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Combinational stem cell therapy for critical hindlimb ischemia using dental pulp stem cells and human umbilical vein endothelial cells

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Abstract

Narrowed arteries to limbs provoke critical hindlimb ischemia (CLI). Although CLI results in irreversible sequelae, such as amputation, there are few therapeutic options that could induce formation of new functional blood vessels. Based on the proangiogenic potentials of stem cells, it was examined in this study whether combining dental pulp stem cells (DPSCs) and human umbilical vein endothelial cells (HUVECs) could potentiate therapeutic effects of stem cells for CLI compared with those of DPSCs or HUVECs alone. The combination therapy showed significantly higher blood flow and lower ischemia damage than did the other groups on CLI animal models. The improved therapeutic effects were accompanied by significantly more microvessels in the ischemic tissue than did the other groups. *In vitro* proliferation and tube formation assay showed that VEGF in the conditioned media of DPSCs induced proliferation and vessel-like tube formation of HUVECs. Altogether, our results demonstrated that the combination of DPSCs and HUVECs made significantly better therapeutic effects on CLI via VEGF-mediated crosstalk. This combinational strategy could be used to develop novel clinical protocols for proangiogenic regenerative treatments for CLI.

Introduction

Peripheral artery disease (PAD) is a common circulatory problem involving narrowed arteries to limbs. Critical hindlimb ischemia (CLI) is the most severe clinical symptom among PADs (1) and can lead to ulcers on the leg and feet. Since those ulcers are difficult to heal, many CLI patients have to undergo amputation to prevent secondary damage (2). Current therapies for CLI include intra-arterial stent and bypass surgery (3, 4). However, they have a high risk of restenosis(5), because those therapies only unclog blood vessels without inducing angiogenesis. Therefore, novel therapeutics that can make new functional blood vessels need to be developed for CLI.

Mesenchymal stem cells (MSCs) are the most widely used stem cell type for regenerative medicine in the world (6, 7). Given that the developmental origin of MSCs might be perivascular region, their potential applications for angiogenesis have been suggested (8). Accordingly, MSCs are undergoing Phase I or II clinical trials for CLI at several clinical sites (9, 10). However, it is still controversial that MSCs could differentiate into functional endothelial cells, which is an essential component for angiogenesis (11). Instead, many studies have proposed that there are paracrine pro-angiogenic effects of MSCs (12, 13). Therefore, co-injection of MSCs and endothelial cells (ECs) might upgrade the therapeutic effects of MSCs for CLI.

Dental pulp stem cells (DPSCs) from dental pulp of teeth show MSC-like characteristics and have several advantages for being used for CLI (14, 15). Most importantly, DPSCs have shown significant therapeutic efficacy in preclinical animal models, such as for spinal-cord injury, ischemic stroke, and CLI that require angiogenesis as a recovery mechanism (16-18). Moreover, the combination of DPSCs and ECs has shown improved regenerative potentials in various pathological conditions(19-21). DPSCs could be collected from extracted infantile teeth and then stored for a long time, which would enable autografts of DPSCs for CLI patients (22). Since survival of transplanted cells is important for making functional vessels

with anastomosis in the host (23), autografts would be the most clinically applicable option to transplant stem cells.

The objectives of this study were to compare the therapeutic effects of co-injection of DPSCs and human umbilical vein endothelial cells (HUVECs) in a CLI animal model with those of injection of DPSCs or HUVECs alone and to elucidate molecular mechanisms of treatment effects.

Results

Therapeutic effects of DPSCs and HUVECs in CLI animal model

To evaluate the therapeutic effects, HBSS (negative control), HUVECs, DPSCs, or DPSCs + HUVECs (1:1) were transplanted into CLI animal models (1.0×10^6 cells/ea), intramuscularly, after ligation of femoral artery (Fig. S1). MSC-like characteristics of DPSCs, such as bi-polar morphology (Fig. S2A), expression of MSC-specific markers (Fig. S2B), and differentiation potential (Fig. S2C) were confirmed. Ischemia damage score and blood flow were evaluated by observation and laser doppler imaging (LDI), respectively, at 0, 2, 4, 7, and 14 days post injection (Fig. 1A). Images of the legs (Fig. 1B) revealed that the degree of damage in the DPSCs + HUVECs group was the lowest among the experimental groups, although both the DPSCs and DPSCs + HUVECs groups showed significantly lower scores compared with the HBSS negative control group (Fig. 1C). LDI showed that HBSS injection or HUVECs injection produced significantly less blood flow than did DPSCs injection or co-injection of DPSCs and HUVECs (Fig. 1D). Although the DPSCs group showed recovered blood flow at 14 days, co-injection of DPSCs and HUVECs resulted in significantly higher blood flow than the DPSCs group (Fig. 1E) at 14 days post injection. These data suggested that co-injection of DPSCs and HUVECs had significantly greater therapeutic effects on CLI animal model than did HBSS injection, DPSCs injection, or HUVECs injection.

Treatment mechanisms of co-injection of DPSCs and HUVECs in CLI animal model

To measure the degree of fibrosis and angiogenesis, ischemic hindlimb muscles of the four experimental groups were removed at 14 days post injection. After H&E staining, the degree of inflammation and integrity of muscles were analyzed. In the HBSS group, there was severe inflammation with numerous infiltrated leukocytes. The severity of inflammation in the HUVECs group was similar to that in the HBSS group. In the DPSCs and DPSCs + HUVECs groups, there was less inflammation and damaged muscles than in the other groups (Fig. 2A). The degree of fibrosis was further found by Masson's trichrome staining (Fig. 2B), which showed that the degree of fibrosis was decreased significantly more by the co-injection of DPSCs and HUVECs, followed by DPSCs, HUVECs, and HBSS injection. Importantly, the degree fibrosis of the DPSCs + HUVECs group was significantly lower than that of the DPSCs group (Fig. 2C). The number of microvessels was quantified by immunohistochemistry against CD31. As shown in Fig. 2D, the greatest number of microvessels was observed from the co-injection of DPSCs and HUVECs among the experimental groups. In the quantification of microvessels, the co-injection group had significantly more microvessels than did the HBSS, HUVECs, or DPSCs group (Fig. 2E). These results suggest that co-injection of DPSCs and HUVECs could increase angiogenesis significantly and decrease inflammation and fibrosis of damaged muscles in the CLI animal models.

