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Unsorted human adipose tissue-derived stem cells promote angiogenesis and myogenesis in murine ischemic hindlimb model

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ABSTRACT

We examined the protective effect of unsorted human adipose tissue-derived stem cells (hADSCs) with a short-term culture in endothelial differentiation medium on tissue repair after ischemic injury. hADSCs were isolated from human subcutaneous adipose tissue and cultured *in vitro* in endothelial differentiation medium for 2 wks before transplantation. Cultured hADSCs showed a typical mesenchymal stromal cell-like phenotype, positive for endothelial-specific markers including VE-cadherin, Flt-1, eNOS, and vWF but not CD31. Two hours after ligation of the femoral artery and vein, mice were injected with the unselected hADSCs locally near the surgery site and tested for tissue perfusion and repair. Tissue perfusion rates of the ischemic limbs were significantly higher in the group treated with hADSCs compared with those of the control mice as early as post-operative day 3 (median 195.3%/min; interquartile range, 82.0–321.1 vs. median 47.1%/min; interquartile range, 18.0–58.7; $p = 0.001$ by Friedman two-way analysis). Subsequently, the mice treated with hADSC showed better prognosis at 4 wks after surgery, and the histological analysis revealed increased vascular density and reduced muscle atrophy in the hADSC-transplanted limbs. Moreover, hADSC-treated muscle contained differentiated myocytes positive for human NF-κB and myogenin antigen. These results collectively indicate that unsorted hADSCs after a 2-wk-*in vitro* culture have a therapeutic potential in ischemic tissue injury via inducing both angiogenesis and myogenesis.

Introduction

Peripheral occlusive vascular disease is a major health care problem in our aging society. If peripheral vascular occlusion progresses to ischemic ulceration or gangrene, then the risk of limb loss becomes substantial. More invasive interventions such as balloon angioplasty, stenting, and surgical revascularization are eventually considered to prevent devastating amputation of the ischemic limbs. The choice of intervention mainly depends on the anatomy of the stenotic or occlusive lesion (Ouriel, 2001); while longer lesions or small multiple vascular lesions are impossible to be treated with surgical interventions (Aboyans et al., 2006).

Transplantation of mesenchymal stem cells (MSCs) such as bone marrow stromal cells has been considered to induce therapeutic angiogenesis in critically ischemic tissues of various animal models (Higashi et al., 2004; Moon et al., 2006; Shintani et al., 2001). It has been reported that MSCs have a potential to differentiate into skeletal muscle cells, endothelial cells, and vascular smooth muscle cells (Pittenger et al., 1999). Unfortunately, the percentage of MSCs in bone marrow is quite low and decreases with age (Stolzing et al., 2008). Thus, a large quantity of bone marrow is needed to obtain sufficient numbers of stem cells to induce therapeutic angiogenesis. To overcome this problem, a variety of strategies controlling stem cell fate has been investigated; however, the efficacy of cell fate control has not reached to the level for the clinical uses (Lutolf et al., 2009; Xu et al., 2008).

As an alternative source of adult MSCs, adipose tissues also contain MSCs called adipose tissue-derived stem cells (ADSCs), which release potent angiogenic factors such as vascular endothelial growth factor (VEGF) (Rehman et al., 2004). Subsets of ADSCs such as CD34(−), Flk-1(−), or platelet/endothelial cell adhesion molecule 1 (PECAM-1, CD31) (−) cells or PECAM-positive endothelial cells, have been confirmed for the therapeutic efficacy in a murine ischemic hind limb model (Cao et al., 2005; Moon et al., 2006; Nakagami et al., 2005). Unsorted human ADSCs (hADSCs) have also been shown to be efficacious in several animal models including

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ischemic heart and rat hind limb ischemia models (Iwashima et al., 2009). Long-term *in vitro* culture in specialized culture medium has been shown to promote the differentiation of the transplanted ADSCs into mature vessel-forming endothelial cells (Cao et al., 2005) and muscle regeneration (Kim et al., 2006). Preferential differentiation of ADSCs into striated muscle cells has also been documented in a mouse model (Kim et al., 2006; Liu et al., 2007; Yu et al., 2010). Based on these previous reports, we assumed that unsorted hADSCs can be successfully transplanted to treat the ischemic tissue injuries if these cells are subject to preferential differentiation into endothelial cells by *in vitro* culture in endothelial differentiation medium.

