CD34 Hybrid Cells Promote Endothelial Colony-Forming Cell Bioactivity and Therapeutic Potential for Ischemic Diseases

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Objective—Although endothelial progenitor cells (EPCs) have been reported to promote neovessel formation during vascular injury, the function of supporting cells of EPCs and their interaction with EPCs during EPC isolation remain unclear.

Approach and Results—We investigated the functional properties of 2 types of EPCs, also known as endothelial colony-forming cells (ECFCs), CD34-CD34+ cell–derived ECFCs (hybrid-dECFCs) and CD34+ cell–derived ECFCs (stem-dECFCs), isolated using different methods, to elucidate the role of CD34- cell populations as cell-supporting niches. Using EPC colony-forming and insert coculture assays, we found that CD34- accessory cells dynamically modulate hematopoietic stem cell–derived endothelial progenitor commitment via angiogenic cytokines secreted by CD34+/CD11b+ macrophages. On the basis of these findings, we isolated 2 types of ECFCs and investigated their bioactivities. We found that stem-dECFCs showed remarkably retarded cell growth, enhanced senescence, and decreased characteristics of ECFCs, whereas hybrid-dECFCs showed greater proliferative properties but delayed senescence. In a murine hind-limb ischemia model, hybrid-dECFCs showed significantly enhanced blood perfusion, capillary density, transplanted cell survival and proliferation, and angiogenic cytokine secretion compared with stem-dECFCs. In particular, the migratory capacity of hybrid-dECFCs was significantly enhanced, in part mediated via an augmented phosphorylation cascade of focal adhesion kinase and Src, resulting in a highly increased incorporation capacity of hybrid-dECFCs compared with stem-dECFCs. CD34- accessory cells of hybrid-dECFCs might be niche-supporting cells that facilitate cell survival, increase the secretion of angiogenic cytokines, and increase incorporation.

Conclusions—This study provided important insight into blood vessel formation and repair in ischemic diseases for ECFC-based cell therapy. (Arterioscler Thromb Vasc Biol. 2013;33:1622-1634.)

Key Words: CD34 ■ endothelial progenitor cells ■ hind-limb ischemia ■ niche-supporting cells ■ vascular repair ■ vasculogenesis

Stem cells provide fresh cells to replenish blood, bone, epithelial, nervous system, muscle, and various tissues. Stem cells are regulated and maintained by local tissue microenvironments or niches. Since the coining of the concept of the stem cell niche, it has been possible to identify stem cells and niches with increasing precision. However, the importance of the stem cell niche has not been completely recognized because models of stem cell behavior are not well understood. Thus, it is challenging to predict how self-renewal is influenced by external factors. Asahara et al revealed that CD34+ hematopoietic stem cells (HSCs) from peripheral blood mononuclear cells can differentiate into endothelial lineage cells, and many researchers have shown that these cells, endothelial progenitor cells (EPCs), play a pivotal role in neovascularization; however, the identity of the EPCs is not clear. Differences in the true nature of EPCs and the mechanism of EPC function may be caused by differences in isolation methods. Although studies on EPCs still require optimal isolation and culture conditions, as well as specific phenotypes and characteristics for EPC identification, multiple cultured or unselected subpopulations have been shown to improve neovascularization in animal models, indicating that unselected EPCs are excellent candidates for developing therapeutic approaches for vascular diseases.

CD34 is a 105- to 120-kDa cell-surface glycoprotein that functions as a cell-to-cell adhesion factor selectively expressed on stem, progenitor, and vascular endothelial cells (ECs) within hematopoietic systems. Generally, EPCs, also known as endothelial colony-forming cells (ECFCs), can be obtained by isolating CD34-expressing cells from mononuclear cells (MNCs) or by long-term culture of MNCs in EC-selective media. Both human CD34+ stem cell–derived...
ECFCs (referred to as stem-dECFCs in this study) and MNC-derived ECFCs (referred to as hybrid-dECFCs in this study) contribute to vascular growth and have been assessed for the treatment of ischemic diseases in early phase clinical studies. However, few studies have investigated how isolated human ECFCs affect postnatal vasculogenesis using stem-dECFCs or hybrid-dECFCs. In particular, it is still unknown how human CD34+ cells influence EPC behavior. In view of the recent report that HSCs support the differentiation and proliferation of ECs and EPCs during embryonic vascular development and angiogenesis, we hypothesized that human CD34+ cells function as niche-supporting cells that control the features of human ECFCs from human umbilical cord blood (HUCB) during ECFC culture in EC-selective media and that they might be linked to distinct characterization for neovascularization, according to types of human ECFCs.

In the present study, we addressed whether human CD34+ accessory cells (niche-supporting cells) can facilitate the cell survival, incorporation capacity, and endothelial differentiation of ECFCs. The results provide important insights in blood vessel formation and repair in ischemic diseases for ECFC-based cell therapy.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Influence of CD34+ Accessory Cells on the Endothelial Lineage Commitment of CD34+ Cells

To investigate the role of accessory cells on the endothelial lineage commitment of CD34 stem cells, we first performed an EPC colony-forming assay using human CD34+, CD34−, or hybrid CD34+/CD34− cells, developed in our laboratory as described previously. The mixture ratio of hybrid CD34+ and CD34− cells was 1.7% CD34+ and 98.3% CD34− cells, which are approximately the ratio of CD34+ and CD34− cells in MNCs isolated from HUCB. CD34+ cells and hybrid cells were differentiated into 2 EPC colony shapes, small EPC-CFUs and large EPC-CFUs. Small EPC-CFUs were composed of round adhesive cells, and large EPC-CFUs were composed of spindle-shaped cells. In contrast, CD34− cells could not be differentiated into these colony shapes (Figure 1A and 1B). Interestingly, coculture of CD34+ cells and CD34− accessory cells resulted in significantly higher EPC colony-forming capacity of both small EPC-CFUs and large EPC-CFUs (Figure 1C). To determine whether the EC-lineage commitment of CD34+ cells is regulated by paracrine factors secreted by the accessory cells, we performed coculture analysis as follows: CD34+ cells were seeded in the lower compartment of transwells and CD34− cells or isolated CD11b+ macrophages from HUCB were either seeded or not seeded onto the transwell membrane insert for 72 hours (Figure 1D). Interestingly, CD34+ cells indirectly cocultured with CD34+ cells, or isolated CD11b+ macrophage cells showed significantly enhanced EPC-CFUs as compared with CD34+ cells cultured without CD34− cells or macrophage (CD11b+ or CD11b−) on the transwell membrane (Figure 1E). To determine the paracrine factors secreted by isolated CD11b+ macrophage cells, we examined the expression and secretion of stromal cell-derived factor (SDF)-1α and vascular endothelial growth factor (VEGF) as pivotal regulators of EPC commitment by Western blotting and ELISA. As expected, macrophages markedly expressed and secreted SDF-1α and VEGF (Figure 1F and Figure I in the online-only Data Supplement). EPC-CFA supplemented with either SDF-1α or VEGF showed that either SDF-1α or VEGF accelerated the sprouting of large EPC-CFUs, suggesting that 2 pivotal EPC committing factors are secreted by activated macrophages and modulate the differentiation of mature EPCs (Figure 1G and Figure II in the online-only Data Supplement). In addition, fluorescence-activated cell sorter analysis clearly demonstrated that CD34+ cells cocultured with CD34− cells or macrophages expressed more endothelial lineage markers, such as kinase insert domain receptor, C-X-C chemokine receptor 4, and Tie2, than did CD34+ cells cultured without CD34− cells (Figure 1H and Figure III in the online-only Data Supplement).