Pro-angiogenic paracrine factors of DPSCs

Combination of DPSCs and HUVECs significantly increased in the number of microvessels (Fig. 2E) in the CLI animal models, which indicated that DPSCs might exert their therapeutic effects by promoting new vessel formation. *In vitro* tube formation assay, the conditioned media (CM) of DPSCs significantly increase the tube formation of HUVECs (Fig. 3A). Moreover, the *in vitro* proliferation of HUVECs was significantly induced by the CM of

DPSCs (Fig. 3B). Those results suggested that paracrine mediators of DPSCs provoke new vessel formation of HUVECs. Accordingly, high levels of pro-angiogenic factors such as VEGF and HGF were measured in the CM of DPSCs by ELISA assay (Fig. 3C). Compared with the CM of neural stem cells (NSCs) and Warton's Jelly-derived MSCs (MSCs), the CM of DPSCs showed significantly higher level of VEGF. Although the concentrations of HGF in the CM of MSCs and DPSCs were similar each other, they were much higher than that of NSCs. In contrast, the levels of TNF-α were not different among the CM of NSCs, MSCs, and DPSCs (Fig. 3C). Functionally, phosphorylation of Akt and Erk1/2 in HUVECs was increased by the treatment of the CM of DPSCs (Fig. 3D). The results indicated that the paracrine mediators from DPSCs such as VEGF and HGF mediate the pro-angiogenic effects of DPSCs.

Pro-angiogenic effects of DPSCs mediated by VEGF

To confirm the paracrine mediator of pro-angiogenic activities of DPSCs, bevacizumab(Sigma-Aldrich), a VEGF-neutralizing antibody was utilized. When bevacizumab was added to the CM of DPSCs, increased tube formation (Fig. 4A) and proliferation (Fig. 4B) of HUVECs by the CM of DPSCs disappeared, which indicated that VEGF is the major paracrine factor that make the pro-angiogenic effects of DPSCs. Accordingly, phosphorylation of Akt and Erk1/2 was not induced by the CM of DPSCs when VEGF in the CM was neutralized by bevacizumab (Fig. 4C and D).

Discussion

Stem cell therapies are emerging as alternative therapeutic options for CLI (24). MSCs are the most widely used in clinical trials for CLI, but the efficacy may not be enough to be developed commercially (25). In this study, we preclinically demonstrated that therapeutic effects of stem cells can be potentiated significantly when two or more kinds of stem cells

are combined (26, 27) . Use of multiple sources of stem cells might be inferior economically, which could be compensated by improved isolation and/or primary culture techniques.

Perivascular cell-like characteristics of MSCs (28) can be identified by the expression of pericyte markers, such as NG2, PDGFRβ, CD146, and α-SMA (29). DPSCs also have perivascular cell-like characteristics that may play important roles in *in vitro* and *in vivo* angiogenesis in this study. Our finding, that DPSCs alone could not create functional microvessels *in vitro* and *in vivo*, in agreement with a previous report (30), suggests that paracrine effects of DPSCs might not be enough to treat CLI. In contrast, when DPSCs and HUVECs were co-injected into CLI animal models, they provoked significantly increased numbers of microvessel-like structures. These results also agree with a previous report (31). A previous report has suggested that VEGF/VEGFR, PDGF-BB/PDGFR-α and/or SDF-1/CXCR4 axis might relay communication between DPSCs and HUVECs (23). In this research, VEGF might be the critical paracrine factor, which mediates the paracrine pro-angiogenic activities of DPSCs.

The injection route of stem cells is important clinically, since it can affect the efficacy of stem cells (32). In the clinical trials of CLI, stem cells were transplanted via intramuscular or intra-arterial injection routes. In this study, DPSCs and HUVECs were transplanted intramuscularly at three points (Fig. S1). Weak points of intramuscular injection include possible leakage, uneven injection, the number of cells per site, and differences of injection sites for each patient with diverse body sizes. In contrast, intra-arterial injection needs to risk the danger of a surgical procedure under anesthesia. Moreover, injected stem cells might act as a new embolus to induce new intraarterial blockages. When intramuscular transplantation methods are optimized, it would be available in clinical trials for CLI.

The number of injected stem cells is another issue for clinical trial, because the efficacy of stem cells could increase with the number of transplanted cells. Human equivalent dose of a chemical drug can be calculated by converting preclinical doses to those for humans based

on a simple equation (33). However, it is not simple to convert a preclinical dose of stem cells into that of clinical trials, because they do not dissolve in blood. In addition, they should have different pharmacokinetics compared with those of chemical drugs. Using the same calculation method as for chemical drugs, the number of co-injected DPSCs and HUVECs in this study (1×10^6 cells for a 20-gram mouse) could be translated into 5×10^8 cells for a 60 kg patient.

Presently, more than 12 clinical trials of cell therapies for CLI can be found at ClinicalTrial.gov. In those trials, various kinds of cells have been hired. However, in most trials, a single kind of stem cells or cells was transplanted for CLI patients. One clinical trial for CLI (NCT00390767) reported to use ECs and smooth muscle cells (SMCs) simultaneously (34), which are isolated from patients' own short vein segments. Those ECs and SMCs are genetically modified to express angiopoietin I and VEGF, respectively. The genetic modifications might intend to increase paracrine interactions between two types of cells or to activate residual stem cells of patients (35). However, the technique has its own disadvantages, such as potential mutagenesis and continuous systemic release of growth factors, which might provoke transformation of cells (36).

