EPCs from young men. Our data for the first time suggest that diminished CXCR4/JAK2 signaling is, at least in part, responsible for the reduction in EPCs-mediated endothelial repair capacity in men with aging. Based on the close association between CXCR4/JAK-2 signaling and EPC reendothelialization capacity, it is of particular importance to enhance CXCR4/JAK-2 signaling upregulation of EPCs from elderly men to augment the endothelium-reparative potential of EPCs and maintain the endothelial integrity.

Physical exercise has been shown to increase the function and number of circulating EPCs in healthy individuals (Hoetzer *et al.*, 2007; Yang *et al.*, 2007; Möbius-Winkler *et al.*, 2009; Walther *et al.*, 2009; Witkowski *et al.*, 2010), which is paralleled with improvement of endothelial function. However, it is not clear whether exercise contributes to the promotion of endothelial repair capacity of EPCs from elderly men and if yes, whether CXCR4/JAK-2 signaling is involved in the functional regulation of EPCs. Therefore, we evaluated the effects of physical exercise on reendothelialization capacity and CXCR4/JAK-2 signaling of EPCs in healthy elderly men. The data presented here showed that physical exercise for 12 weeks significantly enhanced *in vivo* reendothelialization

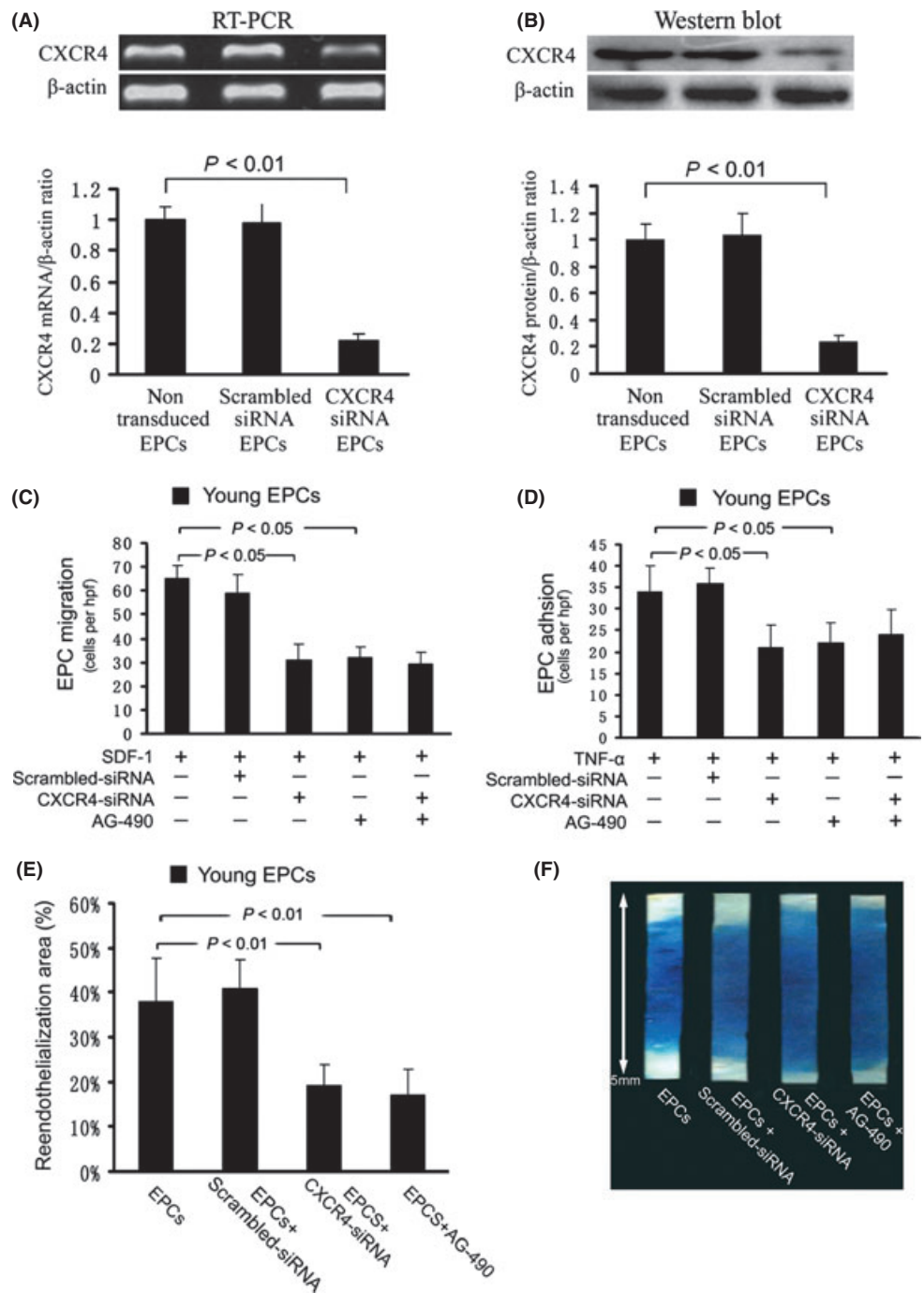


Fig. 4 CXCR4/JAK-2 signaling blockade inhibits *in vitro* function and *in vivo* reendothelialization capacity of endothelial progenitor cells (EPCs) from young men. Representative photographs and quantification analysis of mRNA (A) and surface protein (B) expression of CXCR4 of EPCs ($n = 6$ per group). Quantification analysis of migration (C) and adhesion to human umbilical vein endothelial cells (HUVECs) (D) of EPCs from young men treated with CXCR4-siRNA, Scrambled-siRNA or AG490 ($^{\#}P < 0.05$ 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; $n = 6$ per group). Quantification analysis (E) and