Characterization of Hybrid-dECFCs and Stem-dECFCs From Human Umbilical Cord Blood

As shown above, CD34+ cells cocultured with CD34− accessory cells showed augmented EPC colony-forming capacity and endothelial lineage marker expression than did CD34+ cells cultured without CD34− accessory cells. On the basis of these results, we established a long-term culture of hybrid cells (composed of human CD34+ and CD34− cells) and human CD34− cells to isolate late EPCs, also known as ECFCs. We then characterized hybrid-dECFCs (hybrids between human CD34+ and CD34−-derived ECFCs) and CD34+ stem-dECFCs (human CD34− cell population-derived ECFCs) to assess the function of these cells. To characterize both types of ECFCs, we performed morphological analysis, immunocytochemistry, and fluorescence-activated cell sorter analysis of endothelial-specific markers. As shown in Figure 2A, both types of ECFCs were elongated and had a spindle shape similar to outgrowth ECs, a type of late EPC reported by Ingram et al. Next, we performed immunocytochemistry on 2 types of ECFCs to confirm whether they express endothelial lineage markers, such as CD31, KDR (VEGFFR2), and von Willebrand factor, as well as pivotal functional markers, including endothelial nitric oxide synthase, p-endothelial nitric oxide synthase, and phosphor-protein kinase B. Immunocytochemistry showed that both types of ECFCs expressed endothelial lineage surface markers and pivotal markers of functional ECFCs (Figure 2B). Finally, we analyzed whether the 2 types of ECFCs express several ECFC surface markers, such as CD34, kinase insert domain receptor, C-X-C chemokine receptor 4, c-Kit, and CD144, and hematopoietic lineage markers, including CD11b, CD14, or CD45, by fluorescence-activated cell sorter analysis. These results showed that both types of ECFCs strongly expressed ECFC surface markers (Figure 2C) but did not express hematopoietic lineage markers (Figure IV in the online-only Data Supplement).

Assessment of Proliferation and Senescence of Hybrid-dECFCs and Stem-dECFCs

As shown in Figure 2A through 2C, we confirmed that both hybrid-dECFCs and stem-dECFCs from HUCB...
can differentiate into ECFCs by characterization of the morphology and specific markers of the ECFCs. We then compared the proliferation capacity and senescence of hybrid-dECFCs and stem-dECFCs. First, to assess the proliferation capacity of the 2 types of ECFCs, we compared the results of Western blotting to detect cell-cycle signaling molecules, including cyclin-dependent kinase-2, cyclin E, cyclin-dependent kinase-4, and cyclin D1, with those of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. The expressions of cyclin-dependent kinase-2 and cyclin E in hybrid-dECFCs decreased after passage 20, whereas the expression in stem-dECFCs gradually decreased after passage 14 (Figure 2D). We attempted unsuccessfully to expand stem-dECFCs of passage 16 to the next passage. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay and bromodeoxyuridine...
(5-bromo-2′-deoxyuridine) cell proliferation assay were then used to assess the proliferation capacity of hybrid-dECFCs and stem-dECFCs. The proliferation capacity was significantly lower in stem-dECFCs compared with hybrid-dECFCs (Figure 2E and Figure V in the online-only Data Supplement). These results showed that hybrid-dECFCs have higher proliferation capacity for a longer time during gradual cell passages compared with stem-dECFCs. To evaluate the senescence of the 2 types of ECFCs, we examined the senescence-associated β-galactosidase (SA-β-gal) activity of the 2 types of ECFCs. Stem-dECFCs were more positive than hybrid-dECFCs for SA-β-gal staining when comparing different culture passages (Figure 2F). Moreover,
we investigated the expression levels of the senescence markers p21 and senescence marker protein-30 by Western blotting. p21 is a potent and tight-binding inhibitor of cyclin-dependent kinases, which cooperate with cyclins to phosphorylate key substrates involved in cell-cycle progression. Senescence marker protein-30 expression levels decrease with aging and senescence. In passage 14, p21 expression was highly elevated, and senescence marker protein-30 expression gradually decreased in stem-dECFCs compared with hybrid-dECFCs (Figure 2G). These results demonstrate that stem-dECFCs progressed to senescence faster than hybrid-dECFCs.

Evaluation of the Functional Recovery of the 2 Types of ECFCs After Murine Hind-Limb Ischemia

A murine hind-limb ischemia model was used to assess the postnatal neovascularization of the 2 types of ECFCs. After operative excision of the left femoral artery and its boundary vessels, hybrid-dECFCs, stem-dECFCs (passage 6), or PBS (sham) were locally injected into the ischemic thigh muscle. The blood flow to the limb injected with hybrid-dECFCs or stem-dECFCs was significantly augmented at postoperative days 7, 10, 14, 21, and 28, compared with that in the PBS-injected sham group. When comparing the blood flow between the 2 types of ECFCs using laser-doppler perfusion imaging (Figure 3A), the blood flow of hybrid-dECFCs was significantly increased compared with stem-dECFCs at postoperative days 7, 10, and 14. At postoperative days 21 and 28, however, the recovery of blood flow was improved in the hybrid-dECFC group compared with that in the stem-dECFC group but not statistically significant (Figure 3B). The capillary density of the ischemic tissue was examined by immunohistochemistry analysis of the ischemic tissues with CD31 and α-SMA at postoperative day 28 (Figure 3C through 3F). Immunohistochemistry of CD31 and α-SMA revealed a marked increase in vessel density by transplantation of hybrid-dECFCs or stem-dECFCs compared with the PBS-injected sham. The vessel density was significantly increased in the hybrid-dECFC group compared with the stem-dECFC group.

