Vascular Incorporation of Endothelial Colony-Forming Cells Is Essential for Functional Recovery of Murine Ischemic Tissue Following Cell Therapy


Objective—Cord blood–derived human endothelial colony-forming cells (ECFCs) bear a high proliferative capacity and potentially enhance tissue neovascularization in vivo. Here, we investigated whether the leading mechanism for the functional improvement relates to their physical vascular incorporation or perivascular paracrine effects and whether the effects can be further enhanced by dual-cell–based therapy, including mesenchymal stem cells (MSCs).

Methods and Results—ECFCs or MSCs were lentivirally transduced with thymidine kinase suicide gene driven by the endothelial-specific vascular endothelial growth factor 2 (kinase insert domain receptor) promoter and evaluated in a hindlimb ischemia model. ECFCs and MSCs enhanced neovascularization after ischemic events to a similar extent. Dual therapy using ECFCs and MSCs further enhanced neovascularization. Mechanistically, 3 weeks after induction of ischemia followed by cell therapy, ganciclovir-mediated elimination of kinase insert domain receptor cells completely reversed the therapeutic effect of ECFCs but not that of MSCs. Histological analysis revealed that ganciclovir effectively eliminated ECFCs incorporated into the vasculature.

Conclusion—Endothelial-specific suicide gene technology demonstrates distinct mechanisms for ECFCs and MSCs, with complete abolishment of ECFC-mediated effects, whereas MSC-mediated effects remained unaffected. These data strengthen the notion that a dual-cell–based therapy represents a promising approach for vascular regeneration of ischemic tissue. (Arterioscler Thromb Vasc Biol. 2012;32:e13-e21.)

Key Words: angiogenesis ■ coronary heart disease ■ endothelium ■ ischemia ■ peripheral arterial disease

Diseases of the cardiovascular system remain the leading causes of mortality and still account for more deaths than cancer, chronic lower respiratory diseases, and accidents together.1 Regenerative medicine using stem and progenitor cells from different sources is a rapidly growing area of research aiming for repair or replacement of injured tissues. Some previous cell-based therapies using adult-derived stem and progenitor cells showed significant, albeit modest functional improvements and, therefore, did not fulfill high expectations.2 On the other hand, ethical controversy and immunologic barriers over pluripotent embryonic stem cells hindered their therapeutic application, whereas induced pluripotent cells are still in their infancy and require comprehensive characterization and complete depletion of teratogenic cells before their clinical use. Still, the future use of induced pluripotent cell–derived, patient-specific, highly proliferative ECFCs raises the question of potential security measures, such as the use of suicide genes to stop excessive proliferation.

To date, cell therapy based on the use of autologous endothelial progenitor cells (EPCs) still remains a safe, promising, and innovative therapeutic approach. These cells are capable of enhancing neovascularization after ischemic insults, including limb ischemia, acute myocardial infarction, and cerebral ischemia.3 Major limitations of patient-derived EPCs from peripheral blood represent their low number, modest proliferative and migratory capacity, and limited functional in vivo activity.4 Several approaches have been investigated to further enhance the effects of adult cell therapy, among these the combination of different cell sources5,6 and stimulation through various growth factors.7 Optimization of cell therapy can be achieved only by a comprehensive understanding of the underlying mechanisms contributing to neovascularization. Although basic principles of cell therapy in cardiovascular disease have been demonstrated,8,9 further insights regarding the relevance of the underlying mechanisms are still needed.

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Early endothelial progenitor cells have been shown to participate in ischemia-induced neovascularization via direct differentiation into endothelial as well as pericytic cells\textsuperscript{10–12} and stimulation of host stem cells through delivery of paracrine factors, such as vascular endothelial growth factor.\textsuperscript{13} In the present study, we used endothelial colony-forming cells (ECFCs) as a subset of endothelial progenitor cells with reportedly strong vasculogenic potential\textsuperscript{14} and mechanistically studied their contribution to neovascularization. For this purpose, we used the ganciclovir (GCV)-inducible herpes simplex 1 thymidine kinase (TK) suicide gene driven by the kinase insert domain receptor (KDR, or vascular endothelial growth factor receptor 2) promoter and linked to green fluorescent protein (GFP) as a reporter gene. Using this system, all endothelial-committed KDR\textsuperscript{+} cells can be tracked and, by administration of GCV, selectively eliminated. Moreover, we studied the salutary effects of single- versus dual-cell therapy using a distinct second cell type, namely mesenchymal stem cells (MSCs), alone or in combination with ECFCs, applying the same KDR promoter-based suicide gene approach.

