Angiogenic Mechanisms of Human CD34+ Stem Cell Exosomes in the Repair of Ischemic Hindlimb


Rationale: Paracrine secretions seem to mediate therapeutic effects of human CD34+ stem cells locally transplanted in patients with myocardial and critical limb ischemia and in animal models. Earlier, we had discovered that paracrine secretion from human CD34+ cells contains proangiogenic, membrane-bound nanovesicles called exosomes (CD34Exo).

Objective: Here, we investigated the mechanisms of CD34Exo-mediated ischemic tissue repair and therapeutic angiogenesis by studying their miRNA content and uptake.

Methods and Results: When injected into mouse ischemic hindlimb tissue, CD34Exo, but not the CD34Exo-depleted conditioned media, mimicked the beneficial activity of their parent cells by improving ischemic limb perfusion, capillary density, motor function, and their amputation. CD34Exo were found to be enriched with proangiogenic miRNAs such as miR-126-3p. Knocking down miR-126-3p from CD34Exo abolished their angiogenic activity and beneficial function both in vitro and in vivo. Interestingly, injection of CD34Exo increased miR-126-3p levels in mouse ischemic limb but did not affect the endogenous synthesis of miR-126-3p, suggesting a direct transfer of stable and functional exosomal miR-126-3p. miR-126-3p enhanced angiogenesis by suppressing the expression of its known target, SREPD1, simultaneously modulating the expression of genes involved in angiogenic pathways such as VEGF (vascular endothelial growth factor), ANG1 (angiopoietin 1), ANG2 (angiopoietin 2), MMP9 (matrix metalloproteinase 9), TSP1 (thrombospondin 1), etc. Interestingly, CD34Exo, when treated to ischemic hindlimbs, were most efficiently internalized by endothelial cells relative to smooth muscle cells and fibroblasts, demonstrating a direct role of stem cell–derived exosomes on mouse endothelium at the cellular level.

Conclusions: Collectively, our results have demonstrated a novel mechanism by which cell-free CD34Exo mediates ischemic tissue repair via beneficial angiogenesis. Exosome-shuttled proangiogenic miRNAs may signify amplification of stem cell function and may explain the angiogenic and therapeutic benefits associated with CD34+ stem cell therapy. (Circ Res. 2017;120:1466-1476. DOI: 10.1161/CIRCRESAHA.116.310557.)

Key Words: cardiovascular disease ■ cell transplantation ■ exosomes ■ hindlimb ■ ischemia ■ microRNA ■ stem cells

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In our earlier study, we have established a novel mechanism that human CD34+ cells secrete membrane-bound nanovesicles called exosomes (ie, CD34Exo) that mediate most of the proangiogenic paracrine activity of the cells. We have shown that the exosomes secreted by CD34+ cells were similar to exosomes described in previous reports—in their morphology, in size and shape, in expressing known exosomal protein markers by which the paracrine factors induce vessel growth and functional recovery post-ischemia remain largely undefined.
Novelty and Significance

What Is Known?

• Human CD34+ stem cells have been shown to induce therapeutic neovascularization in preclinical studies and in phase I, II, and III clinical trials.
• One of the key mechanisms by which CD34+ cells induce neovascularization seems to include paracrine secretion.
• Exosomes, secreted nanovesicles, are one of the major components of CD34+ cell paracrine secretion, which have independent angiogenic activity, both in vitro and in vivo.

What New Information Does This Article Contribute?

• Exosomes from CD34+ cells induce therapeutic angiogenesis that are mediated by exosomal angiomiRs such as miR-126-3p.
• Exosomes from CD34+ cells are most efficiently internalized by endothelial cells in the ischemic tissue to induce cell cycle induction, angiogenesis, and proliferation.
• Exosome-shuttled angiomiRs may signify amplification of stem cell function and may explain the angiogenic and therapeutic benefits associated with CD34+ stem cell therapy.

In this study, we addressed a key undefined paracrine mechanism by which human CD34+ stem cells mediate their beneficial effects. Our data show that exosomes, a major component of the paracrine secretion from CD34+ cells, stimulate beneficial angiogenesis in a mouse model of hindlimb ischemia. CD34+ exosomes were selectively enriched with several angiomiRs that are distinct from miRs in nontherapeutic exosomes from CD34− mononuclear cells. Another key discovery is the first evidence of an in vivo trafficking mechanism of CD34+ exosomes and exosome-shuttled angiomiRs. Both of these findings have both scientific and clinical importance. It will lead to a deeper understanding of the mechanisms of stem cell exosomes trafficking and function on one hand and therapeutic interventions on the other.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AngiomiRs</td>
<td>Proangiogenic miRNAs</td>
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<tr>
<td>CD34Exo</td>
<td>CD34+ cell–derived exosomes</td>
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<tr>
<td>CM</td>
<td>Conditioned media</td>
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<tr>
<td>HLI</td>
<td>Hindlimb ischemia</td>
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<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>MNCs</td>
<td>Mononuclear cells</td>
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<td>SPRED1</td>
<td>Sprouty-related, EVH1 domain-containing protein 1</td>
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and in expressing CD34+ cell–specific CD34 protein maker on their surface. Moreover, CD34Exo mimicked the function of their parent cells, at least in part, and induced angiogenic activity both in vitro and in vivo. Exosomes from several different cell types have been shown to carry and transfer selective cytosolic components such as proteins, lipids, and nucleic acids to communicate with cells at the vicinity or at a distance, altering their function. Interestingly, the unique cargo of exosomes is often distinct from the cell of their origin, although they are also known to carry selective cell-specific signature molecules such as parent cell–specific surface proteins or disease-specific signature proteins originating from the parent cells.

In recent parallel investigations, role of exosomes as a mediator of cardiac communication among different cell types in the heart has been studied extensively. Both human and mouse stem and progenitor cell–derived exosomes have been shown to augment myocardial function post-ischemia. Remarkably, cardiac progenitor cell–derived exosomes isolated from neonatal patients were found to have higher regenerative potential for cardiac tissue repair compared with cardiac progenitor cell exosomes from older children. Moreover, expression of certain exosomal cargo, such as miR-126, was significantly lower under high-glucose or diabetes mellitus conditions in human CD34+ exosomes, indicating that the exosomal cargo is dependent on the physiological condition of the cell of their origin. Recent research highlights significant potential of exosomes derived from pericardial fluid and plasma obtained from human heart failure patients to induce therapeutic angiogenesis. In contrary to the beneficial effects shown by the stem cell exosomes, cardiac fibroblast–derived exosomes were shown to disseminate the damaging effects of cardiac remodeling by transferring miR-21* as a paracrine signaling mediator of cardiac hypertrophy. Although most of the current cardiovascular exosome studies have examined the RNA and miRNA content and function, the in vivo uptake mechanisms of exosomes and exosomal miRNAs are largely undefined.

