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Potentiated therapeutic angiogenesis by primed human mesenchymal stem cells in a mouse model of hindlimb ischemia

Background: Human bone marrow-derived mesenchymal stem cells (hMSCs) are advantageous for cell-based therapy to treat ischemic diseases owing to their capacity to secrete various paracrine factors with potent angiogenic activity. **Materials & methods:** In this study, we describe a method to increase secreted levels of VEGF and HGF from hMSCs without genetic modification. **Results:** We demonstrated that transplantation of primed hMSCs into ischemic limbs led to significantly greater improvements in tissue perfusion and limb salvage by increasing capillary formation compared with nonprimed hMSCs. The primed hMSCs also exhibited greater survival *in vivo* and secreted human VEGF and HGF in the ischemic tissue, supporting enhanced angiogenesis and cell survival. **Conclusion:** These findings indicate that priming hMSCs via methods described in this study enhances secretion of critical proangiogenic factors resulting in an enhanced therapeutic effect of cells for the treatment of ischemic diseases.

KEYWORDS: angiogenesis ■ ischemia ■ mesenchymal stem cells ■ paracrine effect ■ transplantation

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Ischemia is a common and fatal human disorder associated with decreased blood supply to certain organs or tissues. Ischemia is caused by pathological changes in the vascular system, such as constriction and obstruction. For example, peripheral arterial obstructive disease, which is mainly primed by atherosclerosis, causes lower-limb ischemia and usually results in amputation. The goal of 'therapeutic angiogenesis' is to treat ischemic disorders, such as limb ischemia and myocardial infarction, by stimulating new blood vessel growth from pre-existing vessels [1–3].

Human bone marrow-derived mesenchymal stem cells (hMSCs) are an ideal cell source for autologous cell-based therapy owing to their self-renewal capacity *in vitro*, high plasticity and low immunogenicity. In addition, hMSCs could be very useful for clinical application since they inhibit the immunological responses and do not need a major histocompatibility match for allogeneic transplantation [4–6]. Transplanting MSCs induces neovascularization and improves blood flow to ischemic limbs in animal models of hindlimb ischemia [7–9]. It has also been demonstrated that growth factors and cytokines released by MSCs promote *in vitro* and *in vivo* arteriogenesis in ischemic tissue through paracrine mechanisms [10].

VEGF was originally shown to play an essential role in promoting angiogenesis during development [11–13]. In addition, VEGF is one of the most important proangiogenic factors involved in therapeutic angiogenesis during

and after ischemia [1,10]. HGF is another potent proangiogenic factor that induces migration and proliferation and inhibits the apoptotic cell death of endothelial cells (ECs) [14].

Our previous studies demonstrated that hMSCs without genetic modification could be induced into Schwann cell-like cells that release higher amounts of VEGF and HGF when compared with control hMSCs [15]. Moreover, these induced hMSCs rendered the tissue micro-environment more favorable for tissue repair by secreting various growth factors that promote cell survival in a model of spinal cord injury [15]. In this study, we investigated whether these primed hMSCs could enhance therapeutic angiogenesis to treat ischemic disease. We demonstrate that bone marrow-derived hMSCs primed to release high amounts of VEGF and HGF *in vitro* and transplanted into a mouse hindlimb ischemia model led to significant improvements in tissue perfusion and limb salvage by enhancing therapeutic angiogenesis. Thus, the present study provides a novel approach to enhance the angiogenic potential of hMSCs without genetic modification to treat ischemic diseases.

Materials & methods

■ Culture of hMSCs

Adult hMSCs purchased from Cambrex (MD, USA) were cultured in DMEM-low glucose (HyClone, UT, USA) with 10% fetal bovine serum (Gibco-BRL, CA, USA) and 1% penicillin–streptomycin (Gibco-BRL). Cells (number

of passages: 4–12) were cultured at 37°C with 5% CO₂.

■ Priming of hMSCs

hMSCs were primed as previously described by our group [15]. Briefly, hMSCs were treated with 1 mM β-mercaptoethanol (Sigma-Aldrich, MO, USA) for 24 h and 0.28 μg/ml all-*trans*-retinoic acid (Sigma-Aldrich) for 3 days. Then, cells were treated with a cocktail containing 10 μM forskolin (Sigma-Aldrich), 10 ng/ml recombinant human bFGF (PeproTech, NJ, USA), 5 ng/ml recombinant human PDGF-AA (PeproTech) and 200 ng/ml recombinant human heregulin-β1 (R&D Systems, ON, Canada) for 8 days.

■ Reverse transcription PCR

The RNA extraction by the TRIzol Reagent (Invitrogen, CA, USA) was followed by DNase treatment and then reverse transcription to cDNA using a M-MLV reverse transcriptase (Invitrogen). Next, the PCR reaction (50 μl) containing 200 nM dNTPs, 100 pM of each primer pair and 0.5 U of Taq DNA polymerase (Takara Bio, Japan) was performed using an aliquot of cDNA (200 ng) as a template. The primer probe sets used for the PCR reactions are described in TABLE 1. The PCR products were electrophoresed on 2% agarose gel, and each transcript abundance was measured and normalized based on GAPDH transcript level.

■ ELISA

After being rinsed four times with phosphate-buffered saline (PBS), control hMSCs or primed hMSCs (4000 cells/cm²) were incubated with serum-free Neurobasal Media (Gibco-BRL) for 18 h. Then, the conditioned media from control or primed hMSCs were centrifuged at 1500 × *g* for 5 min to remove cell debris, and analyzed by the ELISA kit for human VEGF/HGF (Ray-Biotech, GA, USA) based on the manufacturer's instructions.

■ Mouse model of hindlimb ischemia

All procedures involving animals were approved by the Experimental Animal Committee of Clinical Research Institute, Seoul National University Hospital (Republic of Korea). All animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the NIH [101].

