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

Jun Hee Lee, Sang Hun Lee, So Young Yoo, Takayuki Asahara, Sang Mo Kwon

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;33:00-00.)

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
Interestingly, coculture of CD34+ cells and CD34− accessory cells results in significantly higher EPC colony-forming capacity of both small EPC-CFUs and large EPC-CFUs. Small 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 were seeded in the upper compartment of transwells. To determine the paracrine factors secreted by isolated CD11b+ macrophages, 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).

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. Based on 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, immunocytometry, 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 immunocytometry on 2 types of ECFCs to confirm whether they express endothelial lineage markers, such as CD31, KDR (VEGFR2), and von Willebrand factor, as well as pivotal functional markers, including endothelial nitric oxide synthase, p-endothelial nitric oxide synthase, and p-Akt. 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, KDR, CXCR4, 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).
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 Cdk2, cyclin E, Cdk4, and cyclin D1, with those of the MTT assay. The expressions of Cdk2 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 MTT assay and BrdU 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.
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 SA-β-gal activity of the 2 types of ECFCs (passages 6–16). The green cells were counted as positive for senescence (magnification, ×40). G, Hybrid-dECFC (passages 6–20) and stem-dECFC (passages 6–16) lysates containing equal amounts of total protein were analyzed by Western blotting using senescence markers with anti-SMP30 and anti-p21 antibodies. Results are shown as the mean±SEM (*P<0.05 and **P<0.01 vs hybrid-dECFCs). DAPI indicates 4',6-diamidino-2-phenylindole; EBM, ; and SMP, .
to phosphorylate key substrates involved in cell-cycle progression. SMP30 expression levels decrease with aging and senescence. In passage 14, p21 expression was highly elevated, and SMP30 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 differentiation 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 ECFCs (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
migration assay to examine whether c-Src is involved in cell migration. We wounded confluent monolayers of the 2 types of ECFCs pretreated or not pretreated with PP2, a selective inhibitor of Src tyrosine kinases, and measured cell migration to the cell-free area. Hybrid-dECFCs showed significantly increased cell migration compared with stem-dECFCs. As expected, PP2 treatment failed to induce cell migration in both types of ECFCs (Figure 6D and 6E).

Incorporation of the 2 Types of ECFCs in Murine Hind-Limb Ischemia
We next examined the contribution of the 2 types of transplanted EPCs in murine hind-limb ischemia to assess the incorporation potential of ECFCs in murine hind-limb ischemia and whether Src signaling in ECFCs is involved in incorporation into injured vessels. Tissue sections harvested at day 3 after operation from ischemic hind limbs intravenously injected with the 2 types of ECFCs pretreated or not pretreated with PP2 (1×10^6 cells/mouse) were immunostained for HNA (red) and CD31 (green). The 2 types of ECFCs stained with HNA (red) were found in capillaries stained with CD31 (green; Figure 6F), whereas the 2 types of ECFCs pretreated with PP2 showed decreased incorporation (Figure 6F and 6G). When the number of cells incorporated into capillaries was measured, hybrid-dECFCs showed significantly augmented incorporation compared with stem-dECFCs (Figure 6G). Immunohistochemistry revealed that hybrid-dECFCs also contributed more to incorporation into vessels than did stem-dECFCs, whereas PP2-pretreated ECFCs were rarely observed in the vessels (Figure 6H). These findings indicate that hybrid-dECFCs might contribute to incorporation into capillaries and vessels by Src signaling linked to cell motility and migration.