In this study, we have investigated the role of CD34Exo in the beneficial angiogenesis associated with CD34+ cell therapy after ischemic injury by (1) exploring whether CD34Exo induce beneficial effects in the absence of cells or other paracrine factors, (2) identifying the molecular components responsible for the angiogenic and therapeutic function of CD34Exo, (3) studying the in vitro and in vivo uptake mechanisms of CD34Exo.

Methods

Cell Culture and Exosome Isolation

CD34+ cells and the CD34+ cell–depleted mononuclear cells (MNCs) were obtained from Baxter Healthcare Corporation (Deerfield, IL). The cells were purified from mobilized peripheral blood mononuclear cells (AllCells LLC, Emeryville, CA) with an Isolex 300i device (Baxter Healthcare), cell purity (85% to 95%) was determined via flow cytometry. Both CD34+ cells and MNCs (250000 cells/mL) were cultured in X-VIVO 10 serum-free cell culture medium (Lonza, Inc, Allendale, NJ), and ultrapure exosomes were isolated from the conditioned media (CM) by ultracentrifugation and separated from the soluble protein fraction on a sucrose gradient. The exosomes from both cell types were characterized using dynamic light scattering, flow cytometry analysis, electron microscopy, and immunoblotting as described before. Human umbilical vein endothelial cells (HUVECs; Lonza) were maintained in endothelial growth medium-2 (EGM-2; Lonza, Inc) and starved in EBM-2 medium containing 0.25% fetal bovine serum for 24 hours before cell assays were performed.

Mouse Hindlimb Ischemia Model

All animal protocols were approved by the AAALAC-accredited Northwestern University and Icahn School of Medicine at Mount...
Sinai Animal Care and Use Committee. Immunocompromised BalbC mice (8–10 weeks old) were anesthetized with Isoflurane delivered at ≈2%. A ligation was made around the femoral artery, and all arterial branches were removed. A small segment of the artery was then dissected free. Post-ischemia, mice were randomly assigned to receive intramuscular injections of PBS, CD34+ cells, CD34+ cell CM, CD34+ exosomes, CD34+ exosome-depleted CM, or MNC exosomes (MNCExo; all derived from equal number of cells; treatment dose: 5×10⁶ cells/kg). All treatments were injected directly into the ischemic hindlimb in a 20 µL volume and injected at 4 different locations immediately after the surgery.

**Laser Doppler Perfusion Imaging and Scoring for Limb Functional Recovery**

For laser Doppler measurements of the ischemic and control limbs, animals were anesthetized with isoflurane (2%), and laser Doppler perfusion imaging measurements were taken at 7, 14, 21, and 28 days after the hindlimb ischemic surgery. At each time point, tissue perfusion was measured via laser Doppler perfusion imaging, measuring blood flow in both the ischemic and nonischemic limb and reporting results as the ratio of these 2 measurements. Ischemic and nonischemic tissues were harvested at day 28 for histological analyses. Before euthanasia, the mice were injected with 50 µg of BS-1 lectin to identify the mouse vasculature. In addition, motor function and tissue damage was semiquantitatively assessed on postoperative days 7, 14, 21, and 28 by established scoring systems.

**Immunohistology for Quantification of Capillary Density**

At day 28, muscle tissue from the ischemic limb was harvested, fixed in methanol, paraffin embedded, and cross-sectioned (6 µm) for histological immunostaining. Briefly, sections were blocked with 10% donkey serum (30 minutes; room temperature). Tissue slices were stained with primary antibody (Vector Laboratories, Burlingame, CA) against BS-1 lectin, an endothelial cell marker, for 1 hour at 37°C. AlexaFluor-conjugated secondary antibody (Invitrogen Corporation, Carlsbad, CA) was then added and nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories). BS-1 lectin-positive cells were imaged and quantified using fluorescent microscopy (Zeiss).

**miRNA Quantification**

Total RNA from the CD34+ cells, CD34+-depleted MNCs, and their respective exosome preparations were extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol (including a DNase step). Taqman small RNA assay and Taqman quantitative real-time polymerase chain reaction (qRT-PCR) were used to assess

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![Figure 1](http://circres.ahajournals.org/)  
**Figure 1.** CD34+ cell–derived exosomes (CD34Exo) improve revascularization and functional benefits in mouse hindlimb ischemia (HLI).  
A. Day 28 of mouse ischemic hindlimb treated as indicated. B. Day 28 laser Doppler perfusion imaging (LDPI) images showing severely restricted limb perfusion in blue and normal limb in red. C. Quantification of LDPI perfusion. D. Limb motor function and (E) salvage scores of the ischemic limb (in a scale of 1–5; 1-poor and 5-strong); n=10 to 21; *P<0.05.
miRNA and mRNA expressions, respectively. miRNA and mRNA expressions were normalized to U6 or 18s rRNA, respectively.

**miRNA Microarray**

miRNA profiling was completed at Asuragen using Affymetrix miRNA microarrays, and data analysis was performed by Asuragen. CD34+ cells, CD34Exosomes, MNCs (depleted of CD34+ cells), and MNCExo—all isolated from the same donor—were processed and compared for 1100 known human miRNAs. At least 3 healthy donors were used for the experiments (n=3). RNA was isolated as before. The expression level signals were scaled in GCOS 1.2 to give a median intensity of 100. This was done to enable different arrays to be compared. The program Spotfire DecisionSite 8.2 (www.spotfire.com) was used for gene-profiling analysis.

**Locked Nucleic Acid–Mediated miRNA Knockdown**

Anti-miR 126-3p or scrambled control locked nucleic acid (Ambion, Carlsbad, CA) was transfected into CD34+ cells using Lipofectamine RNAiMAX (Invitrogen Corporation) for 16 hours. Cells were washed 3 times and replated in fresh media to remove any external miRs. Cells were incubated for exosome secretion for 40 hours. Exosomes were isolated using the protocol described before and used in an in vitro Matrigel tube formation assay using HUVECs to evaluate their angiogenic properties. Mouse hindlimb ischemia model was used to compare the therapeutic efficacy of miR-126 knockdown (KD) exosomes with the control scrambled miR exosomes. Proangiogenic mir-126-3p expression in both the cells and exosomes were quantified using Taqman qRT-PCR as described before.

**In Vitro Matrigel Tube Formation Assay**

HUVECs (2.0x10^4, serum-starved overnight) were mixed with either PBS (control), or control scrambled CD34Exo or miR 126-3p-KD CD34Exo collected from the CM of 2.0x10^4 CD34+ cells—and seeded into 48-well plates. The plates were previously coated with 150 µL of growth-factor–reduced Matrigel (BD). Tube formation ability of control or exosome-treated HUVECs was examined by phase-contrast microscopy ±2 to 4 hours later. Each condition in each experiment was assessed at least in duplicates. Tube length was measured as the mean summed length of capillary-like structures (>6 cell long) per well, per high-power field using ImageJ software.