Endothelial Differentiation in Murine Hind-Limb Ischemia

To investigate the effect of the 2 types of ECFCs on endothelial differentiation in vivo, the ischemic injury sites were excised at postoperative day 28 for histological analysis of cell engraftment and differentiation. Transplanted EPCs, detected by the expression of a human nuclear antigen (HNA, red), were found in the vessel wall stained with CD31 (green; Figure 4A). Histological analysis revealed that the differentiation was considerably more noticeable in stem-dECFCs than in hybrid-dECFCs. To confirm the difference of endothelial differentiation in vivo, the tube-forming ability was investigated by capillary network formation on Matrigel in vitro. The 2 types of ECFCs could successfully form capillaries on Matrigel (Figure 4B). The area of the tube-like structures of stem-dECFCs was greater than that of hybrid-dECFCs (Figure 4B and 4C), although there were no differences between hybrid-dECFCs and stem-dECFCs in the number and length of the tube-like structures (data not shown). On the basis of the in vitro and in vivo data, the endothelial differentiation capacity of stem-dECFCs was more potently enhanced than the differentiation capacity of hybrid-dECFCs.

In Vivo Proliferation and Survival of the Transplanted EPCs

To evaluate the proliferation and survival of the 2 types of transplanted EPCs (passage 6) in murine hind-limb ischemia, the ischemic injury sites were excised at postoperative day 3 for histological analysis of cell proliferation and survival. Initially, we determined the proliferation of cells in the ischemic injury sites by immunohistochemistry of tissue sections using antiproliferating cell nuclear antigen antibodies (Figure 5A). Immunohistochemistry analysis indicated that the hybrid-dECFC group had a greater number of proliferative cells in ischemic tissue than any other group (ie, normal, sham, and stem-dECFCs; Figure 5B). We then performed immunohistochemistry for HNA and Ki-67 to determine whether the 2 types of transplanted ECFCs could proliferate in ischemic tissues. Proliferation of the ECFCs was confirmed by the presence of HNA- and Ki-67–positive cells (Figure 5C). Proliferative cells in ischemic tissues (ie, HNA and Ki-67 double-positive cells) were significantly more abundant in hybrid-dECFCs than in stem-dECFCs (Figure 5E). Next, we investigated whether the 2 types of transplanted ECFCs could survive in ischemic tissues. Potential for survival was confirmed by the presence of HNA-positive and caspase-3–negative cells (Figure 5D). Apoptotic cells in ischemic tissues (ie, HNA and caspase-3 double-positive cells) were significantly higher in stem-dECFCs than in hybrid-dECFCs (Figure 5F). These results indicated that the proliferation and survival capacity at the ischemic injury sites were substantially higher in hybrid-dECFCs than in stem-dECFCs.

Secretion of Angiogenic Growth Factors by the 2 Types of ECFCs in Murine Hind-Limb Ischemia

As described above, the 2 types of transplanted ECFCs enhanced angiogenesis and arteriogenesis in ischemic injury tissues. To address the angiogenic effects of the 2 types of ECFCs, we examined the secretion of angiogenic factors by the 2 types of ECFCs (passage 6) transplanted in the murine hind-limb ischemia model. Immunohistochemistry for angiogenic cytokines, such as fibroblast growth factor-2, hepatocyte growth factor, SDF-1α, VEGF, and interleukin-8, showed significantly increased expression of these factors in hybrid-dECFCs compared with other groups (normal, sham, and stem-dECFCs; Figure 5G through 5K). Moreover, Western blotting of ischemic tissue lysates proved that transplanted hybrid-dECFCs induced significantly more secretion of angiogenic cytokines at ischemic sites than did any other group (Figure 5L).

Involvement of Focal Adhesion Kinase and Src in the Migration of the 2 Types of ECFCs

After investigating the characteristics and functional differences of hybrid-dECFCs and stem-dECFCs in vitro and in vivo, we confirmed the differences in functional recovery of
the 2 types of ECFCs, as well as their effects on cell migration via focal adhesion kinase (FAK) and proto-oncogene tyrosine-protein kinase (Src) signaling. We examined the expressions of p-FAK and p-c-Src in the 2 types of ECFCs in vitro by Western blotting (Figure 6A). The level of phosphorylation of FAK and c-Src in the 2 types of ECFCs was significantly enhanced in hybrid-dECFCs relative to stem-dECFCs (Figure 6B). To investigate the cellular location of p-FAK and p-c-Src in the 2 types of ECFCs, immunocytochemical staining for p-FAK and p-c-Src was performed. The majority of the p-FAK and p-c-Src proteins in hybrid-dECFCs seemed to be localized to and extending from the cell membrane region, whereas p-FAK and p-c-Src in stem-dECFCs seemed to diffuse in the cytosol (Figure 6C). Next, we performed a
mobilization and the development of functional progeny, these cells have enormous therapeutic potential for vessel repair and neovascularization in ischemic diseases. However, the identification, characterization, and function of EPCs in vascular biology are still subjects of debate. It is unknown whether cultured cells are representative of cells that exist in the bloodstream or whether cultured cells represent an artificial phenotype generated by specific culture conditions. Although the EPC identity is elusive, a detailed functional characterization of EPCs using preclinical models seems to be more relevant than their antigenic phenotype. Accumulating data have demonstrated that human CD34+ cells are useful for the treatment of vascular ischemic disease. Conflicting results have been reported regarding whether human CD34+ cells or CD34− cells constitute the major cell population. Because the isolation of EPCs (according to the presence or absence of CD34) has been controversial, we hypothesized that there might be other characteristics that distinguish the 2 types of late EPCs, also known as ECFCs, according to the presence of CD34.

The EPC colony-forming assay, a novel method to assess the colony-forming potential of EPCs at different differentiation levels, was recently established and allows us to investigate the EPC commitment of CD34+ cells, CD34− cells, or hybrid CD34+/CD34− cells. In the EPC-CFA, hybrid CD34+/CD34− cells were superior in their expansion of small, large, and total EPC colonies compared with only CD34+ cells or CD34− cells. Because the isolation of EPCs (according to the presence or absence of CD34) has been controversial, we hypothesized that there might be other characteristics that distinguish the 2 types of late EPCs, also known as ECFCs, according to the presence or absence of CD34.