Autologous transplantation of stem cells can minimize immune rejection, which could induce restenosis of vessels and/or secondary inflammation. It is more important in the treatment of CLI, since vessels continuously interact with circulating immune cells (37). Although autologous HUVECs can be isolated at birth, ECs are hard to isolate from older patients. In several preclinical studies, ECs were derived from human-induced pluripotent stem cells (iPSCs) (38). However, iPSCs could be an adequate option as a source of autologous ECs when their safety issues, such as teratoma formation after transplantation, are addressed properly (39). Direct conversion could be another solution to acquire autologous ECs (40). Despite of immunological pros of autograft, properties of DPSCs and ECs from various individuals could be different, which could lead unequal therapeutic effects.

Those inconsistencies need to be overcame using potency factors (i.e., VEGF) that guarantee the therapeutic potentials of stem cells.

In this study, we demonstrated that co-injection of DPSCs and HUVECs has significantly better therapeutic effects on CLI than does injection of DPSCs or HUVECs alone. The difference could originate from induced angiogenesis by interaction between DPSCs and HUVECs, which might be mediated by VEGF from DPSCs. The combination strategy for CLI in this study could be used in the development of clinical trial protocols that have better therapeutic effects on CLI.

Study approval

Usage of DPSCs was approved by the Institutional Review Board of Samsung Medical Center (SMC, Seoul, South Korea) (IRB File No. SMC 2016-09-120). Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (SBRI, Seoul, South Korea) with approval number 20180813001.

Further detailed information is provided in the Supplementary Information.

Acknowledgments

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Conflicts of Interest: The authors have no potential conflicts of interest to disclose.

Figures legends

Figure 1. Therapeutic effects of DPSCs and/or HUVECs transplantation for CLI. **(A)** Experimental schedule. **(B)** Images of the legs of CLI animal models. **(C)** The degrees of damage of legs were analyzed and compared. n = 10 for each group. *, $p < 0.05$. **(D)** Blood flow of the legs of CLI animal models was measured by LDI. **(E)** The blood flow was analyzed and compared. n = 10 for each group. *, $p < 0.05$.

Figure 2. Histological analysis of CLI animal models. Ischemic hind-limb muscles were retrieved at 14 days post injection for histological analysis. **(A)** In H&E staining, the severity of muscle degeneration and infiltration of immune cells were analyzed and compared. **(B)** Degree of fibrosis was assessed by Masson's trichrome staining. **(C)** Fibrosis area was quantified from Masson's trichrome staining and compared. **(D)** The number of vessels was assessed by immunohistochemistry against CD31. **(E)** The number of vessels was quantified and compared. n = 10 for each group. *, $p < 0.05$.

Figure 3. Angiogenic paracrine effects of CM of DPSCs. (A) Effects of the CM of DPSCs on the differentiation of HUVECs were analyzed by the *in vitro* tube formation assay. **(B)** Effects of the CM of DPSCs on the proliferation of HUVECs were analyzed. **(C)** Concentration of VEGF, HGF, and TNF-α was quantified by ELISA. n = 10 for each group. *, $p < 0.05$. n.s., not significant. LLOQ = lower limit of quantification. **(D)** Effects of the CM of DPSCs on the signaling pathways of HUVECs were analyzed by western blot analysis.

Figure 4. Proangiogenic effects of DPSCs mediated by VEGF. Effects of the CM of DPSCs on the differentiation (A), proliferation (B), and signaling pathways (C, D) of HUVECs with or without bevacizumab, a VEGF neutralizing antibody were analyzed and compared. *, $p < 0.05$.

References

1. Sprengers RW, Moll FL and Verhaar MC (2010) Stem cell therapy in PAD. Eur J Vasc Endovasc Surg 39 Suppl 1, S38-43
2. Johannesson A, Larsson GU, Ramstrand N, Turkiewicz A, Wirehn AB and Atroshi I (2009) Incidence of lower-limb amputation in the diabetic and nondiabetic general population: a 10-year population-based cohort study of initial unilateral and contralateral amputations and reamputations. Diabetes Care 32, 275-280
3. Adam DJ, Beard JD, Cleveland T et al (2005) Bypass versus angioplasty in severe ischaemia of the leg (BASIL): multicentre, randomised controlled trial. Lancet 366, 1925-1934
4. Farber A, Rosenfield K and Menard M (2014) The BEST-CLI trial: a multidisciplinary effort to assess which therapy is best for patients with critical limb ischemia. Tech Vasc Interv Radiol 17, 221-224
5. Kechagias A, Ylonen K, Kechagias G, Juvonen T and Biancari F (2012) Limits of infrainguinal bypass surgery for critical leg ischemia in high-risk patients (Finnvasc score 3-4). Ann Vasc Surg 26, 213-218
6. Buckley RH (2004) A historical review of bone marrow transplantation for immunodeficiencies. J Allergy Clin Immunol 113, 793-800
7. Martin I, De Boer J, Sensebe L and Therapy MSCCotISfC (2016) A relativity concept in mesenchymal stromal cell manufacturing. Cytotherapy 18, 613-620
8. Al-Khaldi A, Al-Sabti H, Galipeau J and Lachapelle K (2003) Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. Ann Thorac Surg 75, 204-209
9. Diez-Tejedor E, Gutierrez-Fernandez M, Martinez-Sanchez P et al (2014) Reparative therapy for acute ischemic stroke with allogeneic mesenchymal stem cells from adipose tissue: a safety assessment: a phase II randomized, double-blind, placebo-controlled, single-center, pilot clinical trial. J Stroke Cerebrovasc Dis 23, 2694-2700
10. Gupta PK, Chullikana A, Parakh R et al (2013) A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. J Transl Med 11, 143
11. Vittorio O, Jacchetti E, Pacini S and Cecchini M (2013) Endothelial differentiation of mesenchymal stromal cells: when traditional biology meets mechanotransduction. Integr Biol (Camb) 5, 291-299
12. Choi M, Lee HS, Naidansaren P et al (2013) Proangiogenic features of Wharton's jelly-derived mesenchymal stromal/stem cells and their ability to form functional vessels. Int J Biochem Cell Biol 45, 560-570
13. Hoch AI, Binder BY, Genetos DC and Leach JK (2012) Differentiation-dependent secretion of proangiogenic factors by mesenchymal stem cells. PLoS One 7, e35579
14. Bronckaers A, Hilkens P, Fanton Y et al (2013) Angiogenic properties of human dental pulp stem cells. PLoS One 8, e71104
15. Estrela C, Alencar AH, Kitten GT, Vencio EF and Gava E (2011) Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. Braz Dent J 22, 91-98

