Late-Outgrowth Endothelial Cells Attenuate Intimal Hyperplasia Contributed by Mesenchymal Stem Cells After Vascular Injury

Chao-Hung Wang, Wen-Jin Cherng, Ning-I Yang, Li-Tang Kuo, Chia-Ming Hsu, Hung-I Yeh, Yii-Jenq Lan, Chi-Hsiao Yeh, William L. Stanford

Objectives—Mesenchymal stem cells (MSCs) are one of a number of cell types undergoing extensive investigation for cardiac regeneration therapy. It has not yet been determined whether this cell therapy also substantially contributes to vascular remodeling of diseased vessels.

Methods and Results—Human MSCs and a variety of progenitor and vascular cells were used for in vitro and in vivo experiments. Wire-induced vascular injury mobilized MSCs into the circulation. Compared with human aortic smooth muscle cells, MSCs exhibited a 2.8-fold increase in the adhesion capacity in vitro ($P<0.001$) and a 6.3-fold increase in vivo ($P<0.001$). In all animal models, a significant amount of MSCs contributed to intimal hyperplasia after vascular injury. MSCs were able to differentiate into cells of endothelial or smooth muscle lineage. Coculture experiments demonstrated that late-outgrowth endothelial cells (OECs) guided MSCs to differentiate toward an endothelial lineage through a paracrine effects. In vivo, cell therapy with OECs significantly attenuated the thickness of the neointima contributed by MSCs (intima/media ratio, from $3.2\pm0.4$ to $0.4\pm0.1$, $P<0.001$).

Conclusions—Tissue regeneration therapy with MSCs or cell populations containing MSCs requires a strategy to attenuate the high potential of MSCs to develop intimal hyperplasia on diseased vessels. (Arterioscler Thromb Vasc Biol. 2008;28:000-000.)

Key Words: mesenchymal stem cells | intimal hyperplasia | cell therapy | vascular remodeling

Stem cell therapy has a promising future for the treatment of a variety of end-stage cardiovascular diseases such as heart failure, ischemic heart, and peripheral artery diseases. To date, bone marrow (BM) cells or mobilized peripheral mononuclear cells are among the most often used cell populations in clinical settings because of their convenience and autologous properties. Recently, the MAGIC cell randomized clinical trial, however, showed that intra-coronary infusion of peripheral blood stem cells mobilized with granulocyte colony stimulating factor (G-CSF) aggravated restenosis after coronary stenting in myocardial infarction. Based on the heterogeneous populations of these cells and the differentiation capacity of stem cells, uncontrolled differentiation is a critical concern when applying these cells to humans.

Mesenchymal stem cells (MSCs) reside in the BM and can also be mobilized in response to G-CSF stimulation. MSCs from the BM are multipotent and have the capacity to differentiate into cardiomyocytes, endothelial cells (ECs), and smooth muscle cells (SMCs). Currently, MSCs are one of the cell types being used in clinical trials for postmyocardial infarction cardiac regeneration therapy. It has been shown that an intramyocardial injection of autologous MSCs or intravenous administration of MSCs can increase vasculogenesis and improve cardiac function after myocardial infarction in animal experiments and clinical trials. However, abundant evidence suggests that BM-derived circulating precursors can give rise to ECs and SMCs that contribute to vascular repair, remodeling, and lesion formation under physiological and pathological conditions. There is a very high possibility that MSCs residing in therapeutic cell populations can adhere to diseased, angioplastic, or stented vessels with cell administration, causing intimal hyperplasia and eventual restenosis. Herein, we sought to determine the contribution of MSCs to neointimal formation after vascular injury, and to investigate possible therapeutic strategies to modulate this contribution.

Methods

An expanded Materials and Methods section is available in the online data supplement #1 at http://atvb.ahajournals.org.
Animals
Male FVB wild-type, athymic nude (Jackson Laboratory, Bar Harbor, Me) and eGFP transgenic mice (FVB background) that ubiquitously express enhanced green fluorescent (GFP) (Level Biotechnology)15 were bred and maintained in the Laboratory of Animal Experiments at Chang Gung Memorial Hospital. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Chang Gung Memorial Hospital Faculty of Medicine, and the experiments were conducted according to the Guidelines of the American Physiological Society.