Six-week-old (20–22 g) BALB/c nude mice (Orientbio, Sungnam, Republic of Korea [102]) were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (20 mg/kg) (Bayer Korea, Korea). By using a skin incision with 6-0 silk (Ethicon, NJ, USA), the femoral artery and its branches were tied. Additionally, the external iliac artery and all of the above arteries were tied. We amputated the femoral artery from its proximal site as a branch of the external iliac artery to the terminal point into which it divides into two parts, which are called the saphenous and popliteal arteries [16]. For the sampling of the lower calf muscles in the ischemic limb, the mice were sacrificed by exposing them to an overdose of anesthetics at day 14 after hindlimb ischemia.

■ hMSC transplantation into hindlimb ischemia

Mice with ischemic limbs were randomly allocated into three groups that received the following injections: vehicle alone; nonprimed control hMSCs; or primed hMSCs. Immediately after surgery, mice were injected intramuscularly using 27-gauge tuberculin syringes at two sites within the gracilis muscle in the medial thigh with hMSCs (50,000 cells/mouse) suspended in 60 μl of vehicle or vehicle alone. We have found that intramuscular injection of total cell numbers corresponding to 50,000 cells is optimal for 6-week-old (20–22 g) BALB/c nude mice. This was previously optimized by our group in a similar experimental model [17].

Table 1. PCR primer pairs.

Gene	Accession number	Primer sequence	Predicted size (base pairs)	Annealing temp. (°C)	Cycle no.	Ref. no.
VEGF	X62568, +53/+453	F: 5'-gccttgctgctctacctcca-3' R: 5'-caaggccacaggatttt-3'	401	59	33	[43]
HGF	M73239, +1402/+1824	F: 5'-atgctcatggaccttggt-3' R: 5'-gcctggcaagcttcatta-3'	423	59	33	[44]
GAPDH	NM_002046, +628/+1079	F: 5'-accacagtccatgccatcac-3' R: 5'-tccaccacctgttgctgta-3'	452	58	24	[45]

F: Forward; R: Reverse; temp.: Temperature.

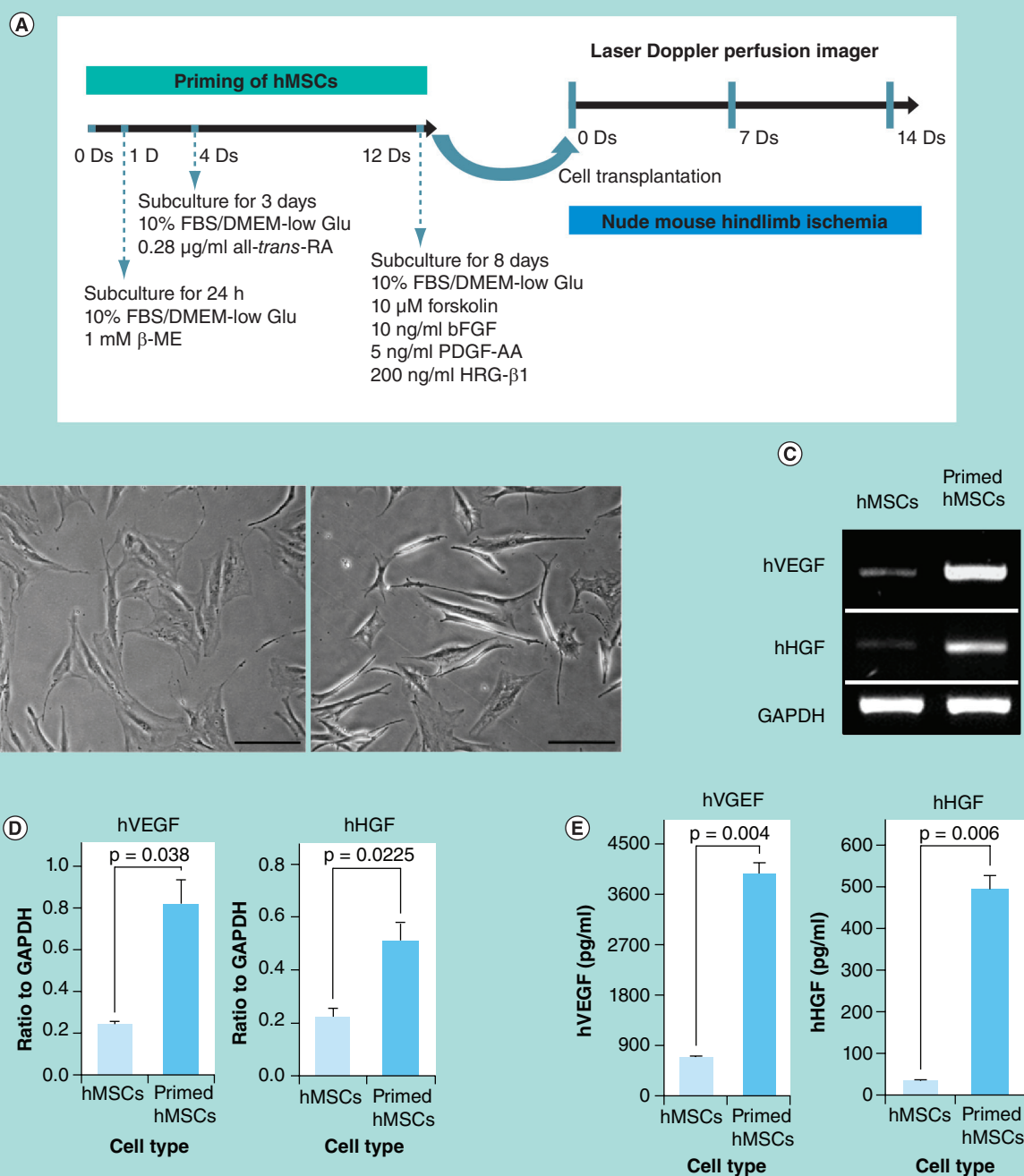


Figure 1. Priming of human mesenchymal stem cells and cell transplantation into a mouse model of hindlimb ischemia.

