**Original Paper**

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2007;20:867-876

Accepted: April 30, 2007

# Direct Comparison of Human Mesenchymal Stem Cells Derived from Adipose Tissues and Bone Marrow in Mediating Neovascularization in Response to Vascular Ischemia

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**Key Words**

Human adipose stromal cells • Human bone marrow stromal cells • Matrix metalloproteinase • Angiogenesis • Hindlimb ischemia

**Abstract**

Background/Aim: Although transplantation of MSC derived from bone marrow or adipose tissues has been shown in proangiogenic action in hindlimb ischemia model of nude mice, little information is available regarding comparison of the angiogenic potency between human adipose stromal cells (hADSC) and bone marrow stromal cells (hBMSC). We compared their therapeutic potential by transplantation of equal numbers of hADSC or hBMSC in a nude mice model of hindlimb ischemia. Methods&Results: One day after creating hindlimb ischemia, mice were randomized to receive hADSC transplantation (hADSC group), hBMSC transplantation (hBMSC group), or vehicle transplantation (Control group). Two weeks after transplantation, the laser Doppler perfusion index was significantly higher in the hADSC group and hBMSC group than in the control group. Comparison between hADSC and hBMSC group showed better recovery of blood flow in hADSC group than in hBMSC group. Conditioned media from hADSC (hADSC-CM) showed better *in vitro* tube formation of hADSC than conditioned media from hBMSC (hBMSC-CM). hADSC showed higher expression of MMP3 and MMP9 than hBMSC. A MMP inhibitor, GM6001, and the transfection of MMP3 or MMP9 siRNA oligonucleotides inhibited in vitro tube formation of hADSC. Transplantation of MMP3 or MMP9 siRNA oligonucleotieds-transfected hADSC showed lower blood flow recovery and higher tissue injury than control oligonucelotide-transfected cells. Conclusion: This study showed that hADSC can be an ideal source for therapeutic angiogenesis in ischemic disease in terms of efficacy, accessibility and available tissue amounts.

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1015-8987/07/0206-0867$23.50/0

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## Introduction

Peripheral vascular disease is a major health care problem in an aging society [1]. An important compensatory response to atherosclerotic obstructive arterial disease is collateral development, a complex process requiring that multiple genes coordinately express their products in an appropriate time-dependent manner [2]. However, the natural capacity of collaterals to remodel and enlarge to compensate for the reduced flow that occurs after occlusion of a major artery is rarely sufficient to restore maximal flow capacity to levels required under various stress-conditions. In the late stages of peripheral vascular disease, progression of tissue hypoperfusion results in ischemic ulceration and gangrene. Unfortunately, amputation is required in more than a third of these patients. Rapid revascularization of injured, ischemic, and regenerating organs is essential for the restoration of their physiological function.

Cell-based therapy becomes promising strategy for the treatment of peripheral artery diseases. Mesenchymal stem cells (MSC) are present in various tissues including bone marrow, peripheral blood and adipose tissues and possess pluripotency and differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells, endothelial cells and vascular smooth muscle cells [3-7]. MSC have been considered as ideal sources of cell therapy because of simple isolation techniques, easy expandability and pluripotency. Bone marrow stromal cells (BMSC), a representative MSC, have been shown to form capillary-like structures in an in vitro Matrigel assay [8] and BMSC transplantation has been shown to induce neovascularization in a rat model of hindlimb ischemia [9].

It has been reported that MSC derived from adipose tissues have similar characteristics of BMSC [10, 11]. We demonstrated that culture expanded hADSC have proangiogenic effect in mouse hindlimb ischemia model and the effect is mediated by paracrine effects of transplanted cells as well as transdifferentiation into endothelial cells [12], and that hADSC can differentiate into smooth muscle-like cells [13]. The purposes of this study were to compare the therapeutic potencies of hBMSC and hADSC transplantation in a mouse model of hindlimb ischemia and to investigate the mechanisms underlying their differences in angiogenic potential. Our data indicate that ADSC show better proangiogenic effect than BMSC, which can be partly explained by differences of MMP3 and 9 expressions.

## Materials and Methods

*Culture of mesenchymal stem cells*

hBMSC and hADSC were isolated and characterized according to the methods described in the previous studies [10, 14]. All protocols involving human subjects were approved by the Institutional Review Boards of Pusan National University. After informed consent, leftover materials (heparinized bone marrow cells and adipose tissues) were obtained from six individuals undergoing total hip arthroplasty and elective abdominoplasty. The patients for adipose tissues were 55 years old male, 24 years old female and 43 years old female. The patients for bone marrow samples were 45 years old male, 48 years old female and 36 years old female. To isolate human adipose tissue stromal cells (hADSC), adipose tissues were washed with phosphate-buffered saline (PBS), and tissues were digested at 37°C for 30min with 0.075% type I collagenase. Enzyme activity was neutralized with α-modified Eagle's medium (α-MEM), containing 10% fetal bovine serum (FBS) and centrifuged at 1,200 x g for 10min to obtain a pellet. The pellet was incubated overnight at 37°C/5% $CO_2$ in control medium (α-MEM, 10% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin). Following incubation, the tissue culture plates were washed to remove residual nonadherent cells and maintained at 37°C 15% $CO_2$ in control medium. Mononuclear cells from bone marrow were separated by centrifugation in a Ficoll-Hypaque gradient (density = 1.077 g/cm$^3$; Sigma, USA), suspended in α-MEM containing 10% FBS and 100 units/ml of penicillin, 100 µg /ml of streptomycin and seeded at a concentration of 1 x 106 cells/cm$^2$. Cultures were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$. When the monolayer of adherent cells has reached confluence, cells were trypsinized (0.25% trypsin; Sigma), resuspended in α-MEM containing 10% FBS, and subcultured at a concentration of 2,000 cells/cm$^2$. For the experiment, we used 3rd-5th passages of MSC.