representative photographs (F) showing reendothelialization area of carotid artery on day 3 after injury after the transplantation of EPCs treated with CXCR4-siRNA, Scrambled-siRNA or AG490 ($^{\#}P < 0.01$ vs. young EPCs without shRNA transduction/AG-490 incubation; $^{*}P < 0.01$ vs. middle-aged/elderly EPCs without shRNA transduction/AG-490 incubation; $n = 6$ per group).

capacity as well as facilitated expression of CXCR4/JAK-2 signaling, which is associated with increased *in vitro* EPC migration and adhesion activity in elderly men. Enhanced reendothelialization capacity of EPCs stimulated by exercise is closely correlated with CXCR4/JAK-2 signaling. To the best of our knowledge, this study for the first time suggests that exercise-modified CXCR4, possible via the phosphorylation of JAK-2, functions as key regulator of reendothelialization capacity *in vivo*. Owing to the fact that ethical reasons limit the usage of specific antagonist of CXCR4 and JAK-2 phosphorylation, we cannot determine the exact signaling pathway *in vivo*. However, the association of above-proposed signaling pathway based on our *in vitro* cell culture experiments is supported

by the closed relationship between CXCR4/JAK-2 signal and *in vivo* reendothelialization capacity of EPCs in elderly men. This is further evidenced by our recent study in which the transplantation of human EPCs overexpressing CXCR4 by gene transfer clearly accelerated reendothelialization in a nude mouse model of carotid artery injury, supporting the notion put forward here obtained by the exercise-mediated upregulation of CXCR4/JAK-2 signal and augmentation of EPC reendothelialization capacity (Chen *et al.*, 2010). Furthermore, flow-mediated vasodilation in the brachial artery is also significantly high in elderly men after exercise, suggesting an improvement of endothelial function probably partially related to the upregulation of EPC function and number with

