Enhanced Angiogenesis by Transplantation of Mesenchymal Stem Cell Sheet Created by a Novel Magnetic Tissue Engineering Method

Masakazu Ishii, Rei Shibata, Yasushi Numaguchi, Tetsutaro Kito, Hirohiko Suzuki, Kazunori Shimizu, Akira Ito, Hiroyuki Honda, Toyoaki Murohara

Objective—Therapeutic angiogenesis with cell transplantation represents a novel strategy for severe ischemic diseases. However, some patients have poor response to such conventional injection-based angiogenic cell therapy. Here, we investigated a therapeutic potential of mesenchymal stem cell (MSC) sheet created by a novel magnetite tissue engineering technology for reparative angiogenesis.

Methods and Results—Human MSCs incubated with magnetic nanoparticle-containing liposomes were cultured, and a magnet was placed on the reverse side. Magnetized MSCs formed multilayered cell sheets according to magnetic force. Nude mice were subjected to unilateral hind limb ischemia and separated into 3 groups. For the control group, saline was injected into ischemic tissue. In the MSC-injected group, mice received magnetized MSCs by conventional needle injections without sheet formula as a control cell group. In the MSC-sheet group, MSC sheet was layered onto the ischemic tissues before skin closure. Blood flow recovery and the extent of angiogenesis were assessed by a laser Doppler blood flowmetry and histological capillary density, respectively. The MSC-sheet group had a greater angiogenesis in ischemic tissues compared to the control and MSC-injected groups. The angiogenic and tissue-preserving effects of MSC sheets were attributable to an increased expression of vascular endothelial growth factor and reduced apoptosis in ischemic tissues. In cultured MSCs, magnetic labeling itself inhibited apoptosis via a catalase-like antioxidative mechanism.

Conclusion—MSC sheet created by the novel magnetic nanoparticle-based tissue engineering technology would represent a new modality for therapeutic angiogenesis and tissue regeneration. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiogenesis ■ ischemia ■ mesenchymal stem cells ■ nanotechnology ■ tissue engineering
system, magnetite Fe₃O₄ nanoparticles with 10 nm in diameter were first taken up by liposomes, creating magnetic cationic liposomes (MCLs) with a positive surface charge. Since the cell membrane is negatively charged, MCLs can easily contact target cells electrostatically, and target cells can be magnetized by fusion with MCLs. Magnetized target cells were then cultured, and cell sheets could be created using external magnetic force. This Mag-TE technology prompted us to construct a variety of cell sheets such as cardiomyocytes, keratinocytes, or hepatocytes.16–18

In the present study, we attempted to construct multilayered 3-D human MSC sheets using the Mag-TE system, and we tested therapeutic impacts of the MSC sheets on ischemia-induced angiogenesis in vivo.

Materials and Methods
Methods in detail are described in the online data supplement files, available at http://atvb.ahajournals.org. Please see the supplemental methods.

Construction of Human MSC Sheets
MSC sheets were constructed using the Mag-TE system. To magnetically label the cells, human MSCs were first incubated with MCLs at a concentration of 100 pg-magnetite/cell. After 4 hours incubation, MSCs (2×10⁶ cells) labeled with MCLs were seeded into a 24-well ultralow-attachment cell culture plate (Corning, NY). Then a cylindrical neodymium magnet (4000 Gauss) was placed on the reverse side of the culture plate to give a vertical magnetic force to the plate, and the cells were cultured for an additional 24 hours. After the culture, the neodymium magnet at the reverse side of the culture plate was removed. A hydrophilic polyvinylidene difluoride membrane covered on top of an Alnico magnet, and the magnet was positioned at the surface of culture medium so that the MSC sheets were attached by the magnetic force and stuck onto the polyvinylidene difluoride membrane-covered magnet surface.19

Mouse Model of Hind Limb Ischemia
Male nude mice (BALB/c nu/nu) at 10 to 12 weeks old were obtained from the SLC Co Ltd (Nagoya, Japan), cared and used in accordance with the guidelines issued by the National Institutes of Health. These mice were subjected to unilateral hind limb ischemia surgery under anesthesia with sodium pentobarbital (30 mg/kg intraperitoneally) as described previously.19