**Methods**

**Human ECFCs**
ECFCs were derived from umbilical cord blood or adult peripheral blood and isolated as described previously.\textsuperscript{15}

**Human MSCs**
MSCs overexpressing human telomerase reverse transcriptase for their unlimited cell growth have been characterized previously and have also been named SCP1.\textsuperscript{16}

**Cloning of GFP-KDR-TK Into the Lentiviral Vector**
KDR-TK cDNA was subcloned in pLenti6/V5-DEST plasmid (Invitrogen, Darmstadt, Germany) by sticky SalI/XhoI ligation. Next, the cytomegalovirus–enhanced GFP cDNA from pLenti6-ikk-cytomegalovirus–enhanced GFP–opA plasmid was inserted into the pLenti6-KDR-TK plasmid by blunt end ligation, obtaining the pLenti6-GFP-KDR-TK plasmid.

**Transduction Efficacy for ECFCs and MSCs Using GFP-KDR-TK**
Successfully transduced ECFCs were selected with 10 \( \mu \)g/mL blasticidin (Invitrogen) for 10 days. Transduction efficiency was assessed by means of GFP expression using flow cytometry. Functional activity of TK was assessed using a cell proliferation assay following GCV treatment for 6 hours (MTS assay; Promega, Madison, WI).

**Hindlimb Ischemia Model**
A severe model of hindlimb ischemia was performed as previously described.\textsuperscript{7}

**Assessment of Perfusion via Magnetic Resonance Perfusion Imaging**
Magnetic resonance imaging experiments were performed on a clinical 3-Tesla whole-body magnetic resonance imaging system (Magnetom Verio, Siemens, Erlangen, Germany). Mice were examined using T1, T2, and time-resolved angiography with interleaved stochastic trajectories sequences. Magnetic resonance imaging perfusion data were analyzed using the perfusion software PMI 0.3.\textsuperscript{17}

**Immunohistochemistry**
Injected human cells were identified by double staining for human leukocyte antigen (Alexa Fluor 647-labeled, Biolegend, San Diego, CA) and lectin from Bandeiraea simplicifolia (fluorescein isothiocyanate labeled, Vector Laboratories, Burlingame, CA). Nuclei were stained with DAPI (Invitrogen), and images were analyzed using scanning confocal microscopy (Leica SP2).

For more information please see the Supplemental Materials and Methods, available online at http://atvb.ahajournals.org.

**Results**

**Phenotyping of MSCs and ECFCs by Flow Cytometry**
ECFCs strongly express the endothelial surface marker CD31 (92.2±2.8\%), as well as KDR (96.2±0.1\%), with the latter being a prerequisite for mediating the proangiogenic effects of vascular endothelial growth factor. Expression of the hematopoietic progenitor/endothelial cell marker CD34 was relatively strong in ECFCs (37.2±3.9\%) (Figure 1A and 1B). MSCs did not express endothelial markers such as CD31, CD34, and KDR (Figure 1B). Although CD31 expression in ECFCs remained stable during in vitro expansion, CD34 decreased during passage of the cells. When ECFCs were constantly passaged at 80% confluence, the fraction of CD34\textsuperscript{+} cells declined from 49.7\% at passage 2 to 3.8\% at passage 8, whereas expression of CD31 remained stable (Figure 1C). Therefore, we focused on the use of the first 3 passages for in vivo experiments.

**ECFCs Show a High Proliferative Capacity In Vitro**
To demonstrate the high and long-standing proliferative capacity of ECFCs, cells were kept in culture until senescence and their cell numbers were recorded and compared with peripheral blood ECFCs. Cells were regularly passaged at 80% confluence. ECFCs showed a significantly longer proliferative activity (\( P<0.05 \)) and also a significantly higher population number (\( P<0.05 \)) after 30 days of culturing (Figure 2A and 2B). After confirming the typical cobblestone appearance of ECFCs (Figure 2C), cell divisions were recorded by time-lapse microscopy to visually demonstrate the high proliferative capacities of ECFCs (Supplemental Video I).