**Exosome Treatment and qRT-PCR of the Hindlimb Ischemia Muscle Tissue**

Hindlimb ischemia (HLI) was induced in mice as described before and randomized to receive treatments of PBS control or CD34+ exosome–scrambled RNA or CD34+ exosome–anti–miR-126-3p in the ischemic limb, 2 injections of 10 µL each, flanking the ischemic tissue. A small piece of muscle flanking the treatment areas was dissected after certain time points. Total RNA was isolated, and expression of miR-126-3p, mouse pri-126, mouse SPRED1 (sprouty-related, EVH1 domain-containing protein 1), and mouse VEGF (vascular endothelial growth factor) and other mRNAs were determined as described before.

**Live Confocal Imaging and Flow Cytometry Analysis of Cy3-Exo–Treated HUVECs and Ischemic Tissue**

Cy3 tagged miR or untagged scrambled control (Ambion) was transfected into CD34+ cells using lipofectamine RNAiMAX (Invitrogen Corporation) for 16 hours. Cells were washed 3 times and replated in fresh media to remove any external miRs. Cells were incubated for exosome secretion for 24 to 40 hours. Intact exosomes were RNase treated at 37°C for 10 minutes to remove any external or surface-bound Cy3. RNase activity was stopped by RNase inhibitor. Exosomes were washed with excess PBS, pelleted, resuspended in PBS, applied to HUVECs (that were plated on coverslips), and imaged immediately using live confocal microscopy.

For analysis of fluorescent-treated ischemic tissue, the exosomes were isolated from a stable dye (rhodamine-PE)–treated CD34+ cells. Ischemic tissues, treated with CD34Exo, were harvested at different time points and immediately processed for histology or flow cytometry analyses. For flow cytometry analysis, tissues were digested into single-cell suspension using collagenase digestion (0.5 mg/mL; 20 minutes at 37°C). The cells were first stained with a live–dead kit, fixed (with 4% paraformaldehyde), washed, and then stained with a cocktail of primary/secondary antibodies specific for endothelial cells or smooth muscle cells or fibroblasts. The stained cells were analyzed by flow cytometry (BD LSR Fortessa with 6 lasers; 16-color analysis, CA). For immunofluorescence studies, mice were injected with lectin (Vector Laboratories; injected by tail-vein injections), hindlimb ischemic limbs, 2 injections of 10 µL each, flanking the ischemic tissue. A small piece of muscle flanking the treatment areas was dissected after certain time points.
tissues were processed immediately after harvesting, stained for lecin, and imaged using Nikon A1R laser scanning confocal microscopy.

Quantified results are presented as mean±SEM; comparisons between groups were evaluated with Student t test or ANOVA, and \( P<0.05 \) was considered significant. Detailed methods for all protocols used in the article are provided in the Online Data Supplement.

**Results**

**Cell-Free CD34Exo Enhance Therapeutic Recovery and Vascular Angiogenesis in a Mouse Model of HLI**

To evaluate the therapeutic efficacy of CD34Exo, we isolated and purified CD34Exo as described previously. Using dynamic light-scattering analysis, we confirmed that isolated CD34Exo fraction is devoid of contaminating protein aggregates and larger extra cellular vesicles (Online Figure I). CD34Exo-depleted CM, MNCExo, or PBS was used as negative control. To study beneficial effects of CD34Exo on tissue repair post-HLI, we created mouse models by ligating femoral artery as described before. We then injected the ischemic limbs with PBS, CD34+ cells, CD34-CM, CD34Exo, CD34Exo-depleted CM, or MNCExo—all isolated from equal number of cells (treatment dose: 5×10^6 cells/kg). Mice treated with CD34+ cells, CD34Exo, or CD34-CM had robust improvements in tissue necrosis, tissue perfusion, limb motor function, and limb salvage compared with controls (Figure 1A through 1E). Interestingly, we observed that the ischemic limbs injected with CD34 Exo, but not with MNCExo, were retaining their limbs and overall muscle mass, which were evident as early as day 7 post-HLI/treatment (Online Table IA and IB). Quantification of limb tissue microcapillary density indicated significant increase in the mice treated with CD34+ cells, CD34Exo, or CD34-CM (Figure 2A and 2B), suggesting enhanced angiogenesis. Interestingly, CD34-CM depleted of CD34Exo (CD34Exo-depleted-CM) lost its beneficial effects (Figures 1 and 2), suggesting that the exosomes in CD34-CM provide most, if not all, of the beneficial effects. Together, these data suggest that cell-free CD34Exo have independent therapeutic activity similar to that of CD34+ cells and that CD34Exo induces angiogenesis.

**CD34Exo Is Enriched With Proangiogenic miR-126-3p and Loss of miR-126-3p Attenuates CD34Exo-Mediated Angiogenesis**

To identify factors responsible for CD34Exo-induced beneficial effects and therapeutic angiogenesis, we examined the exosomal protein and RNA content. Exosomes function as mediators of cell–cell communication by selectively carrying biologically active molecules in the form of proteins and miRNAs. To this end, we extracted protein from CD34Exo and blotted using human angiogenesis protein array that contains known proteins with angiogenesis function (Online Figure II). Angiogenic protein profiling indicated no significant enrichment of proteins in CD34Exo as compared with MNCExo (Figure 3A and 3B). We then isolated RNA from angiogenic CD34Exo and performed microarray profiling of miRNAs comparing that to control nonangiogenic MNCExo. To avoid contaminating RNAs sticking to outside of exosomal membranes, we performed RNase A digestion of intact exosomes...
without altering the quantity or quality of the isolated RNA (Online Figure III). Furthermore, analysis of total exosomal RNA confirmed that CD34Exo was mostly enriched with small RNAs and miRNAs as compared with CD34+ cells (Online Figures IV and V). miRNA microarray analysis revealed that CD34+ cells and CD34Exo had strong similarity in their miRNA composition and had significantly greater expression of several proangiogenic miRNAs (angiomiRs) as compared with MNCs and MNCExo (Figure 3C; Online Figure XI). We determined the most enriched miRNAs in CD34+ cells and MNCs and those in CD34Exo and MNCExo (Online Table II). Interestingly, the exosomes from both cell types were distinctively enriched with certain miRNA species of unknown function compared with the cells and vice versa (Online Figure XII and Table II). The exosome-specific miRNAs may suggest selective packaging of exosomal miRNAs.