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Figure 5. Proliferation, survival, and secretion of angiogenic growth factors of endothelial colony-forming cells (ECFCs) in hind-limb ischemia. At day 3 after surgery, samples harvested from hind-limb ischemic tissues were stained to determine the proliferation, survival, and secretion of angiogenic growth factors of the transplanted ECFCs (passage 6). 

A, Proliferative cells in ischemic injury sites visualized by immunofluorescent staining for proliferating cell nuclear antigen (PCNA; red).

B, Standard quantification of proliferative cells represented as the number of PCNA/4',6-diamidino-2-phenylindole (DAPI) double-positive cells per high-power field. Results are shown as the mean±SEM (*P<0.05 and **P<0.01 vs sham, ##P<0.05 vs CD34+ cell–derived ECFCs [stem-dECFCs]).

C, Proliferative transplanted cells at the ischemic injury sites indicated by human nuclear antigen (HNA; red), Ki-67 (green), and DAPI (blue) triple-positive cells. White color indicates triple-positive cells in the merged images.

D, Apoptotic transplanted cells in ischemic injury shown as HNA (red), caspase-3 (green), and DAPI (blue) triple-positive cells.

E, Standard quantification of proliferative transplanted cells represented as the number of HNA/Ki-67/DAPI triple-positive cells per high-power field.

F, Standard quantification of apoptotic cells represented as the number of HNA/caspase-3/DAPI triple-positive cells per high-power field. Results are shown as the mean±SEM (**P<0.01 vs CD34−/CD34+ cell–derived ECFCs [hybrid-dEPC]).

G–K, Secretion of angiogenic growth factor from transplanted ECFCs in injury sites visualized by fibroblast growth factor (FGF-2; G), hepatocyte growth factor (HGF; H), stromal cell–derived factor (SDF)-1α (I), vascular endothelial growth factor (VEGF; J), and interleukin (IL)-8 (K) staining (green) and HNA staining (red). At day 3 after surgery, samples harvested from hind-limb ischemic tissues were analyzed to confirm the secretion of angiogenic growth factor at the injury sites by Western blotting. Western blots of ischemic tissue homogenates indicated secretion of FGF-2, HGF, SDF-1α, VEGF, and IL-8. Hvf indicates high visual field.
ECFCs via clonal analysis, according to culture methods and analysis system. However, there is no study of the direct comparison of cellular characterization and functionality for distinct ECFCs,20,32 which were also referred to as late EPCs29 or outgrowth ECs.33 On the basis of these findings, we investigated whether CD34− cells play a pivotal role in the commitment of ECFCs and their characteristics during long-term culture to isolate ECFCs. Hybrid-dECFCs and stem-dECFCs (passage 6) plated on microscope cover glasses. The 2 types of ECFCs were fixed and immunostained for p-FAK and p-c-Src (red) to investigate the cellular localization of p-FAK and p-c-Src. The arrows indicate the cellular distribution of p-FAK and p-c-Src in the 2 types of ECFCs. D, Representative pictures of the effect of p-c-Src and PP2 (p-c-Src inhibitor) on ECFC migration. Cell migration was evaluated by the scratching wound-healing assay. The 2 types of ECFCs (passage 6) were pretreated or not pretreated with 10−7 mol/L PP2, and cell migration was then monitored for 24 hours (magnification, ×40). E, Standard quantification of migration cells presented as the number of migration cells per high-power field. Results are shown as the mean±SEM (**P<0.01 vs stem-dECFCs, ##P<0.01 vs ECFCs pretreated with PP2). F–H, At day 3 after surgery, samples harvested from the hind-limb ischemic tissues were analyzed by immunohistochemistry to determine the incorporation of the 2 types of transplanted ECFCs (passage 6) at the injury sites. F, Representative immunostained images for human nuclear antigen (HNA; red) and CD31 (green) showed incorporation into the capillary of the 2 types of transplanted ECFCs pretreated with PP2 or not. The incorporation of transplanted ECFCs was determined by HNA (red), CD31 (green), and 4',6-diamidino-2-phenylindole (DAPI; blue) triple-positive cell staining. G, Standard quantification of incorporated cells represented as the number of HNA (red), CD31 (green), and DAPI (blue) triple-positive cells per high-power field. Results are shown as the mean±SEM (**P<0.01 vs stem-dECFCs, ##P<0.01 vs ECFCs pretreated with PP2). H, Representative images of immunostained HNA (red) and CD31 (green) showing incorporation into vessels of human ECFCs pretreated or not pretreated with PP2. The arrows indicate the incorporation of transplanted ECFCs at the injury sites. Hvf indicates high visual field.
stem-dECFCs were characterized by morphological and endothelial-specific marker analysis. The morphology and endothelial-specific markers of both types of ECFCs were similar, indicating that CD34− cells did not critically alter morphology and endothelial-specific markers during the isolation of ECFCs. To confirm the functional differences between the 2 types of ECFCs, we assessed proliferation capacity, expression of cell-cycle signaling molecules, and senescence and found that hybrid-dECFCs had a higher proliferation capacity and slower senescence progression than did stem-dECFCs. These results indicated that CD34− cells, which functioned as niche-supporting cells during the isolation of ECFCs, in hybrid-dECFCs enhanced the proliferation capacity and delayed senescence of EPCs in vitro, suggesting a pivotal role of human CD34− accessory cells in EPC proliferation and senescence. Next, we evaluated the in vivo recovery after transplantation of the 2 types of ECFCs in hind-limb ischemia. The recovery of blood perfusion was significantly augmented in hybrid-dECFCs compared with stem-dECFCs during the early phase of ischemia (postoperative days 3–14). Hybrid-dECFCs also showed a higher capillary density than stem-dECFCs. On the basis of the proliferation, survival, and secretion of angiogenic cytokines (eg, fibroblast growth factor-2, hepatocyte growth factor, SDF-1α, VEGF, and interleukin-8) characteristics that define ECFCs, hybrid-dECFCs showed better functionality than stem-dECFCs.