**In Vivo Neovascularization Capacity of ECFCs and MSCs**
To assess the in vivo potential of these cells for vascular regeneration, we used a murine model of hindlimb ischemia. First, we studied the tissue distribution of infused ECFCs and MSCs over time. Although both cell types temporarily got trapped in the lung, ECFCs rapidly relocated to the ischemic hindlimb within 6 hours after injection (Figure 3A and 3C). In contrast, MSCs remained in the lung for an extended period of time, with completed relocation to the ischemic hindlimb after 24 hours following intravenous injection (Figure 3B). Immediately after induction of hindlimb ischemia, relative perfusion, assessed as laser Doppler-derived relative blood flow, had decreased to 5.7±0.8\%, indicating strong induction of limb ischemia. After 14 days, relative perfusion spontaneously increased to 25.6±1.9\% in the control group, whereas cell therapy using ECFCs and MSCs separately significantly enhanced recovery of relative perfusion (ECFCs 52.2±4.0\%; MSCs 47.9±2.7\%; both \( P<0.05 \) versus control). Even more importantly, combinational therapy using coinjection of ECFCs and MSCs further and significantly increased relative...
blood flow (70.2±5.8%; P<0.05 versus single treatment). These data suggest that combinational therapy using 2 complementary cell types, human endothelial and mesenchymal progenitor cells, is capable of generating enhanced treatment effects and subsequently leading to significantly better recovery of limb blood flow (Figure 3D).

Positive effects of cell therapy could also be observed by magnetic resonance perfusion imaging. In comparison to control

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Characterization of cord blood–derived human endothelial colony-forming cells (ECFCs) and human telomerase reverse transcriptase–modified mesenchymal stem cells (MSCs). A, Quantification of surface marker expression in ECFCs derived from 3 different donors (n=3–9 analyses). B, Representative cytometry plots for ECFCs and MSCs displaying the surface expression of CD31, CD34, and kinase insert domain receptor (KDR). C, Quantification of ECFC surface marker expression for CD31 and CD34 in relation to passage number. SSC indicates side scatter; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Proliferation kinetics of cord blood (CB)–derived human endothelial colony-forming cells (ECFCs). A, Proliferation of CB-derived ECFCs (n=3) as compared with peripheral blood (PB)–derived ECFCs (n=3) shown as ΔCB-PB population (×10⁶ cells). B, Number of days during which proliferation could be observed (each n=3). C, Representative image of an individual ECFC clone.
mice, mice treated with ECFCs or MSCs demonstrated a lower signal enhancement (SE) of contrast agent during time-resolved angiography with interleaved stochastic trajectories sequences (control: 147 466, ECFCs: 60 206; MSCs: 48 639), which was due to lower vascular permeability compared with the insufficient vessels of control mice (Figure 3E). Mechanistically, we were able to demonstrate a significant increase in smooth muscle actin–positive vessels (Supplemental Figure I) and an enhanced proliferation rate for murine endothelial cells (Supplemental Figure II) in mice receiving dual-cell therapy as compared with mice treated with either single-cell therapy. Moreover, we observed a strong increase in the expression of angiogenic factors in MSCs that were exposed to ECFC-conditioned medium in vitro (Supplemental Figure III).

**Lentiviral Transduction of ECFCs and MSCs With pGFP-KDR-TK**

To obtain further mechanistic insights on how ECFCs and MSCs, separately, enhance blood flow, we lentivirally transduced the cells with a plasmid containing GFP and the suicidal gene herpes simplex virus TK. GFP expression was regulated by a cytomegalovirus promoter, whereas expression of TK was controlled by the endothelial promoter KDR to allow for the specific elimination of all cells with an endothelial phenotype (Figure 4A). To facilitate a thorough analysis of functional mechanisms, high efficiency of transduction was mandatory. By means of resistance to blasticidin (10 μg/mL) and single GFP-transgenic cell picking, we were able to reach a purity of >99% for GFP in transduced ECFCs and MSCs, whereas KDR positivity for ECFCs was sustained (Figure 4B and 4C). To reach 100% purity of the in vivo administered cells, ECFCs and MSCs, separately, were sorted by fluorescence-activated cell sorting before injection, ensuring that indeed all cells with an endothelial phenotype could be eliminated by the suicide gene approach. Because the suicide gene TK is expressed under the control of the endothelial promoter KDR, only transduced cells expressing KDR would be eliminated in the presence of GCV as TK phosphorylates the nontoxic prodrug GCV. Endogenous kinases can then phosphorylate GCV-monophosphate to GCV-triphosphate resulting in DNA chain termination and single-strand breaks and subsequently induction of apoptosis. Indeed, when we measured induction of cell death using MTS assays,
our data confirm in vitro that only endothelial-committed cells such as ECFCs are affected by GCV, whereas MSCs or nontransduced cells remained unaffected (Figure 4D).