Discussion
Because of the capacity of EPCs for proliferation, circulation, 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 or absence 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, suggesting that CD34+ cells have an impact on the differentiation CD34+ cells into EC progenitors. In previous studies, many researchers compared the function and characteristics of different types of EPCs that are different according to culture methods, culture period, or analysis system. Hur et al. established the characterization of early EPCs and late EPCs by means of different culture periods. Yoon et al. characterized early EPCs and late EPCs and reported that the combination of these different cell types shows synergism during neovascularization. Yoder et al. compared the function of EC colony-forming units and
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 proliferating 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 proliferating transplanted cells represented as the number of HNA/Ki-67/DAPI triple-positive cells (white) per high-power field. F, Standard quantification of apoptotic cells represented as the number of HNA/caspase-3/DAPI triple-positive cells (white) 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). L 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 XXX.
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, which were also referred to as late EPCs or outgrowth ECs. 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. 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). 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). 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. 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. 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). 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 XXX.
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.34 ECs and leptin-expressing perivascular stromal cells, 2 functionally important components of the niche, contribute to HSC maintenance.35 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.36 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.37 The FAK-Src complex binds to and phosphorylates several adaptor proteins. The activated FAK-Src complex promotes cell-cycle progression, cell motility, cell survival,37 and angiogenesis.38 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.39 Recently, several studies have highlighted the potential of niche-supporting cells.40–42 CD33/CD31+ T-cells are required for EPC colony formation during early EPC differentiation.40 Spheroid culture of blood MNCs potentiates the expansion of circulating blood HSCs.41 Mesenchymal stem cells support the maintenance of cord blood HSC during long-term ex vivo culture.42 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 niche-supporting cells for the functionality of ECFCs (Figure 7). Hybrid-dECFCs isolated from HUCB consist of CD34+ cells and CD34− cells (Figure 7A). During culture for isolation of ECFCs, the CD34− cells support differentiation, delay senescence, and contribute to the functional improvement of ECFCs through the formation of cell–cell interactions or the production of paracrine factors. In contrast, stem-dECFCs are cells sorted from HUCB, based on the expression of CD34+ (Figure 7B). Stem-dECFCs showed faster endothelial differentiation and lower functionality of ECFCs than hybrid-dECFCs. CD34+ cells seemed more activated for differentiation into ECs because CD34− cells were absent. Several clinical studies have reported that intramuscular implantation of BM-MNC in critical limb ischemia in human induced active neoangiogenesis and histological changes, but transplanted BM-MNC did not exist in patients presenting with critical limb ischemia and may not prevent amputation in some patients with critical limb ischemia. Here, we show a pivotal cue of cell-based therapy. Human ECFCs modulated by CD34-cells as niche-supporting cells during ex vivo culture have greater proliferation capacity and possess the hallmark of EC progenitors compared with BM-MNCs, suggesting that an adequate supply of ECFCs can be provided to patients with ischemic diseases and that this might improve the results of clinical cell-based therapy.

In conclusion, 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 FAK-Src signaling in ECFCs and hence enhance ECFC migration and incorporation activity, as well as ECFC proliferation and survival.

**Sources of Funding**

This work was supported by a grant from the National Research Foundation funded by the Korean government (MEST; 2010–0020260, 2012M3A9C6049720).

**Disclosures**

None.

**References**


---

**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
Jun Hee Lee, Sang Hun Lee, So Young Yoo, Takayuki Asahara and Sang Mo Kwon

Arterioscler Thromb Vasc Biol. published online May 2, 2013;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2013/05/02/ATVBAHA.112.301052

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/05/02/ATVBAHA.112.301052.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL DATA
CD34 Hybrid Cells Promote Endothelial Colony-Forming Cell Bioactivity and Therapeutic Potential for Ischemic Diseases

Jun Hee Lee¹, Sang Hun Lee¹, So Young Yoo¹, Takayuki Asahara², Sang Mo Kwon¹

¹Laboratory for Vascular Medicine & Stem Cell Biology, Medical Research Institute, Department of Physiology, School of Medicine, Pusan National University, Yangsan, 626-870, Korea
²Department Regenerative Medicine Science, Tokai University School of Medicine, Isehara, Kanagawa, 259-1193, Japan

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

*Corresponding authors:
Sang Mo Kwon, Ph.D.
Laboratory for Vascular Medicine & Stem Cell Biology, Medical Research Institute, Department of Physiology, School of Medicine, Pusan National University, Yangsan, 626-870, Korea
Tel: +82 51 510 8070; Fax: +82 51 510 8076; E-mail: smkwon323@hotmail.com