Mouse Femoral Wire-Injury Model
Femoral artery injury was induced by inserting a straight spring wire (0.38 mm in diameter, No. C-SF-15-15, Cook) for more than 5 mm toward the iliac artery.14

Mesenchymal Stem Cells Cultured From Peripheral Blood
To culture mouse MSCs in peripheral blood, pooled whole blood was harvested by cardiac puncture from 20 mice for each experiment. MSCs were cultured in 2 groups of mice, including the study group (48 hours after the femoral artery wire injury) and a control (sham-operated) group. After Ficoll density centrifugation (Histopaque-1083, density 1.083 g/mL), mononuclear cells (3 × 10^6) were plated on 6-cm dishes to culture the MSCs as described above.

Dye-Transfer Assay for Gap Junction Intercellular Communication
Lucifer yellow (LY; M; 457 Da; 0.2%) and rhodamine dextran (RD; M; 10 kDa) were microinjected into cells. LY can penetrate through gap junction channels; however, RD is too large to pass through gap junction channels and therefore served as a tracer dye for cells originally receiving the dye. Transfer of the dye was visualized using a phase-contrast fluorescence microscope (Axioskop; Carl Zeiss, Jena, Germany), in which LY and RD were respectively detected as green and red. DAPI was used to label nuclei (blue). Five minutes after the donor cell was injected, the presence of dye transfer to other cells was estimated. On average, 25 microinjections were tried for each experiment.

Results
MSCs Have a High Capacity for Adhering to the Matrix or the Remodeled Vessel Wall After Injury

The capacity for adhesion was investigated in a variety of human progenitor and vascular cells both in vitro and in vivo. In vitro, compared with other progenitor and vascular cells, MSCs had the strongest adhesion capacity to both fibronectin and collagen (Figure 1A, left and middle panels). Furthermore, mixing OECs with MSCs significantly increased the adhesion of OECs to the coated matrix (Figure 1A, right panel). In vivo, progenitor or vascular cells were directly injected via the abdominal aorta of mice 7 days after the right femoral artery wire injury. MSCs had the best adherence to the injured vessel wall compared with all other types of cells (Figure 1B). No adherence of injected cells was found in either small arterioles or the uninjured left femoral artery, suggesting that the injected cells did not cause cell embolism and were unable to adhere to intact and healthy vascular surfaces. The injected MSCs were also unable to adhere to the vessels 1 day after wire injury (data not shown), suggesting that MSC adhesion required matrix formation on the remodeled injured vessels, or the adherent platelets covered on the injured vascular surface interfered with MSC attachment to the underlying matrix.

Evidence of MSC Mobilization Into the Circulation
Blood G-CSF, VEGF, SDF-1α, and SCF concentrations were measured at serial time points in mice after femoral artery wire injury (supplemental Figure IA). The levels of these blood cytokines, especially G-CSF and VEGF, significantly increased within 24 hours. Blood G-CSF concentrations increased up to 8-fold 12 hours after vascular injury. Then, standard MSC cultivation was performed on mononuclear cells purified from pooled peripheral blood of mice 48 hours...
after wire injury. From the same amount of peripheral blood mononuclear cells, colonies of MSCs in the wire-injury group could be subcultured and maintained for long-term culture (at least 10 passages), whereas colonies from the controls could not be maintained for longer than 2 passages (supplemental Figure 1B). MSCs cultured from the wire-injury group had homogeneous morphologies, were Lin<sup>−</sup>/H<sub>11002</sub>CD45<sup>−</sup>/H<sub>11002</sub>Sca-1<sup>−</sup>/H<sub>11001</sub>CD31<sup>−</sup>/H<sub>11002</sub> (supplemental Figure II), and were multipotent, as they were capable of differentiating into osteocytes (positive for Von Kossa staining, alkaline phosphatase, and bone morphogenetic protein-2) or adipocytes (positive for Oil Red O) in response to different differentiation media (supplemental Figure IC), and into ECs and SMCs (described as follows).

