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Co-administration of adipose-derived stem cells and control-released basic fibroblast growth factor facilitates angiogenesis in a murine ischemic hind limb model

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ABSTRACT

Objective: Adipose-derived stem cells (ASCs) have angiogenic potential owing to their differentiation into endothelial cells and their release of angiogenic growth factors to elicit paracrine effects. In addition, control-released basic fibroblast growth factor (bFGF) sustained with a gelatin hydrogel also supports effective angiogenesis. We sought to determine if co-administration of ASCs and control-released bFGF into murine ischemic limbs facilitates angiogenesis.

Methods: Levels of growth factors in the conditioned media of ASCs cultured with or without control-released bFGF were measured by enzyme-linked immunosorbent assays. A murine ischemic hind limb model was generated and intramuscularly injected with the following: gelatin hydrogel (Group 1), a high number of ASCs (Group 2), control-released bFGF (Group 3), a small number of ASCs and control-released bFGF (Group 4), and a high number of ASCs and control-released bFGF (Group 5). Macroscopic and microscopic vascular changes were evaluated until day 7 by laser Doppler perfusion imaging (LDPI) and histological analyses, respectively.

Results: Secretion of hepatocyte growth factor, vascular endothelial growth factor, and transforming growth factor-β1 (TGF-β1) was enhanced by control-released bFGF. Vascular improvement was achieved in Group 4 and 5 according to LDPI. Hematoxylin and eosin staining and CD31 immunohistochemical staining demonstrated an increase in the vascular density, vessel diameter, and thickness of vessel walls in Groups 4 and 5. Cells positively stained for CD146, α-smooth muscle actin, and TGF-β1 were observed around vessel walls in Groups 4 and 5. Conclusions: These findings suggest that co-administration of ASCs and

control-released bFGF facilitates angiogenesis in terms of vessel maturation in a murine ischemic hind limb model.

Keywords: adipose-derived stem cells, control-released basic fibroblast growth factor, angiogenesis, transforming growth factor-β1, vessel maturation.

INTRODUCTION

Critical limb ischemia (CLI) is a manifestation of peripheral arterial disease that describes patients with typical chronic ischemic rest pain or ischemic skin lesions, namely, ulcers or gangrene. Despite improvements in medical care and revascularization, patients with CLI continue to have a high risk of major amputation below or above the knee and cardiovascular death.[1] Surgical and catheter-based procedures are constantly improving the treatment options for many patients with tissue ischemia; however, diseases mainly affecting arterioles and capillaries will likely never be amenable to surgical or dilatation procedures. For these, cell-based therapeutic strategies remain alternative treatment options. Bone marrow-derived stromal cells have been previously identified as a potential new therapeutic option to induce angiogenesis.[2] Mesenchymal stem cells are also expected to be an effective cell source for vascular regeneration because these cells are considered to be able to differentiate into various cell types and produce a significant amount of vascular growth factors. Adipose-derived stem cells (ASCs) which reside in the stromal vascular fraction of adipose tissues can differentiate into multiple mesenchymal cell types.[3-5] Several studies have shown that transplantation of ASCs into an ischemic hind limb improves blood flow in murine models.[6-9]

In addition, basic fibroblast growth factor (bFGF) induces angiogenesis as a result of its effects on endothelial cell proliferation as well as its role as a chemoattractant and in aiding the proliferation of fibroblasts and epithelial cells. However, the free form of bFGF exhibits limited biological effects because its half-time is too short in vivo. To overcome this, Tabata et al. designed a novel approach with a drug delivery system (DDS) that enabled

controlled release of growth factors in vivo and thus improved the efficacy of growth factor therapy.[10, 11] Several clinical trials, not only for therapeutic angiogenesis in ischemic lower legs but also for tissue repair and regeneration including wound healing, have been conducted with great success.[12-14]

Although administration of a single type of cells or a single growth factor may be effective for angiogenesis as indicated above, one of the major limitations of these studies includes that the size of the newly formed vessels seem to be small in diameter.[15] However, several recent reports demonstrated that the co-administration of stem cells with either endothelial progenitor cells (EPCs) or growth factors or gene transfection are more effective for angiogenesis in terms of not only the vascular density but also the vessel size.[16, 17] Based on this phenomenon, we hypothesized that co-administration of ASCs and control-released bFGF might facilitate angiogenesis in a murine model of hind limb ischemia.

MATERIALS & METHODS

Preparation of control-released bFGF gelatin hydrogels

Gelatin hydrogels with a mean diameter of 59.0 μm (ranging from 29.7 to 86.8 μm) were prepared from gelatin (Nitta Gelatin Inc., Osaka, Japan) as described previously.[10] Gelatin hydrogels with a water content of 95.2%, which degrade in approximately 14 days, were used. Human recombinant bFGF was purchased from Kaken Pharmaceutical Co. (Tokyo, Japan). Gelatin hydrogels slowly release bFGF into the local tissue, and most bFGF remains at the injection site or in the surrounding extracellular matrix. To generate bFGF-impregnated gelatin hydrogels, 30 μg of human recombinant bFGF diluted in 100 μL

of phosphate-buffered saline (PBS) (Gibco-BRL, Grand Island, New York) was dropped onto 0.5 mg of dried gelatin hydrogel microspheres and mixed. A vehicle control was prepared by exposing 0.5 mg of gelatin hydrogels to PBS alone without bFGF.

Isolation of ASCs from adipose tissue

The Animal Care and Use Committee of Juntendo University approved all experiments (approval number: 250157). Under general anesthesia with isoflurane (1–3% for maintenance, up to 5% for induction), ASCs were isolated from the inguinal fat pads of 7–10-week-old wild-type C57BL/6J mice or green fluorescent protein (GFP)-expressing transgenic C57BL/6J mice (C57BL/6J-Tg [CAG-EGFP]; Japan SLC Inc., Shizuoka, Japan) depending on the subsequent experiments. Briefly, subcutaneous adipose tissue was digested with 0.075% collagenase I (Wako Pure Chemical Industries Ltd., Osaka, Japan) under gentle agitation for 45 min at 37°C. The digested tissues were filtered through a 40-μm nylon sieve to remove cellular debris and centrifuged at 200 g for 5 min. The pellet was suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) containing 10% fetal bovine serum (FBS, Gibco-BRL). The cell suspension was centrifuged at 200 g for 5 min. The pellet was resuspended in DMEM containing 10% FBS and plated in 100 mm tissue culture dishes at a density of 1×10^6 cells per plate. These cells were maintained in control medium (DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic) at 37°C and in 5% CO_2.

Measurement of the levels of growth factors in the conditioned medium of ASCs treated

with control-released bFGF

ASCs at passage #2 isolated from C57BL/6J mice were plated in 12-well tissue culture plates at a density of 2.5×10^5 cells per plate and maintained in control medium at 37°C and in 5% CO_2. Twenty-four plates were divided into three groups: Group A, 2.5×10^5 ASCs and 30 μg of control-released b-FGF; Group B, 2.5×10^5 ASCs and 0.5 mg of gelatin hydrogel; and Group C, 2.5×10^5 ASCs only (n=8). Conditioned media were collected at days 2, 4, and 6 to measure the levels of growth factors including HGF, VEGF, and TGF-β1. The concentration of each growth factor was measured using a mouse Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, MN USA) according to the manufacturer's instructions. Briefly, 50 mL of sample was added to a 96-well microplate coated with a monoclonal antibody against the growth factor of interest and incubated for 2 h. After washing with washing buffer, a horseradish peroxidase-conjugated cytokine was added to each well, after which the wells were incubated for 1 or 2 h and washed. The substrate solution was added and incubated for 30 min, and the reaction was terminated by the addition of the stop solution. The concentrations of growth factors were determined by measuring the optical density at 450 nm using a microplate reader (SpectraMax 340PC, Molecular Devices Inc., Sunnyvale, USA).