Interestingly, we found that the endothelial differentiation potential was greater in stem-dECFCs than in hybrid-dECFCs. One possible explanation is the existence of the niche-supporting cell effect on the maintenance of niche environments during the ex vivo culture of hybrid-dECFCs. Stem-dECFCs did not retain niche-supporting cells during the culture period for isolation of ECFCs, and therefore could not maintain the niche condition of the progenitor cells. Accumulating evidence supports these findings: coculture of HUCB cells with human EC as niche-supporting cells allowed remarkable expansion of cells capable of multilineage engraftment and serial transplantation; hallmarks of long-term repopulating HSCs. ECs and leptin-expressing perivascular stromal cells, 2 functionally important components of the niche, contribute to HSC maintenance. If stem/progenitor cells lose the support and regulation provided by the niche, and receive only the specific growth factors provided in the culture media, they rely on exogenous direction and potentially drive differentiation at the expense of self-renewal. Stem-dECFCs might progress to endothelial differentiation more than hybrid-dECFCs during ex vivo culture conditions and may not be able to maintain the status of endothelial progenitors. Another possible explanation is the existence of the niche-supporting cell effect on angiogenic cytokines during the ex vivo culture of hybrid-dECFCs. When stem-dECFCs were transplanted in murine hind-limb ischemia, the recovery of blood perfusion was not as dramatically increased as in hybrid-dECFCs during the early phase of ischemia, although they showed similar improvement at the late phase as did hybrid-dECFCs. To validate whether the delay of recovery is attributable to the secretion of angiogenic cytokines, conditioned media isolated from 2 types of ECFCs after hypoxia condition were injected in the murine hind-limb ischemia model. The recovery of blood perfusion was significantly decreased in stem-dECFC conditioned media-injected group during the early phase and even in the late phase of ischemia. Moreover, foot necrosis occurred in the stem-dECFC conditioned media-injected group (Figure VI in the online-only Data Supplement). These data suggest that the secretion ability of angiogenic cytokines in stem-dECFCs is low, and that stem-dECFCs might possess a predominantly physical contribution to angiogenesis. As a result of the increased progression of endothelial differentiation in stem-dECFCs, stem-dECFCs showed improved tubular forming capabilities in vitro and endothelial differentiation in vivo in hind-limb ischemia.

EC adhesion and mobility are essential for the formation of new blood vessels, processes in which FAK and Src play important roles. The FAK-Src complex binds to and phosphorylates several adaptor proteins. The activated FAK-Src complex promotes cell-cycle progression, cell motility, cell survival, and angiogenesis. Thus, we focused on FAK-Src signaling in the 2 types of ECFCs to elucidate functional differences in migration and angiogenesis. The expression levels of p-FAK and c-Src were significantly increased in hybrid-dECFCs compared with stem-dECFCs. In addition, hybrid-dECFCs showed strikingly enhanced cell migration in vitro and incorporation capacity in vivo compared with stem-dECFCs. After treatment with the Src inhibitor PP2, both cell migration and incorporation capacity were drastically inhibited in 2 types of ECFCs, indicating that FAK-Src signaling is involved in the migration and incorporation of 2 types of ECFCs. These results suggest that hybrid-dECFCs are different from stem-dECFCs in terms of FAK and Src phosphorylation, indicating that niche-supporting cells may activate the phosphorylation of the FAK-Src complex during the isolation of hybrid-dECFCs.

On the basis of these findings, we propose that CD34− accessory cells are important niche-supporting cells for proper hEPC function. Niche-supporting cells provide a protecting environment that isolates stem cells from stimuli, such as differentiation and apoptotic signals. Recently, several studies have highlighted the potential of niche-supporting cells. CD34−/CD31+ T-cells are required for EPC colony formation during early EPC differentiation. Spheroid culture of blood MNCs potentiates the expansion of circulating blood HSCs. Mesenchymal stem cells support the maintenance of cord blood HSC during long-term ex vivo culture. In the present study, we found that CD34− cells, particularly macrophages that secrete either VEGF or SDF-1α on activation, might regulate mature EPC differentiation, demonstrated by a novel EPC-CFU assay, suggesting that crosstalk between incorporated EPCs and recruited macrophages plays a critical role in EPC functionality in vitro. In addition, our studies on 2 types of ECFCs demonstrated that hybrid-dECFCs might be responsible for paracrine effects, whereas stem-dECFCs might have a physical contribution to neovascularization in vivo. Although stem cell active factors, including bone morphogenetic protein 2, bone morphogenetic protein 4, transforming growth
factor-β, brain-derived nerve growth factor, jagged 1 and jagged 2, and angiogenic factors, such as placental growth factor, angiopoietin 2, VEGFA, fibroblast growth factor-2, and platelet-derived growth factor are known, the precise definition of a set of growth factors and cytokines and the cell–cell interaction mechanism between stem/progenitor cells and niche-supporting cells that can expand and maintain stem/progenitor cells during long-term ex vivo culture remains to be determined.

On the basis of our findings, we propose a scheme for the role of CD34− cells as niche-supporting cells for cell survival, incorporation, and endothelial differentiation of endothelial colony-forming cells (ECFCs). CD34+/CD34− cell-derived ECFCs (hybrid-dECFCs) and CD34− cell-derived ECFCs (stem-dECFCs) have different populations of cells. Hybrid-dECFCs are isolated CD34+ cells and niche-supporting CD34− cells, and stem-dECFCs are solely composed of CD34+ cells. A, For hybrid-dECFCs, CD34− cells may give rise to differentiated ECFCs with the support of the CD34− cells, enhancing proliferation, migration, and survival, as well as delaying senescence. B, Although stem-dECFCs also possess the functions of ECFCs, their differentiation and senescence are induced earlier than in hybrid-dECFCs. Because stem-dECFCs populations consist only of CD34+ cells, stem-dECFCs seem more activated for differentiation into ECFCs. Through this activation, stem-dECFCs may enhance their differentiation capacity, such as tube formation, decrease the proliferation capacity and secretion of angiogenic cytokines, as well as accelerate cellular senescence, as compared with hybrid-dECFCs. In conclusion, niche-supporting cells may facilitate cell survival, incorporation capacity, and endothelial differentiation of ECFCs by interacting with CD34+ cells. EC indicates endothelial cells; and FAK, focal adhesion kinase.

