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Smooth muscle progenitor cells from peripheral blood promote the neovascularization of endothelial colony-forming cells

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ABSTRACT

Proangiogenic cell therapy using autologous progenitors is a promising strategy for treating ischemic disease. Considering that neovascularization is a harmonized cellular process that involves both endothelial cells and vascular smooth muscle cells, peripheral blood-originating endothelial colony-forming cells (ECFCs) and smooth muscle progenitor cells (SMPCs), which are similar to mature endothelial cells and vascular smooth muscle cells, could be attractive cellular candidates to achieve therapeutic neovascularization. We successfully induced populations of two different vascular progenitor cells (ECFCs and SMPCs) from adult peripheral blood. Both progenitor cell types expressed endothelial-specific or smooth muscle-specific genes and markers, respectively. In a protein array focused on angiogenic cytokines, SMPCs demonstrated significantly higher expression of bFGF, EGF, TIMP2, ENA78, and TIMP1 compared to ECFCs. Conditioned medium from SMPCs and co-culture with SMPCs revealed that SMPCs promoted cell proliferation, migration, and the *in vitro* angiogenesis of ECFCs. Finally, co-transplantation of ECFCs and SMPCs induced robust *in vivo* neovascularization, as well as improved blood perfusion and tissue repair, in a mouse ischemic hindlimb model. Taken together, we have provided the first evidence of a cell therapy strategy for therapeutic neovascularization using two different types of autologous progenitors (ECFCs and SMPCs) derived from adult peripheral blood.

1. Introduction

Neovascularization is a process essential to improving tissue ischemia and restoring organ regeneration. During the last few decades, cell therapy using stem and progenitor cells has been extensively utilized to achieve therapeutic neovasculogenesis.

Among various stem and progenitor cells, endothelial colony-forming cells (ECFCs) deserve extra attention for their neovasculogenic potential [1,2]. ECFCs are also known as late-outgrowth endothelial cells, blood-derived outgrowth endothelial cells, or endothelial progenitor cells and are a blood-originating endothelial cell-like population that possesses similar gene expression and function to mature endothelial cells. ECFC implantation has been demonstrated to induce de novo neovessels *in vivo* and improve functional recovery in ischemic disease models [3,4]. Although ECFCs have been thought to have comparable neovasculogenic potential to mature endothelial cells, one report demonstrated that that ECFCs have inferior *in vivo* neovasculogenic potential compared to mature endothelial cells [5]. Therefore, many attempts to modulate ECFC activity have been also reported. Priming with growth factors or cytokines [6,7], gene transfer [8], and combined transplantation with other cell types [9,10] have all been proposed to promote the neovasculogenic potential of ECFCs *in vitro* and *in vivo*. In addition to ECFCs, smooth muscle progenitor cells (SMPCs) can also be induced from human blood [11]. SMPCs have been suggested to be associated with atherosclerosis [12]. However, little has been studied regarding other functions of SMPC in neovasculogenesis.

Considering that (1) vessels contain both endothelial cells and vascular smooth muscle cells, (2) neovasculogenesis is the harmonized cellular response of both cell types, both ECFCs and SMPCs might have roles during *in vivo* neovasculogenesis. Moreover, it is clinically feasible to induce both cell types from adult peripheral blood. In the present study, we characterized both ECFCs and SMPCs and tested the hypothesis that transplanted ECFCs and SMPCs synergistically contribute to new vessel formation and improve tissue regeneration after ischemic injury.

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2. Methods

2.1. Cell culture

The Institutional Review Board at the Korea University Anam Hospital approved the study protocols. Adult peripheral blood samples were obtained from volunteers after informed consent was obtained. ECFCs and SMPCs were induced as described previously [11,13]. Briefly, mononuclear cells (MNCs) were separated from blood using Ficoll-Paque (GE-Healthcare). Then, 8.0×10^6 MNCs were plated on collagen-coated 12-well plates (Stem Cell Technologies) and cultured in EGM-2MV (Lonza). For SMPC induction, we supplemented PDGF-BB (50 ng/mL) into EGM-2MV. Purities of ECFCs and SMPCs were defined by cell morphology, immunofluorescent staining, and flow cytometric analysis. Mixtures of ECFCs and SMPCs were not used for the experiments. Normal karyotyping of the adult peripheral blood-derived ECFCs and SMPCs was confirmed (data not shown).