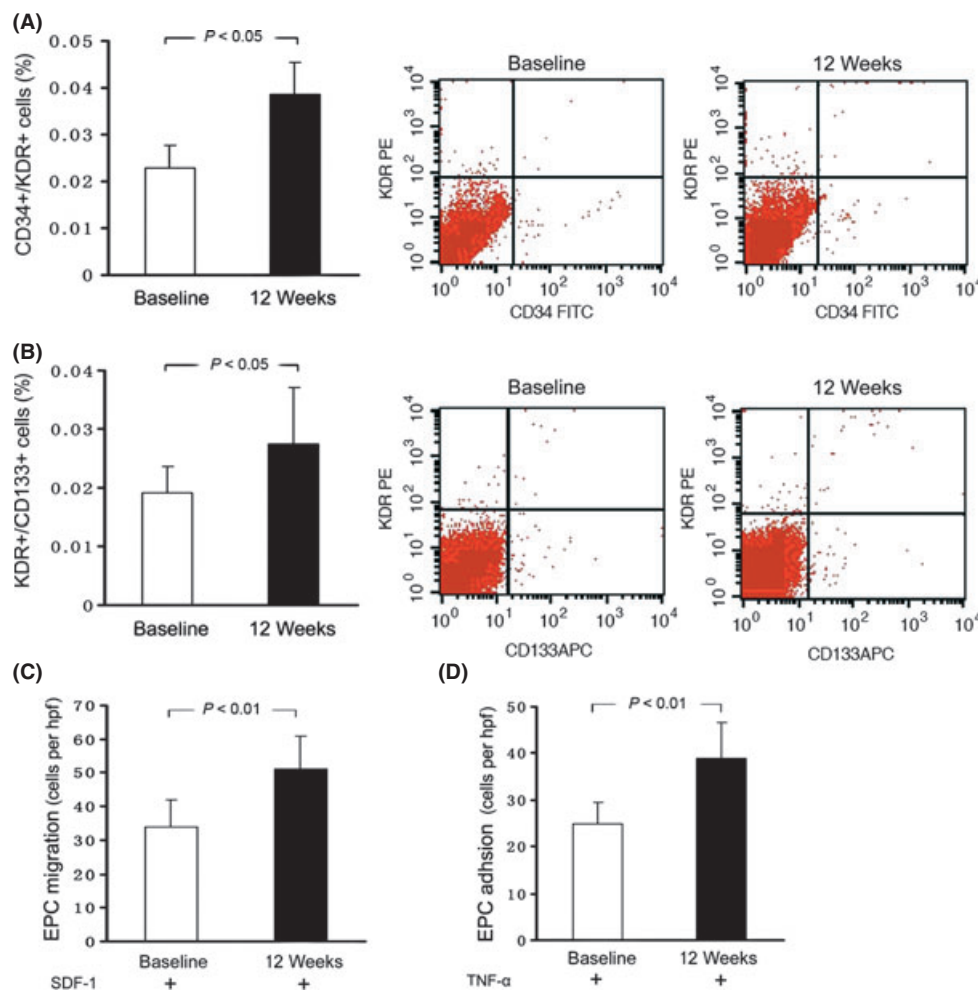


Fig. 5 Effects of exercise on the number and *in vitro* function of endothelial progenitor cells (EPCs) from elderly men. The level of circulating CD34 + /KDR+ (A) or KDR+/CD133+ (B) double positive cells from elderly men before and after exercise. Stromal cell-derived factor-1-induced migration (C) and TNF- α -stimulated adhesion to human umbilical vein endothelial cells (D) of EPCs before and after exercise.

subsequently an enhanced EPC integration into defects of the endothelial cell layer and rejuvenescence of aging endothelial cells consistent to previous studies (Hill *et al.*, 2003; Werner *et al.*, 2003). Collectively, this study, taken with previous investigations, indicates that the beneficial effect of physical exercise on the vascular endothelium is related to the amelioration of the functional properties in human EPCs at least in part via CXCR4/JAK-2 signaling that impairs with aging, by which physical exercise improves endogenous repair mechanism and maintains homeostasis of the vascular endothelium with aging.