16. Leong WK, Henshall TL, Arthur A et al (2012) Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms. Stem Cells Transl Med 1, 177-187
17. Mamidi MK, Pal R, Dey S et al (2012) Cell therapy in critical limb ischemia: current developments and future progress. Cytotherapy 14, 902-916
18. Sakai K, Yamamoto A, Matsubara K et al (2012) Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. J Clin Invest 122, 80-90
19. Dissanayaka WL, Zhan X, Zhang C, Hargreaves KM, Jin L and Tong EHJJoe (2012) Coculture of dental pulp stem cells with endothelial cells enhances osteo-/odontogenic and angiogenic potential in vitro. 38, 454-463
20. Dissanayaka WL, Hargreaves KM, Jin L, Samaranayake LP and Zhang CJTEPA (2015) The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo. 21, 550-563
21. Zhang Y, Liu J, Zou T et al (2021) DPSCs treated by TGF-β1 regulate angiogenic sprouting of three-dimensionally co-cultured HUVECs and DPSCs through VEGF-Ang-Tie2 signaling. 12, 1-17
22. Volponi AA, Pang Y and Sharpe PT (2010) Stem cell-based biological tooth repair and regeneration. Trends Cell Biol 20, 715-722
23. Nam H, Kim GH, Bae YK et al (2017) Angiogenic Capacity of Dental Pulp Stem Cell Regulated by SDF-1alpha-CXCR4 Axis. Stem Cells Int 2017, 8085462
24. Gupta NK, Armstrong EJ and Parikh SA (2014) The current state of stem cell therapy for peripheral artery disease. Curr Cardiol Rep 16, 447
25. Liew A and O'Brien T (2012) Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. Stem Cell Res Ther 3, 28
26. Bak S, Ahmad T, Lee YB, Lee JY, Kim EM and Shin H (2016) Delivery of a Cell Patch of Cocultured Endothelial Cells and Smooth Muscle Cells Using Thermoresponsive Hydrogels for Enhanced Angiogenesis. Tissue Eng Part A 22, 182-193
27. Ban DX, Ning GZ, Feng SQ et al (2011) Combination of activated Schwann cells with bone mesenchymal stem cells: the best cell strategy for repair after spinal cord injury in rats. Regen Med 6, 707-720
28. Shi S and Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18, 696-704
29. Crisan M, Corselli M, Chen WC and Peault B (2012) Perivascular cells for regenerative medicine. J Cell Mol Med 16, 2851-2860
30. Lee KH, Pyeon HJ, Nam H et al (2018) Significant therapeutic effects of adult human multipotent neural cells on spinal cord injury. Stem Cell Res 31, 71-78
31. Dissanayaka WL, Hargreaves KM, Jin L, Samaranayake LP and Zhang C (2015) The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo. Tissue Eng Part A 21, 550-563
32. Van Tongeren RB, Hamming JF, Fibbe WE et al (2008) Intramuscular or combined

intramuscular/intra-arterial administration of bone marrow mononuclear cells: a clinical trial in patients with advanced limb ischemia. J Cardiovasc Surg (Torino) 49, 51-58

33. Reagan-Shaw S, Nihal M and Ahmad N (2008) Dose translation from animal to human studies revisited. FASEB J 22, 659-661
34. Flugelman MY, Halak M, Yoffe B et al (2017) Phase Ib Safety, Two-Dose Study of MultiGeneAngio in Patients with Chronic Critical Limb Ischemia. Mol Ther 25, 816-825
35. Lucitti JL, Mackey JK, Morrison JC, Haigh JJ, Adams RH and Faber JE (2012) Formation of the collateral circulation is regulated by vascular endothelial growth factor-A and a disintegrin and metalloprotease family members 10 and 17. Circ Res 111, 1539-1550
36. Griffin M, Greiser U, Barry F, O'Brien T and Ritter T (2010) Genetically modified mesenchymal stem cells and their clinical potential in acute cardiovascular disease. Discov Med 9, 219-223
37. Tateishi-Yuyama E, Matsubara H, Murohara T et al (2002) Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 360, 427-435
38. Jang S, Collin de l'Hortet A and Soto-Gutierrez A (2019) Induced Pluripotent Stem Cell-Derived Endothelial Cells: Overview, Current Advances, Applications, and Future Directions. Am J Pathol 189, 502-512
39. Kuroda T, Yasuda S and Sato Y (2013) Tumorigenicity studies for human pluripotent stem cell-derived products. Biol Pharm Bull 36, 189-192
40. Ginsberg M, James D, Ding BS et al (2012) Efficient direct reprogramming of mature amniotic cells into endothelial cells by ETS factors and TGFbeta suppression. Cell 151, 559-575