*Hindlimb ischemia model and cell transplantation*

The neovascularization capacity of hMSC was investigated in a murine model of hindlimb ischemia in 8 weekold Balb/C nude mice (Sam:TacN(SD)fbr) [12]. To produce hindlimb ischemia, animals were anesthetized by pentobarbital sodium (0.5mg/g). The proximal portion of the femoral artery including the superficial and the deep branches were ligated twice with 7-0 silk suture, and the overlying skin was closed. After 24 hours, injection of hMSC was performed intramuscularly by injecting cells resuspended in phosphate buffered saline (PBS) at three different sites of the ischemic leg (gastrocnemius, gracilis, and quadriceps muscles). For the tracing of transplanted hMSC, cells were marked with the fluorescent CM-DiI dye (Molecular Probes) before cellular transplantation. After 2 weeks, cutaneous blood flow was measured by a laser Doppler blood flow meter (Laser Doppler Perfusion Imager System, OMEGAFLOW FLO-CI, OMEGAWAVE, INC.). Before scanning was initiated, mice were placed on a heating plate at 37°C.

*Histological evaluation*

Mice were killed after blood flow was determined by a laser Doppler blood flow meter, and perfused with 4% (w/v) paraformaldehyde (Sigma). The skeletal muscles (gastrocnemius, gracilis and quadriceps muscles) from ischemic hindlimb were removed and fixed in neutral buffered 4% (w/v) paraformaldehyde at 4 for 24 hours prior to embedding in paraffin and sectioning. Six transverse muscular sections (5 ìm) of each muscle were deparaffinized and then stained with hematoxylin and eosin (H-E). The proportion of muscle injury was determined by the examination of whole slides at 200 x magnification.

*Gel zymography*

hADSC or hBMSC were cultured and expanded on tissue culture plates in EGM-MV/5% medium or EBM2/5% medium and used for the experiments at passages 0 through 3. At 90% confluence, culture medium was switched to EBM2 and placed for 24 hours. At the end of the incubation period, the conditioned media from hADSC or hBMSC were collected. To assess MMP3 and 9 activity, conditioned media was collected and concentrated 30-fold using an Amicon Centricon (Millipore). The 7.5% acrylamide/bisacrylamide gels were 1mm thick, pH 8.6, and contained 0.1% gelatin (Sigma) incorporated as an MMP9 substrate. Casein gels (12%) contained 0.1% casein (Sigma). The samples were electrophoretically separated. After electrophoresis, the gel was washed at room temperature for 1hr in wash buffer (2.5% Triton X-100 in 50mM Tris-HCl (pH 7.4)) and incubated at 37°C overnight in incubation buffer (50mM Tris-Cl (pH 7.4), 150mM NaCl, and 5mM $CaCl_2$). The gel was stained with 0.2% Coomassie brilliant blue F-250 in mixture of methanol:acetic acid:water(2:1:7) for 2hr, and then destained in the same solution, without dye.

*In vitro tube formation assay*

Thawed Matrigel (Chemicon) was added to 96-well plates and allowed to solidify at room temperature for 1 hour. hMSC were seeded $1x10^4$ cells per well and were induced to differentiate into endothelium by plating in Matrigel-coated 96 well plate with EGM-MV containing 5% FBS for 6 hours. To assess the effects of hADSC-CM and hBMSC-CM on tube formation of hADSC, hADSC were seeded $1x10^4$ cells per well and then incubated with 50% fresh EBM-2/5% FBS medium (control medium) and 50% hADSC-CM or hBMSC-CM/5% FBS. The effect of MMP inhibitor (Chemicon) on hADSC tube formation was assessed by treatment with GM6001 MMP inhibitor (20-30 μM) or DMSO for 5 days. This experiment was performed in triplicate.

*Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) Analysis*

Total cellular RNA was isolated from hADSC and hBMSC that were cultured in α-MEM or hADSC that were cultured in EBM2(Endothelial Basal Medium2)/5% FBS medium or EGM-MV(EBM2 and growth factors (hydrocortisone, hEGF, FGF, VEGF, hFGF-B, R3-IGF-1, ascorbic acid and gentamicin))/5% FBS medium, hADSC-CM/5% FBS or hBMSC-CM/5% FBS for 5 days and reverse transcribed using conventional protocols. cDNA was synthesized in a reaction containing 1 mg of DNase I treated total RNA, oligo dT primer, dNTP, and avian myeloblastosis virus (AMV) reverse transcriptase. PCR amplification was performed using the primer sets. PCR amplification was performed using the primer sets. GAP DH5'-TCC ATG ACA ACT TTG GTA TCG-3', 5'-TGT AGC CAA ATT CGT TGT CA-3', MCP 1 5'-CCC CAG TCA CCT GCT GTT AT-3', 5'-GAG TTT GGG TTT GCT TGT CC-3', MCP 2 5'-TCA GCC AGA TTC AGT TTC CA-3', 5'-ATT TGG TCC AGA TGC TTC AT-3', GCP 2 5'-GTC CTT CGG GCT CCT TGT-3', 5'-AAC TTG CTT CCC GTT CTT CA-3', IL-6 5'-AAA GAG GCA CTG GCA GAA AA-3', 5'- CAG GGG TGG TTA TTG CAT GT-3', MMP 1 5'TAC CCC AAG GAC ATC TAC AG-3', 5'-AAT TCC AGG AAA GTC ATG TG -3', MMP 2 5'-CAG GTG ATC TTG ACC AGA AT-3', 5'-CAT CAT GGA TTC GAG AAA AC-3', MMP 3 5'-ACT CCA CTC ACA TTC TCC AG-3', 5'-TCC CTG TTG TAT CCT TTG TC-3', MMP 9 5'-GGA GTA CTC GAC CTG TAC CA -3', 5'-GTG AAG CGG TAC ATA GGG TA-3', TIM P1 5'-GAC ACC AGA AGT CAA CCA GA-3', 5'-TAG TGA TGT GCA AGA GTC CA -3', TIM P2 5'-TGC AAT GCA GAT GTA GTG AT-3', 5'-TTC CTG CAA TGA GAT ATT CC-3', TIM P3 5'-CTG GGT TGT AAC TGC AAG AT-3', 5'-GGC ATT GAT GAT GCT TTT AT-3', and VEG F 5'-AAG GAG GAG GGC AGA ATC AT-3', 5'-ATC TGC ATG GTG ATG TTG GA-3'. All primer sequences were determined using established GenBank sequences. Duplicate PCR reactions were amplified using primers designed GAPDH as a control for assessing PCR efficiency and for subsequent analysis by agarose gel electrophoresis. As a negative control, total RNA was used for PCR amplification.