There are growing numbers of preclinical studies examining the potential of adipose-derived cells for improving ischemic repair (Cao et al., 2005; Iwashima et al., 2009; Moon et al., 2006). Based on the promise from these animal model studies, few clinical trials have been recently initiated (Hong et al., 2010). In the present study, we addressed an important consideration concerning the use of hADSCs in therapies. Using a standard mouse hindlimb model of ischemic revascularization, we explore the ability of unsorted, short-term cultured hADSCs to improve limb post-ischemic recovery.

Methods

Isolation and culture of ADSCs

An average of 25 mL (ranging from 10 to 50 mL) of abdominal subcutaneous adipose tissue was obtained from seven patients (female, age: 35.5 ± 1.3 yr) who underwent cesarean section with consent according to the guidelines of the ethical committee of Ewha Womans University Mokdong Hospital. The specimens were finely minced using surgical scissors and incubated in a digestion buffer at 37 °C for 2 h. The digestion buffer consisted of 1 g/L collagenase (type II) and 2% BSA in an isolation buffer. The stromal vascular fraction was separated from the adipocyte fraction by low-speed centrifugation and treated with a red blood cell lysis buffer for 5 min. Cells were filtered using a 100-μm nylon mesh filter (BD Bioscience, Franklin Lakes, CA, USA) to remove cellular debris. An average of 2×10^5 cells were obtained and seeded at a density of 3000 cells/cm^2 in endothelium growth media (EGM-2 BulletKit medium, Lonza, Allendale, NJ, USA). After overnight incubation at 37 °C in a humidified atmosphere containing 5% CO_2, each plate was washed extensively with PBS to remove residual non-adherent red blood cells. The resulting stromal vascular fraction showed uniform fibroblast-like shape and maintained at 37 °C 5% CO_2. When the monolayer of adherent cells reached confluence, the cells were subcultured at a density of 3000 cells/cm^2.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

To evaluate the gene expression of endothelial nitric oxide synthase (eNOS), Flt-1, Flk-1 (KDR), VE-cadherin, and von Willebrand factor (vWF), we extracted mRNA using easy-Spin™ Solution (INtRON Biotechnology, Sungnam, Korea), according to the manufacturer's protocol. First-strand cDNA was synthesized using *Maxime* RT PreMix (INtRON Biotechnology) and amplified by *Maxime* PCR PreMix *i*-MAX II (INtRON Biotechnology). PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with the following parameters: 5 min of denaturing at 94 °C, followed by 40 cycles of 30 s of denaturing at 94 °C, 30 s of annealing at 60 °C, 30 s of extension at 72 °C, and a final extension step of 5 min at 72 °C. The primer sequences for the study are described in Supplementary methods. NIH 3T3 fibroblasts were used as a negative control and human umbilical vein endothelial cells (HUVECs) as a positive control. GAPDH served as an internal standard.

Flow cytometric analysis

ADSCs were cultured in EGM-2 medium for 2 wks before fluorescence-activated cell sorter analysis using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences). Cells were harvested in 0.25% trypsin/EDTA and washed in a flow cytometry buffer (PBS containing 0.3% BSA). Cell aliquots (1×10^6 cells) were incubated for 1 h in buffer containing fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies against CD44 (Millipore, CA, USA), CD105 (Abcam, Cambridge, UK), CD31 (R&D Systems, Minneapolis, MN, USA) or vWF (Millipore, CA, USA). As a negative control, cells were stained with an isotype control IgG. Cells were washed once in the flow cytometry buffer and fixed in 200 μL of 4% paraformaldehyde. Each experiment was repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA)

To assess the protein level of VEGF and IL-8 (CXCL-8), hADSCs were maintained for 12 h in EGM-2 basal medium without growth factors (Lonza, Allendale, NJ, USA). Culture supernatants were collected at varying time points and assayed in triplicate for VEGF and IL-8 using ELISA kits (R&D Systems) according to the manufacturer's instruction. Each experiment was repeated three times.