(A) Schematic illustration of the protocol used for the priming of hMSCs and cell transplantation into a mouse model of hindlimb ischemia. (B) Bright-field images of control and primed hMSCs. After priming, hMSCs showed an elongated bipolar shape. Scale bar: 200 μ m. (C) Reverse transcription PCR analysis of hVEGF and hHGF mRNA levels in control hMSCs and primed hMSCs. (D) Quantitation of VEGF and HGF PCR products. The mRNA level of each gene was quantitated by densitometry and normalized to the corresponding GAPDH mRNA level. Each bars represent mean \pm the standard error of the mean of three independent experiments. p-values were obtained by Student's t-test. (E) ELISA analysis for hVEGF and hHGF released by control hMSCs and primed hMSCs. Each bar represents mean \pm the standard error of the mean of three independent experiments. p-values were obtained by Student's t-test. β -ME: β -mercaptoethanol; All-*trans*-RA: All-*trans*-retinoic acid; D: Day; FBS/DMEM: Fetal bovine serum/DMEM; hHGF: Human HGF; hMSC: Human mesenchymal stem cell; hVEGF: Human VEGF.

■ Laser Doppler imaging analysis

In order to measure the serial noninvasive physiological evaluation of neovascularization, a laser Doppler perfusion imager (Moor Instruments,

UK) was used for mapping tissue blood flow by the shift in the laser light frequency. At days 0, 7 and 14 after treatment, mice were observed through serial scanning of surface blood flow

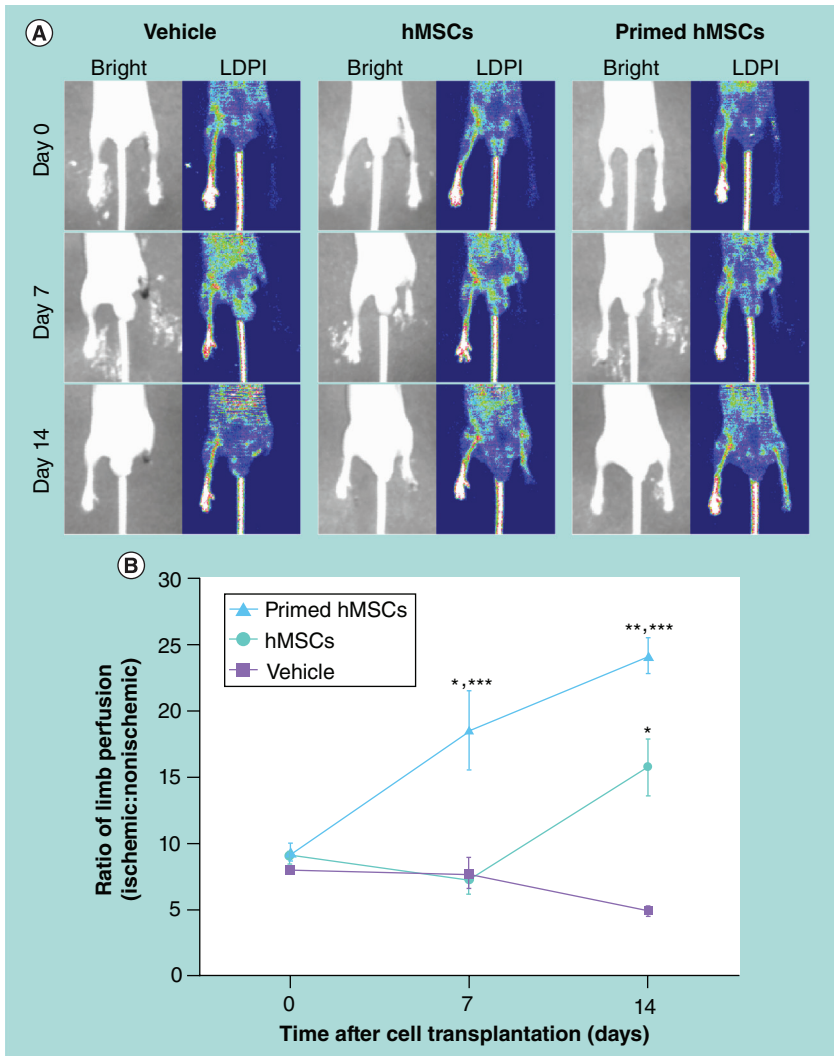


Figure 2. Primed human mesenchymal stem cell transplantation enhances blood flow in ischemic hindlimbs. (A) Serial analysis of LDPI performed at 0, 7 and 14 days after transplantation. Primed hMSC transplantation resulted in greater limb blood perfusion in the ischemic limbs of mice than vehicle injection or hMSC transplantation. (B) The blood perfusion ratio of ischemic limbs measured by LDPI at 0, 7 and 14 days after transplantation. The ratio of ischemic to normal limb blood perfusion was significantly increased in primed hMSC-transplanted mice compared with the vehicle- and hMSC-transplanted mice at 7 and 14 days after transplantation. The bars represent mean \pm the standard error of the mean ($n = 4-5$ mice per group). Please see color figure at www.futuremedicine.com/doi/pdf/10.2217/rme.13.17.

* $p < 0.05$; ** $p < 0.001$ versus vehicle injection group; *** $p < 0.05$ versus hMSC-transplanted group. The significance was determined by one-way analysis of variance with Tukey *post hoc* analysis.

hMSC: Human mesenchymal stem cell; LDPI: Laser Doppler perfusion imaging.

in hindlimbs. To qualify blood flow at the site from the knee joint to the toe, the digital color-coded images were utilized after laser Doppler perfusion imager scanning, and mean values of perfusion were also evaluated. In these digital color-coded images, red is shown in areas with maximum perfusion values, medium perfusion values are indicated in yellow and blue represents areas with lowest perfusion values.