*Real time polymerase chain reaction (PCR)*

cDNA was synthesized in a reaction containing 1 mg of DNase I treated total RNA of control hADSC and hBMSC, oligo dT primer, dNTP, and avian myeloblastosis virus (AMV) reverse transcriptase. Primer sequences to be used in the experiment were as follows: β-actin, 5'-CTG GTG CCT GGG GCG-3', 5'-AGC CTC GCC TTT GCC GA-3'; MMP9 5'-ACC TCG AAC TTT GAC AGC GAC A -3', 5'-GAT GCC ATT CAC GTC GTC CTT A -3'Real time quantitation was predicated on the LightCycler assay, using a fluorogenic SYBR Green I reaction mixture for PCR with the LightCycler Instrument (Roche, Germany). All experiments were conducted three times, and both negative and positive controls were included. Whereas no template negative control ($H_2O$ control) was run with every gene specific primer, the no RT-PCR control was run with only one primer pair, which could amplify contaminated genomic DNA. For each of the primer pairs, the linearity of detection was verified to have a correlation coefficient of at least 0.98 over the detection area, via the measurement of a dilution curve with the cDNA isolated from the hADSC. β-actin mRNA was amplified as an internal control. LightCycler software version 3.3 (Roche Diagnostics) was used to analyze PCR kinetics and calculate quantitative data. A standard curve generated in a separate run was loaded into runs of each samples (without standard curves). Each run included one sample of known

**Fig. 1.** *In vivo* proangiogenic action of hADSC or hBMSC in hindlimb ischemia model. (A) Hindlimb ischemia was induced by femoral artery ligation in nude mice, and animals were treated with either PBS (Cont.) or hADSC or hBMSC one day after ligation. Quantitative analysis with laser Doppler was performed, and data are presented as ratio of perfusion in ischemic leg compared with nonischemic leg over time on day 14. (B-C) The skeletal muscles of hindlimb were taken at day 14 after transplantation. The relative area of injured tissue was measured by microscopic examination of H-E stained paraffin sections. (C) Data represent mean ± SEM (n=5). *p < 0.05, significantly different from control group; # p<0.05, significantly different from hBMSC group.

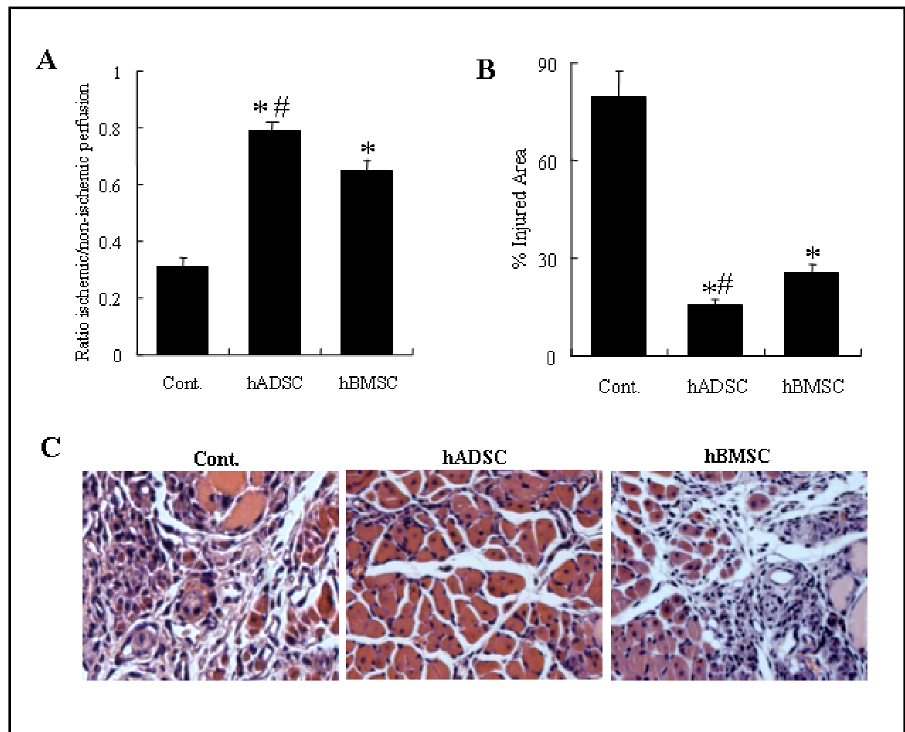
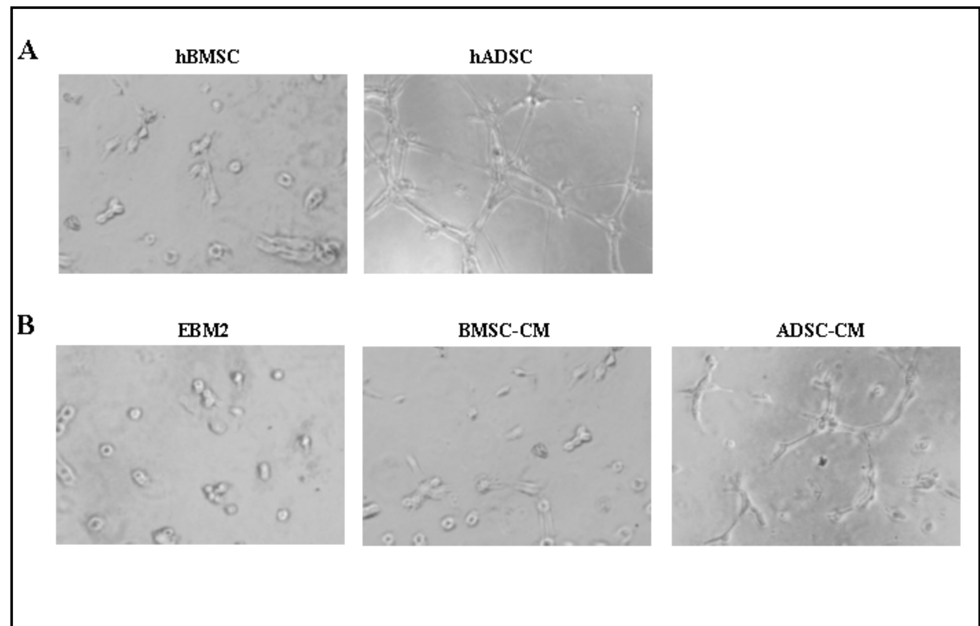