Murine hind limb ischemia model

BalB/cAnNCriBgi-nu nude male mice were obtained from Charles River Japan Inc. (Yokohama, Japan). All mice were 7–8 wks old (15–20 g) at the time of the study. Hind limb ischemia was induced by ligation and excision of the right femoral artery and vein under ketamine–xylazine anesthesia as previously described (Kang et al., 2009a). Animal care and experimental procedures were performed under the approval of the Animal Care Committees of Ewha Womans University Mokdong Hospital. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). For therapeutic angiogenesis studies, mice were divided into two groups after induction of ischemia for intramuscular injection into three separate regions from ankle up to thigh regions with culture medium (20 μL) or hADSC culture for 2 wks (10^6 cells/20 μL medium). Twelve mice were injected with hADSCs while 11 mice were treated with control culture medium. Serial indocyanine green (ICG) perfusion imagings were taken immediately after surgery (before injection of hADSC or medium) and on post-operative days (POD) 3, 7, 14, 21, and 28 as previously described (Kang et al., 2009a).

Near infrared fluorescence imaging

To measure tissue perfusion, we performed ICG perfusion imaging using the near infrared fluorescence imaging system (Vieworks Corp., Sungnam, Korea), as described previously (Kang et al., 2009a). For time-series ICG imaging, mice under ketamine–xylazine anesthesia were injected with an intravenous bolus injection of ICG (0.1 mL of 400 μmol/L; Sigma, St. Louis, MO, USA) into the tail vein. ICG fluorescence images were obtained for 12 min in 1-s intervals immediately after injection. Tissue perfusion rates in both legs were analyzed using a C^{++}-based analysis program according to a mathematical model to calculate the regional (pixel) perfusion rate and transferred into the perfusion map. The reproducibility of the ICG perfusion imaging method was confirmed by calculating intra-class correlation coefficient ($R = 0.94$, $p = 0.03$) and coefficient variation (8.2%). The probability of necrosis of the ischemic limbs was also estimated (Kang et al., 2009a). Briefly, regional perfusion rate of an ischemic limb was measured immediately after surgery, and necrotic regions in the ischemic limbs were assessed on POD 7. The region

number of a given limb was typically around 300–380. Necrosis probability of the ischemic tissue was plotted using the correlation between the regional perfusion rate (average perfusion rate of 2×2 pixels) and the necrosis of the corresponding region (determined at POD 7). Necrosis probabilities were calculated based on the data from 4200 regions of 12 hADSC-treated mice and about 3800 regions of 11 media-treated mice, respectively.

Histological analysis

Vessel density within the calf muscles of the ischemic limbs was quantified by histological analysis. Four weeks after surgery, the ischemic muscles were perfused with 4% (w/v) paraformaldehyde (Sigma) and embedded in paraffin. Calf muscle sections (10 μm thick) were stained with hematoxylin and eosin and an anti-mouse PECAM-1 antibody (Chemicon, Temecula, CA, USA). Proteins reactive with the anti-mouse PECAM antibody were stained with an anti-hamster IgG antibody conjugated with rhodamine (Jackson Laboratories, West Grove, PA, USA). The stained sections were examined by confocal microscopy (Axiovert LSM 510 META; Zeiss, Oberkochen, Germany). Calf muscle sections were also stained with an anti-human NF-κB antibody (Chemicon) or the anti-mouse PECAM antibody, and an anti-rabbit IgG antibody conjugated with FITC (Jackson Laboratories) as a secondary antibody. Finally, calf muscle sections were stained with anti-human myogenin (Lifespan Bioscience, Seattle, WA, USA) and an anti-rabbit IgG antibody conjugated with rhodamine (Jackson Laboratories). The injured area was evaluated semi-quantitatively on the basis of the percentage of parenchymal area round involved in five ×40 fields per H&E stained section ($n=5$).