2.2. Fibrinogen bead assay

Cytodex3™ microcarrier beads (GE Healthcare) were hydrated according to the manufacturer's protocol. Briefly, microbeads were swollen for 3 h in PBS, washed, resuspended in PBS, and then autoclaved at 121 °C for 15 min. ECFCs (1.0×10^6) and microbeads (2.5×10^3) were suspended in EGB2-MV medium and incubated for 4 h with gentle agitation at 15-min intervals. The cell-covered microbeads were mixed in fibrinogen (2 mg/mL, Sigma) and the fibrinogen was then polymerized with thrombin (0.625 U/mL, Sigma). Culture medium was added into the polymerized fibrinogen. Sprouting angiogenesis was analyzed 4 days following the addition of the culture medium.

2.3. In vivo Matrigel plug assay

Cells were mixed with 100 μL Matrigel, and the mixture was implanted subcutaneously into the dorsal side of eight-week-old nude/SCID mice. After 2 weeks, the mice were sacrificed under anesthesia (intramuscular injection of 80 mg/kg ketamine and 12 mg/kg xylazine), and the implanted Matrigels were harvested for histologic analyses.

2.4. Ischemic hindlimb model

All animal care and experimental procedures were performed in accordance with the Guidelines for Animal Care and Use of the Korea University School of Medicine. All protocols were approved by the Administrative Panel on Laboratory Animal Care at the Korea University School of Medicine. Eight-week-old nude/SCID mice underwent surgical ligation of the proximal part of the left femoral artery under anesthesia. Cells were then injected intramuscularly into three sites of the hindlimb adductor muscle. Laser Doppler perfusion images (Moor Instruments, Devon, United Kingdom) were assessed at days 3, 7, 14, 21, and 28. The hindlimb adductor muscle and contralateral adductor muscle were harvested for histologic analyses at day 28.

2.5. Immunofluorescence staining, flow cytometric analysis, semi-quantitative RT-PCR, Matrigel tube-forming assay, Angiogenic cytokine array, cell viability assay, in vitro scratch wound healing assay, adipogenic and osteogenic differentiation

These procedures were performed as described in detail in the Supplementary methods.

2.6. Statistics

Values are presented as means ± standard deviation (SD). Significant differences between the means were determined by analysis of variance followed by the Student–Newman–Keuls test. Significance was set at $p < 0.05$.

3. Results

3.1. Characterization of ECFCs and SMPCs derived from adult peripheral blood

Based on previous literatures [11,13], we successfully induced both ECFC and SMPC derivation from adult peripheral blood (Fig. 1A). ECFCs had a typical cobblestone appearance, similar to mature endothelial cells, and SMPCs were spindle-shaped cells with a pronounced hill and valley pattern when they were confluent (Fig. 1B).

Immunofluorescent staining demonstrated that ECFCs expressed typical endothelial junctional markers, CD31 and CD144, and did not express smooth muscle markers such as vimentin, desmin and αSMA (Fig. 1C). On the contrary, SMPCs did not express CD31 and VE-cadherin, and expressed vimentin, desmin and αSMA.

Flow cytometric analyses also demonstrated that both ECFCs and SMPCs did not express hematopoietic (stem) cell markers (Lineage, CD45, CD117, CD133) (Fig. 1D). Endothelial cell markers (CD144, CD31, Tie2, KDR) were expressed in ECFCs but not in SMPCs. Both ECFCs and SMPCs expressed CD105, which is reported to be expressed in SMPCs as well as in ECFCs [14]. RT-PCR also confirmed the higher expression of endothelial cell-related genes (CD144, vWF, Angiopoietin-2 [Ang2]) in ECFCs and the higher expression of smooth muscle cell-related genes (αSMA, SM22α, Calponin) in SMPCs (Fig. 1E). Moreover, the ECFCs showed typical endothelial cell function, such as Matrigel-induced tubular network formation (Fig. 1F).

3.2. Differential angiogenic cytokine expression in ECFC and SMPC

To compare the expression of angiogenic cytokines and growth factors between ECFCs and SMPCs, a total of 43 angiogenesis-related growth factors and cytokines were analyzed using the angiogenic cytokine array. Angiogenic cytokine array analysis showed that ECFCs and SMPCs have different angiogenic cytokine expression profiles (Fig. 1G). ECFCs expressed EGF, bFGF, GRO, IL-8, MCP1, TIMP1, TIMP2, Ang2, Endostatin, IL-4, MMP1, MMP9, PECAM1, Tie2, μPAR, and VEGFR2. SMPCs expressed EGF, ENA78, bFGF, GRO, IL-8, TIMP1, TIMP2, and μPAR. When the relative expressions were compared between ECFCs and SMPCs, bFGF (2.6-fold), EGF (17-fold), TIMP2 (2.5-fold), ENA78 (not detected in ECFC), and TIMP1 (5.6-fold) revealed higher expression levels in SMPCs, while GRO (2.0-fold), Ang2 (not detected in SMPC), μPAR (2.5-fold), MCP1 (16-fold), and PECAM1 (not detected in SMPC) revealed higher expression levels in ECFCs (Fig. 1H).