**Fig. 2.** Capillary-like tube formation of hADSC or hBMSC in Matri-gel (A) Capillary-like tube formation of hADSC or hBMSC in Matri-gel. Phase contrast micrographs of a vessel-like structure formed by hADSC or hBMSC in EGM-MV medium were taken at 6 hours after plating. (B) Effect of conditioned medium from hADSC (hADSC-CM) or hBMSC (hBMSC-CM) on capillary-like tube formation of hADSC. Phase contrast micrographs of hADSC in EBM2, hADSC-CM or hBMSC-CM were taken at 6 hours after plating on Matri-gel. (Magnification, x 200).




concentration and in the range covered by the standard curve, thus allowing estimation of exact copy numbers by the second derivative maximum method. For each sample, copy numbers of target gene mRNA were divided by those of β-actin mRNA to normalize for target gene mRNA expression and thus avoid sample-to-sample differences in RNA quantity.

*Matrix metalloproteinase down-regulation by RNA interference (RNAi)*

Gene silencing by RNAi was used to down-regulate individual MMPs in hADSC. siRNAs specific for human MMPs (Dharmacon) were synthesized against the following published target sequences for MMP-9: 5'-CAU CAC CUA UUG GAU

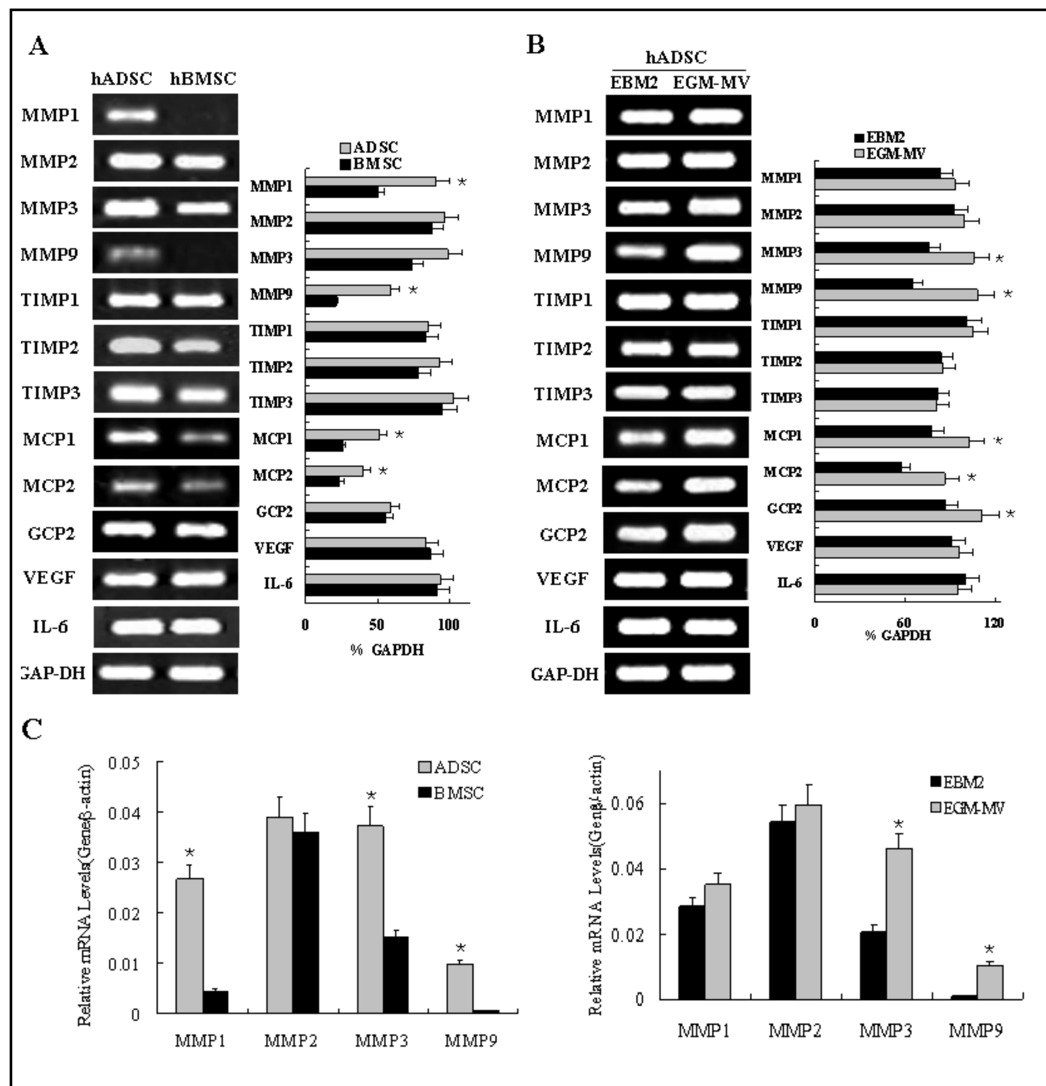



**Fig. 3.** RT-PCR anaylsis of angiogensis-related genes. (A) Comparison of mRNA expression between hADSC and hBMSC. Total RNA were isolated from hADSC and hBMSC that were grown in α-MEM, and. analyzed by RT-PCR for the indicated genes (MCP1, MCP2, GCP2, VEGF, IL-6, MMP1, MMP2, MMP3, MMP9, TIMP1, TIMP2 and TIMP3). (B) Effect of EGM-MV on the expression of angiogenesis-related genes. hADSC or hBMSC were cultured with EBM2/5% FBS medium and EGM-MV/ 5% FBS medium. Total RNA were isolated from control hADSC or hBMSC after 5 days treatment of the medium. (C) Real-time PCR provided a quantitative analysis of increased relative expression