Hind limb ischemic model

A hind limb ischemic model was created in 12–15-week-old male C57BL/6J mice as detailed below. ASCs for transplantation were isolated from GFP-expressing transgenic C57BL/6J mice.

After mice were anesthetized with isoflurane (1–3% for maintenance, up to 5% for induction), the lower half of the body was shaved and prepared with povidone-iodine. The femoral artery and vein, external iliac artery and vein, deep femoral artery and vein, and circumflex femoral artery and vein on the left side were ligated and excised to induce severe hind limb ischemia, which mimics human CLI (Supplemental Fig.).[18, 19] Mice were divided into the following groups according to their treatment: Group 1, gelatin hydrogel only; Group 2, a high number (1×10^6) of ASCs; Group 3, 30 μg of control-released bFGF; Group 4, a small number (1×10^4) of ASCs and 30 μg of control-released bFGF; and Group 5, a high number of ASCs (1×10^6) and 30 μg of control-released bFGF (n=4 each). ASCs and gelatin hydrogel with or without bFGF were injected into the thigh muscle.

Quantitative analysis of blood perfusion by LDPI

To analyze the extent of blood flow improvement in each group, the capillary blood flow of the bilateral hind limb was measured at day 0, 4, and 7 after treatment using "OMEGA ONE" (Omegawave Inc., Tokyo, Japan), which generates a laser Doppler perfusion image. Moreover, quantitative analysis was conducted based on blood flow measurement, which was automatically calculated by the installed software of the system and expressed as the LDPI index, which is defined as the ratio of blood flow in the left hind limb to blood flow in the right hind limb (n=4 each).[19]

Histological analysis

Seven days after treatment, mice were euthanized and perfusion-fixed with 4% paraformaldehyde. In total, 16 sections (6 μm thick at a 3 mm interval in each mouse) prepared from paraffin-embedded transverse-cut tissue samples of the thigh muscle were stained with hematoxylin and eosin. The number and size of vessels and the thickness of vessel walls were observed using a light microscope (DP70, Olympus Co., Tokyo, Japan) at 40-100x magnification. For semi-quantitative analysis, the mean vessel number, vessel diameter and the thickness of the vessel walls were determined by measuring the six different fields in each section using Image J software (National Institutes of Health, Bethesda, Md).

Immunohistochemistry

At day 7, immunohistochemical analyses were performed using a rabbit polyclonal antibody against mouse CD31 (abcam28346, 1:400 dilution; Abcam Inc., Cambridge, United Kingdom), a rabbit polyclonal antibody against αSMA (abcam5694, 1:500 dilution; Abcam Inc.), and a rabbit monoclonal antibody against CD146 (abcam75769, 1:400 dilution; Abcam Inc.) to identify endothelial cells, smooth muscle cells, and pericytes, respectively, with a Ventana BenchMark® GX autostainer (Roche Diagnostics K.K., Tokyo, Japan) and an i-VIEW™ DAB Detection Kit (open secondary protocol) (Roche Diagnostics K.K.) in accordance with the manufacturer's instructions. Briefly, sections were deparaffinized and pretreated with cell conditioning 1 solution for 1 h. Sections were then washed with reaction buffer and incubated with anti-CD31, anti-αSMA, and anti-CD146 antibodies as mentioned above at 37˚C for 32 min. Thereafter, sections were incubated with

biotin-conjugated polyclonal goat anti-rabbit immunoglobulins (E0432 Dako, Glostrup, Denmark) as a secondary antibody at 37°C for 8 min. The secondary antibody was visualized using diaminobenzidine. Sections were counterstained with hematoxylin.

Finally, to investigate vessel maturation, expression of TGF-β1, an indicator of vessel maturation, was analyzed by immunohistostaining.[20] ASCs isolated from GFP-expressing transgenic mice were injected into the ischemic hind limb in the same manner in Groups 2, 4, and 5, with or without control-released bFGF. Several muscle specimens were harvested at day 7, embedded in optimal cutting temperature compound (Sakura Finetec Japan Co., Ltd, Tokyo, Japan) and frozen at -80°C. Cryostat sections (4 μm thick) of tissues were stained with a rabbit polyclonal anti-CD31 antibody (abcam28346, 1:100 dilution; Abcam Inc.) and anti-TGF-β1 antibody (abcam92486, 1:100 dilution; Abcam Inc.). Thereafter, samples were stained with a goat polyclonal secondary antibody against rabbit IgG-H&L (Alexa Fluor® 594) (abcam150080, Abcam Inc.). After fixation, specimens were observed by fluorescence microscopy (Axioplan 2 imaging microscope and Axiovision software, Zeiss Co., Berlin, Germany).

Statistical analysis

Data were expressed as mean ± standard deviation. Multiple groups were compared using the Kruskal-Wallis test and, if necessary, the Mann-Whitney test. All statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA). A P-value of less than 0.05 was considered to be significant.

RESULTS

Levels of secreted growth factors in ASC conditioned medium were increased by control-released bFGF

The levels of HGF at day 2, 4, and 6 were 3.8-, 1.7-, and 1.9-fold higher in Group A than in Group B, respectively. There was a statistically significant difference between Group A and Group B at all time points ($P<.05$). Additionally, the levels of HGF at day 2, 4, and 6 were 2.5-, 2.0-, and 1.9-fold higher in Group A than in Group C, respectively. There was a statistically significant difference between Group A and Group C at all time points ($P<.05$) (Fig. 1A). Similar to HGF, the levels of VEGF at day 2, 4, and 6 were 1.5-, 1.3-, and 1.2-fold higher in Group A than in Group B, respectively. There was a statistically significant difference between Group A and Group B at all time points ($P<.05$). Additionally, the levels of VEGF at day 2, 4, and 6 were 1.7-, 1.3-, and 1.2-fold higher in Group A than in Group C, respectively. There was a statistically significant difference between Group A and Group C at day 2 and day 4 ($P<.05$) (Fig. 1B). Finally, the levels of TGF-β1 at day 2, 4, and 6 were 2.1-, 1.5-, and 1.1-fold higher in Group A than in Group B, respectively. Similar to VEGF, there was a statistically significant difference between Group A and Group B at day 2 and day 4 ($P<.05$). Additionally, the levels of TGF-β1 at day 2, 4, and 6 were 2.0-, 1.7-, and 1.6-fold higher in Group A than in Group C, respectively. There was a statistically significant difference between Group A and Group C at all time points ($P<.05$) (Fig. 1C).

Co-administration of ASCs and control-released bFGF significantly improved blood

perfusion of the ischemic hind limb according to laser Doppler perfusion imaging (LDPI)

Immediately after induction of hind limb ischemia, the ratio of blood flow was 0.59 ± 0.04 in Group 1, 0.60 ± 0.06 in Group 2, 0.63 ± 0.164 in Group 3, 0.61 ± 0.06 in Group 4, and 0.60 ± 0.03 in Group 5. The ratio of blood flow at 4 days after treatment was 0.60 ± 0.1 in Group 1, 0.65 ± 0.10 in Group 2, 0.65 ± 0.15 in Group 3, 0.75 ± 0.11 in Group 4, and 0.84 ± 0.12 in Group 5. Blood perfusion was significantly higher in Group 4 and 5 than in Group 1 at day 4 ($P<.05$). At 7 days after treatment, the ratio of blood flow was 0.67 ± 0.12 in Group 1, 0.74 ± 0.05 in Group 2, 0.64 ± 0.12 in Group 3, 0.79 ± 0.10 in Group 4, and 0.92 ± 0.03 in Group 5. Blood perfusion was significantly higher in Group 5 than in Group 1, 2 and 3 at day 7 ($P<.05$). Blood flow recovery was severely impaired in this animal model and could be markedly improved by co-administration of ASCs with a control-released bFGF gelatin hydrogel (Fig. 2 and 3).