**ECFCs but Not MSCs Contribute to Neovascularization by Vascular Incorporation**

Next, we investigated whether and to what extent the 2 different progenitor cell types contribute to neovascularization by vascular incorporation. We injected intravenously either ECFC-GFP-KDR-TK or MSC-GFP-KDR-TK 24 hours after surgical induction of hindlimb ischemia. Relative limb perfusion was assessed by laser Doppler analysis using the O2C device on day 14 to demonstrate treatment response, followed by 7 days of intraperitoneal injections of either GCV or PBS, and another round of perfusion measurements on day 21. Measurement on day 14 confirmed a similar neovascularization-promoting effect for transduced ECFCs and MSCs, as shown in Figure 3D. Intriguingly, a significant drop in limb perfusion following infusion of GCV was detected only in animals treated with transduced ECFCs (ECFC-PBS 59±2.1% versus ECFC-GCV 35.4±5.2%; P<0.05). The resulting perfusion levels in mice treated with ECFC-GCV were similar to the relative perfusion level of untreated control mice on day 21 (control-PBS 31.4±4.4%; control-GCV 27.9±3.3%) (Figure 5A).

In contrast, treatment of mice that received transduced MSCs did not display any change in perfusion levels (MSC-PBS 60.4±2.9% versus MSC-GCV 63.6±3.2%). As further evidence for significant reduction of limb perfusion following elimination of ECFCs, laser Doppler imaging was used providing consistent data with the laser Doppler analysis obtained with the O2C device (Figure 5B). Next, we applied the same suicide approach to dual-cell therapy in vivo using either ECFC-GFP-KDR-TK along with nontransduced MSCs or vice versa. Indeed, the enhanced neovascularization achieved by dual-cell therapy was significantly attenuated following administration of GCV when ECFC-GFP-KDR-TK and nontransduced MSCs were used (Figure 5C and 5D). In contrast, for MSC-GFP-KDR-TK and nontransduced ECFCs, administration of GCV did not alter perfusion levels. These results demonstrate that GCV specifically eliminated ECFC-GFP-KDR-TK, whereas KDR-TK-transduced MSCs were not affected and therefore did not substantially differentiate into KDR−endothelial progeny.

Therefore, MSCs must have been contributed differently, most likely by pericytic integration or paracrine mechanisms. Indeed, histological evaluation of the harvested tissue revealed high numbers of human ECFCs per mm² (6.6±0.8) as indicated by human-specific HLA class I (Figure 6A–6C). Infused ECFCs had mainly incorporated into the vessel network. Specifically, on day 21 in the absence of GCV, 89.3±4.8% of myofibers in the
ischemic muscles were surrounded by vessels containing ECFCs, and 27.4±2.1% of total endothelial cells in the ischemic muscle were of ECFC origin. In mice treated with GCV for 7 days, only a very few cells per mm² had survived (0.67±0.3) and were rarely incorporated (0.44±0.17% of endothelial cells were of ECFC origin) (Figure 6A–6C).

**Discussion**

In the present study, using a KDR promoter linked to a TK suicide gene approach and histological analysis by confocal laser scanning microscopy, we mechanistically explored the effect of suicide gene-mediated disruption of ECFC progenitor cell therapy. We demonstrated that the effects of ECFC-based progenitor cell therapy in the setting of ischemic injury are mediated by direct vascular incorporation of KDR⁺ endothelial progenitor cells. Our in vivo data demonstrate functional impairment of relative perfusion in the ischemic limb after GCV-induced killing of KDR⁺ ECFCs as assessed by 4 different methods including laser Doppler imaging, O2C laser Doppler assessment, in vivo fluorescence imaging, and magnetic resonance angiography.