Mice were randomly divided into 3 groups. The control group (n=9) received phosphate-buffered saline. The MSC-injected group (n=9) received MSCs magnetically labeled with MCLs (2×10⁶ cells/200 μL in phosphate-buffered saline; n=9) that were directly transplanted into 6 sites in the ischemic limb by a needle injection. The MSC-sheet group (n=9) received the Mag-TE constructed MSC sheet (2×10⁶ cells/sheet) placed on top of the ischemic adductor muscles using an Alnico magnet. After placement of the sheet, skin was closed with a simple interrupted 5 to 0 suture. In additional experiments, MSCs labeled or unlabeled with MCLs (2×10⁶ cells/200 μL in phosphate-buffered saline; n=9 each group) were transplanted into 6 sites in the ischemic limb by needle injections to examine the effects of MCL labeling itself on angiogenesis. We choose to implant MSCs on the ischemic adductor muscles, because adductor muscles are exposed to most severe ischemia after surgery in this model.20

Results
Characteristics of MSCs Labeled With MCLs
We first assessed the characteristics of MSCs magnetically labeled with MCLs by flow cytometry. There was no significant differences in cell size, as assessed by forward scatter (FSC) between MSCs labeled with MCLs and unlabeled MSCs, whereas cytosolic structure as assessed by sideward scatter was apparently changed by the incorporation of magnetic particles within the MSCs (Supplemental Figure IA). We also compared the cell surface markers between MSCs labeled with MCLs and unlabeled MSCs. Cells in both groups expressed CD44 and CD166, but not CD45 (Supplemental Figure IB). To determine sarcomeric F-actin organization, MSCs labeled or unlabeled with MCLs were stained with fluorochrome-conjugated phalloidin. As shown in Supplemental Figure IC, the incorporation of magnetic particles within the MSCs had no effect on actin organization and cell surface area.

We next examined the expression of the angiogenic cytokines using Proteome Profiler array. In MSCs labeled with MCLs and unlabeled MSCs, a variety of angiogenic factors such as aFGF, basic fibroblast growth factor, and VEGF were highly expressed. These expressions did not differ between the two groups (Supplemental Figure ID). Thus, the incorporation of magnetic particles within the cells did not change their phenotype and functionality.

Construction of Human MSC Sheets by the Mag-TE and Application to Nude Mice
Figure 1A shows a macroscopic human MSC sheet constructed on an ultralow-attachment culture plate using the Mag-TE system. The Mag-TE system caused free MSCs to form a sheet-like structure by cell pileup according to the magnetic force after 24 hours incubation (Figure 1B). The MSC sheets had a brown color that was the color of magnetite Fe₃O₄ nanoparticles. Hematoxylin and eosin staining showed that the MSC sheet comprised 10 to 15 layered MSCs with approximately 300 μm in thickness (Figure 1B). Immunofluorescent staining confirmed the expression of gap junction protein connxin 43 within the MSC sheet (Figure 1C).

To further investigate the cellular condition in the human MSC sheet, proliferating cell nuclear antigen and TUNEL stainings were performed. About 40% of MSCs within the sheet were proliferating cell nuclear antigen-positive, and thus these cells were proliferating (Figure 1D). TUNEL-positive–apoptotic cells were virtually not observed at 24 hours after the initiation of the sheet construction (Figure 1E). There were no significant differences in the proportion of TUNEL-positive–apoptotic cells among the upper layer (1.01±0.36%), middle layer (1.18±0.52%), and lower layer (0.99±0.54%) of the MSC sheets.

Figure 2A to 2C show serial procedures for harvesting a MSC sheet constructed by the Mag-TE system. A neodymium magnet under the culture plate was removed. Then, an Alnico magnet covered with a hydrophilic polyvinylidene difluoride film was positioned at the surface of culture medium (Figure 2A). A MSC sheet was recovered by magnetic force, and it was then placed onto the adductor muscles of mouse hind limb using an Alnico magnet (Figure 2B and C).