3.3. SMPCs enhanced ECFC proliferation, migration, and angiogenic sprouting in vitro

Considering that ECFCs and SMPCs have different angiogenic cytokine expression patterns, we hypothesized that SMPCs could promote the cell proliferation, migration, and angiogenic potential of ECFCs through their complementary angiogenic cytokine expression. We compared the effect of SMPC-conditioned medium (CM) on ECFC proliferation and migration to those of control medium (Control, EGM-2MV) and ECFC-CM. SMPC-CM increased

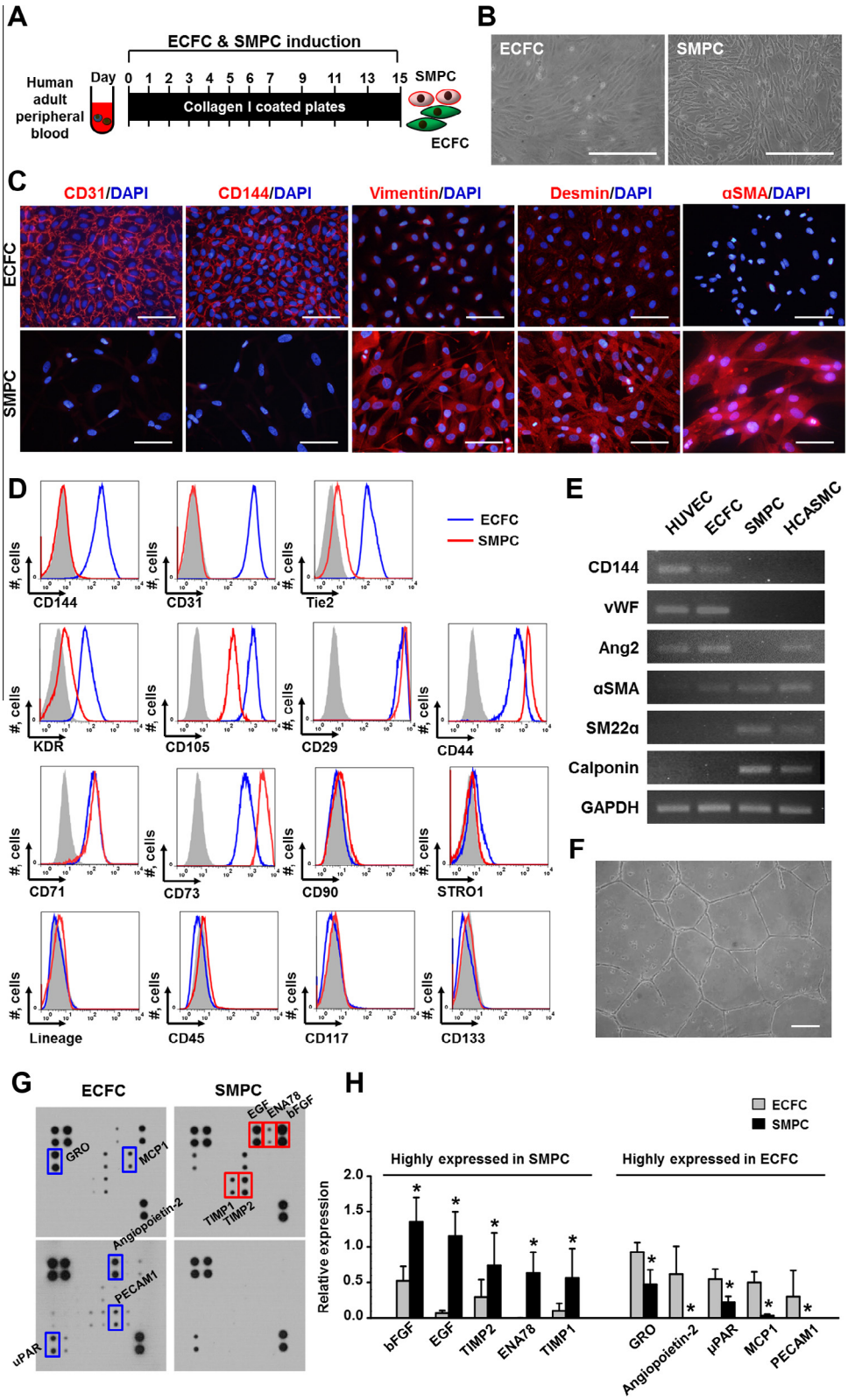

Fig. 1. Characterization of ECFCs and SMPCs derived from adult peripheral blood. (A) ECFC and SMPC induction scheme from adult peripheral blood. (B) Phase contrast images of ECFCs and SMPCs. Scale bars represent 500 μm. (C) Immunofluorescence images showing CD31, CD144, Vimentin, Desmin, and αSMA expression in ECFCs and SMPCs. Nuclei were stained with DAPI. Scale bars represent 100 μm. (D) Representative FACS analyses of CD144, CD31, Tie2, KDR, CD105, CD29, CD44, CD71, CD733, CD90, STRO1, Lineage, CD45, CD117, and CD133 in ECFCs and SMPCs. (E) RT-PCR analysis of CD144, vWF, Ang2, αSMA, SM22α, and Calponin expression in human umbilical vein endothelial cells (HUVECs), ECFCs, SMPCs, and human coronary artery smooth muscle cells (CASMCs). (F) Tubular network formation of ECFCs in Matrigel. Scale bars represent 500 μm. (G) Representative angiogenic cytokine array panels of ECFC and SMPC lysates. Among 43 growth factors and cytokines, GRO, MCP1, Ang2, PECAM1, and μPAR (blue boxes) were expressed higher in ECFCs. EGF, ENA78, bFGF, TIMP1, and TIMP2 (red boxes) were expressed higher in SMPCs. (H) Comparison of bFGF, EGF, TIMP2, ENA78, TIMP1, GRO, Ang2, μPAR, MCP1, and PECAM1 expression between ECFCs and SMPCs. Relative angiogenic cytokine expression was measured by densitometry. Each group, $n = 3$. $^{*}p < 0.05$ versus