Co-administration of ASCs and control-released bFGF induced a high vascular density with increased vessel diameter and thicker vessel walls

The mean number of vessels counted in each group was 8.6 ± 2.2 in Group 1, 9.0 ± 1.4 in Group 2, 21.3 ± 1.5 in Group 3, 19.5 ± 2.5 in Group 4, and 21.5 ± 1.3 in Group 5. The number of vessels was significantly higher in Groups 3, 4, and 5, compared with Groups 1 and 2 ($P<.05$). The mean caliber of vessels measured in each groups was 5.5 ± 2.1 μm in Group 1, 7.8 ± 1.9 μm in Group 2, 6.4 ± 1.6 μm in Group 3, 13.8 ± 3.4 μm in Group 4, and 15.0 ± 2.7 μm in Group 5. The mean caliber of the vessels was significantly larger in both Groups 4 and 5 compared with Groups 1, 2 and 3 ($P<.05$). The mean thickness of vessel

walls measured in each groups was 2.2 ± 0.3 μm in Group 1, 3.3 ± 0.5 μm in Group 2, 2.2 ± 0.2 μm in Group 3, 4.0 ± 0.4 μm in Group 4, and 4.1 ± 0.3 μm in Group 5. The vessel walls were significantly thicker in Groups 2, 4, and 5, compared with Groups 1 and 3 ($P<.05$) (Fig. 4a-h). Furthermore, small numbers of transplanted ASCs, which are positive for GFP, were detected through the fluorescent microscope in Groups 2, 4, and 5 (Fig. 5). Small particles of gelatin hydrogel were also observed in the section of Groups 1, 3, 4, and 5 (data not shown). Finally, atrophic or necrotic changes of the muscle fibers accompanied by the inflammatory cell infiltration were recognized more or less in all groups. However, the extent was less in Groups 4 and 5 than in Groups 1, 2, and 3.

Immunohistochemical analysis

Consistent with findings in hematoxylin and eosin-stained sections, CD31-positive cells were observed in each vessel. The caliber of the vessels was larger in Groups 4 and 5 than in Groups 1, 2, and 3 (Fig. 4i-m and Fig. 6 upper row). Immunohistostaining mainly detected α-smooth muscle actin (αSMA) circumferentially in vessel walls in Groups 2, 4, and 5, all of which were administered ASCs (Fig. 6 middle row). CD146, a pericyte marker, was expressed in Groups 2, 3, 4, and 5. However, CD146 staining was stronger in Group 5 than in the other groups (Fig. 6 lower row).

Finally, immunohistostaining detected TGF-β1 (shown in red in Fig. 7a-c) around the walls of vessels positively stained for CD31 (shown in red in Fig. 7d-f) in Groups 4 and 5, but not in Group 2, suggesting that co-administration of control-released bFGF with ASCs enhanced the expression of TGF-β1.

DISCUSSION

Recent developments in the fields of cell-based therapy and regenerative medicine promise the repair of the blood supply in patients with CLI for whom either endovascular therapy or bypass grafting procedures are unavailable. Several reports have indicated that autologous bone marrow stromal cells are an effective cell source for therapeutic angiogenesis.[2, 15] However, there are drawbacks to the use of such cells because bone marrow procurement is painful and yields a low number of mesenchymal stem cells. By contrast, cells with similar characteristics to bone marrow-derived stem cells exist within adipose tissue, termed ASCs.[3, 5] Adipose tissue is plentiful and liposuction procedures are relatively safe with minimal patient discomfort; therefore, adipose tissue is considered to be a promising source of stem cells.[21]

Preclinical studies have shown the angiogenic potential of ASCs as demonstrated by the finding that murine hind limb ischemia can be improved by intramuscular injection of such cells.[7, 8, 22] Moreover, the survival rate of skin flaps can be extended by subcutaneous injection of ASCs owing to the differentiation of ASCs toward endothelial cells and by their release of angiogenic growth factors.[23] In addition to in vivo studies, in vitro studies showed that a hypoxic culture environment could enhance secretion of VEGF from ASCs 5-fold in comparison to ASCs cultured in standard culture conditions, indicating that ASCs play a pivotal role in angiogenesis, particularly in ischemic conditions.[8] More recently, phase I clinical trials have been performed in which patients with non-revascularized CLI were administered autologous cultured ASCs.[24] However, it remains uncertain whether the

major mechanism via which ASCs support angiogenesis is their differentiation into endothelial cells or their paracrine effects.[25] In this study, small numbers of transplanted ASCs existed in the injected site at day 7 after transplantation, which is consistent with the previous consensuses by many researchers. Recently, Agrawal et al. clearly showed that approximately 90% and 95% of the ASCs implanted subcutaneously in a mouse model resulted to be undetectable by days 10 and 21, respectively because of phagocytosis by hot macrophage.[26] Therefore, these facts imply that the effect of angiogenesis may be mainly due to several growth factors releasing from the injected ASCs.

bFGF has a powerful angiogenic property and is a potential cytokine expressed in bone marrow in response to ischemia.[27] However, the half-life of the free form of bFGF is less than 1 hour, and bFGF is water-soluble and therefore easily eliminated from the applied site via diffusion. To overcome this, Tabata et al. designed a novel approach with a DDS that enabled controlled release of a single growth factor by using a gelatin hydrogel as a vehicle in vivo and thus improved the efficacy of growth factor therapy.[10, 28] A clinical trial using control-released bFGF to improve chronic limb ischemia in patients with intractable ulcers was performed without any adverse events.[12] Furthermore, the angiogenic efficacy of control-released bFGF does not significantly differ from that of bone marrow-derived cells.[14]

We hypothesized that co-administration of ASCs and control-released bFGF might facilitate angiogenesis in a murine ischemic hind limb model based on several previous findings. First, several studies have documented positive effects of various growth factors, including bFGF,[29] epidermal growth factor,[30] platelet-derived growth factor (PDGF),[31] and tumor

necrosis factor-α[32], on the proliferation of ASCs. Of these cytokines, FGF-2 is a particularly effective growth-stimulating factor and is considered as a factor for the long-term propagation and self-renewal of ASCs via the extracellular signal-related kinase 1/2 signaling pathway.[29] Second, co-administration of human ASCs and human EPCs at a ratio of 1:4 together with collagen/fibronectin matrix into NOD/SCID mice dramatically induces angiogenesis by several fold in comparison to implants containing either ASCs or EPCs alone because ASCs may play a role in the stabilization of endothelial networks by eliciting similar effects as pericytes.[16] As expected, secretion of growth factors, including HGF, VEGF, and TGF-β1, from ASCs was enhanced by control-released bFGF in vitro, and intramuscular delivery of both ASCs and control-released bFGF significantly improved blood supply in vivo. In our in vitro study, normalization of cell numbers or total protein content was not carried out. However, our results showed that the expression of HGF, VEGF, and TGF-β1 were increased after only 2 days of culture in Group A, which is significantly different compared with the other two groups. A similar result has recently been reported which describes that the additives of bFGF in ASC culture have a potential of increasing of paracrine effect of ASCs in a short time.[33] These findings strongly suggest that the cell proliferation is not only a factor of increasing expression of growth factors. Normalization of cell numbers at each time point will be the next steps in order to evaluate the growth factor secretion more precisely.