In the MSC-injected group, cell suspensions were transplanted by needle injections. These cells were engrafted into the muscles with a limited area, and some cells were still present at 3 weeks after the transplantation (Figure 2D and E). In the MSC-sheet group, a cell sheet was successfully
engrafted, and new blood vessels were formed within the sheets in the MSC sheet group (Figure 2F and G). Furthermore, to identify the transplanted cells in ischemic tissue, we assessed the human lamin mRNA levels, which are nuclear membrane structural component, in ischemic tissue at day 7 after transplantation using real-time PCR. The mRNA levels of human lamin were significantly higher in MSC sheet group than MSC-injection group (Figure 2H).

Augmentation of Ischemia-Induced Revascularization by the MSC Sheet Transplantation

We next examined whether transplantation of MSC sheet could augment ischemia-induced angiogenesis using a mouse hind limb ischemia model. Figure 3A shows representative laser Doppler blood flow images of hind limb blood flow immediately after the ischemia surgery and at different time points thereafter. Quantitative analysis revealed that the MSC-injected group showed a significant increase in limb blood perfusion at days 7, 10, 14, and 21 after surgery compared to the control group (Figure 3B). Moreover, a greater degree of blood perfusion was observed in the ischemic limb of the MSC-sheet group compared to the MSC-injected group (Figure 3B).

To further investigate the extent of angiogenesis at the microcirculation level, capillary density and arteriolar density were also measured in histological sections harvested from the ischemic adductor and gastrocnemius muscles. Figure 3C shows a quantitative analysis revealing that, on postoperative day 21, the tissue capillary and arteriolar densities in ischemic muscles were significantly increased in the MSC-injected group and the MSC-sheet group compared to the control group. The capillary and arteriolar densities tended to be greater in the MSC-sheet group compared to the MSC-injected group (Figure 3C).
MSC Sheet Transplantation Increased the VEGF Expression

We investigated whether transplantation of MSC sheet stimulated the expression of VEGF in the ischemic hind limb tissues. At postoperative day 7, mouse VEGF mRNA levels increased in the MSC-injected group and the MSC-sheet group compared to the control group. Expression of mouse VEGF mRNA was also greater in the MSC-sheet group than in the MSC-injected group (Supplemental Figure IIA). Human VEGF originating from transplanted cells was investigated (Supplemental Figure IIB). Human VEGF mRNA level was increased significantly in the MSC-injected group and MSC-sheet group compared to the control group at day 3 and day 7. The expression of human VEGF mRNA was greater in the MSC-sheet group than in the MSC-injected group at day 7 (Supplemental Figure IIB). Furthermore, to confirm whether implanted MSC-sheet secretes VEGF protein in the ischemic tissues, frozen sections from ischemic tissues of mice were stained with VEGF monoclonal antibodies. VEGF was detected in the cytoplasm of skeletal myofibers in the ischemic muscles, and this expression level was higher in the MSC-sheet group than in the MSC-injected group consistent with the mRNA levels (Supplemental Figure IIC). Transplantation of MSCs did not affect plasma VEGF protein levels (Supplemental Figure IID).

Transplantation of MSC Sheets Inhibited Skeletal Muscle Cell Apoptosis in Ischemic Tissues

To evaluate the cell viability after the MSC sheet transplantation, we performed TUNEL staining in the ischemic tissues. Representative photographs of TUNEL-positive nuclei in the ischemic muscles are shown in Supplemental Figure IIIA. Quantitative analysis revealed a significantly lower proportion of TUNEL-positive apoptotic cells in the ischemic muscles of the MSC-sheet group compared to the control and MSC-injected groups after hind limb surgery (Supplemental Figure IIIB).