ECFCs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ECFC proliferation compared to Control (1.5-fold) and ECFC-CM (1.4-fold) (Fig. 2A). An *in vitro* wound healing assay also showed that SMPC-CM promoted ECFC migration toward the wound region compared to Control (2.0-fold) and ECFC-CM (1.6-fold) after 24 h (Fig. 2B and C). Next, we performed an *in vitro* angiogenic sprouting assay using microbeads. ECFC-coated microbeads were embedded in fibrinogen and cultured with Control, ECFC-CM, and SMPC-CM. SMPC-CM significantly increased ECFC sprouting from microbeads compared to Control (15-fold) and ECFC-CM (2.3-fold) (Fig. 2D and F). When SMPCs or ECFCs were co-cultured onto the top of the fibrinogen containing ECFC-coated microbeads, co-culture with SMPCs also significantly increased ECFC sprouting compared to Control (3.7-fold) and ECFC co-culture (1.3-fold) (Fig. 2E and G).

3.4. The combination of ECFCs and SMPCs induces robust in vivo neovascularization

To explore the *in vivo* angiogenic potential of ECFCs augmented by SMPCs, we performed the Matrigel plug assay. Matrigels mixed with ECFCs or SMPCs or ECFCs and SMPCs were implanted subcutaneously into nude-SCID mice (Fig. 3A). The implanted Matrigel plugs were harvested after 2 weeks. The implanted Matrigels mixed with ECFCs or SMPCs were transparent. However, those mixed with ECFCs plus SMPCs were reddish, suggesting robust induction of neovascularization (Fig. 3B). Indeed, H&E staining showed blood vessels were rarely observed in the implanted Matrigel mixed with ECFCs or SMPCs, but numerous neovessels containing red blood cells were noted in the implanted Matrigel mixed with ECFCs plus SMPCs (Fig. 3C). Immunofluorescent staining also demonstrated αSMA+ mature vessels only in the implanted Matrigel mixed with ECFC plus SMPCs (Fig. 3D). These data suggested that transplantation of combined ECFCs and SMPCs could successfully induce *in vivo* neovascularization.