Contribution of MSCs to Intimal Hyperplasia

To investigate whether MSCs contribute to neointimal formation in vivo, studies were performed using 2 models, “cell therapy” and “physiological” models. In the cell therapy model, BM-derived MSCs from eGFP mice were injected via the tail vein into wild-type littermates 7 days after femoral artery wire injury (Figure 2). One day after the injection, scattered eGFP MSCs were found on the surface of injured vessels (Figure 2A). Four weeks after cell injection, clusters of eGFP MSCs were identified in the neointima (Figure 2B). Most of the eGFP MSCs were also α-SMA<sup>−</sup>-positive. On average, 31%±15% of cells in the neointima were from the injected eGFP MSCs.

In the physiological model, eGFP MSCs and radioprotective whole eGFP-negative BM cells were transplanted into the tibia and the via tail vein, respectively, of wild-type littermates after 1000 cGy of whole-body irradiation (Figure 3A). Two months after MSC BM transplantation, femoral artery wire injury was performed. Four weeks later, the injured vessels had developed remarkable intimal hyperplasia, which contained a significant amount of eGFP-positive cells indicating the contribution of BM MSCs (39%±17%) (Figure 3B, lower panel, D28). In addition, flow cytometry and fluorescent microscopy proved successful engraftment of eGFP MSCs in the BM (Figure 3A).

Differentiation of MSCs Into Neointimal Cells

In mice after intra-BM eGFP MSC transplantation, the eGFP<sup>+</sup> MSCs in neointima differentiated into a few different cell types. Some of the eGFP<sup>+</sup> cells were α-SMA<sup>−</sup>, some were von Willebrand factor (vWF)<sup>−</sup> or triple positive, and the others were pure eGFP<sup>+</sup> on day 21 after the wire injury (Figure 3B, upper panel, D21). However, when using CD31 as a marker of highly differentiated ECs, only cells over the surface of the neointima were eGFP<sup>+</sup>CD31<sup>−</sup> (Figure 3B, middle panel, D21). These findings suggested that mobilized BM MSCs underwent a differentiation process into either SMC- or EC-lineage cells. When injured vessels were investigated at late time points such as 4 weeks after wire injury, immunostaining of vWF and α-SMA was located only on the surface and the body of the neointima, respectively, suggesting that the differentiation processes had ceased (Figure 3B, lower panel, D28).

Modulation of the Contribution of MSCs to the Neointima by Cell Therapy

The findings described above raise the possibility of manipulating the differentiation of MSCs, which had adhered to the injured vascular surface, into the endothelial lineage to achieve early reendothelialization. Thus, we attempted to use cell therapy to modulate MSC differentiation by mixing eGFP mouse MSCs with human EPCs or OECs. Using the
tail vein injection model, cell therapy with OECs significantly attenuated the contribution of eGFP MSCs and non MSC-derived SMCs to intimal hyperplasia (intima/media ratio, from 3.2 ± 0.4 to 0.4 ± 0.1, *P* < 0.001) (Figure 4A, upper panel and Figure 4C). eGFP cells were either purely α-SMA-positive or purely vWF-positive in the neointima 4 weeks after femoral artery wire injury. A subset of ECs was also positive for HLA-ABC, a marker specific for humans (Figure 4A, lower panel). These findings suggested that OEC therapy causes early re-endothelialization either by OECs themselves or by guiding the injected eGFP MSCs to differentiate into ECs. However, EPC therapy or OEC alone was unable to significantly attenuate the thickness of intimal hyperplasia (Figure 4B and 4C). To investigate the speed of reendothelialization, en face immunostaining was performed on the entire femoral artery 10 days after wire injury. There was more extensive endothelial covering of the injured femoral arteries in the “MSC+OEC” group, compared with the controls (Figure 4D).