Recently, it was reported that magnetic force itself elicits an intrinsic antioxidative peroxidase-like activity.21 We, thus, examined whether MCLs could reverse the oxidation reaction of DAB mediated by H2O2 (Supplemental Figure IIII). To test the effects of MCLs on peroxidase-like activity and cell viability, MSCs labeled or unlabeled with MCLs were subjected to glutathione-depleting agent L-buthionine-[S,R]-sulfoximine (BSO) for 48 hours in vitro. Glutathione-depletion by BSO led to an increase in the ratio of trypan blue-positive-dead cells in MSCs. In MSCs labeled with MCLs, however, BSO-induced cell deaths were markedly attenuated (Supplemental Figure IIII). Treatment with BSO increased the expression of proapoptotic protein Bax by 1.6-fold, and this effect was also attenuated by the MCL labeling (Supplemental Figure IIIIE). Thus, the MCL labeling itself likely contributes to the cell survival action after MSC sheets transplantation via their peroxidase-like antioxidative activity. We also analyzed the effect of the MCL labeling itself on blood flow recovery of ischemic muscles in WT mice on postoperative day 0, 3, 7, 14, and 21. The MSCs labeled with MCLs-injected group showed a significant increase in limb flow at 7, 14, and 21 days after hind limb surgery compared with the MSCs unlabeled with MCLs-injected group and the control group (Supplemental Figure IIIF). Supplemental Figure IIIF shows a quantitative analysis revealing that, on postoperative day 21, the tissue capillary and arteriolar densities in ischemic muscles were significantly increased in the MSCs labeled with MCLs-injected group and the MSCs unlabeled with MCLs-injected group compared to the control group. The capillary density, but not arteriolar density, was significantly greater in the MSCs labeled with MCL-injected group compared to the MSCs unlabeled with MCL-injected group (Supplemental Figure IIIG).

Localization of Transplanted MSC Sheets at Chronic Phase

We examined whether in vivo implanted MSC sheet could differentiate into endothelial cells at chronic phase. At 6 weeks after transplantation, a large number of newly formed capillary and mature blood vessels invaded into the MSC...
sheet (Supplemental Figure IVA). Immunofluorescence staining revealed that implanted MSC sheet expressed anti-mouse α-SMA-positive cells and anti-mouse CD31-positive cells (Supplemental Figure IVA). However, anti-human CD31 and anti-human α-SMA-positive cells were not detected (Supplemental Figure IVB). These results indicate that implanted MSC-sheet component did not directly differentiate into vascular endothelial cells.

**Discussion**

In the present study, we have used a new modality for TE, termed Mag-TE system, and successfully created human MSC sheet comprising 10 to 15 layered cells with approximately 300 μm in thickness. The Mag-TE system enabled us to recover the MSC sheet en block with an Alnico magnet, and we could transplant it to the mouse hind limb ischemia model under conditions that well preserved cell-to-cell connection. The transplanted MSC sheet was successfully engrafted into the ischemic tissues, stimulated revascularization, and inhibited host skeletal muscle cell apoptosis in response to limb ischemia. Thus, our new methodology, the Mag-TE system, would provide a novel modality in the field of regenerative medicine using cell sheets.

A number of clinical trials have supported the safety and efficacy of therapeutic angiogenesis by cell transplantation for patients with severe PAD and myocardial infarction. In various animal models and clinical trials, the conventional methods for cell transplantation were direct injection of free cell suspensions with syringe needles. However, this method has several disadvantages, such as cell loss caused by leakage of injected cell suspension, lower cellular forming rate, and tissue damages. Studies reported that cells administered by a direct syringe injection were lost for a short period and their viability was significantly reduced proportion of TUNEL-positive apoptotic cells. However, it has been shown that the sheet comprising 4 or more layers causes the central necrosis due to lack of oxygen supply. In addition, to fabricate the multilayered sheets, they extend the culture by 1 week. In the present study, we could create the MSC sheet comprised 10 to 15 layered MSCs with approximately 300 μm in thickness. We also fabricated the multilayered sheets for only 24 hours. Thus, constructed MSC sheet by our system is highly viable, proliferative, and convenient in means.

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The present study has several limitations. First, we did not assess whether MSC sheet created by the magnetic nanoparticle-based tissue engineering technology elicited a foreign body reaction or not. Nude mice (BALB/c nu/nu) were used for this study to avoid immunologic rejection of the transplanted MSCs. Second, the surgical layering of MSC-sheets labeled with MCL on a muscle surface is fundamentally different from a simple MSC injection. There might be obvious hindering factors associated with placing cells on top of target tissue such as the requirement for surgery with collateral damage. Third, the link between VEGF and apoptosis after MSC sheet transplantation has not been clarified. It has been reported that VEGF induces cell survival in various cell types including endothelial cells, whereas the MCL labeling itself contributed to the cell survival via their peroxidase-like antioxidative activity in the present study. Thus, MSC-sheet might exert both direct and indirect antiapoptotic actions on the ischemic tissue. Future experimental studies will be required to elucidate the link between VEGF and apoptosis in the ischemic tissue after MSC sheet transplantation. Finally, we did not assess the dose-response relationship for MSCs in our ischemic animal model.