**Figure 4.** Loss of miR-126 in CD34+ cell–derived exosomes (CD34Exo) results in loss of its angiogenic function in vitro and in vivo. A, miR-126 expression in CD34+ cells or exosomes isolated from CD34+ cells, treated as indicated (n=3 to 6). B, Tube formation of human umbilical vein endothelial cells on Matrigel, treated as indicated; *P<0.05, n=3. C, Experimental scheme illustrating CD34Exo injection to ischemic (I) or nonischemic (NI) hindlimbs. D, Day 28 of mouse ischemic hindlimb treated as indicated and laser Doppler perfusion imaging (LDPI) images showing severely restricted limb perfusion in blue and normal limb in red. E, Quantification of LDPI perfusion. F, limb motor function and (G) salvage scores of the ischemic limb (in a scale of 1–5; 1-poor and 5-strong). H and I, Quantification of ratio of capillary density between I and NI limbs; *P<0.001 vs scrambled control cells or scrambled-control-Exo; n=6 to 8.
that may be involved in exosome-specific signaling and function in target cells.

MiR-126-3p was one of the most enriched miRNAs in both CD34+ cells and CD34Exo and was one of the most differentially expressed miRNA between CD34+ cells/CD34Exo and MNCs/MNCExo (Figure 3C). Further, it was significantly enriched in CD34Exo compared with CD34+ cells (Figure 4A). Interestingly, expression of miR-126-3p was lower in CD34Exo from chronic myocardial infarction patient-derived CD34+ cells compared with CD34Exo from healthy individuals, although the difference was not statistically significant (Online Figure XIII). To address whether miR-126-3p contributes to CD34Exo function, we knockdown (KD) its expression in CD34+ cells using anti-miR-126-3p or a scrambled miR control; the exosomes isolated from miR-126KD cells had reduced expression of miR-126-3p (miR-126-KD-Exo) compared with the exosomes isolated from scrambled control-treated cells (scrambled-control-Exo; 99% decrease; Figure 4A). To measure the angiogenic activity of miR-126-KD-Exo, we treated HUVECs with either miR-126-KD-Exo or scrambled-control-Exo and quantified the tube length in an in vitro Matrigel tube formation assay. We observed a significant decrease in tube length in miR-126-KD-Exo–treated HUVECs compared with scrambled-control-Exo–treated HUVECs (Figure 4B; Online Figure IX). These results indicate that angiomiR-126-3p present in CD34Exo is a potential mediator of CD34Exo-mediated angiogenic function in vitro.

**CD34Exo Directly Transfers miR-126-3p to Regulate Gene Expression in Ischemic Hindlimb Tissue**

To gain further mechanistic insights into the role of exosomal miR-126-3p in CD34Exo-induced therapeutic angiogenesis, we injected mouse ischemic hindlimb with either miR-126-KD-Exo or scrambled-control-Exo and measured limb function and capillary density (Figure 4C). miR-126-KD-Exo–treated ischemic hindlimbs had significantly lower limb perfusion, limb motor function, and limb salvage compared with the scrambled-control-Exo (Figure 4D through 4G). Similarly, the capillary density as measured by lectin-positive cells was significantly lower in the miR-126-KD-Exo–treated ischemic hindlimbs (Figure 4H and 4I). This indicates that knocking down of exosomal miR-126-3p abrogated the angiogenic and therapeutic potential of CD34Exo.

Next, to investigate whether the observed angiogenesis was mediated directly by the transfer via CD34Exo or indirectly as a result of de novo synthesis in the mouse ischemic tissue, we quantified mature miR-126-3p (Exo-transferred plus mouse tissue synthesized) and mouse-specific primary miR-126-3p (pri-miR-126; only mouse tissue synthesized) in the ischemic limbs treated with PBS, miR-126-KD-Exo, or scrambled-control-Exo. We detected significantly high level of miR-126-3p in the scrambled-control-Exo–treated limbs compared with miR-126-KD-Exo–treated limbs (Figure 5A). On the contrary, there was no difference in the levels of mouse-specific pri-miR-126 levels between hindlimbs treated with PBS, scrambled-control-Exo, or miR-126-KD-Exo (Figure 5B), suggesting that there was no de novo synthesis of miR-126-3p in the mouse limb post-CD34Exo treatment. Therefore, the observed increase in mature miR-126-3p post-CD34Exo treatment was most likely because of the direct transfer of mature miR-126-3p from the cargo of CD34Exo.

We then tested whether CD34Exo-mediated enhanced angiogenic activity could be because of the regulation of known miR-126-3p target mRNAs in mouse hindlimbs. In line with this hypothesis, we observed a significant reduction in SPRED1 mRNA expression, a direct target of miR-126-3p, in scrambled-miR-CD34Exo, but not in miR-126-KD-Exo–treated HLI tissues compared with other treatment groups (Figure 5C). Conversely, we observed increased expression of proangiogenic mRNAs including VEGF, ANG1 (angiopoietin 1), and MMP9 (matrix metalloproteinase 9) by several folds in the scrambled-control-Exo–treated HLI tissues (Figure 5D through 5F). Similar gene expression pattern was observed for additional genes involved in angiogenic pathways (Online Figure X). Notably, miR-126-KD-Exo treatment failed to induce angiogenic mRNA expression compared with PBS treatment in these ischemic hindlimbs. Collectively, these data suggest that CD34Exo directly delivers miR-126-3p to ischemic tissues by inducing angiogenic gene expression.
Uptake and Transfer of CD34Exo and Its miRNA Content In Vitro and In Vivo

To investigate the direct transfer of miRNAs via CD34Exo, we used fluorescently (Cy3)-tagged miRNA and isolated exosomes containing Cy3-miR. We treated CD34+ cells with Cy3-miR and isolated Cy3-miR–containing exosomes from the CM of those cells. We eliminated nonspecific exosomal surface-bound Cy3-miR by treating the isolated CD34Exo with RNase A. Flow cytometry analysis confirmed that Cy3-miR is contained in CD34Exo (Figure 6A and 6B). Next, we treated HUVECs with Cy3-miR-CD34Exo or with a nonfluorescent control-miRNA-CD34Exo prepared in the same way. Live confocal microscopic imaging shows presence of Cy3-positive punctate cytosolic vesicles only in the Cy3-miR-CD34Exo–treated HUVECs but not in the controls (Figure 6C). To study the in vivo uptake of CD34Exo in the ischemic hindlimb, we used PE-CD34Exo isolated from the CM of rhodamine-PE-tagged CD34+ cells and treated PE-CD34Exo or an unstained Exo control to the ischemic hindlimb, digested the tissue to prepare single cell suspensions, and analyzed for the presence of PE fluorescence in the cells using flow cytometry (at 2 hours) and histology at 12 hours (Online Figure XI). To identify the individual cell types uptaking CD34Exo, we used known cell type–specific antibodies. Our data revealed that PE-CD34Exo were uptaken most efficiently by endothelial cells as compared with smooth muscle cells and fibroblasts (Figure 6D and 6E). Interestingly, we observed the appearance of division in PE-CD34Exo-uptaken lectin-positive endothelial cells in the ischemic hindlimb (Figure 7A). In this line, CD34Exo-positive endothelial cells displayed different scatter properties than control PBS-uptaken endothelial cells (Figure 7B), suggesting that CD34Exo possibly induced cell cycle changes in those cells. To confirm involvement of cell cycle regulators in CD34Exo-injected tissue, we tested the expression of Cyclin genes at day 28 post-CD34Exo injection in the ischemic limbs. We identified increased Cyclin A and Cyclin B mRNA expressions in hindlimbs treated with CD34Exo as compared with PBS control (Figure 7C and 7D; Online Figure XII). Collectively, our data suggest that CD34Exo from human CD34+ progenitor cells carry and transfer stable and functional miR-126-3p to activate angiogenic and cell cycle–related gene expression in the mouse ischemic hindlimb tissue (Figure 8).