Statistical analyses

Data are expressed as means ± SD except for a perfusion rate data (median ± interquartile range) because of non-normal distribution ($p=0.023$ by Shapiro–Wilk test). For analysis of the perfusion rate in the ischemic hind limbs, a Friedman two-way test was performed for daily increases. Mann–Whitney *U* test analyses and Wilcoxon matched pairs signed-rank tests were performed for inter or intra-group comparisons of perfusion rate. Analysis of variance (ANOVA) test was used to analyze the differences of concentration of VEGF and IL-8 between groups. Student's two-tailed *t*-test was used for the analysis of vessel and Myogenin-positive cell density. The difference between samples was considered statistically significant if the *p* value was less than 0.05.

Results

Phenotypic changes of hADSCs by long-term culture

To find out the optimal *in vitro* culture condition of hADSCs, we first investigated the morphological and biochemical changes induced by the long-term culture of hADSCs in EGM-2 medium. Cultured hADSCs maintained a fibroblast-like morphology up to 7 wks after *in vitro* culture (Fig. 1A). Expression of endothelial-specific markers such as VE-cadherin, Flt-1, KDR, and eNOS markedly increased by cultured hADSCs at 2 wks of *in vitro* culture, although the expression levels were lower compared to HUVECs. On the contrary, another endothelial-marker, vWF was constitutively expressed by primary hADSCs, and the expression was not changed by long-term *in vitro* culture (Fig. 1B). Besides surface cell markers, secretion of VEGF and IL-8 (CXCL-8) was significantly increased during the 7-wk culture period (ANOVA test $F_{6,14}=8.443$, $p=0.001$ and $F_{6,14}=3.239$, $p=0.042$, respectively, Fig. 1C). VEGF expression was significantly higher at 2 wks of culture (1063.3 ± 219.6 pg/10^6 cells) compared with the 1- and 3-wk values (143.0 ± 240.1 and 963.3 ± 120.45 pg/10^6 cells, respectively). IL-8 secretion increased slightly around 3 wks of culture (481.0 ± 352.5 pg/10^6 cells), and both VEGF and IL-8 decreased after 3 wks. Analysis of surface protein expression in the 2-wk-cultured cells revealed no significant increase in CD31 (PECAM-1) or vWF, factors known to be involved in angiogenesis (Fig. 1D). MSC markers,