of mRNAs (MMP1, 2, 3 and 9) between hADSC or hBMSC and EBM2 or EGM-MV treated hADSC. The values were normalized using β-actin as an internal control. Data represent mean ± SEM of the relative ratio to β-actin signal of the corresponding samples (n=4). *P<0.05, significantly different from hBMSC or EBM2 data.

CCA A-3' [15], and MMP-3: 5'AUG AAG AGU CUU CCA AUC CUU-3' [16]. As a negative control, On-Target plus non targeting siRNA was transfected. Cells were grown to confluence, harvested, and counted. The cells were nucleofected with siRNAs using the Amaxa system according to the manufacturer's instructions using human MSC nucleofector solution and program U23.

*Statistical analysis*

All results are presented as mean ± SEM. Comparisons between groups were analyzed by use of t test or ANOVA for experiments with more than 2 subgroups. Post hoc range tests and pairwise multiple comparisons were performed with the t test with Bonferroni adjustment. Probability values of P<0.05 were considered statistically significant.

## Results

*Comparison of hADSC and hBMSC for proangiogenic action*

Blood perfusion of the ischemic hindlimb was considerably impaired 2 weeks after surgery (Control group, Fig. 1A). hADSC or hBMSC ($10^6$ cells) were transplanted into ischemic hindlimb of nude mice one day

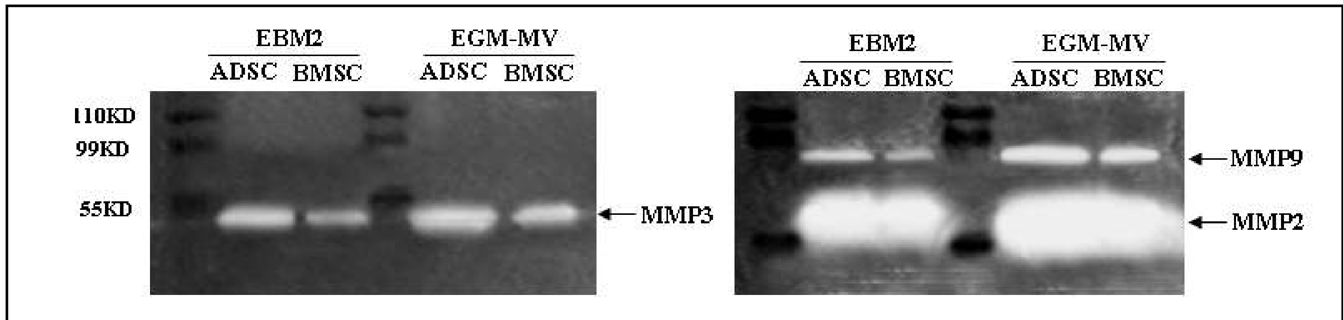



**Fig. 4.** MMP activity of hADSC and hBMSC by Zymography. (A) MMP3 and MMP9 activity were confirmed by casein and gelatin zymography from hADSC or hBMSC cell culture media after 24hrs with or without EGM-MV (n=3).

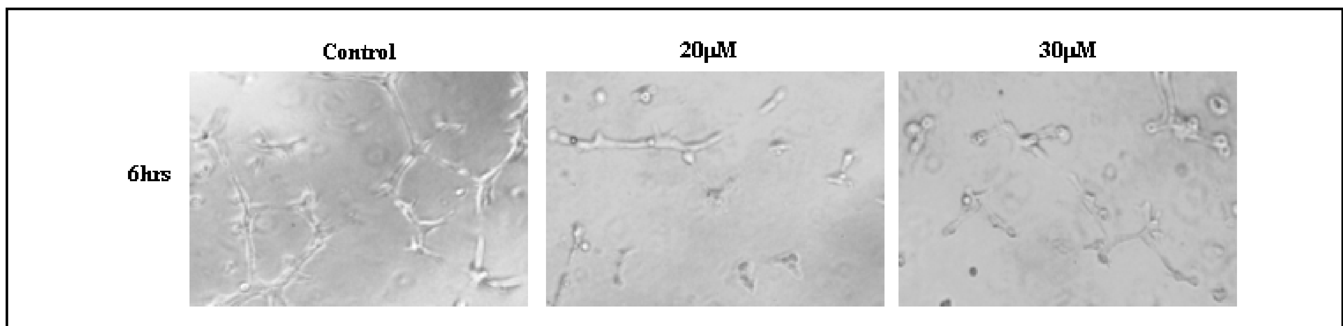



**Fig. 5.** Effect of GM6001 on Capillary-like tube formation of hADSC. The effect of a MMP inhibitor on tube formation of hADSC was assessed by the treatment with GM6001 (20 or 30 μM) or DMSO (Cont.). (Magnification, x 200).