We measured hindlimb perfusion as the ratio of ischemic to nonischemic in order to avoid data variation on account of ambient condition.

Immunofluorescence

Tissue sections from the lower calf muscles of ischemic and healthy limbs were harvested 14 days after cell transplantation and embedded in paraffin (Tissue-Tek; Sakura, CA, USA). Sections were deparaffinized and then subjected to antigen retrieval with citrate buffer (Dako, Denmark). Blocking was performed by incubating sections for 1 h with 5% normal goat serum in PBS. Staining of capillaries in the ischemic regions were achieved by overnight incubation at 4°C with goat polyclonal anti-PECAM-1 (Santa Cruz Biotechnology, CA, USA) at 1:100 final dilution in blocking buffer. After this, the sections were washed three times with PBS and then incubated with donkey anti-goat conjugated with Alexa Fluor 555 at 1:500 final dilution (Invitrogen). Transplanted human cells in the sections were identified by incubating with mouse monoclonal anti-human nuclei (1:500; Chemicon, CA, USA).

To examine the expression of human-specific VEGF and HGF in ischemic tissues, sections were incubated with rabbit monoclonal anti-human VEGF (1:50; Abcam, UK) and mouse monoclonal anti-human HGF (1:50; R&D Systems). After washing three-times, sections were incubated with goat anti-mouse conjugated with Alexa Fluor 546 (1:200; Invitrogen) or goat antirabbit conjugated with Alexa Fluor 488 (1:200; Invitrogen). Cells were counterstained with 4',6-diamidino-2-phenylindole (Santa Cruz Biotechnology) for nuclear staining. The images were captured using a laser-scanning confocal microscope, Fluoview FV300 (Olympus, Japan).

Statistical analysis

Quantitative data are presented as the mean \pm the standard error of the mean. The unpaired t-test was used to compare continuous variables. One-way analysis of variance with Tukey *post hoc* analysis was utilized to compare ≥ 3 groups with regard to continuous response variables. All statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad Software Inc., CA, USA). p -values < 0.05 were considered significant.

Results

Characterization of primed hMSCs

Our previous study has reported that induction of hMSCs into Schwann cell-like cells promotes secretion of various growth factors. Interestingly, they secreted high amounts of VEGF

and HGF [15]. These factors directly functioned as neurotrophic factors for both Neuro2A cells *in vitro* and spinal nerves in an organotypic spinal cord slice culture [15]. Since VEGF and HGF are also angiogenic factors, we sought to determine whether the transplantation of these primed cells has therapeutic effects for ischemic diseases using the mouse hindlimb ischemia model, as described in FIGURE 1A. Here, we used nude mice to avoid immunological complications from xenogenic cell transplantation [18].

Cells used in this study were defined as MSCs based on conventions set forth by the International Society for Cellular Therapy based on fact that they express CD105, CD73 and CD90, and differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* [19]. Nonprimed control hMSCs exhibited a flattened symmetrical fibroblast-like morphology (FIGURE 1B). However, after induction into Schwann cell-like cells, primed hMSCs showed an elongated bipolar Schwann cell-like shape (FIGURE 1B). After priming, VEGF and HGF mRNA levels in primed hMSCs significantly increased by up to 3.4-fold ($p < 0.05$) and 2.3-fold ($p < 0.05$) compared with control hMSCs, respectively (FIGURE 1C & 1D). These increases were also noted at the protein level in the cell culture media by ELISA analysis, as previously described [15]. VEGF and HGF levels were 3974.7 ± 213.5 and 493.7 ± 35.3 pg/ml, respectively, for primed hMSCs and 682.0 ± 18.5 and 36.4 ± 1.2 pg/ml, respectively, for control hMSCs (FIGURE 1E). Taken together, this indicates that there was a significant induction of VEGF and HGF in primed hMSCs when compared with control hMSCs ($p < 0.05$).

■ Blood perfusion in ischemic limbs & ischemic limb salvage is improved by primed hMSC transplantation

We investigated whether transplanting primed hMSCs had an effect on blood flow to the ischemic area of mice by Laser Doppler perfusion imaging analysis (FIGURE 1A & 2A). Seven days after transplantation, the relative ratio of blood flow (ischemic limb to normal limb) was significantly higher in primed hMSC-transplanted mice ($18.59 \pm 2.99\%$; $p < 0.05$) than in mice transplanted with nonprimed hMSCs ($7.23 \pm 0.61\%$) and vehicle-injected mice ($7.62 \pm 1.40\%$; FIGURE 2B). No difference in the relative ratio of blood flow was observed between hMSC-transplanted and the vehicle-injected groups. Fourteen days after transplantation, both hMSC-transplanted ($15.80 \pm 2.18\%$; $p < 0.05$) and primed hMSC-transplanted ($24.22 \pm 1.38\%$; $p < 0.001$)

groups showed significant increases in the relative ratio of blood perfusion compared with the vehicle group ($4.92 \pm 0.39\%$; FIGURE 2B). In addition, a significant difference in the relative ratio of blood flow was observed between control hMSC- and primed hMSC-transplanted groups ($p < 0.05$) (FIGURE 2B). Taken together, this indicated that blood perfusion in ischemic limbs was improved greatly in the primed hMSC-transplanted group compared with that in the vehicle- and control hMSC-transplanted group.

The ischemic limb salvage was also improved greatly by primed hMSC transplantation. Intramuscular transplantation of primed hMSCs reduced the rate of limb loss compared with that in the vehicle injection and control hMSC-transplantation groups (SUPPLEMENTARY FIGURE 1; see online www.futuremedicine.com/doi/suppl/10.2217/rme.13.17). The mice receiving vehicle injection underwent limb loss (four of five) or severe limb necrosis (one of five). Despite the increased relative ratio of blood perfusion compared with the vehicle group 14 days after transplantation, all mice receiving control hMSC transplantation exhibited limb necrosis (four of four) but not limb loss. By contrast, four of five mice that received primed hMSC transplantation retained their limbs with minimal necrosis (SUPPLEMENTARY FIGURE 1).