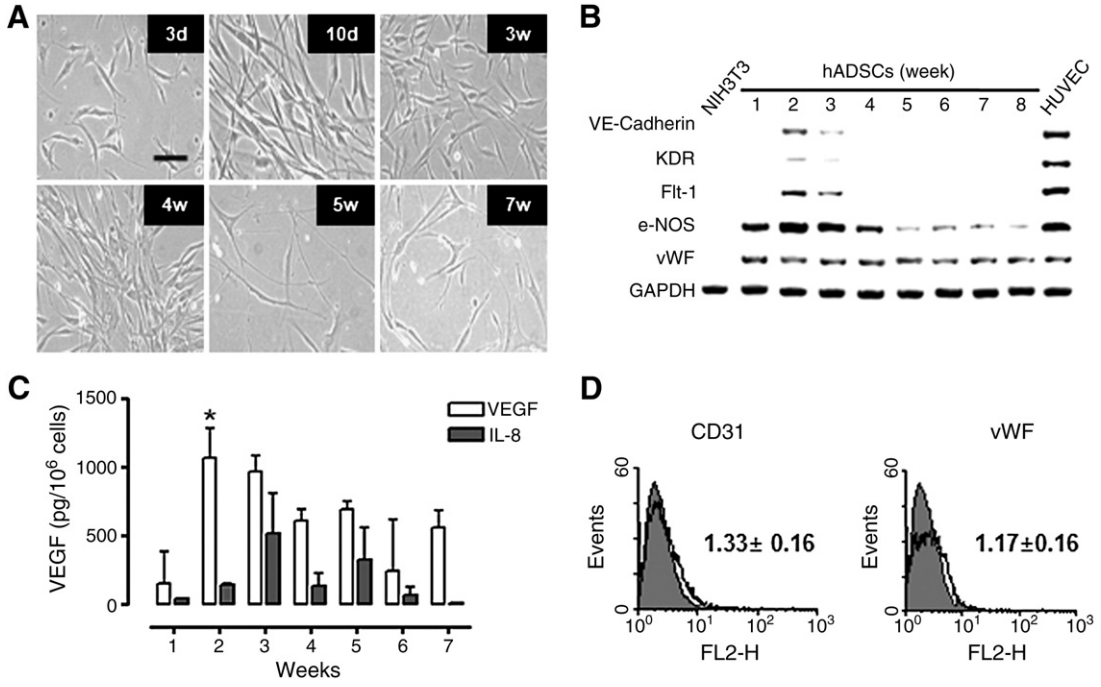

Fig. 1. Characterization of phenotypic changes of hADSCs by prolonged *in vitro* culture. (A) Changes in the morphology of cultured hADSCs. All images were obtained 3 d after seeding or subculture. Scale bar = 50 μm. (B) Differential expression pattern of endothelial cell-specific genes in the cultured hADSCs by RT-PCR. (C) ELISA analysis for secretions of VEGF and IL-8. Data represent mean ± SD. *$p=0.008$ vs. VEGF at 1-wk culture by Student's two-tailed *t*-test. (D) PECAM and vWF expression from hADSCs at 2 wks of culture. Representative histograms are demonstrated in black line, and the respective controls are shown as gray shaded areas. The geomean fluorescence intensity (mean ± SD) of positive signal is shown when the geomean value of each control was normalized as 1.

such as CD44 and CD105, were still highly expressed in 95.4% of the 1-wk-cultured cells and 84.2% of the 2-wk-cultured cells (see Supplementary data online, Fig. S1). These results indicate that long-term *in vitro* culture of unsorted hADSCs in EGM-2 medium induced expression of endothelial markers and cytokines associated angiogenesis at the maximal level around 2 to 3 wks after culture; while most of ADSCs retain the mesenchymal phenotype up to 2 wks after *in vitro* culture.

Protective effect of hADSCs in ischemic limbs

Since we have observed that 2-wk-*in vitro* culture of hADSCs induced an optimal expression of endothelial markers, we have utilized 2-wk-cultured unsorted hADSCs to determine the protective effect in ischemic injuries *in vivo*. To test the efficacy in ischemic tissue injury and repair, we measured the tissue perfusion rates of the ischemic limbs from POD 0 to 28 in groups treated with hADSCs and medium (Fig. 2A) using a NIR fluorescence imaging technique which can measure tissue perfusion quantitatively (Kang et al., 2009a). In the hADSC-treated limbs, the perfusion rates were significantly increased on POD 3 (median 195.3%/min; interquartile range, 82.0–321.1), 7 (median 191.6%/min; interquartile range, 128.8–408.7) and 14 (median 621.9%/min; interquartile range, 431.6–716.8) compared with POD 0 (median 47.1%/min; interquartile range, 18.0–58.7; $p = 0.03$ by Friedman two-way analysis). The perfusion rates of the medium-treated control group were also slightly increased (media 39.4%/min; interquartile range, 15.8–83.7 on POD 3 and median 118.3%/min; interquartile range, 70.7–171.9 on POD 7); however, the degree of perfusion improvement was significantly lower compared to hADSC-treated group. Concordantly, treatment with hADSCs markedly improved tissue perfusion and induced a favorable prognosis in the ischemic limbs compared to the control group even though the initial perfusion rates of the ischemic limbs were comparable (Figs. 2B and C).