**Figure 5.** Human endothelial colony-forming cells (ECFC)–based and dual-cell therapy in the presence or absence of ganciclovir (GCV) in a murine model of hindlimb ischemia. A, Nuclear magnetic resonance imaging: nu/nu mice underwent hindlimb surgery and were treated with PBS (n=9), ECFC–green fluorescent protein (GFP)–kinase insert domain receptor (KDR)–thymidine kinase (TK) (n=10), or mesenchymal stem cell (MSC)-GFP-KDR-TK (n=9) 24 hours after surgery. On day 14, animals received either PBS or GCV for 7 days by intraperitoneal injection. Treatment with GCV induces selective cell death in cells harboring active KDR promoters. On day 21, relative hindlimb perfusion was measured via O2C (oxygen to see) laser Doppler analyzer. B, Representative laser Doppler images illustrating limb perfusion (HLI indicates limb with induced ischemia; control, nonischemic limb). The perfusion signal is subdivided into 6 different intervals, each displayed as a separate color. Low or no perfusion is displayed as dark blue, and the highest perfusion interval is displayed as red. C, Perfusion assessed by in vivo imaging system after dual-cell therapy in response to KDR-driven TK suicide gene expression. Shown is the quantitation of relative perfusion in nude mice treated with dual-cell therapy expressing the TK suicide gene in ECFCs but not in MSCs or vice versa. D, Representative pictures of mice injected with indocyanogreen, indicating perfusion following assessment by in vivo imaging system.
In the present study, we used ECFCs (also termed late EPCs) as a late outgrowing subset of early EPCs. Rehman et al. showed that early EPCs strongly express the monocyte marker CD14, as well as the leukocyte marker CD45, suggesting that these cells still bear a monocyte/macrophage phenotype. This could be related to the still strong heterogeneity of the cultured cells at the early time point of harvesting, insufficient endothelial commitment of the cells, or contamination with proinflammatory cells. Stimulated by ongoing discussions about the true identity of heterogeneous EPCs, Ingram et al. recently identified a distinct subpopulation of endothelial progenitor cells using a clonogenic assay as a functionally advanced isolation process. These cells were named ECFCs, were shown to contain clonally expanded cells, and are capable of undergoing multiple population doublings without senescence. Moreover, in contrast to these newly identified ECFCs, regular EPCs reproducibly failed to form functional vascular networks in collagen plugs. Therefore, instead of using peripheral blood-derived early EPCs, we used cord blood-derived ECFCs for the present study. Alternatively, for autologous cell therapy it is conceivable to use optimized patient-derived cells before their administration, as we have recently shown that ECFCs isolated from patients with diabetes can be functionally rescued by pretreatment with globular adiponectin (gAcrp). Cord blood–derived ECFCs have previously been reported to enhance neovascularization using in vivo hindlimb ischemia models. However, the underlying mechanisms have remained elusive to date. Therefore, we set out to selectively eliminate ECFCs 2 weeks after infusion into a hindlimb ischemia model to assess whether selective killing of KDR+ ECFCs would lead to disruption of a newly created vascular network and subsequent decline in limb perfusion. This would indicate physical incorporation of the cells. Lack of measurable decline of limb perfusion following GCV treatment would be most consistent with temporary paracrine modes of action, namely secretion of proangiogenic factors. In addition, alone or as dual therapy along with ECFCs, we used KDR− MSCs. GCV-induced abolishment of limb perfusion would indicate differentiation of KDR− MSCs into KDR+ cells incorporating into the host vasculature, whereas lack of decline in limb perfusion suggests temporary paracrine effects. Most likely these paracrine effects are required only in the early stages of the neovascularization process. Although several authors have proposed paracrine effects as the most important mechanism for augmentation of neovascularization, others have shown that physical incorporation plays a major role in the setting of EPC-based cell therapy using a ubiquitous promoter. Indeed, the ongoing controversy regarding the mechanisms of action for EPCs may again be related to the rather vague characteristics of early EPCs, as well as the still missing definition of the true stem/progenitor cell responsible for improved neovascularization. Here, our data for homogeneous, highly proliferative, and endothelial-committed ECFCs using an endothelial-specific
KDR promoter further corroborate the impact of physical incorporation as the driving mode of action for vascular cell therapy. Calculating the total number of ECFCs captured in the upper hindlimb reveals that approximately one third of the injected ECFCs can be found in the upper limb muscle within 24 hours of cell infusion. This high recruitment rate and the virtually complete abrogation of the ECFC-mediated therapeutic effect by a KDR–driven suicide gene approach provide compelling evidence for the functional incorporation of ECFCs into the vascular system. However, it remains surprising that a relatively small number of cells is actually capable of generating such a strong therapeutic effect, even when taking into account that the homed ECFCs will further expand during the subsequent days and weeks.

To show the mechanism of angiogenesis in bone marrow cell therapy, Yoon et al30 used the suicide model TK linked to endothelial-, smooth muscle–, or cardiac-specific promoters and demonstrated that predominant differentiation into both endothelial and smooth muscle cells contributed to improved cardiac function after induction of myocardial infarction. Although in this study intracelullar endothelial nitric oxide synthase was used as an endothelial-specific promoter, the TK suicide gene in our system was driven by the endothelial cell surface marker KDR (vascular endothelial growth factor receptor 2) promoter. Whereas the study of Yoon et al investigated differentiation of a heterogeneous pool of bone marrow cell into endothelial cells, we analyzed the perfusion-enhancing mechanism of a pure population of endothelial-committed ECFCs.