Figure 7. Proposed scheme for the role of CD34+ niche-supporting cells for cell survival, incorporation, and endothelial differentiation of endothelial colony-forming cells (ECFCs). CD34+/CD34− cell–derived ECFCs (hybrid-dECFCs) and CD34− cell–derived ECFCs (stem-dECFCs) have different populations of cells. Hybrid-dECFCs are isolated CD34+ cells and niche-supporting CD34− cells, and stem-dECFCs are solely composed of CD34+ cells. A, For hybrid-dECFCs, CD34− cells may give rise to differentiated ECFCs with the support of the CD34− cells, enhancing proliferation, migration, and survival, as well as delaying senescence. B, Although stem-dECFCs also possess the functions of ECFCs, their differentiation and senescence are induced earlier than in hybrid-dECFCs. Because stem-dECFCs populations consist only of CD34+ cells, stem-dECFCs seem more activated for differentiation into ECFCs. Through this activation, stem-dECFCs may enhance their differentiation capacity, such as tube formation, decrease the proliferation capacity and secretion of angiogenic cytokines, as well as accelerate cellular senescence, as compared with hybrid-dECFCs. In conclusion, niche-supporting cells may facilitate cell survival, incorporation capacity, and endothelial differentiation of ECFCs by interacting with CD34+ cells. EC indicates endothelial cells; and FAK, focal adhesion kinase.
activation of FAK-Src signaling in ECFCs and hence enhance ECFC migration and incorporation activity, as well as ECFC proliferation and survival.

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Disclosures
None.

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### Significance

Endothelial progenitor cells (EPCs) have been reported to promote neovessel formation during vascular injury. However, the function of supporting cells of EPCs and their interaction with EPCs during EPC isolation remain unclear. We investigated the functional properties of 2 types of EPCs, also known as endothelial colony-forming cells (ECFCs), CD34+/CD34− cell–derived ECFCs (hybrid-dECFCs) and CD34+ cell–derived ECFCs (stem-dECFCs), isolated using different methods, to elucidate the role of CD34+ cell populations as cell-supporting niches. We suggest that niche-supporting CD34− cells facilitate cell survival, angiogenic cytokine secretion and incorporation capacity, and preserve the progenitor status of ECFCs. In particular, niche-supporting cells affect the activation of focal adhesion kinase-Src signaling in ECFCs and hence enhance ECFC migration and incorporation activity, as well as ECFC proliferation and survival. This study provided important insight into blood vessel formation and repair in ischemic diseases for ECFC-based cell therapy.
CD34 Hybrid Cells Promote Endothelial Colony-Forming Cell Bioactivity and Therapeutic Potential for Ischemic Diseases
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SUPPLEMENTAL DATA

CD34 Hybrid Cells Promote Endothelial Colony-Forming Cell Bioactivity and Therapeutic Potential for Ischemic Diseases

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Running title: Role of CD34+ Accessory Cells for ECFC Commitment

Key Words: endothelial progenitor cells, CD34, niche-supporting cells, vasculogenesis, vascular repair, hind-limb ischemia

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Supplemental Materials and Methods

Enzyme-linked immunosorbent assay (ELISA)
The concentration of SDF-1α and VEGF was assessed in TNF-α-activated macrophage culture supernatants. SDF-1α and VEGF levels were determined by Quantikine ELISA Human SDF-1α Immunoassay and Quantikine ELISA Human VEGF Immunoassay (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions.

Immunodepletion of SDF-1α and VEGF from TNF-α activated macrophage conditioned media
For immunoprecipitation of SDF-1α and VEGF from TNF-α activated macrophage conditioned media (TNF-α-CM), TNF-α-CM was incubated with protein A/G-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) immobilized with anti-SDF-1α (Santa Cruz Biotechnology), anti-VEGF (Santa Cruz Biotechnology) or control mouse antibodies for 1 h at 4°C. Immune complexes absorbed to protein A/G-Agarose beads were precipitated by centrifugation. Supernatants were collected and immediately used for experiments.

ECFC conditioned media generated under hypoxia injection in a hind-limb ischemia murine model
For hypoxic conditions, 2 types of ECFCs (passage 6) under serum free condition were incubated in a hypoxia chamber maintain low oxygen tension (2% O2, 5% CO2, and balanced with N2) for 24 h. After the incubation under hypoxic condition, ECFC conditioned media (CM) were collected and used experiments. All procedures were performed in accordance with the policies of the Pusan National University of Korea institutional animal care and use committees. The hind-limb ischemia murine model was induced by ligating the proximal femoral artery and boundary vessels of 8-week-old Balb/C nude mice. No later than 6 h after operation, 2 types of ECFC CM or normal media (sham) were injected via intramuscular injection in the ischemic thigh area to assess the blood perfusion. 2 types of ECFC CM and normal media were injected daily for 7 day and the every 3 days until postoperative 28 day.

Supplemental Figure legends

Supplemental Figure I. The secretion of angiogenic cytokines in TNF-α treated or untreated CD34+ cells-derived macrophages. Concentrations of SDF-1α and VEGF in macrophage culture supernatants treated with TNF-α or not were determined by ELISA. Concentrations of SDF-1α and VEGF were significantly higher in macrophage culture supernatants treated with TNF-α compared with untreated with TNF-α. (* p < 0.05 and ** p < 0.01 vs. macrophage culture supernatants untreated TNF-α).

Supplemental Figure II. Effect of TNF-α activated macrophage culture supernatants on EPC-CFUs through immunoprecipitation of SDF-1α and VEGF. In response to stimuli of complete TNF-α activated macrophage culture supernatants, the frequency of large EPC-CFUs was significantly increased. (** p < 0.01 vs. normal EPC-CFA media untreated with TNF-α activated macrophage culture supernatants, anti-VEGF and anti-SDF-1α)

Supplemental Figure III. FACS analysis on EPC-CFU. Expression of endothelial lineage markers for KDR, CXCR4, and Tie2 on transwell-cultured cells with or without macrophage cells were measured by FACS analysis.

Supplemental Figure IV. Expression of hematopoietic lineage markers and CD144 on ECFC. Expression of hematopoietic lineage markers for CD11b, CD14, and CD45 and ECFC marker for CD144 on 2 types of ECFCs was measured by flow cytometry. 2 types of ECFCs expressed CD144, but did not express CD11b, CD14, and CD45.
Supplemental Figure V. BrdU cell proliferation assay on 2 types of ECFCs. 2 types of ECFCs (passage 6) were treated with VEGF (100 ng/mL), and the cell proliferation was examined via the BrdU cell proliferation assay after 24 h. The proliferation capacity was significantly increased in hybrid-dECFCs as compared with stem-dECFCs. (** p < 0.01 vs. stem-dECFCs).