■ Primed hMSC transplantation increases angiogenesis in ischemic limbs

To examine whether transplantation of primed hMSCs promoted angiogenesis in ischemic limb tissues, we further analyzed mouse vasculature by immunohistochemical staining for PECAM-1 after sacrificing mice 14 days after transplantation (FIGURE 3A). To measure capillary density, quantitative analysis of PECAM-1 density was performed (FIGURE 3B). Then, the integrated optical density was normalized to the control hMSC-transplantation group, and expressed as the mean \pm the standard error of the mean. A significant difference in the capillary density was observed between control hMSC- and primed hMSC-transplanted groups ($p < 0.05$) indicating that primed hMSC transplantation significantly enhanced angiogenesis in ischemic limbs (FIGURE 3B).

■ Transplanted hMSCs remain in ischemic areas

We investigated whether transplanted cells remained in ischemic tissues 14 days after transplantation (FIGURE 4). Immunofluorescent staining for human nuclei revealed that human nuclei-positive cells were present in both control

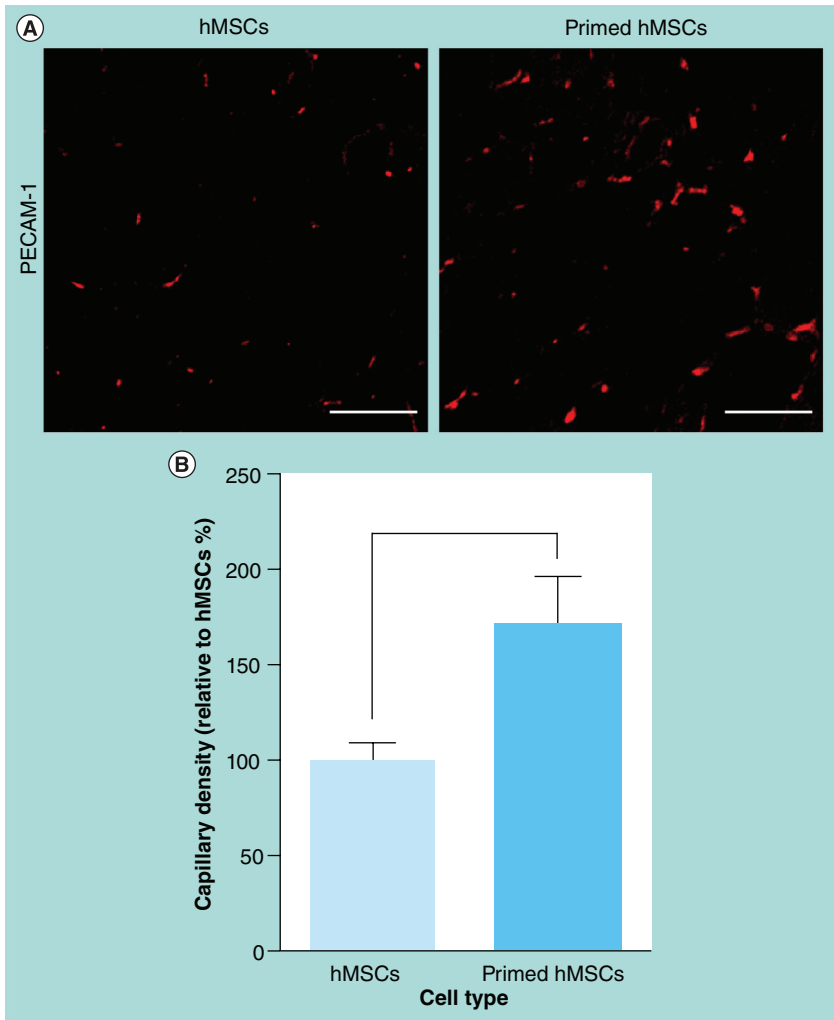


Figure 3. Enhanced angiogenesis in ischemic limb tissues by primed human mesenchymal stem cell transplantation. (A) Representative confocal images of PECAM-1 immunofluorescence in control hMSC- and primed hMSC-transplanted ischemic hindlimbs 14 days after transplantation. Transplantation of primed hMSCs significantly induced the generation of new capillaries in the ischemic limbs compared with control hMSC transplantation. Scale bar: 50 μ m. (B) Quantitative analysis of relative PECAM-1-positive capillary density in control hMSC- and primed hMSC-transplanted ischemic hindlimbs 14 days after transplantation. The bars represent the mean \pm the standard error of the mean (n = 4 mice/group). The p-value was generated by the Student's t-test. Please see color figure at www.futuremedicine.com/doi/pdf/10.2217/rme.13.17. hMSC: Human mesenchymal stem cell.

hMSC- and primed hMSC-transplanted limb tissues but not in vehicle-injected limb tissues (FIGURE 4A). Interestingly, a significant difference in the number of remaining human cells per area of 0.27 mm² was observed between control hMSC- and primed hMSC-transplanted tissues (p < 0.05) indicating that primed hMSC survived better in ischemic limbs (FIGURE 4B).

■ Increases in VEGF & HGF in the transplanted hMSC

We next investigated whether human VEGF and HGF were increased in the ischemic

tissues transplanted with primed hMSCs (FIGURE 5A & 5B). Immunohistochemical staining for human VEGF and HGF demonstrated significant levels of both factors in the group transplanted with primed hMSCs; whereas there was undetectable to low levels of these factors in the sham and mice transplanted with nonprimed hMSCs (FIGURE 5A & 5B). These results suggest that primed hMSCs survived after transplantation and secreted high levels of human VEGF and HGF to enhance angiogenesis in the ischemic tissues.