Figure 6. Uptake and transfer of CD34+ cell–derived exosomes (CD34Exo) and exosomal miRNAs by endothelial cells in vitro and in vivo. Flow cytometry analysis of CD34+ cells (A) or exosomes isolated from CD34+ cells (B), transfected as indicated. C, Confocal image of human umbilical vein endothelial cells treated with Cy3-miR-Exo. D, Flow cytometry analysis of single cell suspensions from posts ischemic hindlimb tissue injected with R-PE-CD34Exo at 2 h; E, Percentage of cells uptaking exosomes (PE) from total number of each cell types quantified from (D). n=3 to 5; *P<0.05.
Discussion

The molecular underpinning of paracrine factor-induced neovascularization that contributes to the therapeutic benefits of human CD34⁺ cell therapy after tissue ischemia is largely unknown. Here, we demonstrate for the first time that CD34Exo constitutes a key component of the paracrine secretion from CD34⁺ cells that promotes ischemic tissue repair. Cell-free CD34Exo were independently therapeutic and improved microcirculation in the ischemic limb, similar to CD34⁺ cells in a mouse model of HLI. Our results provide further insights into the molecular mechanisms underlying the angiogenic activity of CD34Exo by demonstrating that angiomiRs such as miR-126-3p, which are enriched in CD34Exo, have a profound effect on their angiogenic activity. The distinct combination of angiomiRs enriched in CD34Exo may provide a plausible explanation for their superior angiogenic and therapeutic efficacy compared with MNCExo, which is also consistent with enhanced potency of CD34⁺ exosomes may exceed beyond angiogenesis. Moreover, our previous work has shown that exosomes from sonic hedgehog-modified CD34⁺ cells activated angiogenic sonic hedgehog signaling in other cell types. The complete molecular content of CD34Exo and the mechanism of their cell-specific uptake still remains to be fully characterized in future studies.

In patients with coronary artery disease, the plasma levels of miR-126-3p is significantly reduced, indicating a dysfunctional endothelium in those patients. In patients with acute coronary syndrome, miR-126-3p is downregulated across the coronary circulation, implicating a high level of consumption of miR-126-3p in transcoronary passage of patients. These data suggest an essential role of miR-126-3p in regeneration of angiogenic components in exosomes or in the exosome-treated limbs. We do not rule out contribution from other miRNAs or factors in the repertoire of CD34Exo, alone or in combination, to the angiogenic and therapeutic activity of CD34Exo. In agreement, miR-10a and miR-130, enriched in CD34Exo, have antifibrotic activity suggesting that the overall benefits of CD34⁺ exosomes may exceed beyond angiogenesis. Moreover, our previous work has shown that exosomes from sonic hedgehog-modified CD34⁺ cells activated angiogenic sonic hedgehog signaling in other cell types. The complete molecular content of CD34Exo and the mechanism of their cell-specific uptake still remains to be fully characterized in future studies.

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and repair of ischemic tissues. Therefore, CD34Exo-mediated delivery of miR-126-3p or other angiomiRs to the damaged tissues may provide a promising therapeutic strategy as miRNAs are likely to be more stable within the exosome membrane and uptaken efficiently to target cells. Moreover, miRNAs directly supplied in the systemic circulation or injected into the tissues are prone to RNase digestion and, therefore, are less stable in vivo. Although exosomes are known to play a role in angiogenesis during cancer metastasis, in immune regulation, or in disease pathogenesis, their contribution to vessel growth under ischemic cardiovascular conditions is largely unknown. Our findings have unveiled a unique mechanism of revascularization that highlights the contribution of horizontal transfer of angiomiRs from human CD34+ stem cells to endothelial cells that mediate cell-cell communication. Exosome-shuttled angiomiRs may signify efficient amplification of stem cell function and may explain the results of clinical trials demonstrating significant functional improvement from a modest number of transplanted CD34+ cells that are retained in the ischemic tissues. Moreover, we are beginning to propose CD34Exo therapy as a suitable cell-free alternative to CD34+ cell therapy. Exosomes have great potential to overcome certain limitations of cell therapies for cardiovascular diseases, such as retention, viability, and functional impairment of transplanted cells in the unfavorable ischemic environment, while retaining the benefits.

Recent cell therapy approaches have shown promising results to augment cardiac function and quality of life by several means including reduced fibrosis, enhanced vascular angiogenesis, replacement, and repair of damaged myocardial tissue. Emerging evidence highlights a significant contribution from different stem cell types conferred by paracrine mechanisms mediated through exosomes and their miRNA repertoire. Our results identified CD34Exo as a key therapeutic component in the paracrine secretion of human CD34+ stem cells. We have demonstrated a novel mechanism by which cell-free CD34Exo mediates ischemic tissue repair via beneficial angiogenesis. Thus, the benefits of CD34 cell therapy on functional recovery could be induced primarily through CD34Exo-mediated transfer of angiomiRs such as miR-126-3p to endothelial cells in the ischemic tissue. This new knowledge can be used to refine existing treatments and to develop alternative therapeutic approaches that may benefit patients with cardiovascular diseases.

Acknowledgments

We gratefully acknowledge Dr Delara Motlagh from Baxter Healthcare for providing the CD34+ cells, Dr CS Thaxton for providing the dynamic light scattering machine, Meredith Millay, Aiko Ito, and Sheila Murphy for cell culture, animal surgeries, and histology.

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Disclosures

None.