**Coculture Experiment: Cell Therapy Guides the Fate of MSCs**

To clarify the effect of cell therapy on MSC differentiation, coculture experiments were performed by coculturing eGFP mouse MSCs with human OECs. MSCs did not express the endothelial marker, vWF, when cultured alone. However,
when eGFP MSCs were cocultured with OECs, MSCs began expressing vWF by D14 (supplemental Figure IIIA). Further experiments were performed to address whether the effect of coculturing occurs through direct cell–cell interactions or via a paracrine effect. When the culture medium was enriched with endothelial growth factors (EGM-2), MSCs in both the coculture group and the MSC-only group transcribed a variety of EC-specific mRNAs (supplemental Figure IIIIB). However, when culture medium without any growth factor was used (EBM-2), only MSCs in the coculture group transcribed KDR mRNA on D7 and D14 (supplemental Figure IIIIC). A dye-transfer assay for gap junction intercellular communication was performed on the cocultured cells. Microinjection to either MSCs or OECs demonstrated only OEC-OEC and MSC-MSC dye transmission with no intercellular communication between OECs and MSCs (supplemental Figure IIID). Immunostaining demonstrated no connexin-43 formation between OECs and MSCs (supplemental Figure IIIIE).

**Discussion**

We have demonstrated that BM-derived MSCs can be mobilized after vascular injury and that they have a high potential to participate in the remodeling processes of the injured vasculature. In vitro and, particularly, in vivo, MSCs exhibited a strong capacity for adhesion to either the coated matrix or remodeled vessel wall after injury. On adhesion, a substantial proportion of MSCs proliferated and differentiated into SMCs and ECs in the neointima. Cell therapy with OECs modulated the differentiation of MSCs toward an endothelial-like lineage, leading to early reendothelialization and attenuation of intimal hyperplasia.

Over the past few years, we have witnessed a paradigm shift in our understanding of the underlying principles governing intimal hyperplasia in response to vascular injury. The process, formerly ascribed to a local medial vascular smooth muscle response, appears to be partially and systemically governed by cells from the BM. The MAGIC clinical trial performed G-CSF mobilization of BM stem cells in patients with acute myocardial infarction who underwent coronary stenting. In fact, the study ended prematurely because patients receiving G-CSF experienced an unexpectedly high rate of restenosis at the stent site. A close correlation between the gain in neointimal volume and improvements in systolic function was noted in the cell infusion group, suggesting that MSCs have a higher potential to differentiate into cells with a muscular phenotype, compared with HSCs. As shown in our study, vascular injury caused an approximately 8-fold increase in blood G-CSF concentrations along with modest elevations in other cytokines such as SCF, SDF-1α, and VEGF, providing an environment potentially optimal for MSC mobilization. Being able to culture MSCs from the peripheral blood of the wire-injured mice lends further support to the evidence for MSC mobilization.

The origins of cells contributing to intimal hyperplasia are diverse including local SMCs, BM-derived vascular progenitors, stem cells in the adventitia, as well as additional cell types. Because a tremendous amount of medial vascular SMCs underwent apoptosis in the animal model adopted in this study, a significant portion of vascular repair depends on systemically mobilized cells. Using the entire BM transplantation model with an irradiation dose of 1000 cGy, most of the reconstituted BM cells were hematopoietic cells. However, the estimated D0 (the radiation dose that reduces survival to 37%) of MSCs is 1.3 to 1.4 Gy, and 1 Gy already induces death in a significant portion of MSCs. Previously, Fukuda et al showed that reconstituted BM cells contained non-hematopoietic cells when an irradiation dose of 1050 cGy was used. Using an intra-BM MSC transplantation model, they also demonstrated that MSCs, but not HSCs, in the BM contributed to the regeneration of myocardial tissue after myocardial infarction. All of these findings support the hypothesis that MSCs can participate in the process of post wire-injury vascular remodeling. In line with this, our data showed that MSCs seeded onto the surface of injured vessel wall 7 days after wire injury and proliferated in a nodular or clustered pattern. These adherent MSCs proliferated and differentiated into both SMC- and EC-like cells. Because only a small portion of MSCs were reconstituted in our intra-BM transplantation model, the proportion of intimal hyperplasia contributed to by MSCs was probably more than that estimated by our study.

In an undifferentiated state, MSCs do not express EC surface markers such as CD31 or CD34. However, recent work has shown that altering culture conditions can render MSCs capable of differentiating into ECs. All these attributes make MSCs an interesting cell phenotype for investigation in light of their potential to differentiate into mesoderm-derived ECs and their ability to differentiate in...
vivo into ECs.\textsuperscript{24,25} As shown in our study, although coculture with OECs may help MSCs express EC phenotypes, VEGF-enriched medium alone can achieve this effect as well, in line with findings by Oswald et al.\textsuperscript{26} Because both our dye-transfer assay and connexin-43 immunostaining suggested no direct communication between OECs and MSCs, the influence of OECs on MSC differentiation is suggested to occur through a paracrine effect.