Discussion

Our study demonstrates that hMSCs without genetic modification can be primed *in vitro* to secrete copious amounts of specific angiogenic growth factors that promote therapeutic angiogenesis *in vivo*. Both mRNA and protein levels of VEGF and HGF increased significantly in primed hMSCs when compared with those in control hMSCs. Most importantly, primed cells survived 14 days after being transplanted *in vivo* and secreted high levels of human VEGF and HGF in the ischemic tissues. In this study, transplanting nonprimed hMSCs also induced capillary formation and improved blood flow to ischemic limbs, as previously reported [7–9]. However, transplanting primed hMSCs led to a significant improvement in tissue perfusion and limb salvage by increasing capillary formation.

In our previous report, we observed consistency in blood flow recovery of ischemic hindlimbs from day 14 to 21 after transplantation [17]. Similarly, in this study, 14 days after transplantation, both hMSC-transplanted (p < 0.05) and primed hMSC-transplanted (p < 0.001) groups showed significant increases in the relative ratio of blood perfusion compared with the vehicle group (FIGURE 2B). In addition, a significant difference in the relative ratio of blood flow was observed between control hMSC- and primed hMSC-transplanted groups (p < 0.05) (FIGURE 2B). Thus, the longer time period was not included in this study.

MSCs are multipotent and can differentiate into various mesodermal cells such as osteoblasts [20–22]. In addition, MSCs have the potential to transdifferentiate into vascular smooth muscle cells and pericytes [23]. However, it has been suggested that the paracrine properties of MSCs have a greater effect on tissue repair than transdifferentiation by modulating the tissue microenvironment [24]. Our previous study demonstrated that the growth factors secreted

by primed hMSCs directly facilitate axonal regeneration while, at the same time, protecting the resident cells at the site of tissue injury in an *ex vivo* model of spinal cord injury [15]. This study also suggests that the paracrine action of angiogenic factors secreted by primed hMSCs plays a greater role in therapeutic angiogenesis than transdifferentiation of these cells into ECs and/or direct participation in angiogenesis.

Angiogenesis and vasculogenesis are the major mechanisms involved in ischemic tissue neovascularization [25]. Angiogenesis is the formation of new blood vessels from pre-existing vessels, whereas vasculogenesis is the formation of new blood vessels when there are no pre-existing vessels. Our results suggest that endogenous ECs begin sprouting to form capillaries in response to VEGF and HGF secreted by primed hMSCs. However, it would be interesting to examine whether primed hMSCs transdifferentiate into endothelial precursor cells (EPCs) and/or ECs to form new blood vessels in response to local cues, although the probability is very low. Furthermore, the possibility that vasculogenesis also occurs by a mechanism in which endogenous EPCs migrate and differentiate in response to angiogenic factors secreted by primed hMSCs to form new blood vessels cannot be excluded. Several studies have reported that stem cells can either directly enhance angiogenesis by inducing neovascularization [26] or indirectly enhance angiogenesis by secreting various angiogenic and antiapoptotic factors [10].

VEGF stimulates proliferation, migration and survival of ECs. It is also possible that VEGF secreted by primed cells can mobilize bone marrow-derived EPCs into neovascularization sites [27]. As EPCs can differentiate into ECs [28], it is likely that they can be incorporated into growing vessels and complement resident ECs to sprout new vessels [1]. However, the exact role of these cells in therapeutic angiogenesis has not been clearly identified. Therefore, whether EPCs are recruited to ischemic areas by VEGF and other factors secreted from primed cells remains to be determined. Nevertheless, significant increases in capillary density were observed at the sites of primed cell transplantation. This suggests that the paracrine action of primed hMSCs plays an important role in therapeutic angiogenesis in the ischemic tissues.

The VEGF family comprises seven members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PGF [29].

Among the seven isoforms of VEGF-A, the primed cells secrete VEGF-A₁₆₅, which is the most abundant and biologically active form [15,30–32]. hMSCs express the VEGF receptors NP1 and NP2, which bind VEGF₁₆₅ [33]. Thus, autocrine effects of VEGF may increase