Because we and others14 had already demonstrated that ECFCs represent a potent and homogeneous cell population most suitable for stem cell-based therapy, we decided to examine the contribution of ECFCs to improve perfusion in the setting of tissue ischemia and could clearly exhibit their functional incorporation as endothelial cells.

For the present study, we also used well-characterized MSCs16 as a distinct cell source for regeneration of ischemic tissue. These cells were picked from individual single clones and overexpress telomerase, therefore composing a stable, homogeneous, and fast growing population most suitable for mechanistic studies.16 As isolation of MSCs from the bone marrow can be a rather painful procedure, bears infectious risks for the patient, and usually gives rise to only a small percentage of MSCs known to proliferate rather slowly, this alternative and unlimited source of MSCs may indeed prove useful for clinical applications, which usually require large numbers of cells. Certainly this would require further preclinical tests to exclude safety risks related to overexpression of telomerase.31 MSCs have been reported to differentiate in vitro into various cell types, including chondrocytes, adipocytes, and osteoblasts,32 but there is also increasing evidence that these cells also harbor proangiogenic potential.33 Indeed, a first phase 1 trial using allogeneic adult human MSCs after acute myocardial infarction has recently been initiated.34 Although this clinical study was rather designed to assess feasibility and safety, MSC-based cell therapy has already been shown to be superior to placebo treatment.

Although some investigators have described paracrine effects as the driving mechanism for the observed improvements in tissue perfusion,35–37 others observed pericytic engraftment as an essential mechanism.6,38 Interestingly, Melero-Martin et al6 reported that in a Matrigel plug model, MSCs differentiated into smooth muscle cells only after coinplantation with endothelial progenitor cells, whereas neither MSCs nor endothelial progenitor cells alone were able to adopt a smooth muscle cell phenotype. These data suggest that ECFC-based cell therapy might also profit from simultaneous MSC administration by smooth muscle cell-enhanced vascular stability. Using ECFCs and MSCs in our study, we could clearly demonstrate that these distinct cell types were able to enhance neovascularization individually, but their combined use resulted in further enhanced recovery of blood flow. Studying the expression of putative paracrine factors in MSCs exposed to ECFC-conditioned medium, we observed upregulation of a wide range of cytokines involved in tissue neovascularization. Based on our previous results demonstrating a concerted action of a diverse set of cytokines in the process of homing and vascular incorporation of ECFCs, this reciprocal activation of the 2 cell populations could represent an important mechanism for enhancing response to cell-based therapy.7 Although future studies will need to further dissect this intricate interplay of MSCs and ECFCs in vivo by the use of independently inducible suicide genes in ECFCs and MSCs, we here were able to demonstrate distinct mechanisms for ECFCs and MSCs with complete abolishment of ECFC-mediated effects, whereas MSC-mediated effects remained unaffected. These data strengthen the notion that a dual-cell–based therapy represents a promising approach for vascular regeneration of ischemic tissue to improve the clinical course of patients with cardiovascular diseases.

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Disclosures

None.

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In the article by Schwarz et al, which appeared in the February 2012 issue of the journal *Arterioscler Thromb Vasc Biol*. 2012;32:e13-e21. DOI: 10.1161/ATVBAHA.111.239822, a correction was needed.

The fourth author’s name was spelled incorrectly. The correct name is Iker Rodriguez-Arabaolaza.

The authors apologize for the error.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/32/2/e13.
Supplementary Figure 1: Expression of smooth muscle cell antigen (SMA) in ischemic hindlimbs of nude mice treated with ECFC, MSC, or dual therapy with ECFC and MSC, measured by histological analysis two weeks after induction of ischemia.
Supplementary Figure 2: Proliferation of host-derived endothelial cells. Proliferation was assessed by Ki67 expression in the nuclei of murine CD31+ endothelial cells in consecutive sections of hindlimbs of nude mice treated with ECFC, MSC, dual therapy with ECFC and MSC, or ganciclovir (GCV) measured by histological analysis two weeks after induction of ischemia.
Supplementary figure 3: Expression of pro-angiogenic growth factors in mesenchymal stem cells (MSC) in the presence of endothelial colony forming cell (ECFC)-derived conditioned medium (SN) measured by QPCR.
**Supplement Material**