Takayuki Asahara, M.D., Ph.D.
Department Regenerative Medicine Science, Tokai University School of Medicine, Isehara, Kanagawa, 259-1193, Japan
Tel.: +81 463 93 1121; Fax: +81 463 95 0961; E-mail: asa777@icc.u-tokai.ac.jp
Supplemental Materials and Methods

**Enzyme-linked immunosorbent assay (ELISA)**
The concentration of SDF-1α and VEGF was assessed in TNFα-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 TNFα activated macrophage conditioned media (TNFα-CM), TNFα-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**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1α (pg/ml)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

**VEGF**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1600</td>
</tr>
</tbody>
</table>

**VEGF**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1400</td>
</tr>
</tbody>
</table>

**SDF-1α**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1α (pg/ml)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

**VEGF**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1600</td>
</tr>
</tbody>
</table>

**SDF-1α**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1α (pg/ml)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

**VEGF**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1400</td>
</tr>
</tbody>
</table>

**SDF-1α**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1α (pg/ml)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

**VEGF**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1400</td>
</tr>
</tbody>
</table>

**SDF-1α**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1α (pg/ml)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

**VEGF**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1400</td>
</tr>
</tbody>
</table>

**SDF-1α**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1α (pg/ml)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

**VEGF**

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/ml)</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1400</td>
</tr>
</tbody>
</table>
Supplemental Fig. II

Graph showing the effect of different treatments on CD34+ cell-derived EPC-CFUs, with bars representing Small EPC and Large EPC. The horizontal axis shows the treatments: Mφ sup., Anti-VEGF, and Anti-SDF-1α, with '-' indicating absence and '+' indicating presence. The vertical axis represents the number of cells.

- ** indicates statistical significance.
Supplemental Fig. III

CD34+ with Macrophage

- **KDR**
  - CD34+: 10.84%
  - CD34+ with Macrophage: 19.6%

- **CXCR4**
  - CD34+: 39.02%
  - CD34+ with Macrophage: 57.9%

- **Tie2**
  - CD34+: 2.32%
  - CD34+ with Macrophage: 5.9%

CD34
Supplemental Fig. IV

A

Hybrid-dECFC

CD11b 0.1% 0% 0.1% 98.9%

CD14

CD45

CD144

B

Stem-dECFC

CD11b 0.2% 0.2% 0.2% 98.8%

CD14

CD45

CD144
Supplemental Fig. V

VEGF Fold increase

<table>
<thead>
<tr>
<th>VEGF</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybrid</td>
<td>-dECFC</td>
<td>Stem</td>
<td>-dECFC</td>
<td></td>
</tr>
</tbody>
</table>

**
Supplemental Fig. VI

A

0 Day
28 Day

Sham

Hybrid
-dECFC CM

Stem
-dECFC CM

B

Ratio of blood perfusion

Time (Days)

C

Sham
Hybrid
-dECFC CM

Stem
-dECFC CM

D

Distribution (%)

Limb Salvage
Foot Necrosis
Limb Loss

0
20
40
60
80
100

Sham
Hybrid-dECFC CM
Stem-dECFC CM

**
##
**
##
*
**
##
##

0
3
7
10
14
21
28

0.0
0.2
0.4
0.6
0.8
1.0

Sham
Hybrid-dECFC CM
Stem-dECFC CM

**
##
**
##
#
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/Flk-1, 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 × 10⁴ 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 Pharmlingen), 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 Pharmlingen), and anti-human CD45 (BD Pharmlingen). 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-diphenyl-tetrazolium 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 EBM-2 medium supplemented with 1% FBS and VEGF (100 ng/mL) or EBM-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% CO₂, 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 enhance 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 40x objective lens for 24 h. 4-amino-5-(4-chlorophenyl)-7-(t-butyl)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 × 10⁵ cells/mouse) to assess blood perfusion and capillary density, or via intravenous injection in the tail vein (1 × 10⁶ 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**