Two reports have demonstrated the usefulness of a combination of ASCs and bFGF in comparison to either of these alone for skeletal muscle regeneration and angiogenesis to improve hind limb ischemia.[34, 35] Hwang et al. showed that co-administration of human

ASCs and bFGF incorporated into a hydrogel could improve fast-twitch muscle contraction with less fibrosis in a murine muscle laceration model. According to their detailed analyses, some ASCs differentiated into skeletal muscle cells as the muscle underwent revascularization and reinnervation.[34] Bhang et al. showed that transplantation of human ASCs with local bFGF delivery using heparin-conjugated poly (L-lactide-co-glycolide) nanospheres suspended in fibrin gel significantly enhanced microvessel formation and improved blood perfusion in ischemic limbs followed by eventual limb salvage.[35] In this study, bFGF significantly enhanced the viability and proliferation of ASCs and reduced their apoptosis, and also enhanced the angiogenic efficacy of transplanted ASCs, likely by enhancing their secretion of paracrine factors. Although we did not investigate the effects of control-released bFGF on the proliferation or apoptosis of ASCs, upregulation of angiogenic growth factor secretion is consistent with our findings.

Another finding of our study that should be discussed is that co-administration of ASCs and control-released bFGF increased the caliber of vessels and the thickness of vessel walls. Traktuev et al. reported that ASCs possess properties of pericytes and can stabilize endothelial networks in vitro and in vivo.[16, 36] In addition, Bhang et al. speculated that ASCs might play a role in stabilizing vascular networks by acting as pericytes based on the finding that most ASCs were found in the vicinity of microvessels.[35] Likewise, our immunohistochemical findings revealed that expression of αSMA and CD146, which are representative markers of mural cells including smooth muscle cells and pericytes, respectively, was clearly detected particularly in Groups 4, and 5, suggesting that co-administration of ASCs and control-released bFGF facilitate angiogenesis with vessel

maturation and stabilization. In general, the maturation of nascent vasculature requires the recruitment of mural cells, generation of an extracellular matrix (ECM) and elastic laminae, specialization of the vessel wall for structural support, and regulation of vessel function. At least four molecular pathways are considered to be involved in these processes: PDGF-B/PDGF-B receptor-β, sphingosine-1-phosphate-1/endothelial differentiation sphingolipid G-protein-coupled receptor-1, angiopoietin 1/tyrosine kinase with immunoglobulin-like and EGF-like domains 2, and TGF-β.[37] Of these pathways, TGF-β1, a multifunctional cytokine, promotes vessel maturation by stimulating ECM production and by inducing the differentiation of mesenchymal cells into mural cells.[37-39] In our in vitro study, the levels of TGF-β1 at days 2 and 4 were significantly higher in the conditioned medium of ASCs that were treated with control-released bFGF than in that of ASCs that were not. Furthermore, TGF-β1 was found around the walls of vessels only in Groups 4 and 5. Taken together, our findings suggest that increased release of TGF-β1 from ASCs is induced by control-released bFGF, after which TGF-β1 localizes around vessel walls, stimulates the production of ECM, and eventually contributes to maturation of the vasculature. The detailed mechanism underlying vessel stabilization and maturation induced by co-administration of ASCs and control-released bFGF should be further investigated.

CONCLUSIONS

Co-administration of ASCs and control-released bFGF facilitates angiogenesis with vessel maturation and stabilization in a murine ischemic hind limb model. Although further

investigations will be necessary for exploring more details, our findings might be expected to assist in CLI treatment, chronic wound healing, and vascular tissue engineering in the future.

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DISCLOSURES

None.

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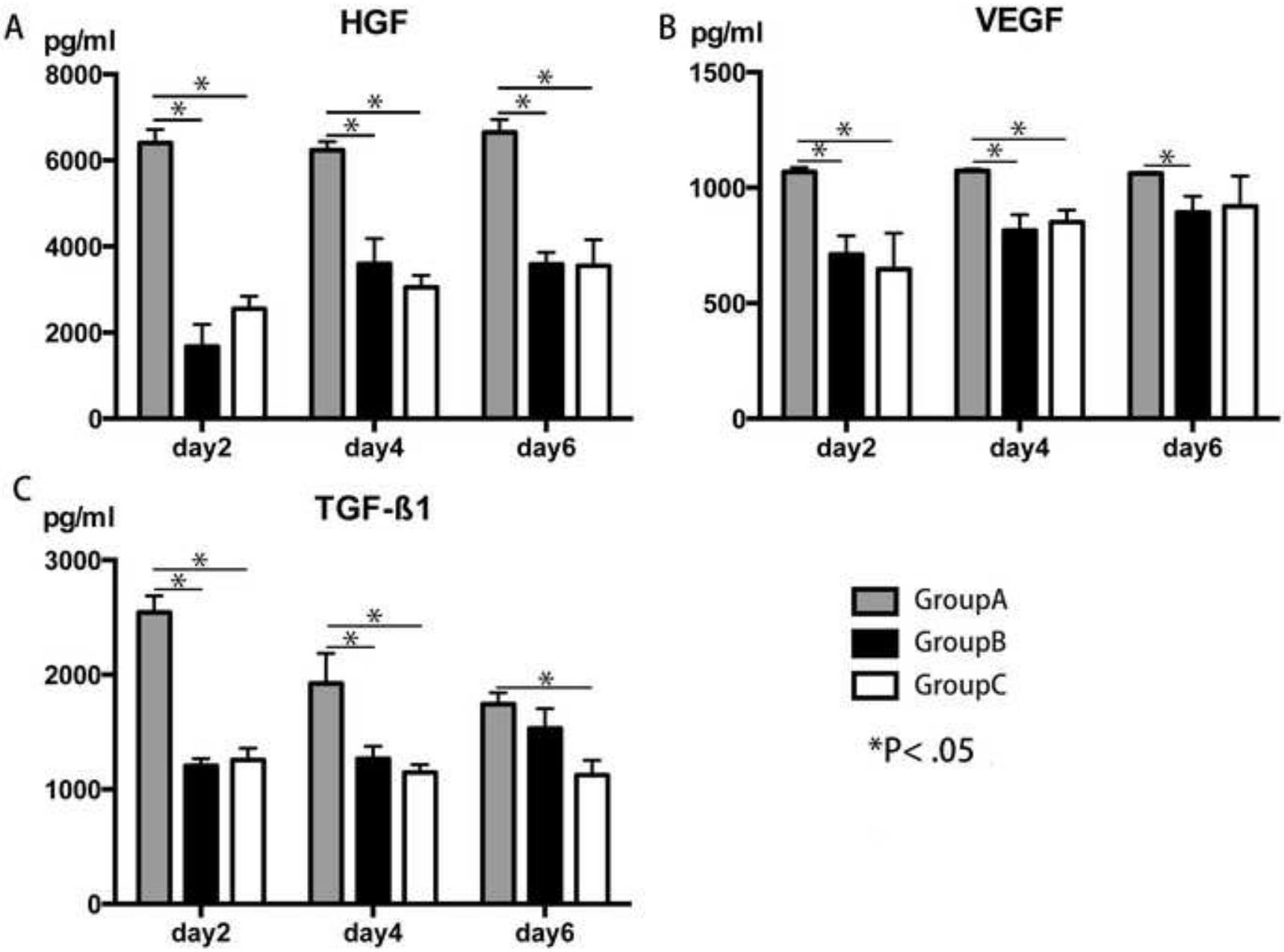