Necrosis of the ischemic limbs usually occurred between POD 3 and 7, which coincide with the period when tissue perfusion significantly increased in the ADSCs-treated group. All but two of the ischemic limbs treated with medium showed complete amputation; while transplantation of hADSCs significantly reduced the incidence of autoamputation (Fig. 3A). To investigate the protective effect of hADSCs on tissue necrosis of ischemic limbs in detail, we analyzed the probabilities of necrosis in regions with the same perfusion rate, and compared between hADSC-treated group and media-treated group (Fig. 3B). In the hADSC-treated group, the necrosis probability was significantly lower for the corresponding regions of the control group with same initial perfusion rate.

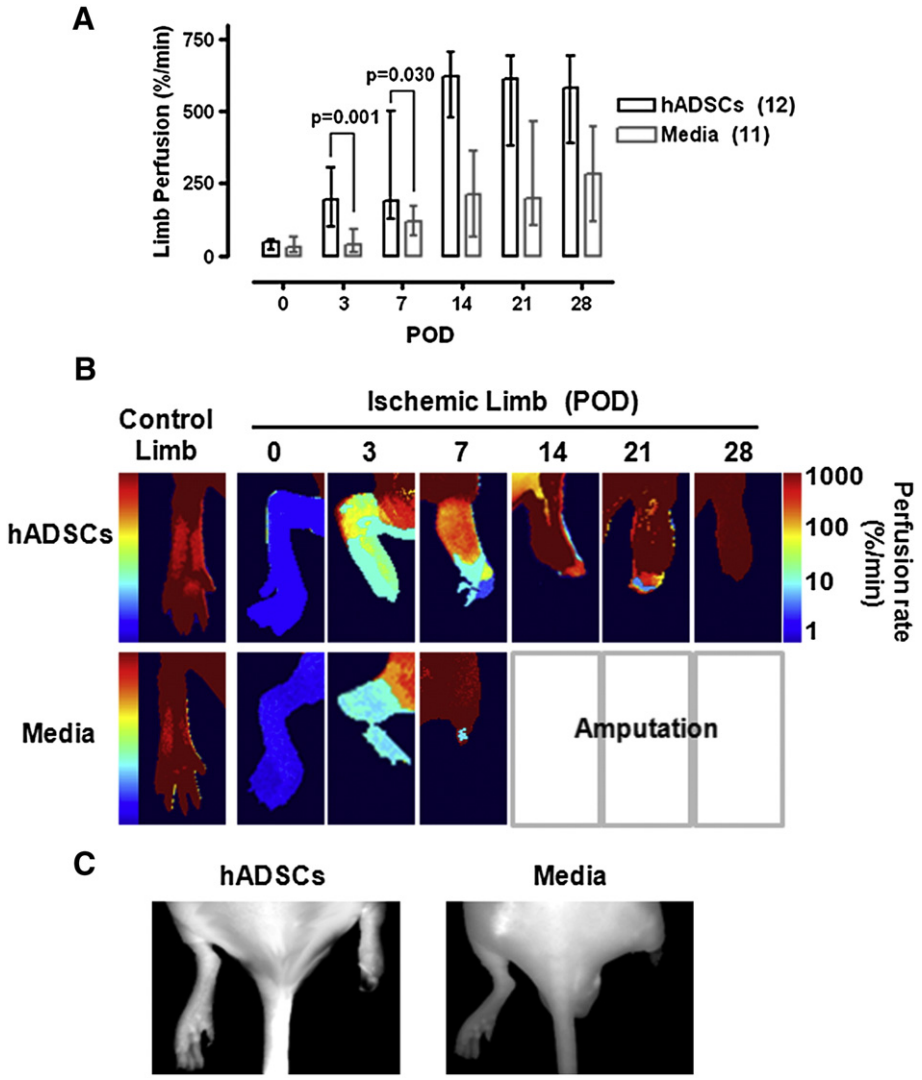

Fig. 2. Therapeutic effect of the unselected ADSCs on recovery of tissue perfusion. (A) Comparison of limb perfusion between the hADSC-transplanted and medium-treated groups. *p* values were estimated using the Mann–Whitney *U* test. (B) Perfusion maps of the ischemic hind limbs after surgery. Representative hADSC- and medium-treated ischemic hind limbs. (C) Representative picture of gross morphology on POD 28.

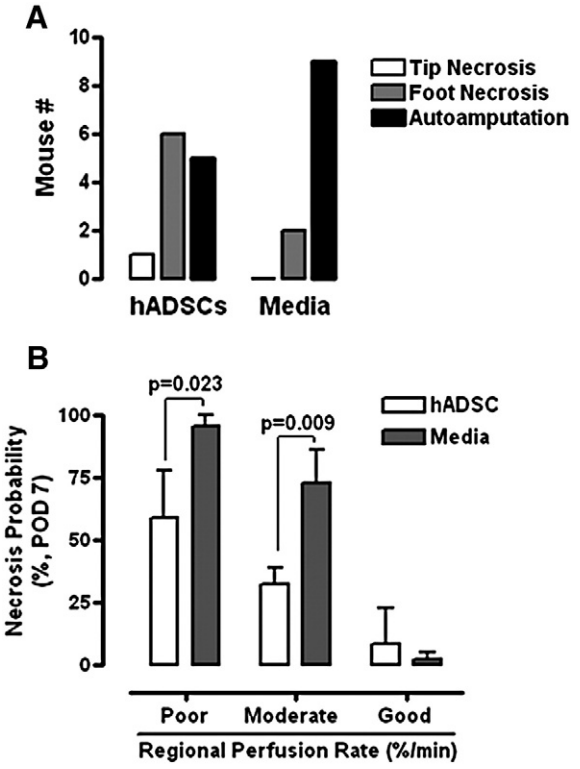