Supplemental Figure VI. Assessment on functional recovery for ECFC conditioned media in the hind-limb ischemia. (A) Laser-doppler perfusion imaging (LDPI) analysis of the improvement in recovery of the blood flow in the ischemic limb of the sham (basic media-injected group), hybrid-dECFCs conditioned media (CM)-injected group, and stem-dECFCs CM-injected groups (n=5). (B) Perfusion ratio obtained by dividing the blood flow of the ischemic (left) limb by that of the non-ischemic (right) limb. LDPI measured days 0, 3, 7, 10, 21, and 28 post-surgery. Results are shown as the mean ± SEM (* p < 0.05 and ** p < 0.01 vs. sham, #: p < 0.05 and ##: p < 0.01 vs. stem-dECFCs CM). (C) Representative three different outcomes (limb loss, limb salvage, and foot necrosis) of mice injected normal media (sham), hybrid-dECFC CM, and stem-dECFC CM at day 28. (D) Rate of the three outcomes in each group at 28 day.

Supplemental Figure VII. Immunohistochemistry on antibody controls using secondary antibodies only. To present control on antibodies, immunohistochemistry were performed by secondary antibodies only for goat anti-rabbit IgG (Alexa Fluor 488) and goat anti-mouse IgG (Alexa Fluor 594).
Supplemental Fig. I

**SDF-1α**

- **TNF-α**
  - -
  - +

**VEGF**

- **TNF-α**
  - -
  - +

SDF-1α (pg/ml)

VEGF (pg/ml)
Supplemental Fig. II

**CD34+ cells-derived EPC-CFUs (number)**

- Small EPC
- Large EPC

<table>
<thead>
<tr>
<th>Condition</th>
<th>Small EPC</th>
<th>Large EPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mφ sup.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anti-SDF-1α</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Notes:**

- **Small EPC** and **Large EPC** bars represent the number of CD34+ cells-derived EPC-CFUs.
- The graph shows the effect of different conditions on the number of EPC-CFUs.
- The conditions include Mφ sup. (Macrophage supernatant), Anti-VEGF, and Anti-SDF-1α.
- The data is presented as mean ± standard error of the mean (SEM).
- **Small EPC** bars are shown in white, and **Large EPC** bars are shown in black.
- The conditions are marked with - (negative) or + (positive).
- The graph indicates a significant increase in the number of Large EPC-CFUs under certain conditions, as indicated by the ** symbol.
Supplemental Fig. III

CD34+ with Macrophage

KDR

CXCR4

Tie2

CD34
Supplemental Fig. V

Fold increase

**

VEGF

Hybrid -dECFC

Stem -dECFC

Fold increase

VEGF - + - +

Hybrid -dECFC

Stem -dECFC

**
Supplemental Fig. VI

A
0 Day 28 Day
Sham
Hybrid -dECFC CM
Stem -dECFC CM

B
Ratio of blood perfusion
0.0 0.2 0.4 0.6 0.8 1.0
Time (Days)
0 3 7 10 14 21 28
Sham
Hybrid-dECFC CM
Stem-dECFC CM

C
Sham Hybrid -dECFC CM Stem -dECFC CM

D
Distribution (%)
100 80 60 40 20
Sham Hybrid -dECFC CM Stem -dECFC CM

Limb Salvage
Foot Necrosis
Limb Loss

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Materials and Methods

Isolation, cell culture, and characterization of hybrid-dECFCs and stem-dECFCs
HUCB was supplied from Pusan National University Yangsan Hospital (PNUYH). HUCB samples were collected from fresh placentas with attached umbilical cords. MNCs were isolated from HUCB by density gradient centrifugation with Ficoll separating solution (Amersham Biosciences, Uppsala, Sweden). The CD34+ cell fraction was isolated from the MNCs using the Magnetic Activated Cell Sorting (MACS) System (CD34+ Microbead Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions (purity; more than 98%). The freshly isolated MNC cells or the CD34+ cell fraction were cultured on 100-mm dishes coated with 1% gelatin (Sigma, St. Louis, MO, USA) and cultured in endothelial basal medium (EBM) 2 (Lonza, Walkersville, MD, USA) supplemented with 5% fetal bovine serum (FBS), human vascular endothelial growth factor (hVEGF), human basic fibroblast growth factor (bFGF), human epidermal growth factor (hEGF), human insulin-like growth factor 1 (hIGF-1), ascorbic acid, and GA-1000 (EGM-2 medium). After 4 days, non-adherent cells were discarded, and fresh culture medium was added. Cultures were maintained for another 3 days and subjected to long-term culture to form spindle-shaped colonies (14–21 days) by replenishing with EGM-2 medium. The medium was changed daily for 7 days, and then every 2 days until the first passage. Hybrid-dECFCs and stem-dECFCs were identified as double-positive cells by fluorescence microscopy using endothelial lineage markers. For the characterization of several surface and pivotal functional markers, immunofluorescence staining was performed using the following antibodies: goat polyclonal anti-platelet-endothelial cell adhesion molecule 1 (anti-PECAM-1) (CD31), rabbit polyclonal anti-KDR/Fk1, and rabbit polyclonal anti-von Willebrand factor (anti-vWF) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and rabbit monoclonal anti-phospho-Akt (p-Akt), rabbit monoclonal anti-endothelial nitric oxide synthase (anti-eNOS), and rabbit polyclonal anti-phospho-eNOS (p-eNOS) from Cell Signaling Technology (Beverly, MA, USA).

EPC colony-forming assay
Human CD34+/CD34-, CD34+, and CD34- cells isolated from HUCB were cultured in methylcellulose-containing medium M3236 (StemCell Technologies, Vancouver, Canada) with 20 ng/mL stem cell-derived factor (Kirin, Tokyo, Japan), 50 ng/mL VEGF (R&D Systems, Minneapolis, MN, USA), 20 ng/mL interleukin (IL)-3 (Kirin, Tokyo, Japan), 50 ng/mL bFGF (Wako, Osaka, Japan), 50 ng/mL EGF (Wako, Osaka, Japan), 50 ng/mL IGF-1 (Wako, Osaka, Japan), 2 U/mL heparin (Ajinomoto, Tokyo, Japan), and 10% FBS on a 35-mm dish for 8 days. The cell density for each sample was 1 × 10^4 cells/dish. The EPCs were identified as small EPC colony-forming units (CFUs) or large EPC-CFUs by visual inspection using a microscope under 40× magnification. Small EPC-CFUs were composed of round adhesive cells, and large EPC-CFUs were composed of spindle-shaped cells.

Co-culture analysis
Co-culture analysis was performed in 12-well Millicell Cell Culture Plates (0.4 μm pore size; Millipore, Billerica, MA, USA) using the same media used in EPC-CFA. Human CD34+ cells isolated from HUCB were seeded in the lower compartment of the transwell, and human CD34 cells or macrophages isolated from HUCB were either seeded or not seeded on the transwell membrane inserts. To measure the EPC-CFU potential of CD34+ cells after co-culture, transwell inserts were removed after 72 h. The EPC-CFU ratio of CD34+ cells was tested by cell counting.