It should be pointed out that this study has several limitations. First, at least two populations of EPCs have been commonly differentiated, that is, early EPCs and late outgrowth EPCs obtained after several weeks of culture (Hirschi *et al.*, 2008; Shantsila *et al.*, 2008). Given that early EPCs are rather more frequent in number, they play a particularly important role in the endothelial repair processes. Therefore, in this study, we were focused on the regulation of physical exercise on CXCR4 signaling and its relation to reparative capacity of early EPCs. However, the influence of physical exercise on the reparative capacity of late EPCs remains to be further elucidated. Second, although we reported that the exercise-induced upregulation of CXCR4/JAK-2 signaling contributed to the improvement of EPCs-mediated endothelial reendothelialization in persons with aging, however, the exact molecular mechanisms underlying the effect of physical exercise on CXCR4/JAK-2 signaling is not clear. Further study is under investigation in our laboratory to answer this question. Finally, whether

the mechanisms other than CXCR4 signaling may also have a beneficial impact on EPCs-mediated endothelial reendothelialization deserves further exploration.

In summary, this study demonstrates that physical exercise can attenuate the age-related reduction in endothelium-reparative capacity of EPCs in healthy men by increasing CXCR4/JAK-2 signaling, providing novel insight into the favorable impact of physical exercise on the vascular endothelium. Physical exercise is an effective nonpharmacological intervention strategy for combating endothelial injury associated with aging and contributes to the prevention and treatment of CVD in humans.

Experimental procedures

Subject characteristics and study protocol

Young (26.3 ± 3.15 years, $n = 22$) and elderly (67.8 ± 3.38 years, $n = 25$) healthy male subjects without clinical evidences of cardiovascular risk factors and without concomitant medications were enrolled into the study. The clinical characteristics were summarized in Table 1.

All subjects underwent a modified Bruce treadmill endurance-type exercise protocol according to Exercise Standards for Testing and Training of American Heart Association (5.5 metabolic equivalents for young group, and 4.5 metabolic equivalents for elderly group) (Fletcher *et al.*, 2001). Subjects in the training group performed treadmill training 30 min

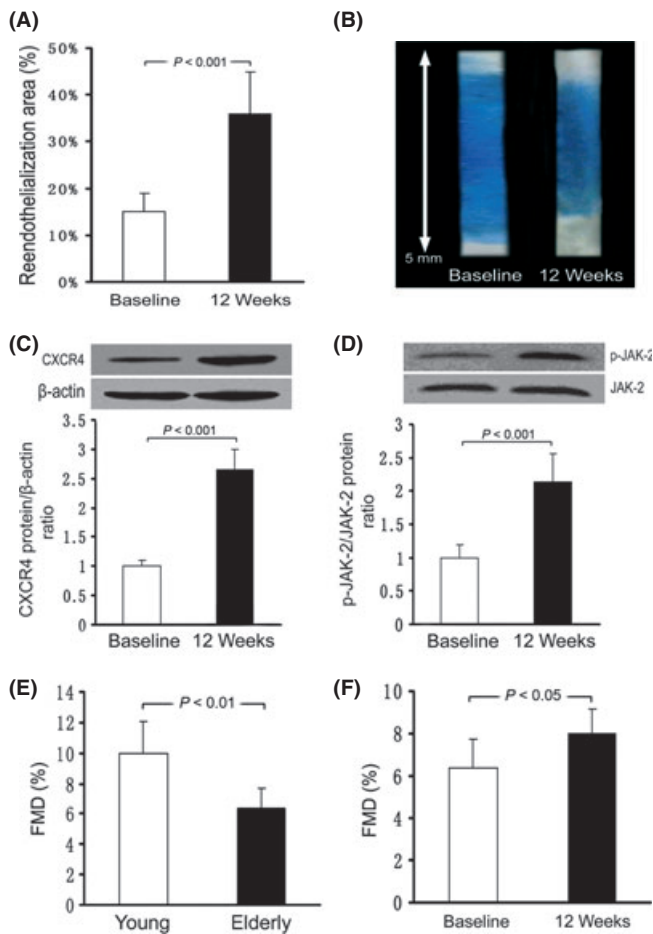


Fig. 6 CXCR4/JAK-2 signaling contributed to exercise-accelerated reendothelialization capacity of endothelial progenitor cells (EPCs) and endothelial function. Quantification analysis (A) and representative photographs (B) of reendothelialization area at day 3 after carotid injury in nude mice with the transplantation of EPCs from elderly men before and after exercise. Representative photographs and quantification analysis of surface CXCR4 (C), p-JAK-2 and JAK-2 (D) protein expression of EPCs from elderly men before and after exercise. Flow-mediated dilation between young and elderly men (E), and of elderly men before and after exercise (F).