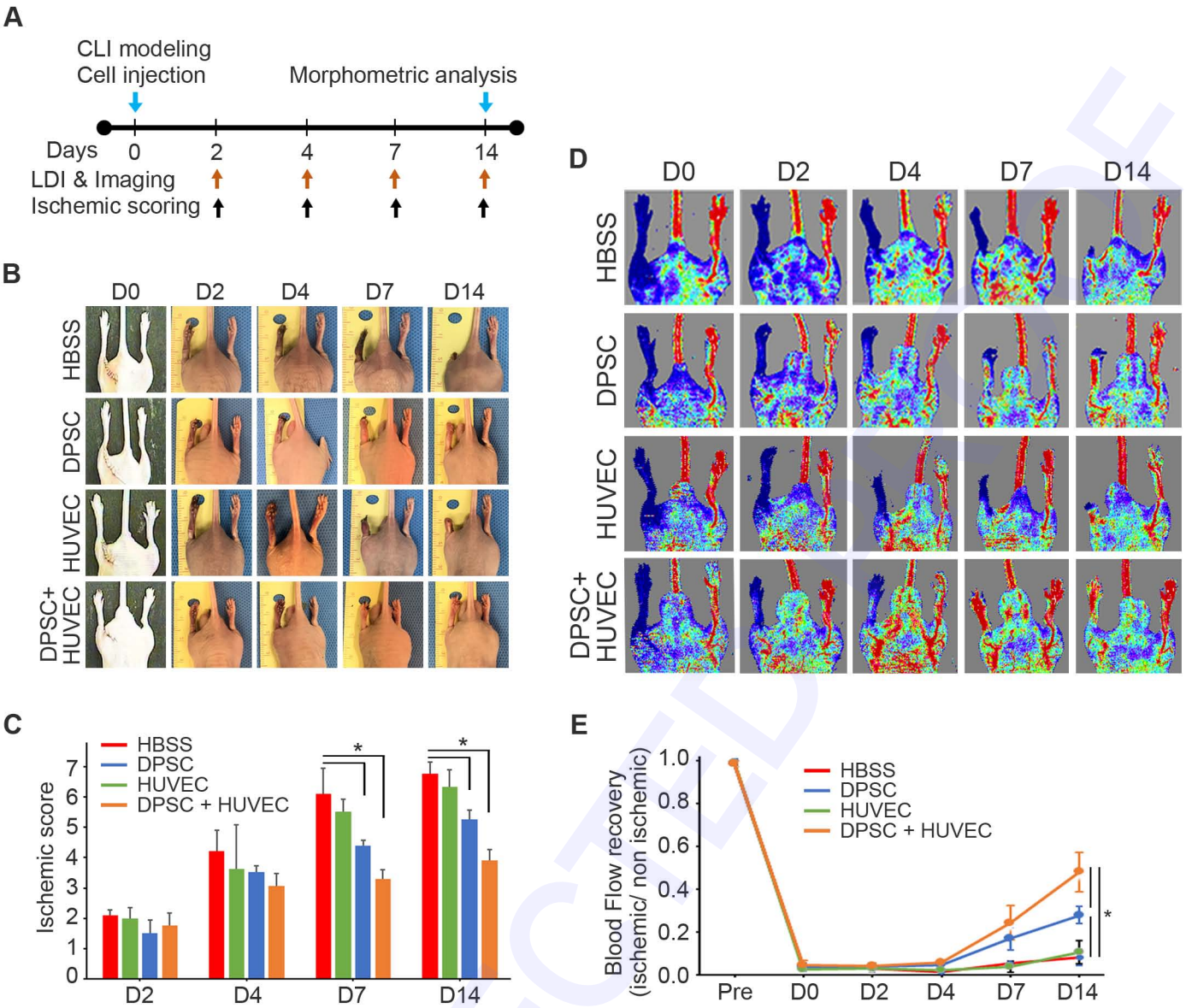


Figure 1

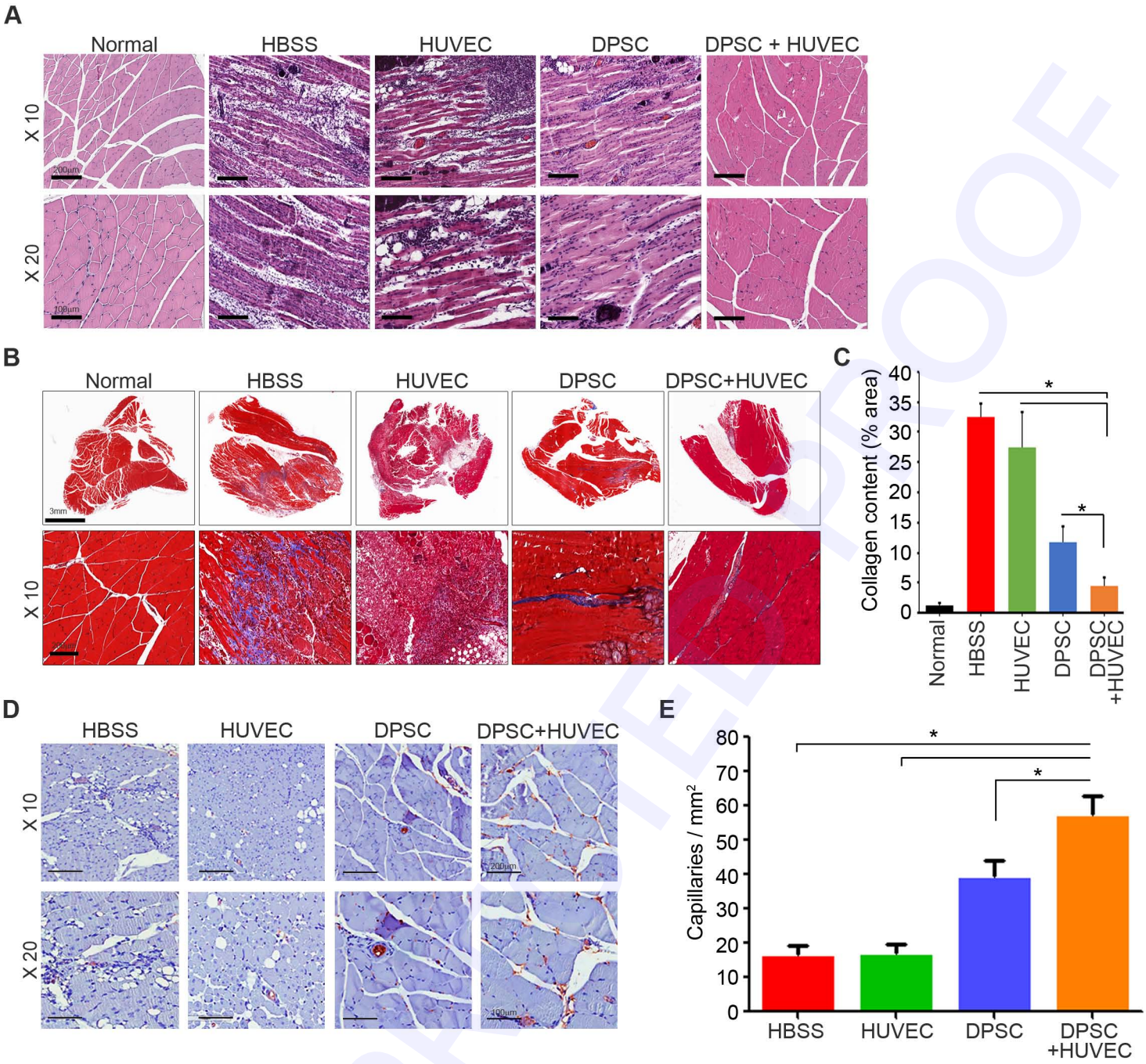


Figure 2

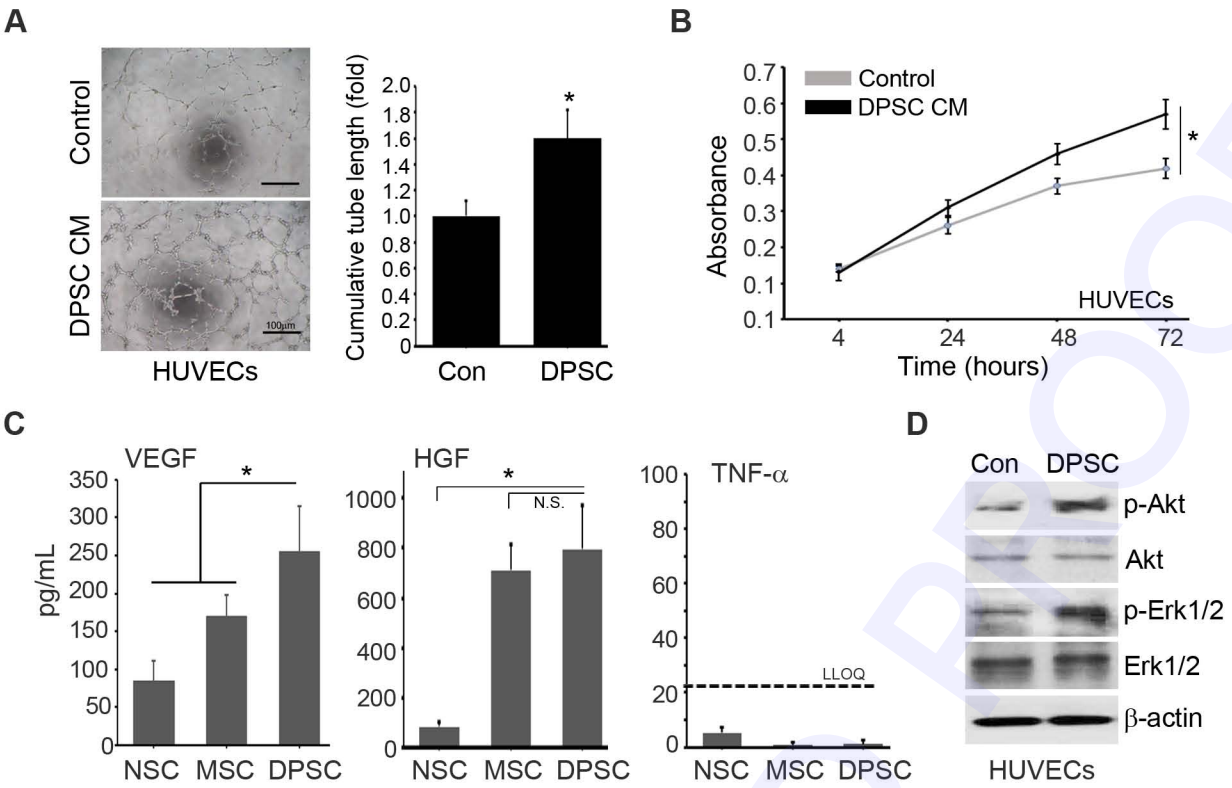


Figure 3

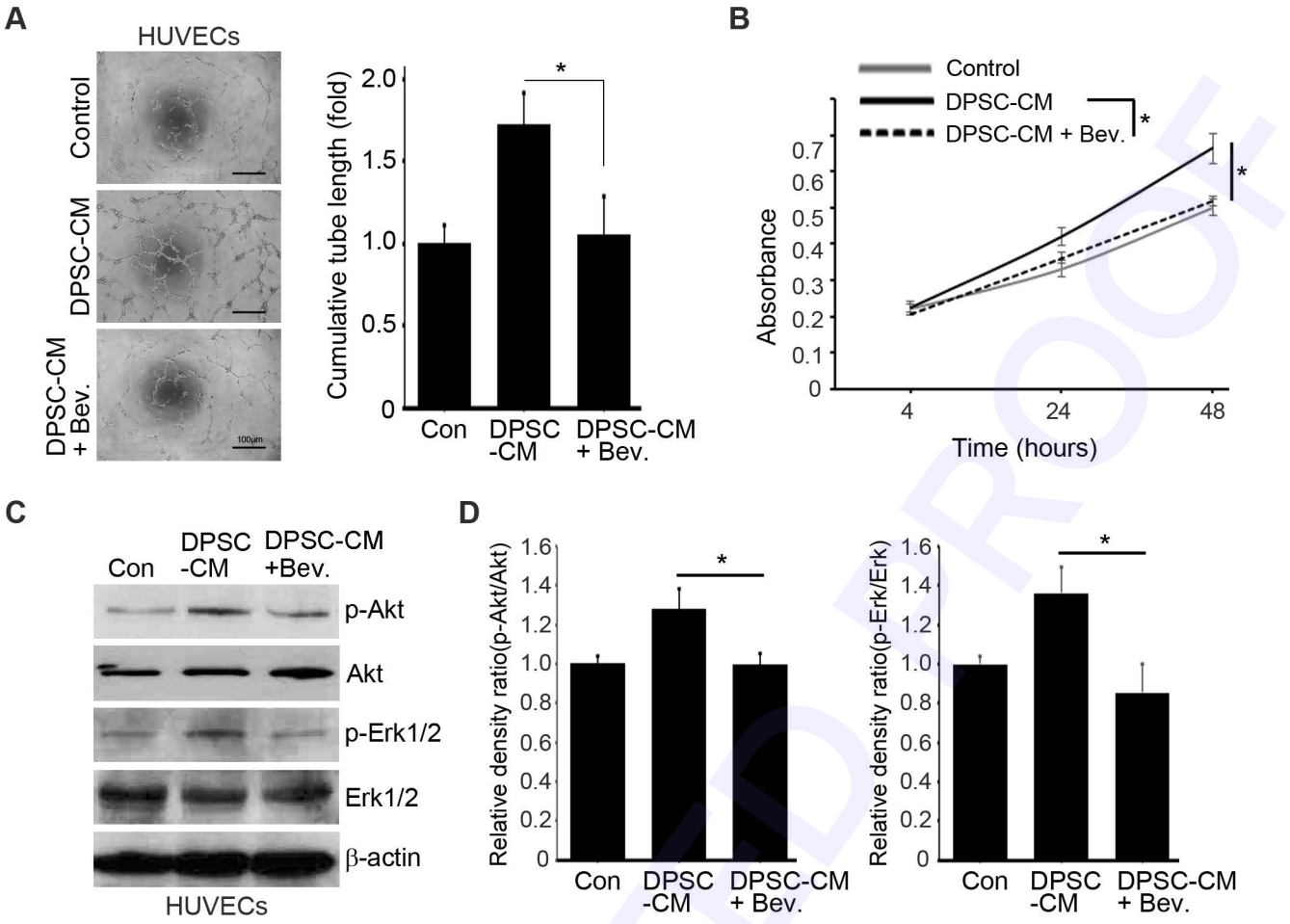


Figure 4

Supplementary Materials and Methods

Cell culture

Third molar teeth were acquired with informed consent. For the primary culture of DPSCs, human dental pulp was extracted and then mechanically and chemically dissociated in an enzyme solution containing 1 mg/mL of Collagenase type I (Gibco, Grand Island, NY, USA) and 2.4 mg/mL of Dispase (Gibco) at 37℃ for one hour. After enzyme inactivation with α-MEM (Gibco) supplemented with 10% FBS (Gibco), cells were washed twice with α-MEM. After being filtered with 70 μm mesh (BD Falcon, Bedford, MA, USA), a single-cell suspension was maintained in α-MEM supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVECs) were purchased from Promocell (Heidelberg, Germany) and cultivated in endothelial cell growth medium (Promocell) with 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) in 5% CO_2 at 37℃. All experiments were conducted using DPSCs at in vitro passage 5 (P5), and HUVECs at P6.

CLI animal model

Animal experiments were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and followed protocols approved by the IRB at the Samsung Medical Center (Seoul, Korea). After the Balb/c-nu mice were anesthetized by intraperitoneal injection of 30 mg/kg Zoletil (Virbac Korea, Seoul, South Korea) and 10 mg/kg Rompun (Bayer Korea, Seoul, South Korea), the hindlimb skin was removed to make an incision according to femoral artery location (approximately 1 cm in length). After dissection, the proximal and distal end points of the femoral artery were ligated tightly using a 6-0 silk (Ailee, Busan, South Korea). The femoral artery between the ligation points was then removed.