Flow cytometry analysis
Hybrid-dECFCs and stem-dECFCs were determined by flow cytometry analysis using labeled EC markers, anti-human KDR (BD Pharmingen, San Jose, CA, USA) and anti-human CD144 (BD Pharmingen), HSC markers, anti-human CD34 (BD Pharmingen), anti-
human CD133 (eBioscience, San Diego, CA, USA), anti-human CXCR4 (BD Pharmingen), anti-human Tie2 (BD Pharmingen), and anti-human c-Kit (DakoCytomation, Glostrup, Denmark), and hematopoietic lineage markers, anti-human CD11b (BD Pharmingen), anti-human CD14 (BD Pharmingen), and anti-human CD45 (BD Pharmingen). Stained cells were analyzed by two-color flow cytometry using a fluorescence-activated cell sorter (FACS) (BD FACSCanto II, San Jose, CA, USA). The percentage of stained cells was determined after comparing the sorted cells with matched isotype controls.

**Cell proliferation assay**

Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, hybrid-dECFCs and stem-dECFCs (from passage 6, $5 \times 10^3$ cells/well) were seeded in 96-well plates in complete EGM-2 medium. After overnight incubation, the cells were serum-starved in EBM-2 medium supplemented with 1% FBS for 12 h. The cells were then cultured in EGM-2 medium supplemented with 1% FBS and VEGF (100 ng/mL) or EGM-2 medium supplemented with 1% FBS as a control. After 1, 2, and 3 days of culture, the medium was removed, and MTT (5 mg/mL, Sigma) was added to each well. The cells were then incubated at 37°C for 4 h. The color was extracted with dimethyl sulfoxide (DMSO) at 37°C for 20 min. The relative viable cell number was determined by measuring the absorbance at 540 nm (OD$_{540}$) at room temperature. To evaluate the cell proliferation of both hybrid-dECFCs and stem-dECFCs, the MTT assay, as described above, was performed at passages 6, 8, 10, 12, 14, 16, 18, and 20. The BrdU incorporation assay was performed using the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology) according to the manufacturer's protocol.

**Tube formation assay**

Matrigel (50 μL/well; BD biosciences, Bedford, MA) was added to 96-well plates and incubated at 37°C. Hybrid-dECFCs and stem-dECFCs ($2 \times 10^4$ cells/well) were plated separately on Matrigel, then cultured at 37°C with 5% CO$_2$, and monitored frequently by phase contrast microscopy. Once tube formation was observed, images were recorded.

**Western blot analysis**

Total protein from hybrid-dECFCs and stem-dECFCs was extracted using RIPA Lysis Buffer. The protein concentration was measured using the bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL, USA). Equal amounts of cell lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then electrotransferred to a polyvinylidene fluoride membrane (PVDF; Millipore, Billerica, MA, USA), blocked with 5% nonfat milk, and incubated with the following primary antibodies: mouse monoclonal anti-β-actin, rabbit polyclonal anti-Cdk2, mouse monoclonal anti-Cdk4, rabbit polyclonal anti-cyclin D1, rabbit polyclonal anti-cyclin E, mouse monoclonal anti-SMP30, mouse monoclonal anti-p21, rabbit polyclonal anti-FAK, and mouse monoclonal anti-c-Src, rabbit polyclonal anti-phospho-FAK, and mouse monoclonal anti-phospho-c-Src (Santa Cruz Biotechnology). After incubation with peroxidase-conjugated secondary antibodies, bands were visualized with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences).

**Migration assay**

Hybrid-dECFCs and stem-dECFCs (passage 6) were plated in 6-well plates and grown until confluence in complete EGM-2 medium. The monolayer was then wounded with a cell scraper, and the detached cells were removed by washing with complete EGM-2 medium. Hybrid-dECFCs and stem-dECFCs were incubated in complete EGM-2 medium and observed under a microscope equipped with a 40× objective lens for 24 h. 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d]pyrimidine (PP2), a potent and selective Src family protein tyrosine kinase inhibitor, was purchased from Sigma. EPCs were treated with 10$^{-7}$ M
PP2 to inhibit Src family kinases.

**Immunocytochemistry**
Hybrid-dECFCs and stem-dECFCs grown on a microscope cover glass were washed 3 times in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (USB Corporation, Santa Clara, CA, USA) for 10 min, permeabilized with 0.3% (w/v) Triton X-100 in PBS for 5 min, and then incubated in PBS containing 3% BSA (Sigma) at 37°C for 2 h to block non-specific binding sites. Next, the 2 types of hECFCs were incubated with the following primary antibodies: rabbit polyclonal anti-phospho-FAK and mouse monoclonal anti-phospho-c-Src (Santa Cruz Biotechnology). Immunostained slides were imaged by confocal microscopy (Olympus, Tokyo, Japan).

**Senescence-associated β-galactosidase (SA-β-gal) assay**
Hybrid-dECFCs (passages 6-20) and stem-dECFCs (passages 6–16) were collected and plated onto 6-well plates in complete EGM-2 medium. After overnight incubation, the cells were stained with Senescence β-Galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer’s protocol. After staining, images were obtained using a phase contrast microscope.

**Cell transplantation in a hind-limb ischemia murine model**
All procedures were performed in accordance with the policies of the Pusan National University of Korea institutional animal care and use committees. The hind-limb ischemia murine model was induced by ligating the proximal femoral artery and boundary vessels of 8-week-old Balb/C nude mice. No later than 6 h after operation, hybrid-dECFCs or stem-dECFCs (passage 6) in PBS were transplanted via intramuscular injection into the ischemic thigh area (5 × 10^5 cells/mouse) to assess blood perfusion and capillary density, or via intravenous injection in the tail vein (1 × 10^6 cells/mouse) to assess the incorporation of the 2 types of ECFCs.

**Laser-doppler perfusion imaging (LDPI) of the hind-limb blood flow**
LDPI (Moor Instruments, Wilmington, DE, USA) was used to measure the ratio of the ischemic (left)/non-ischemic (right) limb blood flow at postoperative days 0, 3, 7, 10, 14, 21, and 28, as previously described.¹

**Statistical analyses**
Data are expressed as mean ± standard error of the mean. We performed statistical analyses using Student’s t test with the significance set at a P-value of <0.05.

**References**