3.5. Combined transplantation of ECFCs and SMPCs improves angiogenic repair and regeneration in hindlimb ischemia

Finally, we tested whether the combined transplantation of ECFCs and SMPCs could be effective for angiogenic repair and regeneration of the injured skeletal muscle in an ischemic hindlimb model. We intramuscularly injected PBS (Control), ECFCs (5×10^5 cells/limb), SMPCs (5×10^5 cells/limb), or ECFCs plus SMPCs (ECFC 5×10^5 cells/limb + SMPC 5×10^5 cells/limb) after inducing hindlimb ischemia in nude/SCID mice. Laser Doppler perfusion imaging revealed that only co-transplantation of ECFCs and SMPCs significantly enhanced the blood perfusion of the ischemic hindlimb compared to the other groups (0.28 ± 0.02% in the Control group, 0.24 ± 0.02% in the ECFC group, 0.23 ± 0.05% in the SMPC group, and 0.38 ± 0.09% in the ECFC plus SMPC group, $p < 0.05$) (Fig. 4A and B). Immunofluorescent staining also revealed that ECFC plus SMPC injection increased the capillary density compared to other groups (8.7 ± 6.6% in the Control group, 16.2 ± 7.0% in the ECFC group, 16.8 ± 7.3% in the SMPC group, and 29.7 ± 3.4% in the ECFC plus SMPC group, $p < 0.05$) (Fig. 4C and D). In addition, histological analysis showed an obvious decrease in muscle degeneration and fibrosis in the ECFC plus SMPC group (Fig. 4E and F). These findings implied that co-transplantation of ECFCs and SMPCs could facilitate *in vivo* neovascularization and blood perfusion and attenuate muscle degeneration in an ischemic hindlimb.

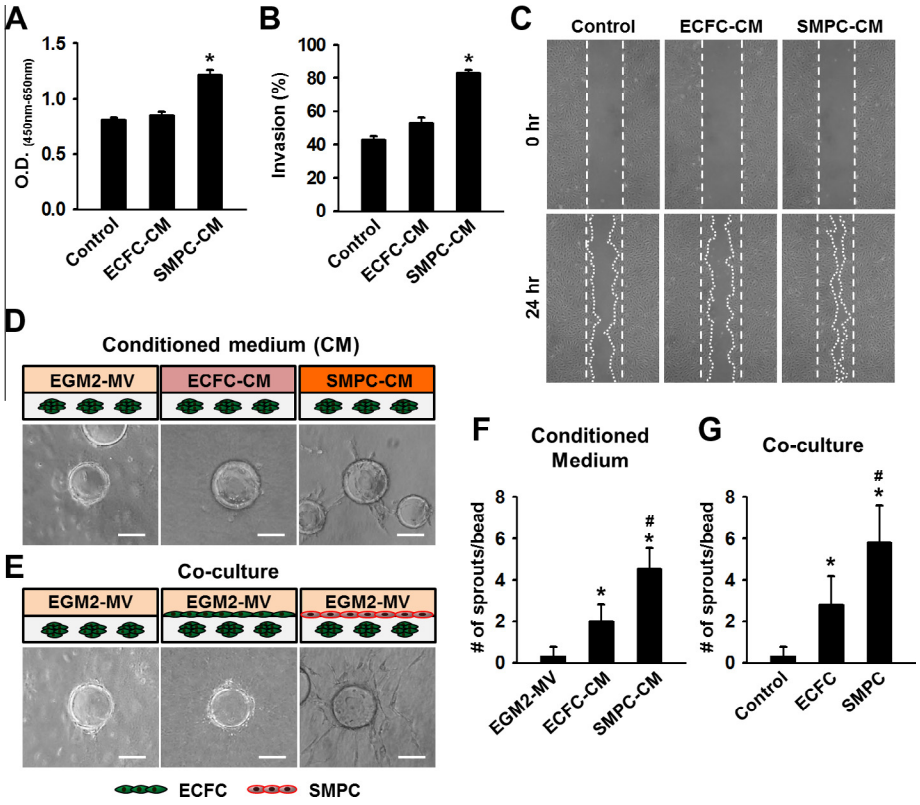

Fig. 2. SMPCs promote the cell viability, migration, and angiogenic potential of ECFCs. (A) Relative cell viability. ECFCs were cultured for 2 days with unconditioned OptiMEM (Control), OptiMEM conditioned by ECFCs (ECFC-CM), or OptiMEM conditioned by SMPCs (SMPC-CM). Each group, $n = 3$. $^*p < 0.05$ versus Control. (B and C) *In vitro* wound healing assays. Confluent ECFCs were cultured with Control, ECFC-CM, or SMPC-CM after scratch wounding. (B) Percentage of invasion. Area of wound at 0 h is regarded as 100%. Each group, $n = 3$. $^*p < 0.05$ (C) Representative phase contrast images showing invasion of ECFCs into wound regions at 24 h. Scale bars represent 100 μm. (D–G) Fibrinogen microbead assay. ECFCs attached to microbeads in fibrinogen were cultured with Control, ECFC-CM, or SMPC-CM (D and F) or co-cultured with ECFCs or SMPCs (E and G). (D and E) Representative phase contrast images showing ECFCs sprouting from microbeads. Scale bars represent 100 μm. (F and G) Number of sprouts per bead. Each group, $n = 3$. $^*p < 0.05$.