Fig. 3. Therapeutic effect of ADSCs on tissue necrosis of ischemic limbs. (A) Comparison of necrosis level in the ischemic hind limbs. Tip necrosis, toe necrosis; foot necrosis, limb necrosis lower than ankle; autoamputation, limb necrosis level upper than ankle. (B) Correlation between the probability of necrosis and regional perfusion rates. *X* axis was expressed as the perfusion rate of corresponding region: poor (lower than 15%/min), moderate (16–120%/min), and good (higher than 120%/min).

Transplantation of hADSCs promotes angiogenesis and myogenesis in ischemic hind limbs

The postmortem histological analysis performed 4 wks after surgery demonstrated that the number of PECAM-positive vessels increased significantly in the hADSC-transplanted group compared with the control group (Fig. 4A). Quantitative analysis showed a 2.2-fold increase in vessel density in the hADSC-treated group (Fig. 4B). To determine whether the transplanted cells directly constituted vascular structures, we performed immunohistochemistry using an anti-PECAM-1 antibody and an antibody specific for human NF-κB. Human NF-κB-positive cells form a vessel-like structure, positive for endothelial-marker PECAM-1 only in hADSC-transplanted ischemic tissues (Fig. 4C). The staining pattern of the mouse PECAM-1 and human NF-κB suggested that transplanted hADSCs might have been transformed into mature endothelial cells or might have formed fusion cells with preexisting mouse endothelial cells, because only several cells stained with human NF-κB antibody were observed.