Cell transplantation

The experimental groups received Hank's balanced salt solution (HBSS) (Welgene) injection, DPSCs injection, HUVECs injection, or a co-injection of DPSCs and HUVECs (1:1). A total of 1.0×10^6 cells was resuspended in 80 μL HBSS and then injected intramuscularly into the skeletal muscles of induced ischemic hindlimb (Fig.S1) according to the previous report (1). Ten mice were used in each group.

***In vivo* assessment of hindlimb ischemic damages**

Semiquantitative assessment of hindlimb ischemia damage was done using a clinical score (0 = No difference from the normal, 1 = Mild discoloration, 2 = Moderate discoloration, 3 = Severe discoloration or subcutaneous tissue loss of necrosis, 4 = Toes amputation, 5 = Plantar amputation, 6 = Ankle amputation, 7 = Complete amputation) as in previous report (2). In addition, serial non-invasive physiological evaluations of blood perfusion using a Laser Doppler perfusion imager (Moor Instruments, Devon, UK) were performed at 0, 2, 4, 7, 9, 11, and 14 days post-injection. Color-coded digital images were calculated to quantify blood flow in ischemic hindlimbs as compared to that of the opposite normal hindlimbs. Mean perfusion values were analyzed using moor LDI Image Review Ver 5.3 (Moor Instruments).

Histological analysis and immunohistochemistry