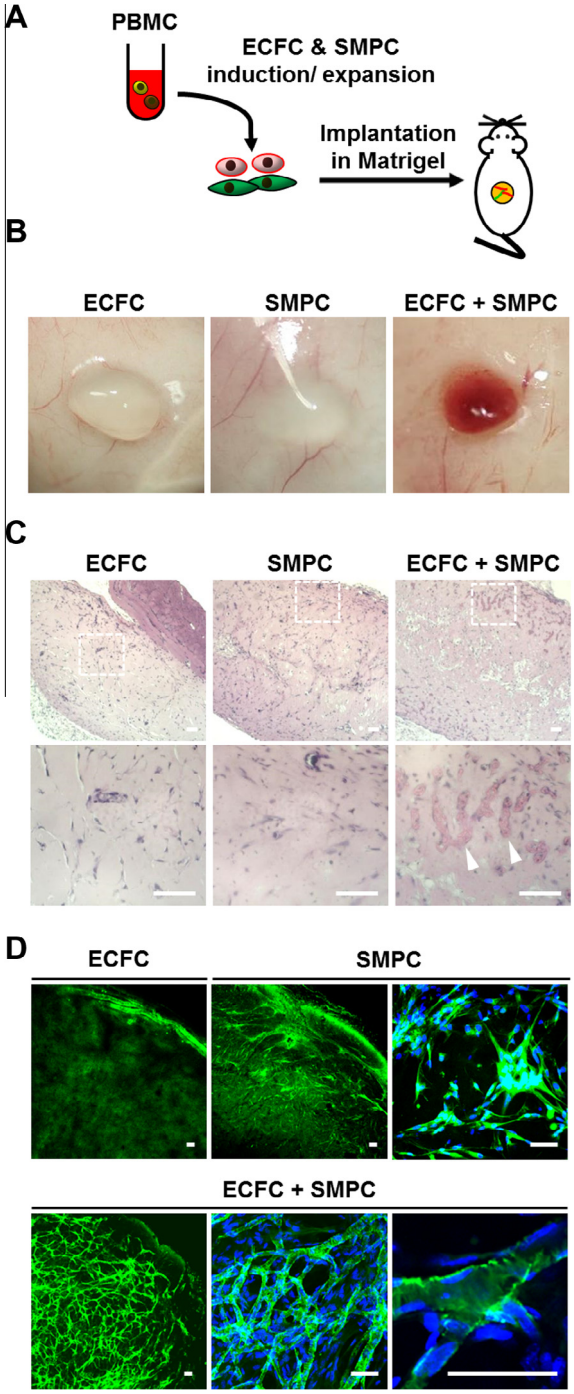

Fig. 3. Co-transplantation of ECFCs and SMPCs induced robust angiogenesis *in vivo*. (A) Scheme of the *in vivo* Matrigel plug assay. ECFCs and SMPCs were induced and expanded from adult peripheral blood. ECFCs or SMPCs mixed in Matrigel were implanted subcutaneously into the dorsal flank of nude/SCID mice. The implanted Matrigel was harvested 2 weeks later. (B) Gross appearance of the implanted Matrigels. (C) Matrigel sections stained with H&E. Arrowheads indicate blood-containing vessels. (D) Whole-mount immunofluorescence images stained for αSMA+(green) neovessels in the implanted Matrigel. Nuclei were stained with DAPI. Scale bars represent 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The novelties of this present study were that (1) both ECFCs and SMPCs were induced from adult human peripheral blood, and (2) co-transplantation of ECFCs and SMPCs synergistically achieved therapeutic neovasculogenic potential in an ischemic animal model.

The neovasculogenic process in ischemic injury involves harmonized cellular interplay among various cell types, including endothelial cells, vascular smooth muscle cells, hematopoietic cells, and other surrounding mesenchymal cells [15]. Endothelial cells and vascular smooth muscle cells are major cell types composing vascular structure. Recent studies have demonstrated the therapeutic potential of cell therapy using both endothelial cells and vascular smooth muscle cells in diseased animal models [16–18]. However, it is not feasible to obtain clinically sufficient amounts of both endothelial cells and vascular smooth muscle cells from patients. ECFCs, a subtype of EPCs derived from human blood, have been known to have cellular characteristics similar to mature endothelial cells *in vivo* [3,4]. SMPCs induced from human blood have been shown to have phenotypic and functional characteristics similar to vascular smooth muscle cells [11,14]. Foubert et al. demonstrated that human umbilical cord blood-originating EPCs and SMPCs synergistically promoted neovascularization in a mouse ischemic hindlimb model [19]. Therefore, cell therapy using both autologous ECFCs and SMPCs, induced from adult peripheral blood, could be a promising strategy to achieve therapeutic neovascularization.