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Angiogenic Mechanisms of Human CD34+ Stem Cell Exosomes in the Repair of Ischemic Hindlimb

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Supplemental Material

Angiogenic Mechanisms of Human CD34\textsuperscript{+} Stem Cell Exosomes in the Repair of Ischemic Hindlimb

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Supplemental Methods

Cell culture and exosomes isolation
CD34+ cells and the CD34+-cell–depleted mononuclear cells (MNCs) were obtained from Baxter Healthcare Corporation (Deerfield, IL, USA). The cells were purified from mobilized peripheral-blood mononuclear cells (AllCells LLC, Emeryville, CA, USA) with an Isolex 300i device (Baxter Healthcare); cell purity (85-95%) was determined via flow cytometry. Both CD34+ cells and MNCs (250,000 cells/mL) were cultured in X-VIVO 10 serum-free cell-culture medium (Lonza Inc. Allendale, NJ) cultured as described before1. Ultra-pure exosomes were isolated from the conditioned media by ultracentrifugation procedure and separated from the soluble protein fraction on a sucrose gradient. The exosomes from both cell types were characterized using dynamic light scattering, flow cytometry analysis, electron microscopy and immunoblotting as described before1, 2. Human umbilical-vein endothelial cells (HUVECs) (Lonza Inc. Allendale, NJ) were maintained in endothelial growth medium-2 (EGM™-2; Lonza Inc.) and starved in EBM-2 medium containing 0.25% fetal bovine serum for 24 hours before cell assays were performed.

Mouse hindlimb ischemia model
BalbC male nude mice (8–10 weeks old) were anesthetized with Isoflurane delivered at approximately 2%. All animals were placed on a warm circulating water pad to maintain body temperature throughout the procedure. Prior to the ischemic procedure and immediately following it, measurements of blood flow in both thighs were taken as a baseline and to confirm ischemia. The left thigh region was surgically prepped with betadine followed by alcohol. The depth of anesthetic plane was assessed by lack of toe pinch reflex and a 5-mm incision was made on the left thigh region. A ligation was made around the femoral artery and all arterial branches were removed. A small segment of the artery was then dissected free. Post ischemia, mice were randomly assigned to receive intramuscular injections of PBS, CD34+ cells, CD34+ cell conditioned media, CD34+ Exosomes, CD34+ exosome-depleted conditioned media, or MNC exosomes (all derived from equal number of cells; treatment dose: 5x10^6 cells/kg). The treatments were applied directly into the ischemic hindlimb in a 20-µl volume and injected at 4 different locations. The connective tissues of the subcutis were closed with interrupted 6-0 polypropylene suture and the skin closed with wound clips or 6-0 polypropylene suture. Prior to recovery from anesthesia, each animal was administered Buprenex (0.2 mg/kg IP) and meloxicam solution (0.001 mg/g) was administered in the water for up to ten days post-operatively to minimize any pain as a result of surgery.

Laser-Doppler Perfusion Imaging (LDPI) and scoring for limb functional recovery:
To determine tissue revascularization and functional recovery with different treatments after critical hindlimb ischemia, animals were assessed for tissue perfusion by Laser Doppler Perfusion Imaging (LDPI), and scored for limb salvage, and limb motor functions. For laser Doppler measurements of the ischemic and control limbs, animals were anesthetized with Isoflurane (2%) and LDPI measurements were taken at 7, 14, 21, and 28 days following the hindlimb ischemic surgery. At each time point, tissue perfusion was measured via LDPI, measuring blood flow in both the ischemic and nonischemic limb and reporting results as the ratio of these two measurements. All LDPI measurements were taken on a 37 °C heating pad to control body temperature. Ischemic and non-ischemic tissues were harvested at day 28 for histological analyses. Before sacrifice, the mice were injected with 50 µg of BS-1 lectin to identify the mouse vasculature. In addition, motor function and tissue damage was
semiquantitatively assessed on postoperative day 7, 14, 21, and 28 by established scoring systems. The limb motor function was scored as follows—1: no limb use; 2: no foot use, limb use only; 3: restricted foot use; 4: no active toe use (spreading), foot use only; and 5: unrestricted limb use. Limb salvage (i.e. no tissue necrosis) was scored as follows—1: limb amputation; 2: foot amputation; 3: toe(s) amputation; 4: necrosis, nail loss only; and 5: full recovery.

Statistical Analysis: Comparisons among all groups were assessed by a one-factor analysis of variance (ANOVA) followed by the Holm-Sidak test when ANOVA P<0.05. All values are expressed as mean ± SEM, and a P value less than 0.05 was considered statistically significant.

Immunohistology for quantification of capillary density
At day 28, muscle tissue from the ischemic limb was harvested, fixed in methanol, paraffin-embedded, and cross-sectioned (6 μm) for histological immunostaining. Briefly, sections were blocked with 10% donkey serum (30 min, room temperature). Primary antibodies for BS-1 lectin (Vector Laboratories, Burlingame, CA, USA), an endothelial cell marker, were diluted in PBS containing BSA, and applied to tissue slices for 1h at 37 °C. Sections were stained for BS-1 lectin antibody with AlexaFluor-conjugated secondary antibodies (Invitrogen Corporation, Carlsbad, CA, USA) and nuclei were counterstained with DAPI ((Vector Laboratories, Burlingame, CA, USA). Slides were imaged using fluorescent microscopy (Zeiss), and BS-1 lectin positive cells were quantified from three independent sections either from ischemic or, non-ischemic limb from each animal. At least 10 high powerfield images per condition were counted and averaged. Values are reported as the ratio of capillary density in the ischemic to non-ischemic limb. P < 0.05 was considered as statistically significant.

MicroRNA quantification
Total RNA from the CD34+ cells, CD34+-depleted MNCs, and their respective exosome preparations were extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol (including a DNase step). RNA concentrations were verified on a NanoDrop Spectrophotometer (NanoDrop) and the quality of total RNA was assessed using Agilent 2100 Bioanalyzer Pico Chips (Agilent). Equal amounts of RNA (5ng) were reverse transcribed using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) using a specific miRNA primer to generate cDNA for use with individual Taqman MicroRNA Assays (Applied Biosystems). Real-time Reactions were performed in triplicate on a 7500FAST Real-Time PCR system (Applied Biosystems). C \text{t} values were averaged and normalized to the U6 RNA (e.g., RNU6B). Experiments were performed with an n = 3–6. Relative expression was determined by the ddC \text{t} comparative threshold method.

MicroRNA Microarray
MiRNA profiling was completed at Asuragen using Affymetrix miRNA microarrays. The data analysis was performed by Asuragen CD34+ cells, CD34Exosomes, MNCs (depleted of CD34+ cells), MNCExosomes- all isolated from the same donor were processed and compared for 1100 known human microRNAs. Atleast three healthy donors were used for the experiments (n=3). RNA was isolated as before. The expression level Signals were scaled in GCOS 1.2 to give a median array intensity of 100. This was done to enable different arrays to be compared. The program Spotfire DecisionSite 8.2 (www.spotfire.com) was used for gene-profiling analysis. The details are described below.