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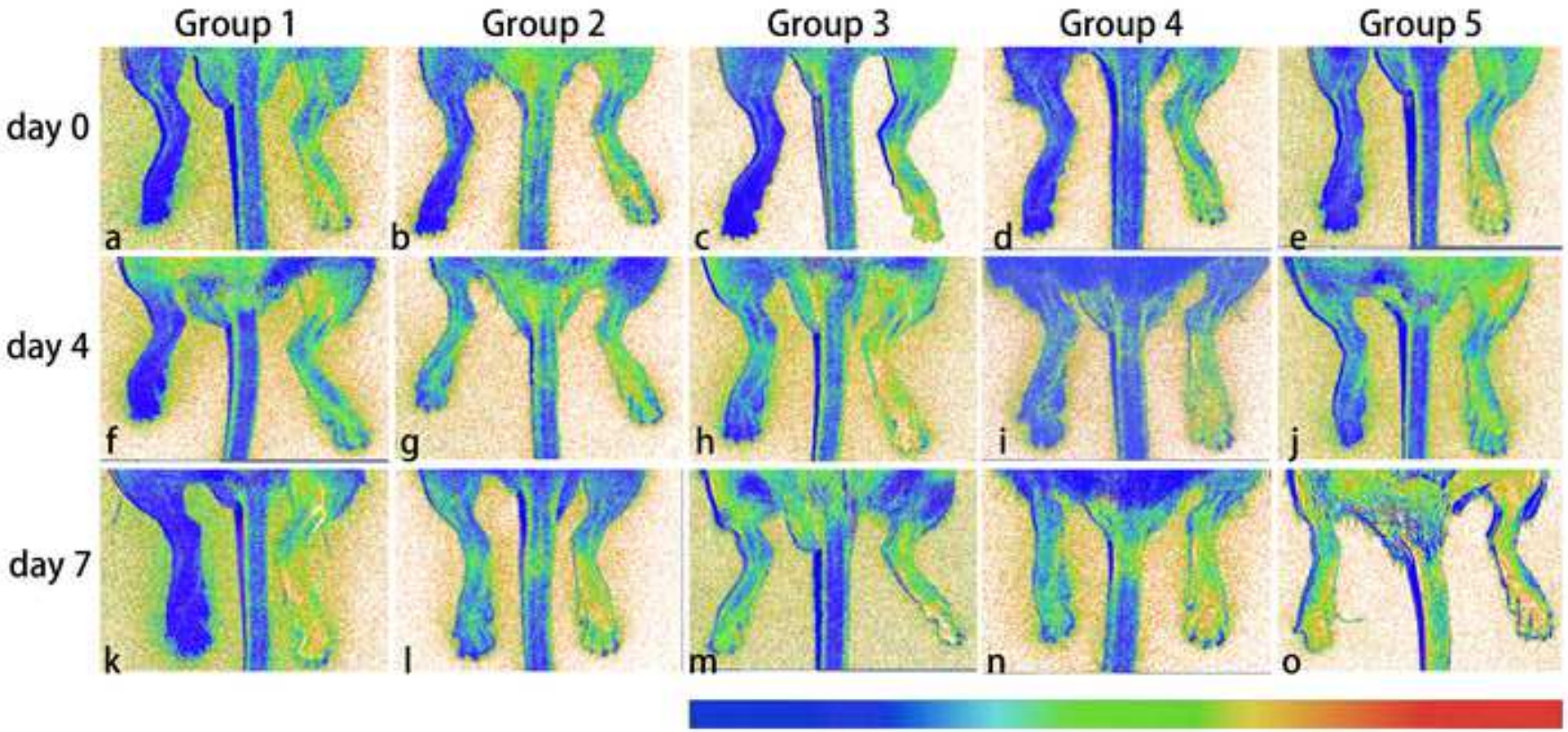

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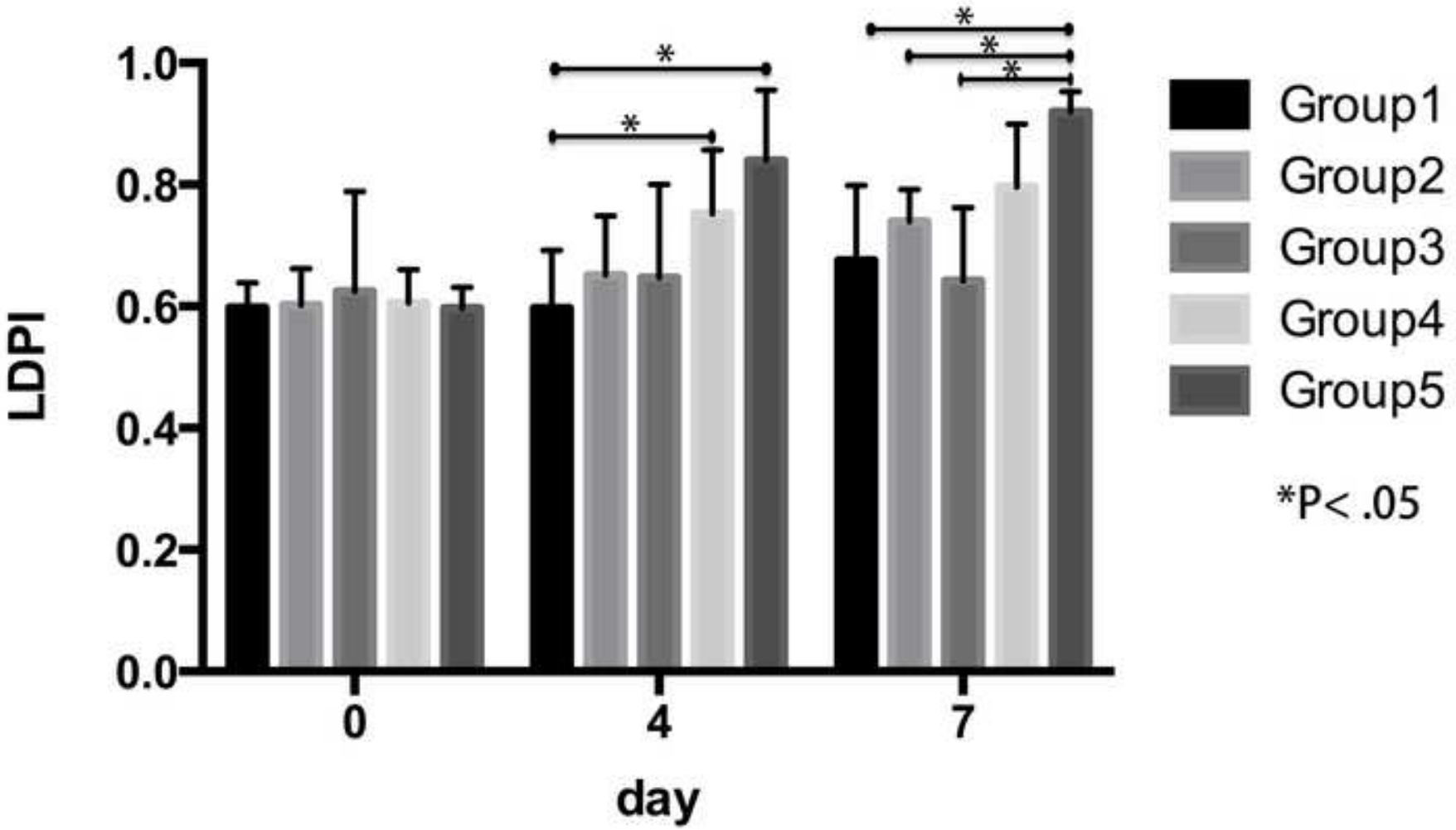