In conclusion, our human MSC sheet constructed by a novel magnetic force-based TE technology (i.e., Mag-TE system) was successfully engrafted into the ischemic tissues of nude mice and promoted revascularization. Transplantation of MSC sheet may become a novel therapeutic modality for regenerative medicine in ischemic limb as well as cardiac diseases.

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**Disclosures**

**References**


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Supplemental Methods

Cell Culture

Human MSCs were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). MSCs in passages 3 to 4 were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS), and antibiotics (100U/ml penicillin G, and 100 µg/ml streptomycin) under humidified air with 5 % CO₂ at 37 °C. 1

Flow cytometric analysis

MSCs labeled with MCLs were characterized by flow cytometric analysis. Flow cytometric analysis was performed using the fluorescence-activated-cell sorter (FACS) Caliber instrument (Becton Dickinson) and Cell Quest software (BD Biosciences). MSCs (5x10^5 cells) labeled or unlabeled with MCLs (100 pg-magnetite/cell) were incubated for 30 minutes at 4°C with monoclonal antibody against CD44-FITC, CD45-FITC and CD166-PE (BD Biosciences), isotype identical antibodies served as controls (BD Biosciences).

Angiogenic factor protein array

Cell lysates from MCLs (100 pg-magnetite/cell) labeled or unlabeled with MSCs were tested using Proteome Profiler Human Angiogenesis Array Kit (R&D Systems). The samples were analyzed according to the manufacturer’s instructions. Nitrocellulose membranes, spotted 55 different angiogenesis related antibodies in duplicate, were incubated with the each cell lysate and a cocktail of biotinylated detection antibodies.
Any protein/detection antibody complex present is bound by its cognate immobilized capture antibody on the membrane. Following a wash to remove unbound material, Streptavidin-horseradish Peroxidase and chemiluminescence detection reagents are added sequentially. The developed film was further scanned by Fujifilm Luminescent Image Analyzer LAS-4000 and densitometric analysis on the array image was performed using Image J software.

**Laser Doppler blood flow analysis**

We measured the ratio of the ischemic/normal hind limb blood flow using a laser Doppler blood flowmetry (LDBF) (Moor LDI, Moor Instrument Inc.) as described previously. To minimize variations due to ambient light, blood flow was expressed as the ischemic/normal hind limb LDBF ratio. Also, a heating pat was used to maintain the body temperature of mice at a same level throughout the experiments.

**Immunohistochemical analysis**

To determine sarcomeric F-actin organization, MSCs labeled or unlabeled with MCLs were stained with fluorochrome-conjugated phalloidin (Alexa488-Phalloidin; Molecular Probes, Eugene, OR) and nuclei were stained with the DNA binding dye 4,6-diamino-2-phenylindole (DAPI) (Dojin, Kumamoto, Japan). The area of cells was measured using NIH Image J software (National Institutes of Health Service Branch).

For histological evaluation, MSC sheets were washed with PBS, fixed in 4 % paraformaldehyde and embedded in paraffin. Sections with 5µm in thickness were
stained by hematoxylin and eosin. To examine the cell proliferation and apoptotic cell
death within the MSC sheets, proliferating cell nuclear antigen (PCNA) and terminal
dUTP nick end labeling (TUNEL) stainings were also performed. To examine the
presence of gap junctions, MSC sheets were subjected to immunofluorescence staining
with anti-connexin43 monoclonal antibody (SantaCruz Biotechnology, Santa Cruz, CA,
USA). Tissue capillary and arteriole density in adductor and gastrocnemius muscles of
mice were measured by histochemical staining with anti-mouse CD31 (BD Biosciences)
and anti-mouse α-SMA (Sigma, St. Louis, MO, USA). The number of capillary
endothelial cells and α-SMA positive cells were counted under fluorescence microscopy
(x200). Five fields from the 5 different muscle samples of each animal were randomly
selected for the capillary and arteriole density analysis. Frozen sections were also
stained with anti-VEGF antibody (Thermo Scientific).