daily for 3 days per week for a period of 12 weeks. Peripheral venous blood samples were obtained for EPC isolation from young and elderly subjects before and after exercise. Informed consents were obtained from all study subjects before enrollment. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China).

EPC culture, identification, and materials

Endothelial progenitor cells were isolated and cultured as previously described (Asahara *et al.*, 1997; Chen *et al.*, 2010; Giannotti *et al.*, 2010). After 7 days of culture, EPCs were defined as cells dually positive for 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine (DiI)-acetylated low-density lipoprotein (acLDL) uptake (Invitrogen, Carlsbad, CA, USA) and BS-1 lectin binding (Sigma-Aldrich, St. Louis, MO, USA) and endothelial markers were examined by flow cytometry analysis using CD31 (BD Pharmingen, San Diego, CA, USA), von Willebrand factor

(vWF) and kinase-insert domain receptor (KDR) (R&D Systems Inc, Minneapolis, MN, USA) as previously described (Chen *et al.*, 2010). Based on the isolation and cultivation protocol, the adherent mononuclear cells were identified as early EPCs (Chen *et al.*, 2010; Giannotti *et al.*, 2010). Circulating EPCs were evaluated by the number of CD34+/KDR+ and KDR+/CD133 cells per 100 thousands PBMCs by flow cytometry analysis (Beckman-Coulter, Fullerton, CA, USA) as previously described (Fadini *et al.*, 2008). Janus kinase-2 inhibitor AG490 (Alexis, Plymouth Meeting, PA, USA) was used as blocking agents in this study.

EPC migration *in vitro*

Endothelial progenitor cells migration was determined using a modified Boyden chamber. In brief, a total of 2×10^4 EPCs were isolated, resuspended in 250 μ L EBM-2, and pipetted at the seventh day in the upper chamber of a modified Boyden chamber (Costar Transwell[®] assay, 8 μ m pore size; Corning, NY, USA). The chamber was placed in a 24-well culture dish containing 500 μ L EBM-2 supplemented with either PBS, or 100 ng mL⁻¹ SDF-1. After 24 h incubation at 37°C, transmigrated cells were counted by independent investigators blinded to treatment randomly.

EPC adhesion to endothelial cells *in vitro*

A monolayer of HUVECs was prepared 48 h before the assay by plating 2×10^5 cells in each well of a four-well plate. Human umbilical vein endothelial cells were pretreated with or without 1 ng mL⁻¹ tumor necrosis factor- α (TNF- α ; Peprotech Inc, Rocky Hill, NJ, USA) for 12 h. Then, 1×10^5 CM-DiI (CellTracker[™] CM-DiI; Invitrogen)-labeled EPCs were added to each well and incubated for 3 h 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 randomly.

RT-PCR and western blot analysis

Total RNA was extracted with the mRNA abstraction kit (Takara Biotechnology, Dalian Co., Ltd., Dalian, Liaoning, China). RT-PCR was carried out by the routine two-step method. The primer CXCR4 A (sense) is 5'-TCTTCCTGCCACCATCTACTC-3', and the primer CXCR4 B (antisense) is 5'-GTAGATGACATGGACTGCCTTGC-3'. Endothelial progenitor cells protein was harvested by cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Protein extracts were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Roche, Indianapolis, IN, USA). The following antibodies were used: rabbit anti-CXCR4 antibody (1:1000, abCAM; Cambridge, UK), rabbit anti-actin antibody (1:2000; Cell Signaling Technology), rabbit anti-phospho-JAK-2, and anti-JAK-2 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 JAK-2, EPCs were preincubated with 100 ng mL⁻¹ SDF-1 for 10 min before protein harvesting.