In this study, ECFCs and SMPCs induced from adult peripheral blood were characterized according to their morphologic and immunophenotypic differences. ECFCs or SMPCs could be selectively induced by separate small scale cell culture, although ECFCs and SMPCs were induced simultaneously in many cases. We used highly homogenous cell populations of ECFCs or SMPCs in the present study by confirming their marker expression through immunofluorescence staining and flow cytometry. Both ECFCs and SMPCs in this present study had typical cell morphologies and surface marker expressions to the previous literatures [3,14,20]. However, mesenchymal stem cells induced from adult peripheral blood also have spindle-shaped morphology similar to smooth muscle cells and the potential to differentiate into other mesenchymal lineages, including adipocyte and chondrocyte, although these lineages rarely exist in peripheral blood [21]. SMPCs in the present study did not express mesenchymal stem cell markers, such as CD90 and STRO1, and failed to induce adipogenic and osteogenic differentiation, suggesting that SMPCs could be different from mesenchymal stem cells derived from blood.

Growth factors and cytokines are involved in the intercellular communication between endothelial cells and vascular smooth muscle cells [22]. PDGF-B and TGF-β1 recruit vascular smooth muscle cells. Ang1 has roles in vascular maturation and angiogenesis. Foubert et al. demonstrated that umbilical cord blood-derived EPCs and SMPCs augmented neovascularization through the Ang1/Tie2 signaling pathway [19]. However, Ang1 was hardly expressed in either ECFCs or SMPCs in the present study. Instead, bFGF, EGF, ENA78, TIMP1, and TIMP2 were highly expressed in SMPCs. bFGF was reported to promote EPC proliferation and differentiation through FGFR1 [23]. EGF was reported to be an essential growth factor for EPCs [24]. ENA78 was reported to be an angiogenic factor [25]. TIMP1 was reported to potentiate blood vessel formation [26]. On the other hand, TIMP2 was reported to suppress angiogenesis [27,28]. This suggests that adult peripheral blood-derived ECFCs and SMPCs might have different neovasculogenic mechanisms from umbilical cord blood-derived ECFCs and SMPCs. Therefore, in addition to Ang1, other growth factors and cytokines expressed highly in SMPCs might have roles in proliferation and migration, as well as angiogenesis, of ECFCs.

To validate the angiogenic effects of SMPCs, we performed various functional assays *in vitro* and *in vivo*. Conditioned medium from SMPCs effectively promoted the proliferation, migration, and *in vitro* angiogenesis of ECFCs. Co-culture of ECFCs and SMPCs in a