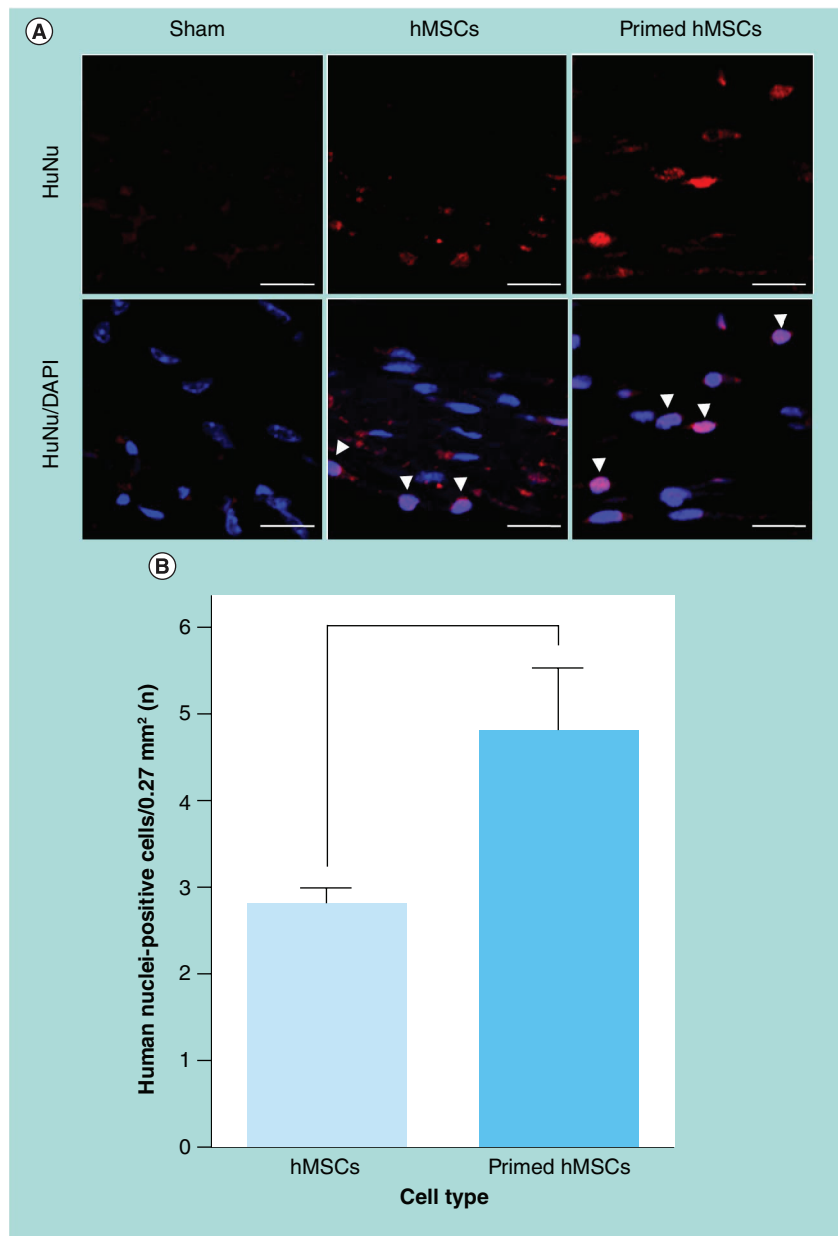


Figure 4. Transplanted human mesenchymal stem cells survive in ischemic limb tissues. (A) Representative confocal images of immunostained human nuclei in the sham-, hMSC- and primed hMSC-transplanted ischemic hindlimbs 14 days after transplantation. Transplanted human cells were detected with Alexa Fluor 546-conjugated antibodies (red). The nuclei were visualized by DAPI staining (blue). Arrowheads indicate the immunostained HuNu. Scale bar: 20 μ m. (B) Number of remaining human cells per unit area. Primed hMSCs survived better in ischemic limbs than control hMSCs. The bars represent mean \pm the standard error of the mean. The p-value was generated by the Student's t-test. Please see color figure at www.futuremedicine.com/doi/pdf/10.2217/rme.13.17. DAPI: 4',6-diamidino-2-phenylindole; hMSC: Human mesenchymal stem cell; HuNu: Human nuclei.

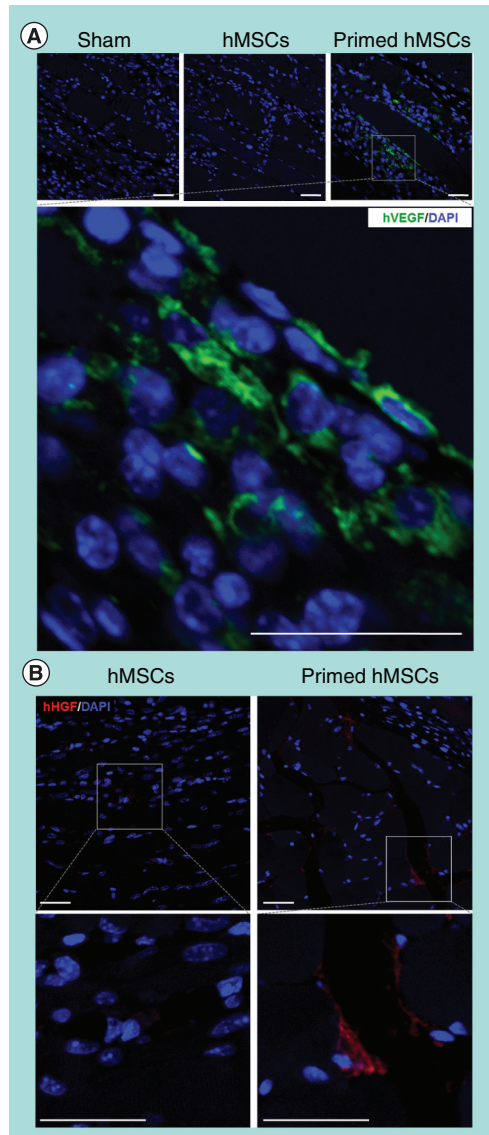


Figure 5. Human VEGF and HGF secretion from transplanted primed human mesenchymal stem cells in ischemic hindlimb tissues. (A & B) Representative confocal images of **(A)** hVEGF immunofluorescence (green) or **(B)** hHGF immunofluorescence (red) in the sham-, control hMSC- and primed hMSC-transplanted ischemic hindlimbs 14 days after transplantation. Significant expression of hVEGF and hHGF was only detected in primed hMSC-transplanted ischemic limb tissues, whereas low levels of hVEGF and hHGF were observed in the hMSC-transplanted limb tissues. In each panel, expression of hVEGF or hHGF in boxed areas appears at higher magnification in the lower panels. The nuclei were visualized by DAPI staining (blue). Scale bar: 25 μ m. Please see color figure at www.futuremedicine.com/doi/pdf/10.2217/rme.13.17. DAPI: 4',6-diamidino-2-phenylindole; hHGF: Human HGF; hMSC: Human mesenchymal stem cell; hVEGF: Human VEGF.

survival of transplanted cells in ischemic tissue. This could explain why the primed hMSCs survived better than controls. In addition, our previous study showed that primed hMSCs secrete other growth factors, such as insulin-like growth factor-binding proteins and stem cell factor, at higher levels than control hMSCs [15]. Thus, these factors may also play an important role in increasing cell survival. We have previously reported that cell cycle arrest occurs in primed hMSCs [15]. Therefore, the increase in survival of primed hMSCs in ischemic tissue is primarily due to increased cell survival rather than increased cell proliferation.

The gene transfer of *VEGF* or *HGF* improves angiogenesis and limb salvage in patients with limb ischemia [25,34,35]. Interestingly, the combination of HGF and VEGF potentiates the angiogenic effect through a more robust proliferation and chemotactic response of ECs than either growth factor alone [36,37]. A recent study demonstrated that the combined VEGF and HGF gene therapy leads to a robust angiogenic effect in ischemic skeletal muscle [38]. However, gene delivery using viral vectors or plasmid DNA has safety and low efficacy problems for clinical application [39].