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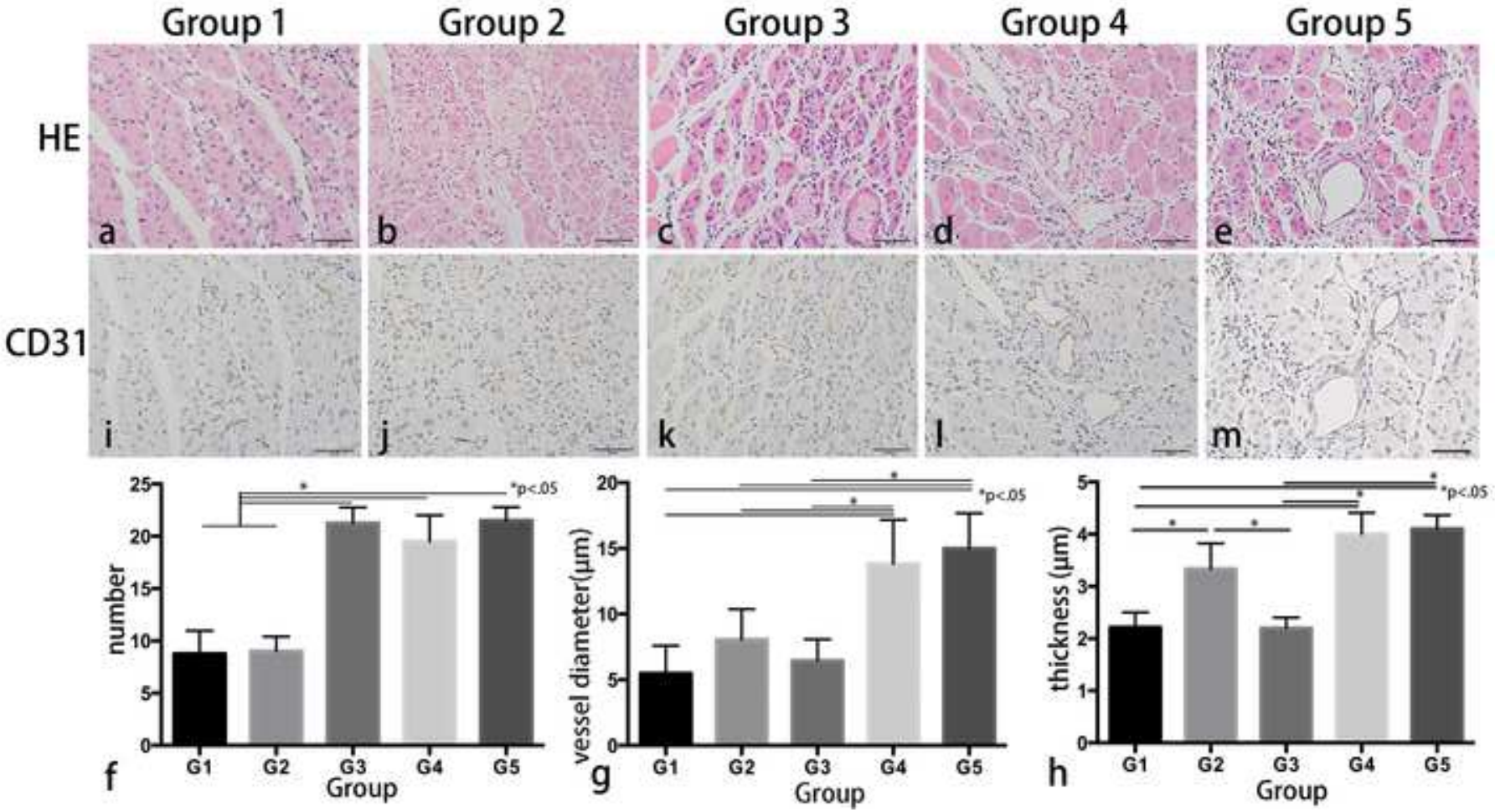

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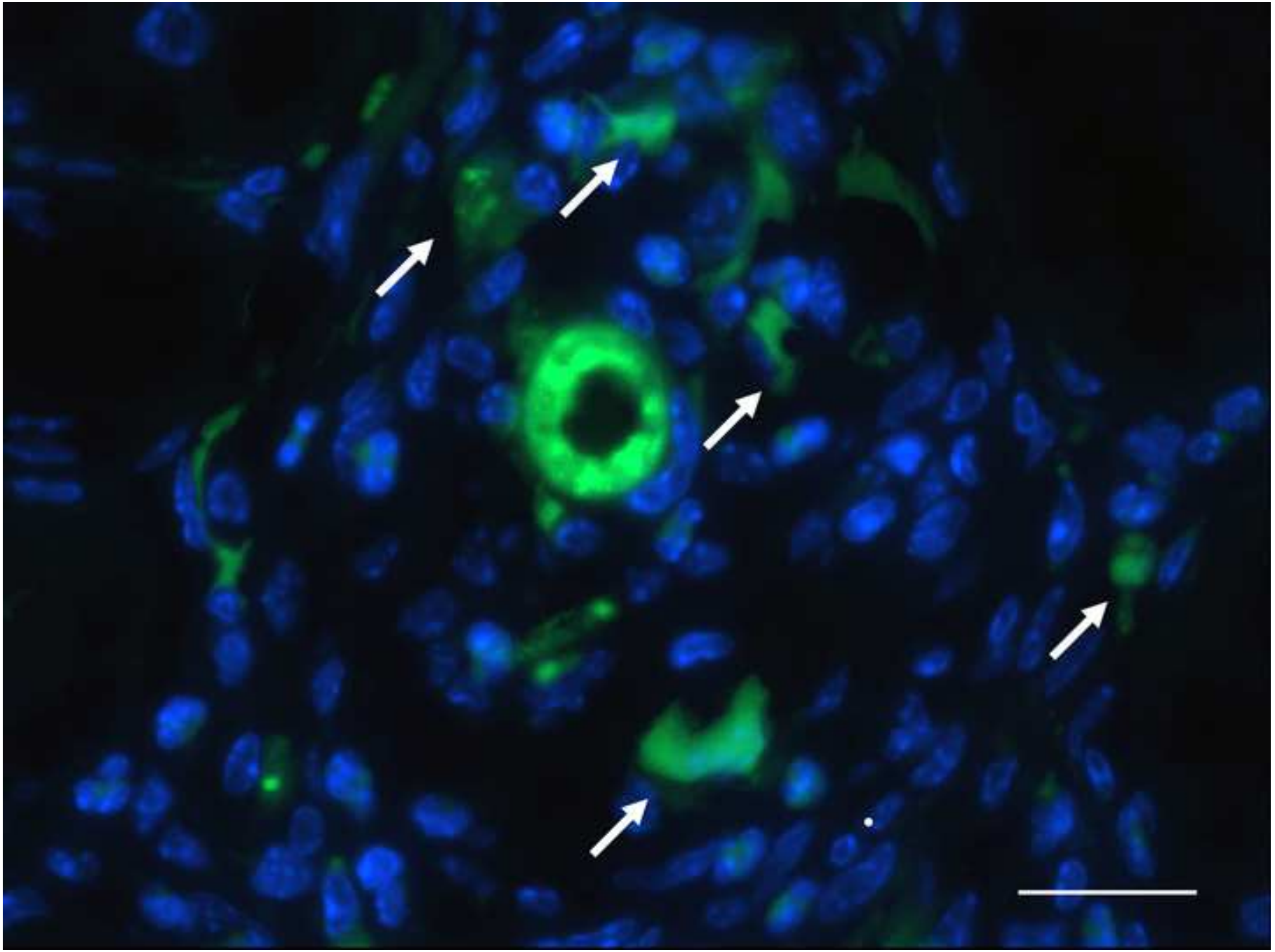