after the ligation of femoral artery. Two weeks after cell transplantation, improvement of hindlimb ischemia was observed in the hADSC and BMSC groups compared with the PBS-injected control group. Quantitative analysis demonstrated that the LDPI index was highest in the hADSC group, followed by the hBMSC group and Control group (Fig. 1A). Histological examination of ischemic limb showed that hADSC and hBMSC transplantation protected atrophic and necrotic damage of muscle. Quantitative analysis demonstrated that the extent of muscle injury was lowest in the ADSC group, followed by the hBMSC group and Control group (Fig. 1 B and C).

To understand the mechanisms of differences between hADSC and hBMSC, we compared *in vitro* tube formation in matrigel between hADSC and hBMSC. hADSC showed better formation of tube-like structure than hBMSC in EGM-MV/5% FBS medium (Fig. 2A). Next, we examined the effect of conditioned media on tube formation of MSC in Matrigel. We obtained conditioned media by incubating confluent hADSC or hBMSC with EBM2 for 48 hours. hADSC failed to form tube-like structure in EBM2/5% FBS medium. When hBMSC conditioned media (hBMSC-CM) containing 5% FBS was added to Matrigel culture of hADSC, their tube formation was decreased compared with culture in hADSC conditioned media (hADSC-CM) containing 5% FBS (Fig. 2B).

*Role of MMP-3 and MMP-9 in the proangiogenic action of hADSC*

To understand the molecular mechanisms of hADSC-CM-induced increase in capillary-like tube formation of hADSC, we compared the angiogensis-related genes and cytokines expression of hADSC and hBMSC. hADSC showed higher expression of MCP1 and 2, MMP1, 3, and 9 than hBMSC (Fig. 3A). For the quantitation of MMP1 ,2, 3,and 9 expression, we performed real time PCR. Real time PCR analysis confirmed higher expression of MMP1, 3,and 9 gene in

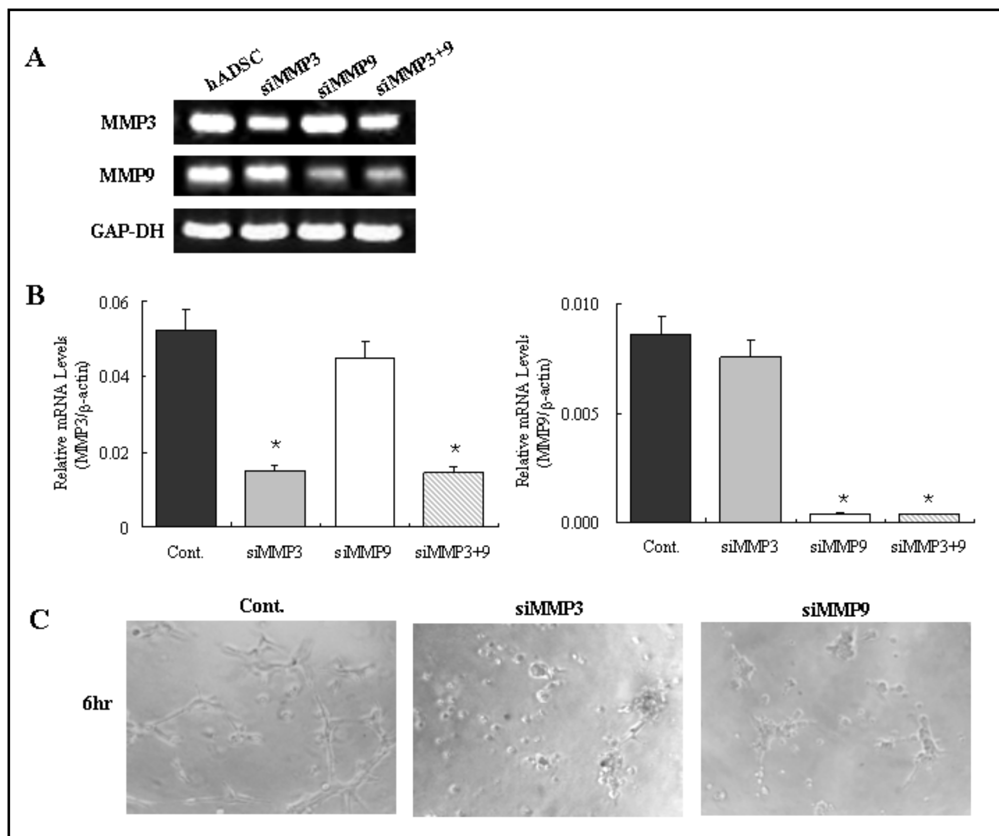



**Fig. 6.** Effect of MMP3 or MMP9 siRNA oligonucleotides on Capillary-like tube formation of hADSC. (A) RT-PCR analysis of MMP3 or MMP9 expression in siRNA oligonucleotides-transfected hADSC. Total cellular RNA was isolated from siRNA oligonucleotides-transfected hADSC after 72hrs. (B) Real time PCR analysis showed that MMP3 or MMP9 siRNA oligonucleotides effectively inhibited MMP3 or MMP9 expression in hADSC. The values were normalized using β-actin as an internal control. *$P<0.05$ compared with control siRNA-transfected cells. (C) hADSC that were transfected with MMP3 (siMMP3) or MMP9 (siMMP9) siRNA oligonucleotides were analyzed for capillary-like tube formation in Matrigel (Magnification, x 200).