Regarding approaches to attenuate neointimal formation, numerous medical therapeutic strategies, including cell therapy, have been investigated. Current concepts support the hypothesis that the earlier that reendothelialization is achieved, the less neointima that forms. Although OECs alone have an inadequate capacity for adhesion, mixing OECs with MSCs is herein suggested to increase the adhesion of OECs to injured vessel walls via the mediation of MSCs. It has been reported that early EPCs secrete more VEGF than do OECs.\textsuperscript{27} However, the influence of EPCs on MSC differentiation appears to be much less than that of OECs. One of the possible reasons is that the life-span of early EPCs is much shorter than that of the late OECs (A brief introduction to early EPCs and OECs is given in the online supplement #2.).\textsuperscript{27} Our data suggest that a coinfusion of OECs and MSCs may help achieve early reendothelialization. These findings can also explain the discrepancy among reports regarding the adverse effects of stem cell therapy on atherosclerosis,\textsuperscript{6,28} because there is a wide variation in the amount of endothelial progenitors in harvested BM cells and in mobilized cells after G-CSF treatment in different individuals.

In summary, our data clearly demonstrated the potential of stem cell therapy to contribute to atherosclerosis or poststenosing restenosis. MSCs, either spontaneously mobilized from BM or delivered directly by cell therapy, are 2 possible sources of cells in intimal hyperplasia. The amount of MSC mobilization depends on the effect of cytokines released in response to the severity of the vascular injury. However, cell therapy containing an MSC population has a direct impact on neointimal formation. Although the contribution of MSCs and the repair processes carried out by local SMCs after vascular injury are individualized in different subjects, interactions between OECs and MSCs were demonstrated herein to accelerate reendothelialization with beneficial effects on regulating local SMCs and MSCs, leading to remarkable attenuation in intimal hyperplasia. In a clinical setting, it is suggested that before cells are applied to the target organs, cell manipulation should be attempted either by combined-cell therapy or by medical intervention to raise the content of endothelial progenitors.

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Disclosures

None


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Supplements

Supplement #1:

Methods

Animals

Male FVB wild-type, athymic nude (Jackson Laboratory, Bar Harbor, ME) and eGFP transgenic mice (FVB background) that ubiquitously express enhanced green fluorescent (GFP) (Level Biotechnology, Taipei, Taiwan)\(^1\) were bred and maintained in the Laboratory of Animal Experiments at Chang Gung Memorial Hospital. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Chang Gung Memorial Hospital Faculty of Medicine, and the experiments were conducted according to the Guidelines of the American Physiological Society.

Mouse femoral wire-injury model

To test the contribution of MSCs to neointimal formation, a mouse femoral artery wire-injury model was adopted since this model is best suited for stem cell studies of post-injury vascular remodeling. Transluminal mechanical injury of the femoral artery was carried out under a dissecting microscope. Briefly, either the left or right femoral artery was exposed by blunt dissection. The accompanying femoral nerve was carefully separated, but the femoral vein was isolated from the artery.\(^2\) The femoral artery and vein were looped together proximally and distally with 6-0 silk sutures for temporary vascular control during the procedure. A small branch between the rectus femoris and vastus medialis muscles was isolated, and looped proximally and ligated distally with 6-0 silk sutures. The vein and connective tissues around the artery were carefully removed with microsurgical forceps. The exposed muscular branch artery was dilated by topically applying one drop of 1% lidocaine hydrochloride. A transverse arteriotomy was performed on this branch. A straight spring wire (0.38 mm in diameter, no. C-SF-15-15, Cook, Bloomington, IN) was
carefully inserted into the femoral artery toward the iliac artery. The wire was left in place for 1 min to denude and dilate the artery. Then, the wire was removed, and a silk suture looped at the proximal portion of the muscular branch artery was secured. Blood flow to the femoral artery was restored by releasing the sutures placed in the proximal and distal femoral portions. The skin incision was closed with 5-0 silk sutures. At different time points, the femoral arteries were excised, fixed in OCT compound (TissueTek, Tokyo, Japan), and used for the immunofluorescence studies.