Sample and array processing. Samples for miRNA profiling studies were processed by Asuragen Services (Austin,TX), according to the company’s standard operating procedures.
Upon receipt, the purity and concentration of human total RNA samples were determined by absorbance readings at 260 and 280 nm using a NanoDrop® ND-1000 UV spectrophotometer, and qRT-PCR for a discrete panel of miRNAs was performed as a surrogate measure of overall miRNA abundance, using TaqMan® microRNA assays (Life Technologies, Inc.). Following incoming sample quality control (QC) assessment, the 3’ ends of RNA molecules in total RNA samples were labeled with biotin according to the company’s standard protocol. Labeled RNA (100 ng total RNA per sample) was purified and hybridized to Affymetrix GeneChip® miRNA Arrays (Affymetrix, Santa Clara, CA). Hybridization, washing, staining, imaging, and signal extraction were performed according to Affymetrix-recommended procedures. Arrays were scanned on an Affymetrix GeneChip® Scanner 3000 7G.

**Signal processing.** The signal processing implemented for the Affymetrix miRChip is a multi-step process involving probe specific signal detection calls, background estimate and correction. For each probe, a global RMA background correction is performed with Median Polish summarization. Arrays within a specific analysis experiment are normalized together using Quantile normalization. Detection calls are based on a Wilcoxon rank-sum test of the miRNA probe signal compared to the distribution of signals from GC-content matched anti-genomic probes.

**Normalization.** A global Variance Stabilization Normalization (VSN) procedure is applied to normalize the data. The VSN method models the quadratic relationship between the variance of microarray data and the signal intensity, and transforms the data such that the variance is roughly constant. This transformation replaces the use of the logarithms and has the following properties: 1. comparable to the logarithmic transformation at the high end of the intensity spectrum; 2. roughly linear at lower end of intensity spectrum; 3. valid for negative after-background-subtraction values; 4. results in mild compression of values at lower end of spectrum and, as a result, the differences in VSN transformed values cannot be directly reverted to fold changes.

**Statistical Differential Analysis.** Test for the presence of genes differentially expressed across groups.

**Multiple Group Comparison:** We use the ANOVA model to test the null hypothesis (no variation in the mean gene expression within groups) in order to identify genes whose mean expression values are significantly different across groups. In addition to ANOVA, we use the Linear Models for Microarray data (LIMMA, Smyth (2004)) package. The LIMMA package allows for the comparison of the large data sets that are generated with microarray experiments for differential expression analysis. An empirical Bayes model (as implemented by the LIMMA R package) is used to reduce the variance of individual probes or genes towards a common value (also augmenting the degrees of freedom for the individual variance estimates) Smyth (2004).

**Pair-wise Comparison:** For each pair of treatments, an ANOVA contrast of the difference between the associated model coefficients is carried out for every gene, with multiplicity correction applied to the resulting p-values to control the false discovery rate (FDR) at 0.05 (a description of FDR is outlined below). Empirical Bayes methods were used to shrink individual probe-/gene-variances towards a common value.
Multiplicity Correction Methods: To keep the overall false positive rate less than the p-value cut-off of 0.05, we use a method described by Benjamini & Hochberg (Benjamini & Hochberg (1995)) to adjust for the overall rate of false discovery (FDR) which allows us to control the number of false positives and create statistically reliable gene lists.

Analysis Software. The analyses in this report have been performed in R (http://www.r-project.org).

LNA-mediated miRNA knockdown
Anti-miR-126-3p, or, scrambled control LNA (Ambion, Carlsbad, California, U.S) was transfected into CD34+ cells using Lipofectamine RNAiMAX (Invitrogen Corporation, Carlsbad, CA, USA) for 16h. Cells were washed 3 times and re-plated in fresh media to remove any external miRs. Cells were incubated for exosomes secretion for 40h. Exosomes were isolated using the protocol described before and used in an in vitro Matrigel tube formation assay using HUVECs to evaluate their angiogenic properties. Mouse hindlimb ischemia model was used to compare the therapeutic efficacy of miR-126-KD-Exo with the control Scrambled-Control-Exo. Pro-angiogenic miR-126-3p expression in both the cells and exosomes were quantified using Taqman qRT-PCR assay as described before.

In-vitro Matrigel tube formation assay
HUVECs (2.0x10^5, serum-starved overnight) were seeded with either PBS (control), or, with exosomes from the conditioned media of 2.0x10^4 CD34+ cells treated with either scrambled miRNA, or, anti-miR-126-3p into 48-well plates. The plates were coated with 150 µl of growth-factor–reduced Matrigel™ (BD). Tube formation ability of control, or, treated HUVECs was examined by phase-contrast microscopy about 2-4 hours later. Each condition in each experiment was assessed at least in duplicates. Tube length was measured as the mean summed length of capillary-like structures per well, per high-power field.

Exosomes treatment and qRT-PCR analysis of the HLI muscle tissue
Anti-miR-126-3p, or, scrambled control LNA was transfected to human CD34+ cells as described before. Exosomes were isolated from the conditioned media of cells with each treatment. HLI was induced in mice as described before and randomized to receive treatments of either PBS control, or, CD34+ exosomes-scrambled RNA, or, CD34+ exosomes-anti miR-126 in the ischemic limb; two injections of 10 µl each, flanking the ischemic tissue. A small piece of muscle flanking the treatment areas was dissected after certain time points. Total RNA was isolated from the tissue using Qiagen microRNA isolation kit and expression of miR-126-3p, mouse pri-126, mouse Spred-1 and mouse VEGF was determined as described before. Data is presented is as mean ± SEM. P<0.05 was considered as statistically significant.

Live Confocal Imaging and Flow Cytometry Analysis of Cy3-Exo Treated HUVECs and Ischemic tissue
Cy3 tagged miR, or, untagged scrambled control (Ambion, Carlsbad, California, U.S) was transfected into CD34+ cells using Lipofectamine RNAiMAX (Invitrogen Corporation, Carlsbad, CA, USA) for 16h. Cells were washed 3 times and re-plated in fresh media to remove any external miRs. Cells were incubated for exosomes secretion for about 24-40h. Exosomes were isolated using the protocol described before; intact exosomes were RNase-treated at 37°C for 10min to remove any external or, surface-bound Cy3. RNase activity was stopped by RNase-
inhibitor. Exosomes were washed with excess PBS, pelleted, re-suspended in PBS, applied to HUVECs (that were plated on coverslips) and imaged immediately using live confocal microscopy. The untagged control was processed in parallel in the same way as the exosomes with labeled Cy3-miRNA. For flow cytometry analysis, exosomes were tagged to 4micron latex beads. Transfected cells and bead-tagged exosomes were analyzed by flow cytometry (BD LSR Fortessa) for presence of Cy3-miRNA as described before.