hADSC than in hBMSC. Then, we determined whether the expression ofthe genes that show differential expression between hADSC and hBMSC can be altered by the exposure in EGM-MV media compared with in EBM2 media, because hADSC can form tube-like structure in EGM-MV media not in EBM2 media (Fig. 2). RT-PCR and quantitative real time PCR analysis showed that hADSC exposed to EGM-MV for 5 days markedly increased MCP1 and 2, MMP-3 and MMP9 compared with hADSC that were cultured in EBM2($p < 0.05$) (Fig. 3B, C). We measured MMP3 and 9 activities in conditioned media by zymography. Conditioned media were obtained from hADSC or hBMSC to be cultured in EBM2 or EGM-MV media for 5 days. The conditioned media obtained from hADSC showed higher MMP3 or 9 activity than that from hBMSC in EBM2 media (Fig 4). However, MMP2 activity did not show any significant difference between the conditioned media from hADSC and hBMSC. The conditioned media obtained from hADSC or hBMSC which were cultured in EGM-MV showed higher MMP3 or MMP9 activity than that from hADSC or hBMSC which were cultured in EBM2 media.

To determine the role of MMP on tube formation of hADSC, the effect of GM6001, a MMP inhibitor, on tube formation of hADSC was examined. GM6001 (20 and 30 μM) inhibited tube formation of hADSC (Fig. 5). We determined the role of MMP3 and 9 by RNA interference technique. Transfection of hADSC with siRNA MMP3 or MMP9 oligonucleotides was performed by nucleofection. RT-PCR and real time PCR analysis showed that MMP3 or MMP9 siRNA oligonucleotides effectively inhibited MMP3 or MMP9 expression in hADSC (Fig. 6A and B). However, MMP3 siRNA did not affect MMP9 expression and MMP9 siRNA did not affect MMP3 expression, indicating their specificity. MMP9 siRNA oligonucleotide-transfected hADSC showed less capillary tube formation than control siRNA-transfected cells (Fig. 6C). Then we determined the proangiogenic action of siMMP-transfected hADSC in hindlimb ischemia model. In hindlimb ischemia model of nude mice the blood flow recovery in the group that was transplanted with MMP3 or MMP9 siRNA transfected hADSC was significantly lower than that in the group that was transplanted with control siRNA transfected hADSC (Fig. 7A). In the animals transplanted with MMP3 siRNA oligonucleotides or MMP9 siRNA oligonucleotides-treated hADSC, the injured area of skeletal muscle was significantly higher than that in the