**Human endothelial colony forming cells (ECFC).** Umbilical cord blood was obtained after informed consent has been given. The study was approved by the Medical Faculty Ethics Board of the University of Munich. ECFC were isolated as described previously [15]. Briefly, mononuclear cells were isolated from human cord blood by density-gradient centrifugation with Ficoll separating solution (Pancoll, Pan Biotech, Aidenbach, Germany). After isolation, 8-12 x 10^6 mononuclear cells were plated in 6-well tissue culture plates precoated with type 1 rat-tail collagen (Becton Dickinson Labware, Bedford, MA) and cultured with endothelial basal medium-2 supplemented with 10% FBS and EGM-2 SingleQuots (hEGF, hFGF-B, VEGF, ascorbic acid, hydrocortisone, Long R3-IGF-1, heparin, gentamicin/amphotericin) (Lonza, Walkersville, Maryland). After 24 hours, non-adherent cells and debris were removed. For the following 7 days, medium was changed daily then every other day until colonies appeared. Colonies were picked and further expanded for experiments.

**Human mesenchymal stem cells (MSC).** MSC overexpressing human telomerase reverse transcriptase (hTERT) for their unlimited use have been characterized previously and are named SCP1 [16]. Briefly, MSCs from human bone marrow aspirates (Cambrex Corporation, East Rutherford, NJ) were lentivirally transduced with the cDNA hTERT for immortalization. MSC were cultured in α-MEM medium (Invitrogen, Darmstadt, Germany) supplemented with 10% FCS and 1% Penicillin/Streptomycin and medium was changed every third day.

**Population doubling curves.** To determine proliferation capacity of cord blood-derived ECFC, cells were kept in culture until no further expansion was detectable and cell numbers were counted at 80% confluence.

**Flow cytometry.** Surface expression of CD31, CD34, and vascular endothelial growth factor receptor 2 (VEGF-R2 = KDR) was assessed by flow cytometry. Cells were incubated for 15 minutes with Flebogamma (Grifols, Langen, Germany) to prevent unspecific binding of antibodies. Subsequently,
cells were stained using antibodies against CD31-FITC or APC (Becton Dickinson, BD), CD34-PE (BD), and biotinylated KDR (Relia Tech, Braunschweig, Germany) with Streptavidin-APC (BD) as secondary antibody. Isotype-identical antibodies served as controls. Each analysis included 50,000 gated events. Samples were analyzed using the FACSCalibur and FACS Canto II devices. Data were analyzed with CELLQuest-Pro and FACS DIVA software (both BD).

*Cloning of GFP-KDR-TK into the lentiviral vector.* KDR-TK cDNA (kindly provided by Dr. Ying Wang, Department of Medical Ultrasonics, The First Affiliated Hospital, Sun Yat-Sen University, China), was subcloned in pLenti6/V5-DEST plasmid (Invitrogen) by sticky SalI/XhoI ligation. The correct sequence of the resulting pLenti6-KDR-TK was confirmed by digestion and sequencing (Sequiserve, Vaterstetten, Germany). Next, the CMV-eGFP cDNA from pLenti6-IKK-CMV-eGFP-opA plasmid was inserted into pLenti6-KDR-TK plasmid by blunt end ligation obtaining pLenti6-GFP-KDR-TK plasmid. The correct sequence of the resulting pLenti6-GFP-KDR-TK was confirmed by digestion and gel electrophoresis.

*Lentiviral transduction of target cells.* The ViraPower lentiviral expression system™ (Invitrogen) was used for lentivirus production according to the manufacturer’s instructions. The DNA-Lipofectamine 2000 complexes were added to a T-225 tissue culture flask containing 293FT cell suspension (24 x 10^6 total cells). Forty-eight hours after transfection, the virus containing supernatant was harvested. The viral stocks were stored in aliquots at -80°C.

**Transduction of ECFCs and MSC with GFP-KDR-TK lentivirus.** Briefly, viral supernatant was added in a 1:2 dilution in normal growth media in the presence of 8 µg/ml polybrene (hexadimethrine bromide, Sigma, Munich, Germany). One day after transduction, the medium was replaced by fresh, nonviral medium. Finally, the cells were tested for a presence of viral particles by using HIV-1 p24 ELISA kit (PerkinElmer, Wiesbaden, Germany). Only virus-free cells were transfer to S1-laboratory and used in the following experiments.
**Transduction efficacy for ECFC and MSC using GFP-KDR-TK.** Successfully transduced ECFC were selected with 10 µg/ml blasticidin (Invitrogen) for 10 days. Eventually, we picked single GFP-transgenic cells to generate GFP-transgenic clones. Since MSC were already lentivirally transduced and selected with blasticidin at that time, single green fluorescent cells were picked and expanded. The purity of the obtained cell populations was assessed by means of GFP expression using flow cytometry. Functional activity of thymidine kinase was assessed by MTS assays following ganciclovir treatment for 6 hours (Promega, Corporation, Madison, WI).