For analysis of fluorescent-treated ischemic tissue, the exosomes were isolated from a stable dye, Rhodamine-PE-treated CD34+ cells. The dye gets integrated into the plasma membrane of the cells, and is secreted via exosomes. CD34+ cells were incubated with the dye O/N and excess dye was washed off 3 times. The cells were re-plated to secrete exosomes for about 24-40h. The exosomes were isolated and treated to the ischemic tissues as described before. Tissue was harvested at 2h, or, 12h as indicated and immediately processed for histology, or, flow cytometry analyses. For flow cytometry analysis, the tissues were digested into single cell suspension using collagenase digestion (0.5mg/ml, 20min at 37°C), the cells were first stained with a live-dead kit, fixed (with 4% PFA), washed, a cocktail of antibodies (Mouse CD31-FIT; alpha-sarcomeric actin with Alexa-647 secondaries; FSP1 with Brilliant violet 421 secondaries) were added and incubated for 30 min on ice. The unbound antibodies/secondaries were washed off, and cells were analyzed by flow cytometry (BD LSR Fortessa with six lasers, 16-color analysis, CA, USA). For fluorescent studies, cells, exosomes, and digested tissues were kept in dark during processing. For immunofluorescence studies, mice were injected with lectin (Vector laboratories, CA, USA; injected by tail-vein injections), hindlimb tissues were processed immediately after harvesting, stained for lectin and imaged using Nikon A1R laser scanning confocal microscopy.

Supplemental References:


Online Figure I. Dynamic light scattering (DLS) analysis of the clarified conditioned media (CD34-CM) from human CD34+ cell culture before and after exosomes isolation. The CM was successfully separated into vesicular exosomes-enriched fraction (CD34Exo) taken from the pellet, and non-vesicular soluble fraction called CD34Exo-depleted-CM, taken from the supernatant after ultracentrifugation.
**Online Figure II.** List of proteins with angiogenic function as present in human angiogenesis protein array blot.
Online Figure III. RNA is present in the lumen of CD34Exo. A, Isolated intact exosomes were digested with RNase A, the reaction was stopped by adding RNase inhibitor; B, total RNA and C, 260/280 and 260/230 ratio was compared before and after the RNase A treatment.
Online Figure IV. CD34Exo are significantly enriched with microRNAs. Bioanalyzer analysis of total RNA from CD34+ cells and CD34Exo using total RNA chip (A-D).
Online Figure V: Total number and nucleotide length of microRNAs detected by microarray analysis
Online Figure VI: A & B, Volcano plot comparison of CD34+ Cells with CD34Exo, and MNCs with MNCExo. C & D, MiRNA expression profiles of CD34+ Cells and MNCs and their corresponding exosomes shown using volcano plot.
Online Figure VII: Principal component analysis comparison of CD34+ Cells with CD34Exo, and MNCs with MNCExo.
Online Figure VIII: A, Relative miR-126-3p expression normalized to U6 in human CD34+ exosomes isolated from G-CSF-mobilized healthy and patient-derived CD34+ cells, B, Mean miR-126-3p values from healthy and patient CD34Exo (re-plotted from Online Figure VIIIA)
Online Figure IX: Loss of miR-126-3p results in loss of angiogenic function of CD34Exo.
Quantification of tube length of HUVECs on Matrigel treated with either PBS, or CD34Exo isolated from Scrambled-Control-Exo or miR-126-KD-Exo; P<0.05, *vs. PBS, †vs. scrambled-miR; n=5
Online Figure X: CD34Exo induce angiogenic gene expression in mouse HLI tissues. Expression of A, mANG2 and B, mTSP1 mRNA normalized to 18s in mouse HLI tissue homogenates.
CD34+ cells in culture + R-PE

(R-PE integrates to the plasma membrane)

MVB

Exosomes become fluorescent

Fluorescent exosomes isolated from CM

PE-CD34Exo

Hindlimb Ischemia

Rx Exo

Tissue harvest (gastrocnemius muscle harvested)

Single cell suspension

Flow Cytometry

Histology

**Online Figure XI.** Fluorescent R-PE-tagged CD34+ exosomes or non-fluorescent control exosomes were injected to mouse ischemic hindlimb immediately after HLI surgery; tissue was harvested both from ischemic and non-ischemic (NI) limb after 2h (for flow cytometry) and 12h (for histology) and analyzed for the presence of PE fluorescence. A compatible antibody cocktail (tagged with FITC, BV-421, Alexa-647) was used to identify different cell types by flow cytometry analysis. For histology, the tissue was processed immediately and sections were images using a confocal microscope.
Online Figure XII. CD34Exo induce gene expression changes in mouse HLI tissues.

Expression of **A**, Cyclin D and **B**, Cyclin E mRNA normalized to 18s in mouse HLI tissue homogenates.
Online Figure XIII. Binding of miR-126-3p. A, As known previously, 3'UTR of SPRED-1 directly binds to miR-126 seed (blue box) sequence with high affinity; B, ANG1, ANG2, MMP9, VEGFA, and TSP1 are not predicted as direct targets of miR-126 by TargetScan, DIANA-microT and miR map algorithms.
Online Table IA: Ischemic hindlimbs treated with CD34Exo show higher muscle mass and limb retention, Day 7

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<th>Limb Salvage Score</th>
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<th>MNC Exo</th>
<th>CD34+ Exo</th>
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Online Table IB: Ischemic hindlimbs treated with CD34Exo show higher muscle mass and limb retention, Day 28

<table>
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Online Table I. Ischemic hindlimbs treated with CD34Exo show higher muscle mass and limb retention (limb salvage (LS) score in a scale of 1-5; 1- complete limb loss and 5-full recovery). Ischemic hindlimbs received CD34+ Exo, MNC Exo or PBS control treatment. At day 7 (A) and day 28 (B), tissue damage was semi-quantitatively assessed by established scoring systems. Limb salvage (i.e. no tissue necrosis) was scored as follows: 1, limb amputation; 2, foot amputation; 3, toe(s) amputation; 4, necrosis, nail loss only; 5, full recovery.
Online Table II: MicroRNA expression in CD34\(^+\) cells, CD34Exo and MNC and MNCExo analyzed by miRNA microarray:

*Note: Fold changes calculated here do not directly reflect the actual fold-changes between the samples.

### Online Table II A: MicroRNAs enriched in CD34\(^+\) cells as compared to MNCs

<table>
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<tr>
<th>miRNA</th>
<th>*Fold Change</th>
<th>FDR P-value</th>
</tr>
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<td>hsa-miR-126-3p_st</td>
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### Online Table II B: MicroRNAs enriched in MNCs as compared to CD34\(^+\) cells

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<th>*Fold Change</th>
<th>FDR P-value</th>
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<tbody>
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<td>hsa-miR-150_st</td>
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# Online Table IIC: MicroRNAs enriched in CD34Exo compared to MNCExo

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# Online Table IID: MiRNAs enriched in MNCExo compared to CD34Exo

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### Online Table IIE: microRNAs enriched in CD34\(^+\) cells as compared to CD34Exo

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### Online Table IIF: MicroRNAs enriched in CD34Exo as compared to CD34\(^+\) cells

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