**Cell culture**

Early endothelial progenitor cells (EPCs), late-outgrowth endothelial cells (OECs), smooth muscle progenitor cells (SPCs), and MSCs (mouse) were cultured as previously described. Human aortic endothelial cells (HAECs) and aortic smooth muscle cells (HASMCs) (Cascade Biologics, U.S.A.) were respectively cultured in media 200 and 231 with cell growth supplements. Mouse ECs (SVEC4-10) were purchased from ATCC (CRL-2181) (Rockville, MD) and cultured with DMEM. MSCs were expanded within MesenCult MSC culture medium (StemCell Technologies, Vancouver, Canada) and used at passage 4 to 5. For the co-culture experiments, numbers of MSCs and OECs or EPCs were plated at a 1:9 ratio. Immunostaining and RT-PCR were performed at different time points after co-culturing.

**Adherence capacity**

*In vitro*, collagen type I (Rockland) (50 µg/mL) or fibronectin (50 µg/mL) was coated onto 24-well plates for 2 h at 37 °C. Wells were blocked with 1% BSA in PBS for 15 min, and cells (5 × 10^5) were added to each well and allowed to attach for 1 h as previously described. In an additional adhesion assay, OECs (5 × 10^5, labeled with 5-chloromethylflourescein diacetate (CMFDA), green dye, Molecular Probes) were mixed with MSCs (5 × 10^5) to test whether the MSCs enhanced the adherence of
OECs to the matrix. Attached cell numbers were randomly counted in four areas of each well.

*In vivo*, 1 x 10^6 cells (either from eGFP mice or from human cells) were injected via the abdominal aorta into mice (either FVB wild-type or nude mice) 7 days after femoral artery wire injury. One day after the cell injection, the femoral artery was harvested to estimate the amount of cell adhesion by immunofluorescence staining. For human cells, CellTracker Green CMFDA was used for cell tracking.

**Mouse bone marrow harvesting**
To harvest BM, animals were sacrificed by cervical dislocation, the femurs were removed, and the BM was flushed into a sterile 15-ml tube using a syringe and a 23-G needle. Single cell suspensions were prepared by passing the material through a 23-G needle in a small amount of growth medium. Cells were washed, and after the process of RBC lysis, were counted, and then were ready for use.

**Intra-BM transplantation of MSCs**
Wild-type FVB mice at 8 weeks of age were lethally irradiated with a total dose of 1000 cGy. After irradiation, the recipient mice received unfractionated BM cells (1 x 10^6) (which served as radioprotective cells) from wild-type mice through a tail vein injection and MSCs (1 x 10^5) from eGFP mice through a tibial BM injection in each leg. Two months after transplantation, the mice were ready for examination. When the mice were sacrificed, engraftment of the eGFP MSCs was confirmed by flow cytometry and culture of BM cells.

**Cell therapy model**
To investigate the contribution of MSCs to neointimal formation, 1 x 10^6 eGFP MSCs were injected into the tail vein of wild-type FVB mice (or nude mice) 7 days after the femoral artery wire injury. To investigate whether the cell therapy attenuated MSC-mediated intimal hyperplasia, MSCs (1 x 10^6) alone or mixed with EPCs or
OECs (1 x 10⁶ for each cell type) were injected via the tail vein of nude mice (1 h after cells were mixed). Vessels were harvested 28 days after the cell injection.