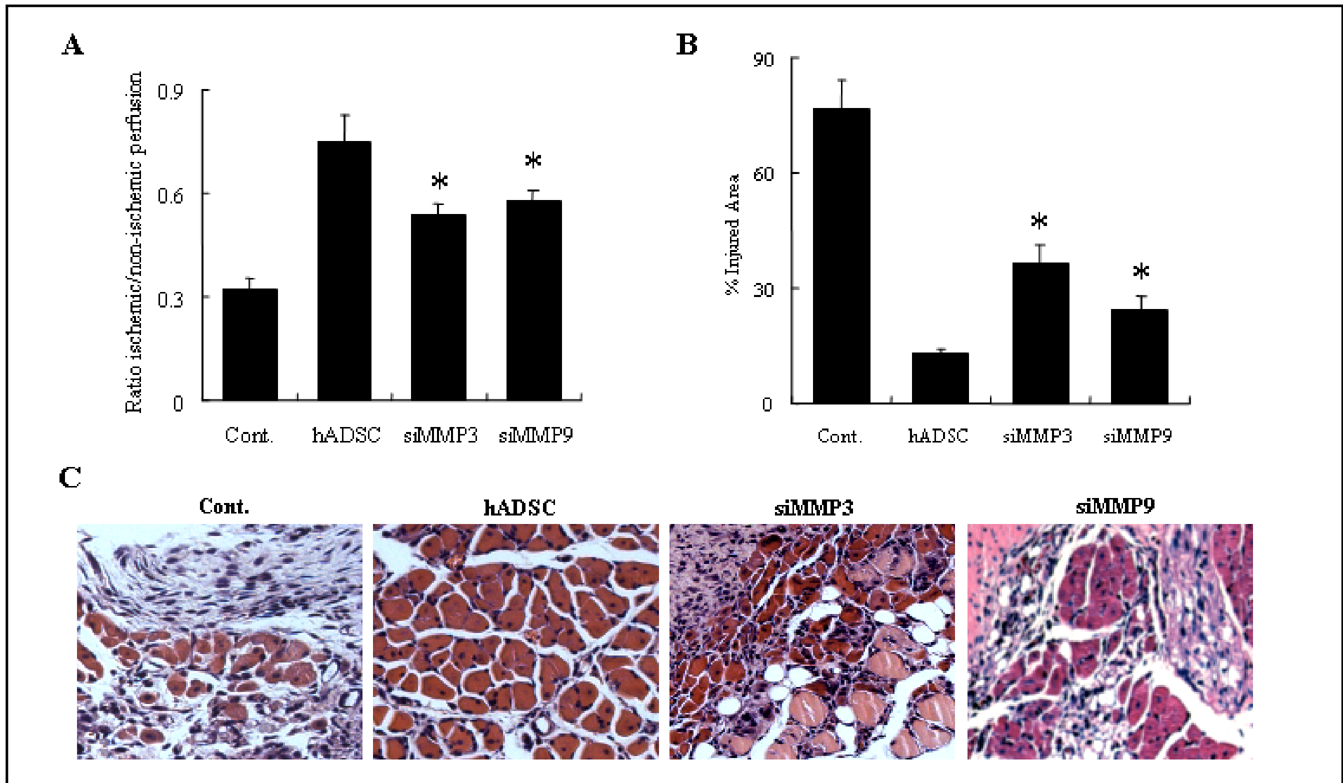



**Fig. 7.** *In vivo* proangiogenic action of MMP3 or MMP9 siRNA oligonucleotide-treated hADSC in hindlimb ischemia model. (A) Hindlimb ischemia was induced by femoral artery ligation in nude mice, and animals were transplanted with control, MMP3 or MMP9 siRNA oligonucleotide-transfected hADSC one day after ligation. Two days before transplantation hADSC were transfected with control, MMP3 or MMP9 siRNA oligonucleotides. Quantitative analysis with laser Doppler was performed at day 14 after transplantation. Data were presented as the ratio of perfusion in ischemic leg compared with nonischemic leg. (B-C) The skeletal muscles of hindlimb were taken at day 14 after transplantation. The relative area of injured tissue was measured by microscopic examination of H-E stained paraffin sections. (B) Paraffin-embedded tissue sections were stained with H-E. (C) Data represent mean ± ΣΕΜ (ν=4). *π < 0.05, significantly different from control siRNA transfected group.

animals transplanted with control oligonucleotides-transfected hADSC (Fig. 7B and C).

## Discussion

Stem cell therapy is a promising approach in cardiovascular medicine. There are several reports [17] of clinical studies in which EPC have been used for neovascularization of ischemic organs. However, the paucity of EPC in the circulation, combined with their putative functional impairment in pathological conditions, limits their therapeutic application. Thus, the identification of suitable sources of angiogenic cells represents a challenge for therapeutic angiogenesis of ischemic tissues.

The use of umbilical cord blood, mobilization of EPC by growth factors, or the local infusion of suspensions of autologous bone marrow cells and mesenchymal stem cells has been suggested as ways in which to circumvent this problem. In fact, earlier studies have shown that hBMSC and hADSC transplantation enhances neovascularization by transdifferentiating into endothelial cells and supplying multiple angiogenic factors such as VEGF, bFGF, and angiopoietin-1 [9, 12, 18]. However, it remains unclear whether the angiogenic potency of hADSC transplantation is comparable or superior to that of hBMSC transplantation. Because hADSC is a more readily available autologous stem cell source than hBMSC, it is important to address whether hADSC have equivalent functionality compared with hBMSC.

In the present study we therefore cultured MSC from adipose tissue and bone marrow with standard conditions and injected equal numbers of ADSC or BMSC into ischemic muscle to compare the therapeutic effects of the two types of cells. Our studies directly comparing infusions of hADSC or hBMSC revealed that hADSC

showed better biologic effects of increased blood flow in the nude mice study model of hind-limb vascular injury, although both hADSC and hBMSC injections significantly increased blood flow in the ischemic leg by days 14 after injury/cell injection. These results suggest that hADSC transplantation is more potent in therapeutic angiogenesis than hBMSC transplantation.

The underlying mechanisms responsible for the superiority of hADSC in therapeutic angiogenesis remain unknown. Recent studies have demonstrated that the angiogenic potential of hADSC and hBMSC is attributed not only to their differentiation into vascular endothelial cells but also to their ability to produce various angiogenic factors, including VEGF and bFGF [9, 12, 19]. In this study hADSC have better tube formation in Matrigel than hBMSC. The data using conditioned media obtained from hADSC and hBMSC indicated that soluble factors derived from hADSC were involved in the process. RT-PCR and real time PCR analysis of angiogenesis-related genes showed that MMP3 and 9 expression is higher in hADSC than in hBMSC. Among them MMP3 or 9 expression was induced in EGM-MV media which induce tube formation in Matrigel, compared with cells grown in EBM-2 media which fail to support tube formation. Therefore, we focused on the role of MMP3 or MMP9 in proangiogenic action of hADSC.

Matrix metalloproteinases (MMP) are a family of $Zn^{2+}$ endopeptidases that regulate cellular activity in various ways. These include extracellular matrix (ECM) degradation, cell adhesion, proteolytic release of ECM sequestered molecules, and shedding of cell surface proteins that transduce signals from the extracellular environment. MMPs are implicated in a wide range of diverse pathological processes that include atherosclerosis, arthritis, cancer, and neurodegeneration [20-24]. MMPs have been shown to play complex and integral roles during both the initiation and resolution phases of angiogenesis in various tissues [25-27]. Among these proteases, MMP-9 (gelatinase B) plays a more specific role in angiogenesis [28]. MMP-3 also has been reported to be involved in angiogenesis [29, 30]. In this study the role of MMP3 and MMP9 in hADSC-induced proangiogenic action was supported by the effect of a MMP inhibitor and MMP3 or MMP9 siRNA oligonucleotides. These findings indicated that MMP3 and 9 expressions play important roles in the proangiogenic action of hADSC, although we have not defined soluble factors that are involved in the induction of their expression in hADSC. Bergers *et al.* (2000) [31] demonstrated that MMP-9 can trigger the angiogenic switch by increasing the bioavailability of the angiogenic growth factor, VEGF (vascular endothelial growth factor), from matrix stores, enabling activation of preexisting VEGF tyrosine kinase receptors on endothelial cells.

It is difficult to obtain hBMSC enough for clinical trial in some cancer patients, although hADSC can be easily isolated from a small amount of adipose tissue and rapidly expand in culture. The data in this study demonstrated that hADSC show better proangiogenic action than hBMSC. Therefore, hADSC transplantation may be one of the most attractive cell therapies in the treatment of critical limb ischemia.

## Acknowledgements

This study was supported by a grant (R012006000105250) from the ministry of science and technology and the MRC program of MOST/KOSEF (R13-2005-009).

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