**Hind limb ischemia model.** Female NMRI nu/nu mice, aged 8 to 10 weeks and weighing 25 g were purchased from Janvier, Le Genest-Saint-Isle, France. All animal experiments were conducted in accordance with institutional guidelines and were approved by the Administrative Panel on Laboratory Animal Care (Government of Upper Bavaria, Germany). For induction of unilateral hind limb ischemia, mice were anesthetized by intraperitoneal injection with a combination of ketamine (150 mg/kg) and xylazine (15 mg/kg). The right femoral artery and vein, including the superficial and the deep branch as well as the superficial epigastric and the external pudenda, were occluded with an electric coagulator. This procedure results in postoperative blood flow levels that are close to the detection limit of the utilized devices (5.69±2.39% relative blood flow) and represents a model of critical limb ischemia. Twenty-four hours after inducing hind limb ischemia, either 5 x 10^5 ECFC or MSC if administered as single cell therapy or 2.5 x 10^5 for each cell type if administered in combination, were intravenously injected. Limb perfusion was assessed post surgery and again after 2 weeks using the O2C laser Doppler blood flow analyzer (Lea Medizintechnik, Giessen, Germany). Relative perfusion was calculated as the ratio of blood flow in the ischemic and the healthy hind limb. After the two-week assessment, in case transduced cells were used in the experiment, mice received 40 mg/kg bodyweight ganciclovir (Hoffmann-La-Roche AG, Grenzach-Wyhlen, Germany) intraperitoneally for seven days and finally limb perfusion was re-measured via O2C laser Doppler.

**Assessment of perfusion via magnetic resonance perfusion imaging (MRI).** MRI experiments were performed on a clinical 3-Tesla whole-body MRI system (Magnetom Verio, Siemens, Erlangen,
Germany) equipped with 32 parallel receiver channels (gradient system: 45mT/m and 200T/m/s). A dedicated small animal whole-body coil for a typical mouse (up to 30 g) was utilized (Rapid Biomedical, www.rapidbiomed.de) covering a length of 80 mm and with an inner diameter of 35 mm. The 2D coil array consisted of 8 elements arranged in two rings of 4 elements each with an average element size of 40 x 29 mm². Mice were anaesthetized and examined with several morphologic and functional pulse sequences, including T1-weighted MRI with isotropic spatial resolution of 0.3 x 0.3 x 0.3 mm³ pre and post administration of a Gadolinium contrast agent. Additionally, proton-density- and T2-weighted MRI with and without parallel imaging was carried out. Contrast media injection was carried out via tail vein; gadolinium DTPA (Gd-DTPA,-BMA, Gadodiamide, 0.1 mmol/kg body weight, Omniscan, Amersham Health, Princeton, NJ) was utilized. A pulse sequence (TWIST: time resolved angiography with interleaved stochastic trajectories) with high time resolution was carried out for contrast enhanced perfusion imaging. This DCE sequence with rapid image acquisition acquired image slices through the selected muscle region with a total acquisition time of 32 min. The entire MRI data collection period lasted about 60 min. MRI perfusion data were analyzed utilizing the perfusion software PMI 0.3 [17].

Assessment of perfusion by in vivo fluorescence imaging. We performed in vivo fluorescence imaging by intravenous injection of the near infrared fluorophore indocyanine green (ICG; Sigma-Aldrich). Images were obtained using an In Vivo Imaging System (IVIS)-200 (Caliper Life Sciences, Hopkinton, MA, USA) and analyzed using the Living Image™ 3.2 software.

Immunohistochemistry. Adductor muscles of the ischemic limbs were harvested for histological analysis. Injected human cells were identified by double staining for human HLA (Alexafluor 647-labeled; Biolegend, San Diego, CA) and lectin from Bandeiraea simplicifolia (FITC-labeled; Vectorlabs, Burlington, CA). Nuclei were stained with DAPI (Invitrogen) and images were analyzed using scanning confocal microscopy (Leica SP2).

Statistical analysis. Results for continuous variables are expressed as means ± SEM. Overall comparison of the treatment groups was performed with the Kruskal-Wallis test followed by post-hoc
pair-wise comparison using the Mann-Whitney test. P values < 0.05 were considered statistically significant. All analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, Illinois).