Although VEGF is the most important angiogenic factor promoting therapeutic angiogenesis, the clinical application has been compromised by the proinflammatory side effects of VEGF (e.g., edema due to increased vascular permeability, increased leukocyte adhesion to ECs and inflammatory response due to adhesion molecule upregulation) [40]. Interestingly, HGF has been reported to inhibit the proinflammatory action of VEGF *in vitro* and *in vivo* by decreasing VEGF-induced leukocyte–endothelial cell interactions and endothelial expression of adhesion molecules. This suppression of VEGF-induced inflammation by HGF is mediated by inhibiting the endothelial NF- κ B pathway [41]. Since the combination of these factors results in a more robust angiogenic response than either growth factor alone without complications of inflammation and edema, it has been suggested that “the dynamic duo of VEGF and HGF” may be an efficient growth factor combination for therapeutic angiogenesis [40]. Other proangiogenic factors, such as IL-6 and monocyte chemoattractant protein-1 are also secreted from hMSCs [42]. This may explain why at 14 days after transplantation, control hMSC-transplanted group showed significant increases in the relative ratio of blood perfusion compared with the vehicle group ($p < 0.05$) (FIGURE 2B).

The advantage of injecting primed MSCs rather than the supernatants of cells is that it is possible to have sustained release of paracrine factors from cells *in vivo*. We have previously reported on sustained release of HGF and VEGF from primed hMSCs [15]. In short, ELISA analyses were conducted after culturing primed hMSCs in growth medium for up to 5 days. The secreted levels of both factors from primed hMSCs remained significantly elevated compared with control hMSCs even after 5 days in culture. In this study, immunohistochemical staining for human VEGF and HGF demonstrated significant levels of both factors in the group transplanted with primed hMSCs; whereas there was undetectable to low levels of these factors in the sham and mice transplanted with nonprimed hMSCs (FIGURE 5A & 5B). These results suggest that primed hMSCs survived after transplantation and secreted high levels of human VEGF and HGF to enhance angiogenesis in the ischemic tissues. In addition, we could not see any 'creased' or partially condensed nuclear morphology in anti-human nuclei staining, indicating the survival of transplanted cells.

In summary, the primed hMSCs used in this study may serve as a cellular vector to deliver important angiogenic factors to ischemic tissue. The factors released from cells are much more stable than recombinant peptides, and can increase survival of transplanted cells by autocrine and paracrine actions to protect residual cells in the ischemic area. Our results also indicate that primed hMSCs express other factors in addition to VEGF and HGF that promote repair and that elevated levels of these factors in primed hMSCs contribute to the enhanced therapeutic benefit. Furthermore, ensuring local trophic support at the ischemic area will enhance therapeutic angiogenesis. Our results suggest that an autologous cell therapy using these primed cells could be a new therapeutic strategy to treat ischemic diseases.

Conclusion

We demonstrate that priming hMSCs via the methods described in this article enhances secretion of critical proangiogenic factors resulting in enhanced therapeutic angiogenesis in an animal model of hindlimb ischemia. Our data recapitulate previous studies showing that hMSC administration restores blood flow in a model of hindlimb ischemia but also demonstrate that primed hMSCs exhibit significantly enhanced therapeutic activity compared

with nonprimed hMSCs in this model. These primed cells could be applied to enhance angiogenic efficacy of stem cell therapy through the synergistic actions with critical angiogenic factors.

Future perspective

Autologous bone marrow-derived MSCs are widely-used cell sources in clinical cell therapy for patients with ischemic diseases, among others. However, autologous cells have limitations, such as poor functionality or vitality, because they are derived from elderly patients. Thus, although they are safely applicable to real-world practice, the therapeutic efficacy would be inherently limited. One way to overcome such a limited functionality or vitality of MSCs from patients is genetic augmentation or modification. However, considering clinical applications, priming of hMSCs with cocktails of growth factors would be more feasible and safer than genetic engineering of the therapeutic cells.

Disclaimer

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Characterization of primed human bone marrow-derived mesenchymal stem cells

- Subconfluent human bone marrow-derived mesenchymal stem cells (hMSCs) were primed with all-*trans* retinoic acid for 3 days, and with a cocktail containing forskolin, bFGF, PDGF-AA and heregulin-b1 for 8 days.
- The primed hMSCs without genetic modification secreted higher amounts of VEGF and HGF compared with control hMSCs.
- Both mRNA and protein levels of VEGF and HGF increased significantly in primed hMSCs when compared with those in control hMSCs.

Potentiated therapeutic angiogenesis in ischemic limb tissues by primed hMSC transplantation

- Transplanting primed hMSCs into ischemic limbs in a mouse model of hindlimb ischemia significantly improved tissue perfusion and limb salvage by increasing capillary formation compared with nonprimed hMSCs.

Transplanted hMSCs survive & secrete VEGF & HGF in ischemic limb tissues

- The primed hMSCs survived better than nonprimed hMSCs in ischemic limbs.
- The primed hMSCs survived after transplantation and secreted high levels of human VEGF and HGF to enhance angiogenesis in the ischemic tissues.

Conclusion

- Our data recapitulate previous studies showing that hMSC administration restores blood flow in a model of hindlimb ischemia.
- Priming hMSCs via the methods described in this study enhances secretion of critical proangiogenic factors resulting in an enhanced therapeutic effect of cells for the treatment of ischemic diseases.
- The primed hMSCs could enhance therapeutic angiogenesis to treat ischemic disease by serving as a cellular vector to deliver two potent proangiogenic factors at the same time.
- The primed hMSCs could be applied to enhance the angiogenic efficacy of cell-based therapy for ischemic diseases through the synergistic actions with critical angiogenic factors.

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