At 14 days post injection, ischemic hindlimb muscles of the four experimental groups were removed, fixed in 4% paraformaldehyde (Biosesang), embedded in paraffin, and sectioned (4 μm in thickness). We used H&E (Sigma–Aldrich, St.Louis, MO, USA) or Masson's trichrome (Sigma–Aldrich) staining for histologic analysis. In Masson's staining, quantification of fibrosis was expressed as average percentage of collagen content present in the field of view. For immunohistochemistry, tissue slides were deparaffinized, hydrated, and then incubated

with a primary antibody against CD31 (Abcam, Cambridge, UK) overnight at 4°C. CD31-positive cells were visualized by 3,3′-diaminobenzidine (DAB) using ChemMate Envision Kits (Dako System, Glostrup, Denmark) according to the manufacturer's protocol. Slides were counterstained with hematoxylin.

Collection of CM

After 2.0×10^5 cells DPSCs at in vitro passage 5 were plated into 55 cm^2 culture dishes and incubated for 2 days. Attached cells were washed twice with PBS and then maintained in serum-free basal medium, α-MEM. After 24 h of culture, CM was collected.

***In vitro* tube formation and proliferation assay**

A total of 2.0×10^4 HUVECs in endothelial-cell growth medium (Promocell) was seeded onto 96-well plates precoated with Matrigel (Corning, NY, USA). Morphologies of tube-like structures were observed after 8 hours. The cumulative tube length in nine random fields was analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA). HUVECs (5.0×10^3 Cells/well) were seeded into a 96-well plate and incubated in endothelial cell growth medium with/without DPSC CM. The cell population was estimated by MTT assay for 72 hours.