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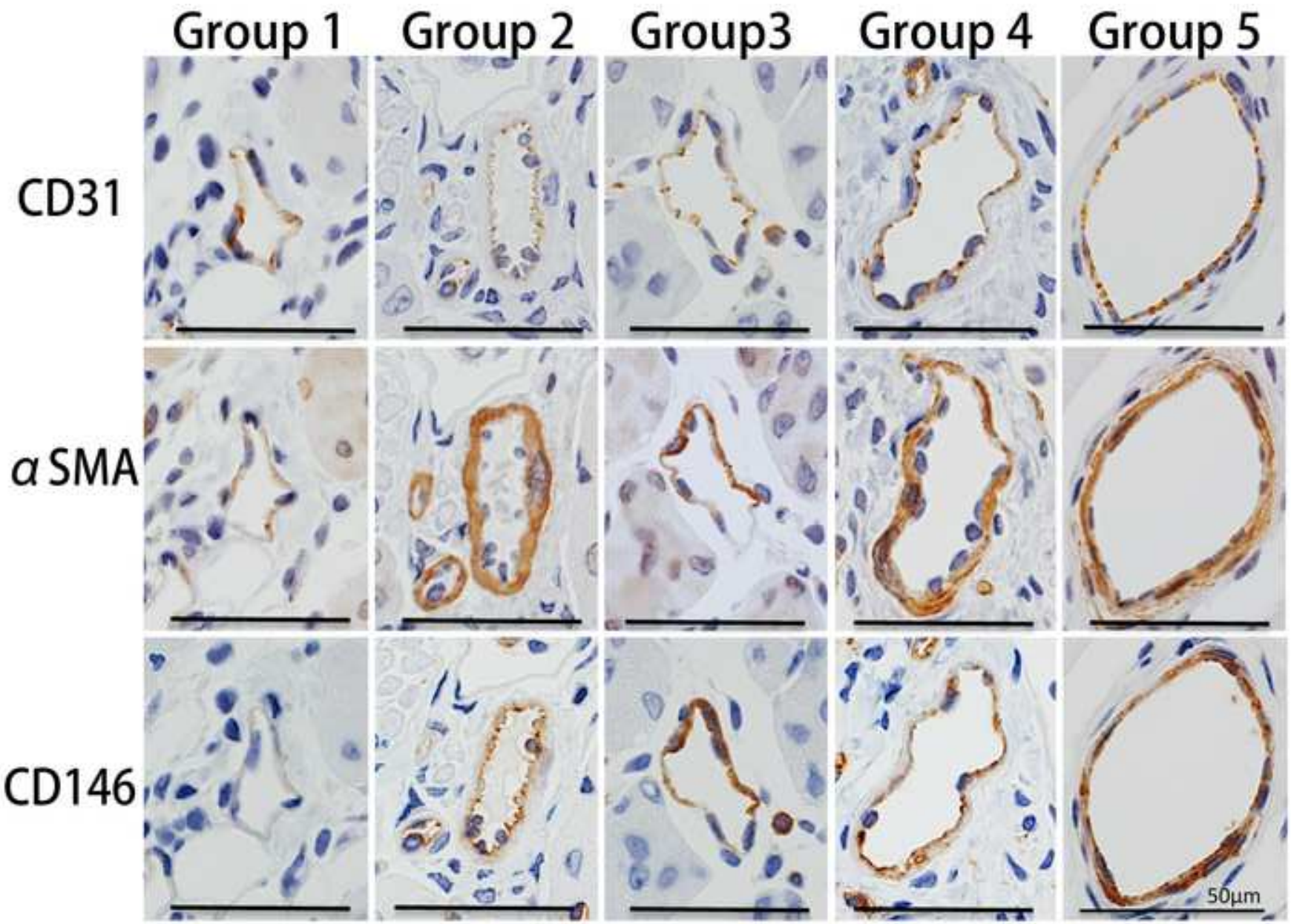

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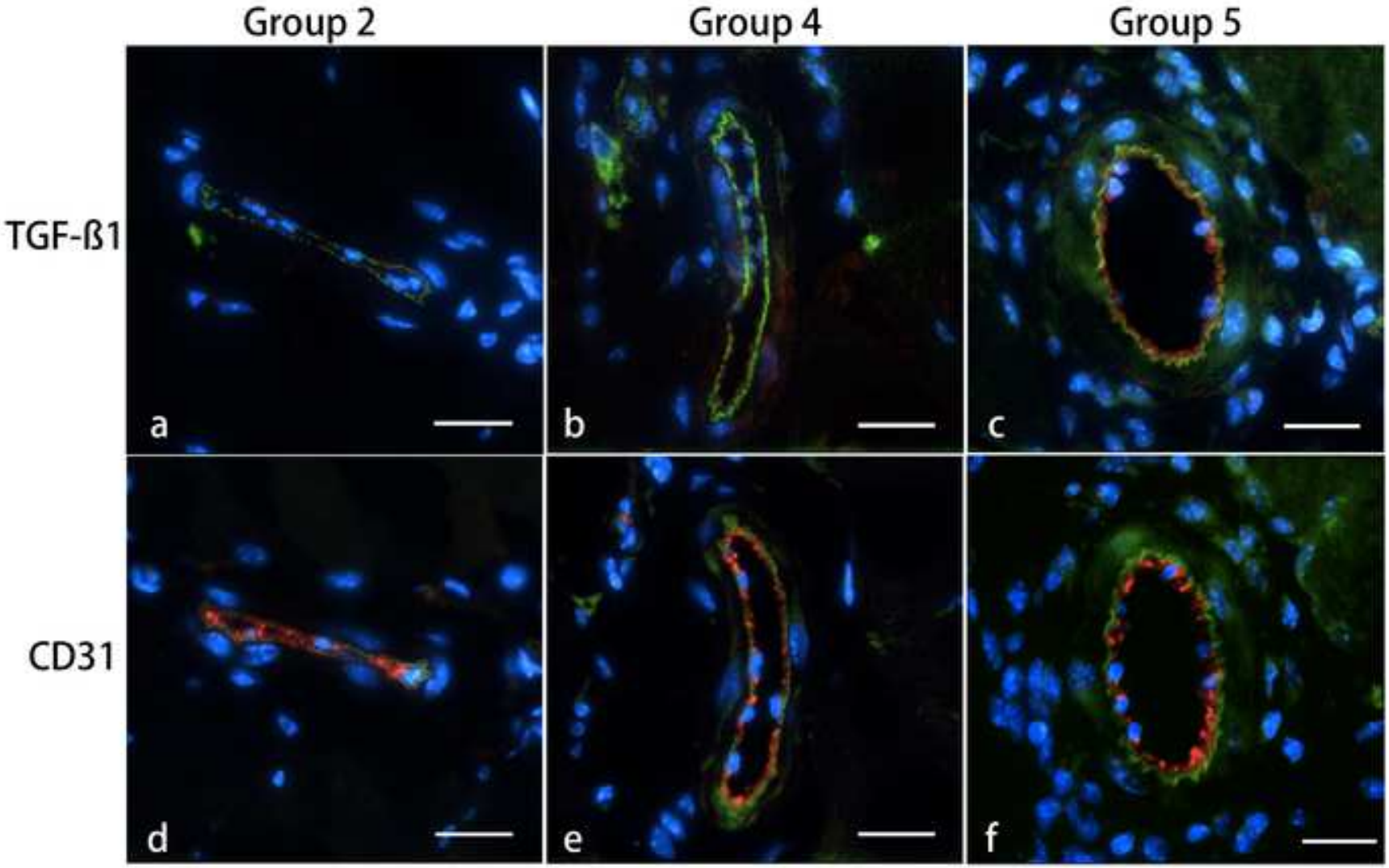