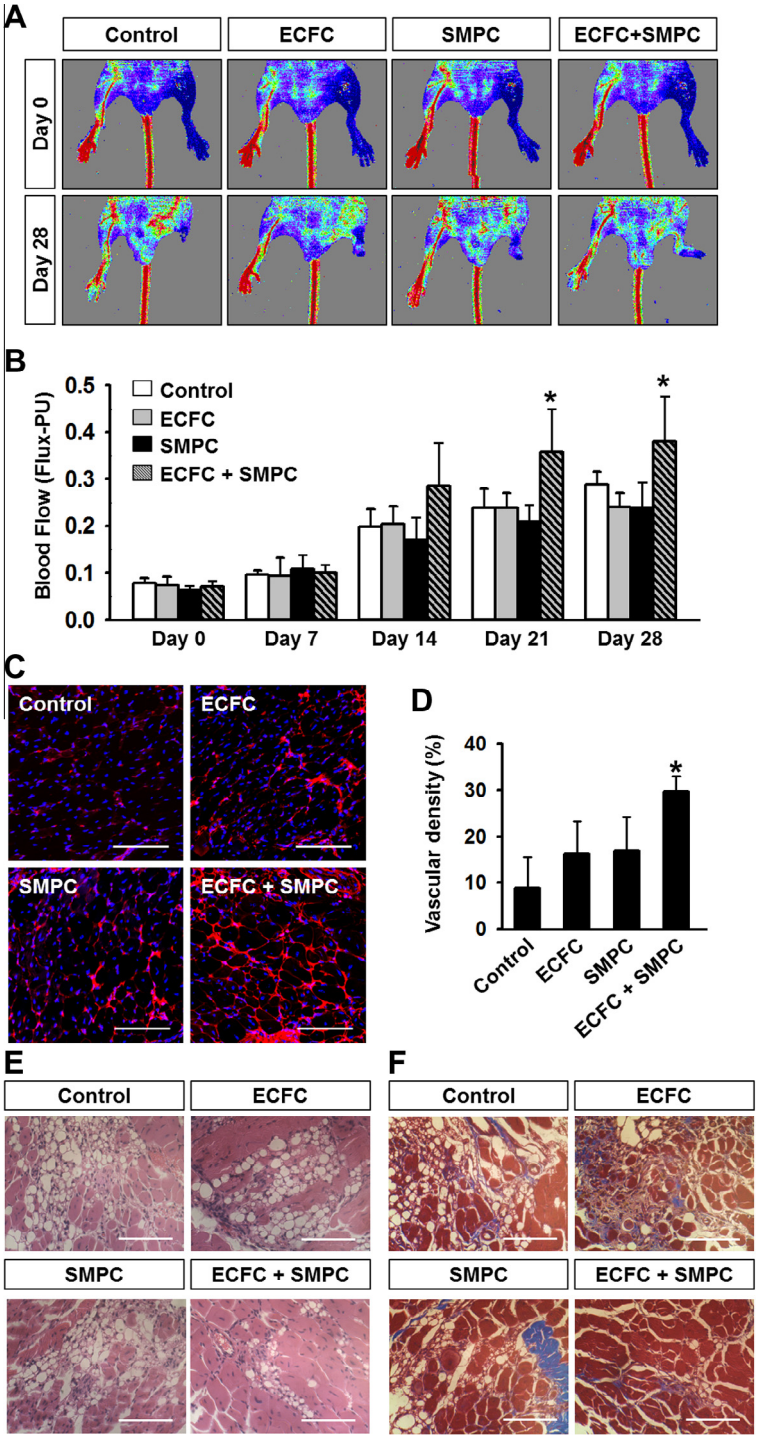

Fig. 4. Co-transplantation of ECFCs and SMPCs improves blood perfusion and attenuates muscle degeneration in an ischemic hindlimb model. PBS (Control), ECFCs, SMPCs, or ECFCs plus SMPCs were injected intramuscularly into the adductor muscle after ischemic hindlimb induction. (A) Representative laser Doppler perfusion images at days 3 and 28 after operation. (B) Recovery of blood perfusion ratio. Each group, $n = 5$ or 6, $p < 0.05$ versus Control. (C) Representative immunofluorescence images showing CD31+ blood vessels of the ischemic adductor muscles. (D) Density of CD31+ blood vessels of the ischemic adductor muscles. Each group, $n = 5$. $^{*}p < 0.05$ versus Control. (E and F) Representative H&E (E) and Masson's trichrome (F) staining images of the ischemic adductor muscles. Scale bars represent 100 μm.

microbead assay demonstrated that SMPCs promoted the *in vitro* vascular sprouting angiogenesis of ECFCs. However, Matrigel plug assays demonstrated that implantation of ECFCs or SMPCs alone failed to induce neovascularization *in vivo*. It suggested that adult peripheral blood-derived ECFCs alone might be insufficient to induce neovessels. Adult peripheral blood-derived EPCs demonstrated inferior *in vivo* neovasculogenic potential compared to mature endothelial cells [5] or umbilical cord blood-derived ECFCs [29]. However, co-transplantation of adult peripheral blood-derived ECFCs and SMPCs successfully achieved robust neovascularization in the present study. Previous investigators have also suggested that co-transplantation with stromal cells should be required for ECFCs to induce neovessels [17,30].

Although Foubert et al. already demonstrated successful therapeutic neovascularization using co-transplantation of human umbilical cord blood-originated EPCs and SMPCs in a mouse

ischemic hindlimb model, it is essential to investigate the therapeutic potential of adult peripheral blood-derived ECFC and SMPC transplantation for autologous cell therapy. In the present study, co-transplantation of adult peripheral blood-derived ECFCs and SMPCs significantly improved blood perfusion and reduced muscle degeneration in a mouse ischemic hindlimb model.

Nevertheless, there are several limitations in this study. The detailed *in vitro* and *in vivo* mechanisms for the promotion of neovascularization by adult peripheral blood-derived ECFCs and SMPCs still must be clarified. Moreover, for clinical applications, the therapeutic potential of ECFCs and SMPCs derived from patients with cardiovascular risk factors should be validated, considering the impaired function of progenitors in such patients [31].

In conclusion, we successfully induced two different types of vascular progenitor cells, ECFCs and SMPCs, from adult peripheral blood. SMPCs promoted the angiogenic potential of ECFCs. Finally, co-transplantation of adult human peripheral blood-derived ECFCs and SMPCs exhibited significant effectiveness in facilitating blood perfusion and tissue repair in an ischemic hindlimb model. This study provides the first experiments using adult peripheral blood-derived ECFCs and SMPCs together to achieve therapeutic neovascularization. This might contribute to the development of a clinically effective autologous cell therapy strategy in the future.

Conflicts of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.061.

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