ELISA assay

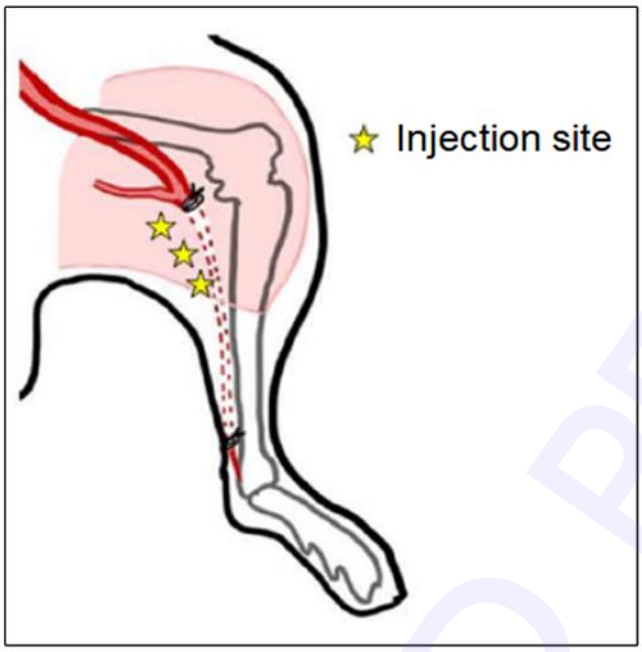
ELISA kits for VEGF (cat. no. DY293B-05), HGF (cat. no. DY294) and TNF-α (cat. no. DY210) (all from R&D Systems, Inc., Minneapolis, MN, USA) were used to determine the concentrations of secretory proteins in the DPSC conditioned media to manufacturer's protocol.

Western blot analysis

To identify a VEGF signaling, we were used p-Akt(Cell Signaling Technology, Danvers, MA, USA), Akt(Cell Signaling Technology), p-Erk1/2(Santa Cruz Biotechnology, Dallas, TX, USA) Erk1/2(Santa Cruz Biotechnology) and β-actin(Santa Cruz Biotechnology) antibody for Western blot analysis. The cells were harvested and lysed in nuclear extraction (NE) buffer (20 mM HEPES (pH 7.6), 20% glycerol, 250 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail (Roche)). Equal amounts of protein were separated through SDS-PAGE and analyzed through immunoblotting with the indicated antibodies.

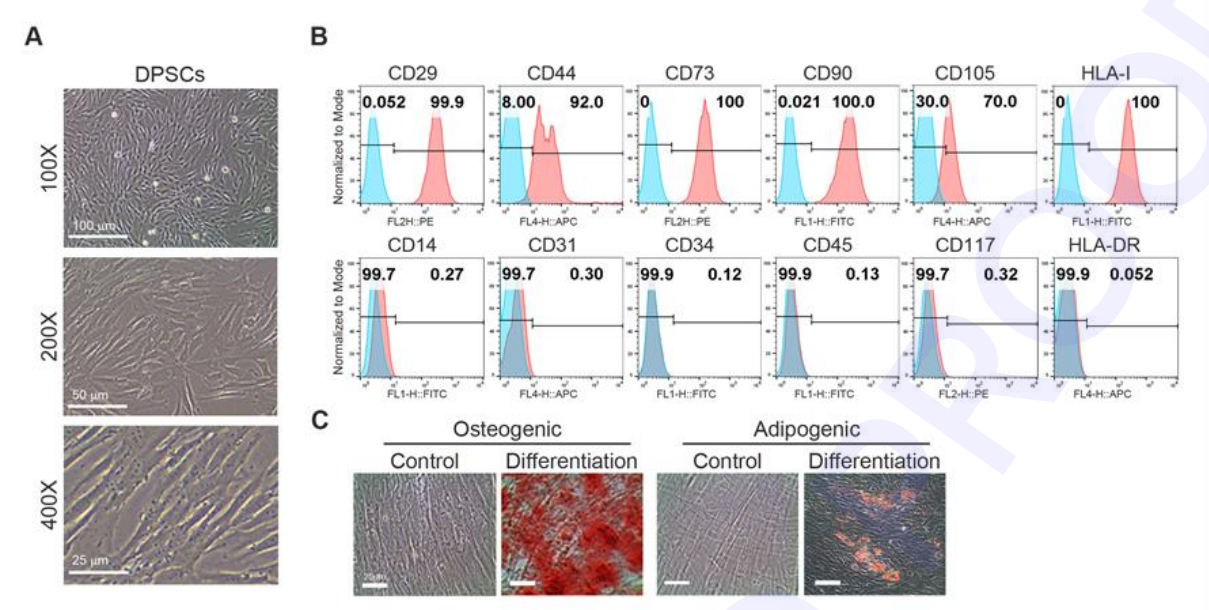
Statistical analysis

We analyzed data using PASW statistics 18 software (SPSS, Inc., Chicago, IL, USA). Values were calculated as means ± standard deviation (SD) or expressed as percentage ± SD of controls. Statistical significance was considered when $p < 0.05$.



Supplementary Figure S1. Injection sites of stem cells.

There were four experimental groups. Cells were injected into mice via intramuscular injection route, A total of 1.0×10^6 cells were injected into each three sites of legs intramuscularly.



Supplementary Figure S2. MSC-like characteristics of DPSCs.

Morphology (A), expression of MSC-specific markers (B), and differentiation potential into osteogenic and adipogenic cells (C) of DPSCs were analyzed.

References

1. Han KH, Kim AK, Kim MH, Kim DH, Go HN and Kim DI (2016) Enhancement of angiogenic effects by hypoxia-preconditioned human umbilical cord-derived mesenchymal stem cells in a mouse model of hindlimb ischemia. Cell Biol Int 40, 27-35

2. Stabile E, Burnett MS, Watkins C et al (2003) Impaired arteriogenic response to acute hindlimb ischemia in CD4-knockout mice. Circulation 108, 205-210