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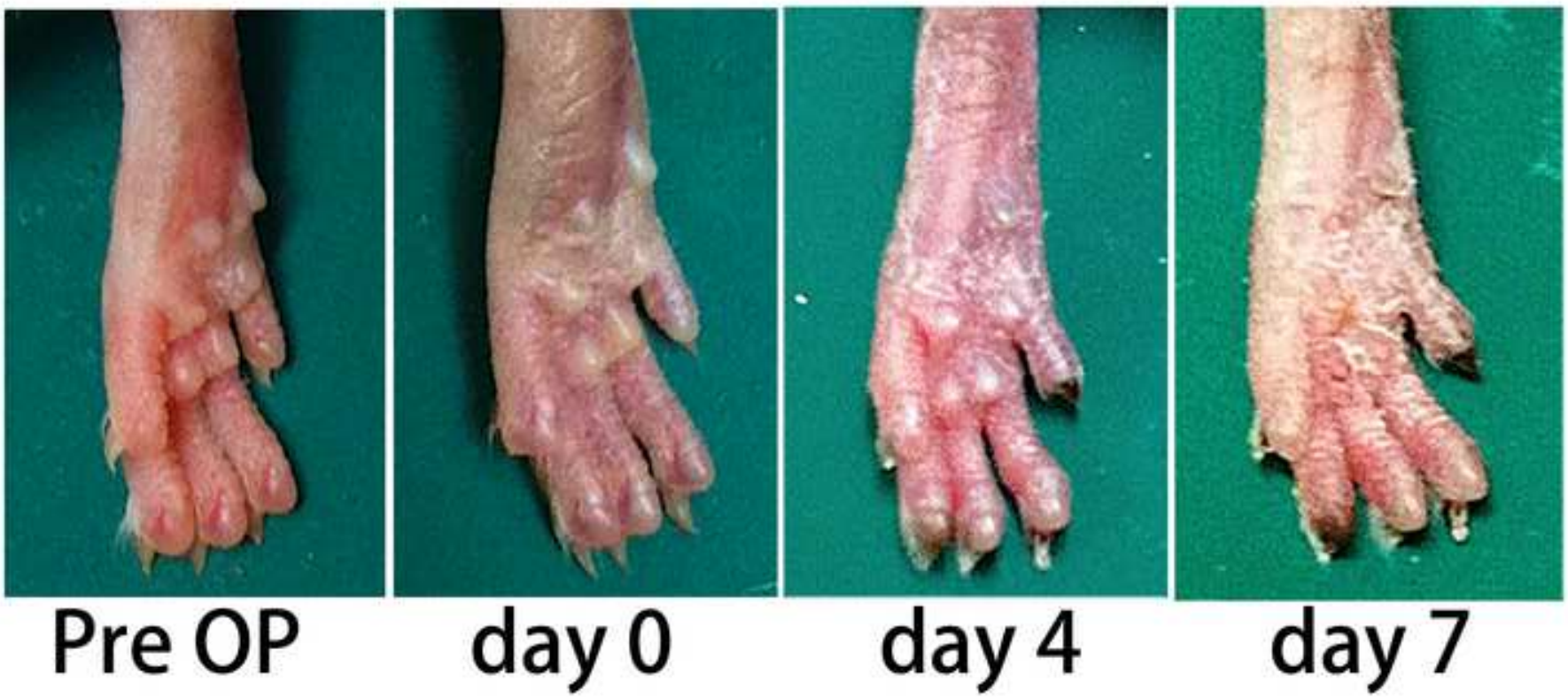

FIGURE TABLE LEGENDS

Figure 1. Concentrations of growth factors secreted from adipose-derived stem cells (ASCs) in specific culture conditions.

(A) The concentration of hepatocyte growth factor (HGF) was significantly higher in the conditioned medium of ASCs treated with control-released bFGF (Group A) than in the other groups at all time points. (B) The concentration of vascular endothelial growth factor (VEGF) in conditioned medium was significantly higher in Group A than in Group B at all time points, and was significantly higher in Group A than in Group C at days 2 and 4. (C) The concentration of transforming growth factor-β1 (TGF-β1) in conditioned medium was significantly higher in Group A than in Group B at days 2 and 4, and was significantly higher in Group A than in Group C at all time points. Asterisks indicate a statistically significant difference between the indicated groups (*P<.05).

Figure 2. Representative image of laser Doppler perfusion imaging of murine hind limbs in each group at day 0 (a–e), day 4 (f–j), and day 7 (k–o) after treatment.

In Group 5, blood perfusion was severely impaired at day 0 (e), increased at day 4 (j), and

restored to near-normal levels at day 7 (o).

Figure 3. Quantitative analysis of blood perfusion recovery by laser Doppler perfusion imaging (LDPI) in each group.

The LDPI index was evaluated as the ratio of blood perfusion in the ischemic hind limb to that in the non-ischemic hind limb. LDPI found a significant improvement in Groups 4 and 5 in comparison to Group 1 at 4 days after treatment and in Group 5 in comparison to Groups 1, 2, and 3 at 7 days after treatment.

Figure 4. Representative images of hematoxylin and eosin (HE) staining and immunohistochemical staining for CD31 in each group at 7 days after treatment.

(a-c) Histologically, the thickness of vessel walls was thicker in Group 2 than in Group 1. In Group 3, the vascular density was higher than in both Groups 1 and 2, whereas the vessel wall thickness was similar to that in Group 1 (bar, 50 μm). (d, e) Co-administration of ASCs and control-released bFGF (Groups 4 and 5) dramatically increased the number and the caliber of vessels and the thickness of vessel walls, which mainly consist of endothelial cells, smooth muscle cells, pericytes, and elastic lamina, in a dose-dependent manner (bar,

50 μm). (f) The number of vessels was significantly larger in Group 3, 4 and 5 than Group 1 and 2 at day 7 (*P<.05). (g) The mean caliber of vessels was significantly larger in Groups 4 and 5 than in Groups 1, 2, and 3 at day 7 (*P<.05). (h) The thickness of the vessel walls was significantly larger in Groups 2, 4 and 5 than in Groups 1 and 3 at day 7 (*P<.05). (i-m) Consistent with HE staining, immunohistochemical staining for CD31 showed obvious differences with the caliber of vessels and the thickness of vessel walls between Groups 1-3 and Groups 4-5 (bar, 50 μm).

Figure 5. Representative image of GFP-positive ASCs in the injected site. A small number of GFP-positive ASCs (arrows) reside particularly around the vessels and the surrounding tissue (bar, 20 μm).

Figure 6. Representative images of immunohistochemical staining for CD31 (upper row), α-smooth muscle actin (αSMA, middle row), and CD146 (lower row) at 7 days after treatment.

The caliber of vessels was larger in Groups 4 and 5 than in Groups 1, 2, and 3. Immunohistostaining mainly detected αSMA circumferentially in vessel walls in Groups 2,

4, and 5, all of which were administered ASCs. CD146 was expressed in Groups 2, 3, 4, and 5. However, CD146 staining was stronger in Group 5 than in the other groups (bar, 50 μm).

Figure 7. Representative images of immunohistochemical staining of transforming growth factor-β1 (TGF-β1, a-c) and CD31 (d-f) in new vessels in Groups 2, 4, and 5 at 7 days after treatment. Immunohistostaining detected TGF-β1 around the walls of vessels positively stained for CD31 in Groups 4 and 5, but not in Group 2. The expression of TGF-β1 was particularly strong in Group 5 and seemed to be located in the outer layer in the vessel wall compared with the layer of the CD 31 positive cells (bar, 20 μm). Green fluorescence indicates green fluorescent protein. Blue fluorescence indicates DAPI.

Supplemental Figure. Representative images of the mouse ischemic hind limb model.

Entire atrophy of the foot and toe tip necrosis was observed at day 4 and 7 post-induction.