**Confocal immunofluorescence staining**

The femoral artery was harvested for immunostaining at different time points after the cell injection. Frozen sections were stained with the following primary antibodies: anti-GFP (Chemicon); Cy3-conjugated anti-α-SMA (Sigma, St. Louis, MO); anti-vWF (DAKO, produced in rabbits); anti-CD31 (BD Pharmingen); anti-connexin-43 (Chemicon); and anti-HLA-ABC (Biolegend) followed by incubation with FITC-, PE-, or Alexa Fluor 647-conjugated secondary antibodies. Slides were mounted using a Prolong Antifade kit (Molecular Probes, Eugene, OR) and observed under a confocal microscope (Leica TCS SP2 AOBs). Nuclei were stained with DAPI or Hoechst 33258 (Sigma). For en face denuded arteries, 3-dimensional (3D) confocal images were used to visualize the area of re-endothelialization. The femoral arteries were harvested 10 days after wire injury (5 days after cell therapy through the abdominal aorta). To measure the re-endothelialized area, arteries were fixed with 4% paraformaldehyde (PFA) and 10% sucrose for 2 h. The artery was opened longitudinally, fixed on a slide and then subjected to immunostaining for eGFP and vWF and 3D confocal microscopic imaging. After the 3-D imaging was compacted into two dimensions, the areas stained positive for vWF and the total femoral artery area were measured, and the percentage areas were calculated.

**Immunoassay for chemokines and cytokines**

Plasma levels of granulocyte colony-stimulating factor (G-CSF), stroma-derived factor 1α (SDF-1α), SCF (stem cell factor), and vascular endothelial growth factor (VEGF) were measured using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) at different time points after the surgery.
Mesenchymal stem cells cultured from peripheral blood

To culture mouse MSCs in peripheral blood, pooled whole blood was harvested by cardiac puncture from 20 mice for each experiment. MSCs were cultured in two groups of mice, including the study group (48 h after the femoral artery wire injury) and a control (sham-operated) group. After Ficoll density centrifugation (Histopaque-1083, density 1.083 g/ml), mononuclear cells ($3 \times 10^6$) were plated on 6-cm dishes to culture the MSCs as described above. The multipotent capacity for differentiation of the cultured mobilized MSCs was also tested.

Mesenchymal stem cell differentiation assay

For osteoblast differentiation, cells were stimulated with dexamethasone ($10^{-8}$ M), L-ascorbic acid (50 μg/ml), and β-glycerophosphate (3.5 mM) in 15% alpha-MEM. Selected culture specimens after 6, 12, and 18 days of growth in the subculture were stained for mineral deposition by the Von Kossa method. The stained cultures were examined using phase-contrast optics. For adipocyte differentiation, adipogenesis was stimulated with dexamethasone ($10^{-6}$ M), insulin (5 μg/ml), IBMX (50 μM), indomethacin (60 μM), and rosiglitazone (10 μM) in 15% alpha-MEM. Cells were fixed on day 14, and adipocytes were visualized by staining with Oil Red O.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Co-cultured cells were harvested at the indicated time points, and total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen). RT-PCR was performed with a OneStep RT-PCR kit (Promega). For each reaction, 1 μg of total RNA served as a template. For amplification, primer pairs specific for mouse vWF (sense primer 5'-CCG GAA GCG ACC CTC AGA-3' and antisense primer 5'-CGG TCA ATT TTG CCA AAG ATC T-3'), mouse Tie-2 (sense primer 5'-TTG AAG TGA CGA ATG AGA T-3' and antisense primer 5'-ATT TAG AGC TGT CTG GCT T-3'), mouse Flk-1 (KDR) (sense primer 5'-AGC TTG GCT CAC AGG CAA CAT CGG-3' and antisense primer 5'- TGG CCC GCT TAA CGG TCC GTA GG-3'), and GFP (sense
primer 5'-AAG TTC ATC TGC ACC ACC G-3' and antisense primer 5'-TGC TCA GGT AGT GGT TGT-3')\(^1\) were used. The bands of the RT-PCR appeared only in mouse ECs not in HAECs, indicating that the primers used were specific for mice. In the RT-PCR for Tie-2, 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min were used. For other genes, 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, were used with a final extension at 72 °C for 10 min. The RT-PCR product of mouse vWF was 130 bp, of Tie-2 was 197 bp, of Flk-1 was 624 bp, and of GPF was 500 bp.

Dye-transfer assay for gap junction intercellular communication

A cell microinjection assay was performed based on a published procedure with modifications.\(^9\) Briefly, Lucifer yellow (LY; \(M_r: 457\) Da; 0.2%) and rhodamine dextran (RD; \(M_r: 10\) kDa) were microinjected into cells. LY can penetrate through gap junction channels; however, RD is too large to pass through gap junction channels and therefore served as a tracer dye for cells originally receiving the dye. Transfer of the dye was visualized using a phase-contrast fluorescence microscope (Axioscope; Carl Zeiss, Jena, Germany), in which LY and RD were respectively detected as green and red. DAPI was used to label nuclei (blue). Five minutes after the donor cell was injected, the presence of dye transfer to other cells was estimated. On average, 25 microinjections were tried for each experiment.