CXCR4 knockdown

The Mission shRNA lentiviral transduction particles were used to knock-down CXCR4 expression of EPCs. Viral transduction was performed per manufacturer's instruction (GeneChem company Ltd, Shanghai, China). The following oligomers were used: CXCR4-siRNA-1, 5'TGGAGG

GGATCAGTATATACA-3'; CXCR4-siRNA-2,5'-GTTTCACTCCAGCTAAC-ACA-3'. Briefly, after 7 days incubation, the lentiviral particles with siRNA targeting CXCR4 gene (CXCR4-siRNA) or nontargeting siRNA (Scrambled-siRNA) were added to the cells for 120 min in culture without serum. The efficiency transfection was almost 95%. RT-PCR and western blot were used to detect the effect of lentiviral transduction, almost 80% CXCR4 gene and protein could be silenced by CXCR4-siRNA transduction, and scrambled-siRNA transduction did not affect CXCR4 gene and protein in EPCs. After transduction, cells were washed with PBS and incubated with EPC medium for 48 h before subsequent experiments.

Animal model and reendothelialization assay

Male NRMInu/nu athymic nude mice (SLAC laboratory animal center, Shanghai, China), aged 8–10 weeks, were used to allow the injection of human EPCs. Animals were anesthetized with ketamine (100 mg kg⁻¹ IP) and xylazine (5 mg kg⁻¹ 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 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.

After 7 days culture, EPCs or shear stress-treated EPCs (15 dyn cm⁻² treatment for 12 h) (5×10^5 cells) were resuspended in 100 μ L of prewarmed PBS (37°C) and transplanted 3 h 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 50 μ L of solution containing 5% Evans blue dye via tail vein injection.

After 7 days culture, EPCs from two groups subjects ($n = 5$ per group) were labeled with CM-Dil (CellTracker™ CM-Dil; Invitrogen) and injected into the tail vein of nude mice with carotid injury to examine the homing to the uninjured and injured carotid artery. After 24 h, the animals were sacrificed, 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- μ m mesh and centrifuged at 290 g for 5 min. The cells were resuspended in 2 mL of FACS buffer and analyzed by the BD FACSCanto II system. A fluorescent microscope (Olympus BX51, Tokyo, Japan) was performed to detect homing of transplanted EPCs to the site of vascular injury in separate experiments with the use of CM-Dil-labeled EPCs.

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.

Endothelial function evaluation

Before and after exercise, FMD in the brachial artery was measured by high-resolution ultrasound (Acuson 128XP/10, Mountain View, CA, USA, with a 7.0 MHz linear-array transducer) to evaluate endothelium-dependent vascular response as previously described (Tao *et al.*, 2007).

Statistical methods

All results are expressed as mean value \pm SD. Statistical significance was evaluated by means of a Student's *t* test. Single linear regression was used to correlate the association between reendothelialization capacity, EPC protein expression, and FMD. A value of $P < 0.05$ was considered to denote statistical significance. For all statistical analyses, we used the SPSS software package (version 17; SPSS Inc., Chicago, IL, USA).

Acknowledgments

This work was funded by National Natural Scientific Foundation (u0732002, 30973535, 30770895 and 30800215) of China 973 program (2012CB517802) of China and the PhD Programs Foundation of Ministry of Education of China (20090171110061).

Author contributions

Jun Tao and Shen-Ming Wang involved in conception, design of data, drafting of the manuscript, and final approval of the manuscript submitted; Wen-Hao Xia, Jing Li and Chen Su contributed to conception, design of data, and drafting of the manuscript; Zhen Yang contributed to conception, design of data and analysis, and interpretation of data; Long Chen involved in analysis and interpretation of data; Fang Wu and Yuan-Yuan Zhang contributed to analysis and interpretation of data; Bing-Bo Yu involved in interpretation of data; Yan-Xia Qiu contributed to analysis of data.

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