To investigated whether implanted MSC sheets could survive, differentiate into
endothelial cells and secrete angiogenic cytokines in ischemic tissues, frozen sections
were stained with anti-mouse CD31 (BD Biosciences), anti-mouse α-SMA (Sigma, St.
Louis, MO, USA), anti-human CD31 and anti-human α-SMA (Epitomics, Inc.,
Burlingame, Ca, USA).

**Reverse Transcription PCR Analysis**

Total RNA was isolated with Trizol (Invitrogen Co, CA, USA) from adductor and
gastrocnemius muscles of ischemic and non-ischemic limbs at day 3, day 7, and day21.
Reverse transcription was performed with 1 µg of RNA, random primers and MMLV
reverse transcriptase (ReverTraAce-α TOYOB, Osaka, Japan). Quantitative real-time PCR was performed with the LightCyclerT System (Roche Diagnostics, IN, USA) and QuantiTect SYBR Green PCR kit. Primers: mouse VEGF: sense, 5’-CAGGCTGCTGTAACGATGAA-3’, and antisense, 5’-AATGCTTTCTCCGCTCTGAA-3’, human VEGF: sense, 5’-AAGGAGGAGGGCAGAATCAT-3’ and antisense, 5’-ATCTGCATGGTGATGTTGGA-3’, human lamin: sense, 5’-GAAGTGGCCATGAGGACTGT-3’ and antisense, 5’-GAGTGTGGGTTCACATCACG-3’, mouse GAPDH: sense, 5’-ATGGTGAGGTCGGTG-3’, and antisense, 5’-ACCAGTGGATGCAGGGAT-3’.

ELISA analysis

Plasma VEGF levels at day 7 after hind limb ischemia were measured by mouse VEGF ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA) according to the manufacturer’s instruction.¹

Peroxidase-like anti-oxidative activity of MCLs and assessment of cell viability

Experiments were carried out using MCLs (11.0 µg-magnetite) in a 0.5 ml reaction mixture comprising 0.05 M Tris-HCl, 0.15 M NaCl, 0.02% H₂O₂, (pH7.5) with 1 mg/ml Di-azo-aminobenzene (DAB) as a substrate. Immediately after the substrate was added, color reactions were measured.² MSCs labeled or unlabeled MCLs (100
pg-magnetite/cell) were treated with 1mM L-buthionine-[S,R]-sulfoximine (BSO) (Sigma-Aldrich, St. Louis, MO, USA). After 48h, cell viability was assessed by trypan blue exclusion.

**Immunoblotting analysis**

To assess the anti-apoptotic effect of MCLs, human MSCs with or without MCLs (100 pg-magnetite/cell) were treated with 1mM BSO for 24h to deplete reduced glutathione (GSH). These cells were then lysed in Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO, USA), and the cell lysate was subjected to immunoblotting, as previously described. The following antibodies were used: rabbit anti-mouse Bax (Upstate Biotechnology Inc. NY, USA), anti-mouse β-actin (Sigma-Aldrich, St. Louis, MO, USA).

**Statistical Analysis**

All data are presented as mean ± SEM. One way ANOVA followed by Bonferroni’s correction was used for the statistical analysis for multiple comparisons among the groups. \( p<0.05 \) was considered statistically significant.

**References for Supplemental Methods**


Supplemental Figure Legends

**Supplemental Figure I.** Characteristics of MSCs labeled with MCLs. (A) Cell size as assessed by forward scatter and cytosolic structure as assessed side scatter between MSCs labeled with MCLs and MSCs unlabeled with MCLs by flow cytometric analysis. (B) Comparison of cell surface markers such as CD44, CD45 and CD166 between MSCs labeled with MCLs and MSCs unlabeled with MCLs. Filled areas indicate staining with a specific antibody, and also open areas show staining with isotype control antibodies. (C) Representative photographs of immunostaining of sarcomeric F-actin with phalloidin in MSCs labeled with MCLs or MSCs unlabeled with MCLs (left panel). Quantitative analysis of cell surface area measured by NIH Image J software from two-dimensional images of 100 cells selected at random (right panel). (D) The expression of the angiogenic cytokines using Proteome Profiler array in MSCs labeled with MCLs and MSCs unlabeled with MCLs. Representative immunoblots (upper panel) and quantitative analysis of relative changes in various angiogenic factors (lower panel).