Statistical analysis

Data were analyzed by one-way analysis of variance. When statistically significant effects were found, Tukey's test was performed to isolate differences between groups. Student's \(t\)-test was used when appropriate. A \(p\) value of < 0.05 was considered significant. All data are presented as the mean ± SEM in the text and figures.

Supplement #2: Two different types of EPCs can be cultured from adult peripheral
blood, namely early EPCs and OECs (also called late EPCs) according to their
time-dependent appearance. Early EPCs with a spindle shape show peak growth at 2
to 3 weeks and die at 4 weeks, whereas OECs with a cobblestone shape appear late at
2 to 3 weeks, show exponential growth at 4 to 8 weeks, and live up to 12 weeks.10
Early EPCs are a heterogeneous group of cells and secrete high levels of cytokines;
however, OECs are quite homogeneous. They share some endothelial phenotypes and
similarly contribute to neovasculogenesis in vivo. Compared to early EPCs, OECs are
closer to mature ECs in phenotype but show surprising migration, and tube-forming
capabilities.

**Supplementary figure legends:**

**Supplemental Figure I.** Evidence of mesenchymal stem cell (MSC) mobilization. A,
Stem cell-mobilizing cytokines significantly increased after wire injury. * p < 0.05, **
p < 0.01 compared to the baseline (0 h) (n = 8-10 in each group). B, Peripheral blood
MSCs were cultured and subcultured to confluence. C, Adipogenic (red) and
osteogenic (brown) potentials of peripheral blood MSCs. Immunostaining was
positive for alkaline phosphatase (ALP, blue) and bone morphogenetic protein-2
(BMP-2, red).

**Supplemental Figure II.** Phenotypes of mesenchymal stem cells cultured from
peripheral blood. Flow cytometry and confocal immunofluorescent analysis show that
they are Lin⁻CD45⁻Sca-1⁺CD31⁻.

**Supplemental Figure III.** Manipulation of mesenchymal stem cell (MSC)
differentiation by late-outgrowth endothelial cells (OECs). A, MSCs from eGFP mice
were co-cultured with human OECs, and immunofluorescent staining was conducted
at serial time points after co-culturing. eGFP, green; α-SMA, blue; vWF, red; eGFP\(^{+}\)α-SMA\(^{+}\), cyan; eGFP\(^{+}\)vWF\(^{+}\), yellow. Arrows indicate smooth muscle-like cells; arrowheads indicate endothelial-like cells. B, RT-PCR for a variety of endothelial phenotype mRNA was also performed at different time points after co-culturing in either EGM-2 (enriched with EC growth factors) (B) or EBM-2 (containing no growth factors) (C) (n = 4). mEC, mouse EC. D, dye-transfer assay: (left panel) OECs, injected dye (*) was transferred to other OECs (green); (middle and right panels) co-culture of OECs (not labeled) and MSCs (labeled with DAPI, blue) for 48h; (middle) dye was injected into OECs (*) and transferred to OECs only, not to MSCs; (right) dye was injected into MSCs (*) and transferred to MSCs only, not to OECs (n = 25 for each experiment). E, After the co-culturing of human MSCs (green) and OECs (white) for 14 days, immunostaining revealed that connexin-43 had only formed between OECs.

References


**A**

**G-CSF**

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**B**

Peripheral blood MSCs

**C**

Adipogenesis

Osteogenesis

**supplementary Fig. I**
supplementary Fig. III

A Co-culture D7 D14

C mMSC mMSC + OEC mMSC

D Dye-transfer assay

D0 D7 D14 D7 D14 D7 D14

KDR

GFP

B

mMSC mMSC + OEC mMSC

KDR

vWF

Tie-2

GFP

mEC HAEC

E

α-SMA: MSC

Connexin-43

vWF: OEC

DAPI: nucleus

Co-culture D14

supplementary Fig. III