**Supplemental Figure II.** (A) Expressions of mouse VEGF mRNA determined by real-time PCR in the PBS control, MSC-injection and MSC-sheet transplantation groups
(n=4 each group).  (B) Expression of human VEGF mRNA was greater in the MSC-sheet group than in the MSC-injected group at day3. (C) VEGF protein expression was higher in the MSC-sheet group than MSC injected group. (D) Plasma concentrations of VEGF protein did not differ among the 3 groups, indicating the transplantation of MSCs did not affect plasma VEGF levels (n=4 each group).

**Supplemental Figure III.** Quantitative analysis of TUNEL positive apoptotic cells in the host ischemic tissues. (A) TUNEL staining images in the ischemic muscles at 21 days after surgery.  (B) The percentages of apoptotic cells were significantly lower in the MSC-sheet group compared to the control and MSC-injected groups. *p<0.05 vs. PBS group; †p<0.05 vs. MSC-injection group.  (C to E) Peroxidase-like activity of MCLs. (C) MCLs reversed oxidation of DAB in the presence of H$_2$O$_2$. (D) GSH depletion by BSO (1mM) treatment caused a significant increase in the cell death ratio in MSCs without MCL labeling. However, MCL labeling rescued human MSCs from cell death. *p<0.05 vs. control MSCs without MCLs ; †p<0.05 vs. MCL (-) BSO group. (E) Bax expression levels increased in MSCs without MCLs after BSO treatment, whereas Bax expression levels did not increase in MSCs treated with MCLs (n=3 each). *p<0.05 vs. BSO 0h group. The effect of the MCL labeling itself on blood flow
recovery of ischemic muscles in WT mice. (F) Quantitative analysis of the ischemic/normal hind limb LDPI ratio in the MSCs labeled with MCLs-injected group, the MSCs unlabeled with MCLs-injected group and the control PBS group. (G) Representative photomicrographs of tissues stained with CD31 and α-SMA in the MSCs labeled with MCLs-injected group, the MSCs unlabeled with MCLs-injected group and the control PBS group.

**Supplemental Figure IV.** Location of transplanted MSC sheets at chronic phase. (A) Section was stained with anti-mouse CD31 (Red), anti-mouse α-SMA (Green) and nuclei (Blue). Lower panels show the high power magnification of area selected in the dotted square (x200). (B) Immunostaining with anti-human CD31 (Red), ant-human α-SMA (Green) and nuclei (Blue).
Supplemental Figure I
Supplemental Figure I
A

![Graph A](image)

B

![Graph B](image)

C

![Images C](image)

D

![Graph D](image)

Supplemental Figure II
**Supplemental Figure III**

**A**
- TUNEL staining images for PBS, hMSC Injection, and hMSC Sheet groups.
- DAPI staining images for the same groups.

**B**
- Bar graph showing TUNEL positive nuclei (%).
- Groups: PBS, hMSC Injection, hMSC Sheet.
- Statistical comparison: N.S., *, †.

**C**
- Image showing MCL (Fe₃O₄) with + DAB, H₂O₂.

**D**
- Bar graph showing trypan blue positive cells (%).
- Groups: Control, BSO 1mM, MCL (-), MCL (+).
- Statistical comparison: *, †.

**E**
- Western blot images for Bax and β-actin at 0h, 2h, 4h, 12h, 24h.
- MCL (-) and MCL (+) conditions with 1 mM BSO.
- Bar graph showing Arbitrary Unit for 1 mM BSO at 0h, 2h, 4h, 12h, 24h.
- Comparison: MCL (-), MCL (+).
Ischemic/normal perfusion ratio

Days after ischemic surgery

PBS
MCL(-) hMSC Injection
MCL(+) hMSC Injection

†
* 
#

0 7 14 21

0.1 0.2 0.3 0.4 0.5 0.6 0.7

Capillary density/mm²

PBS
MCL(-) hMSC Injection
MCL(+) hMSC Injection

P<0.05

CD31
α-SMA
DAPI

PBS
MCL(-) hMSC Injection
MCL(+) hMSC Injection

P<0.05
N.S.

Supplemental Figure III