To test the possibility of myogenic differentiation of transplanted hADSCs, we further examined calf muscle sections using an antibody specific for human myogenin. Interestingly, human myogenin-positive cells were only observed in the hADSCs treated mice (Figs. 5A and B); while we did not observe any human myogenin-positive cells in the control medium-treated group (Fig. 5C). Moreover, there were significant differences in muscle atrophy between the hADSC-transplanted and control groups at 4 wks after ischemia induction: the control group showed larger area of tissue necrosis around the ligated femoral vessels compared with the hADSC-transplanted group (atrophied area, %: 38.8 ± 16.1 vs. 16.7 ± 6.38, $p = 0.035$ by Student's two-tailed *t*-test, Fig. 5D).

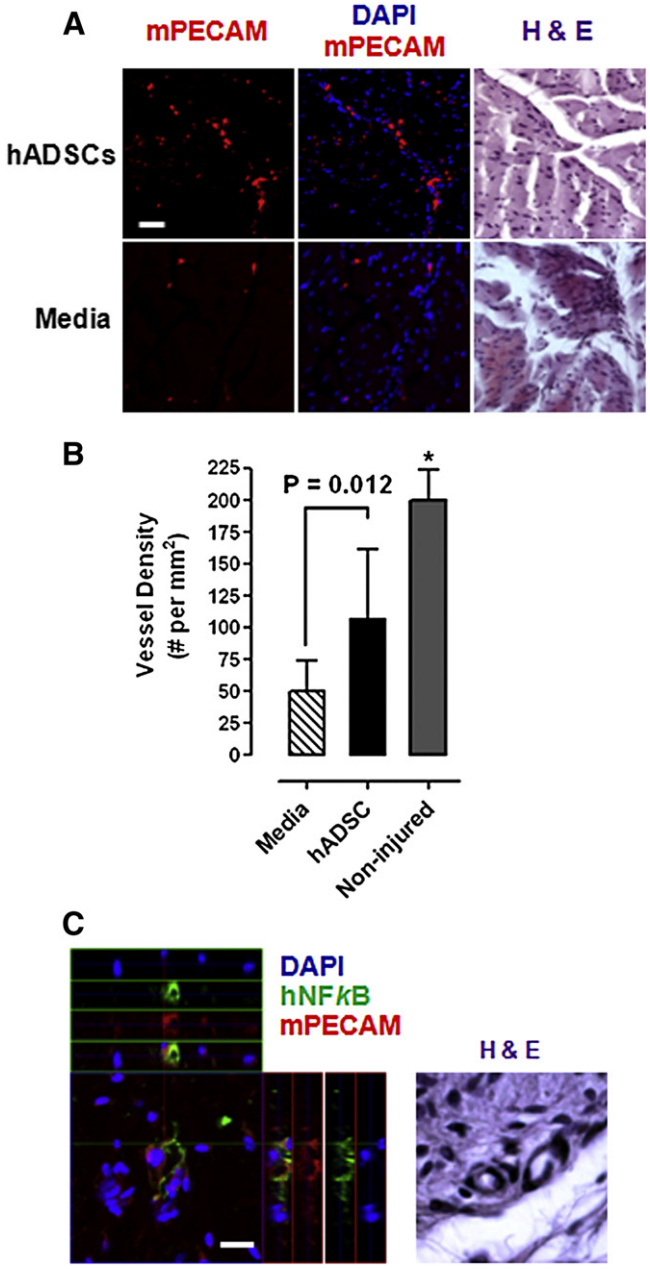

Fig. 4. Induced angiogenesis by transplanted hADSCs. (A) Confocal micrographs of calf muscle sections from ischemic limbs treated with hADSCs or medium. Scale bar = 100 μm. (B) Vascular density 4 wks after hADSCs transplantation. Data from 25 fields of the three mice of hADSC-treated group and media-treated group were averaged, respectively. $^{*}p = 0.001$ vs. hADSC and $p < 0.001$ vs. media control by Student's two-way *t*-test. (C) Z-stack images of the confocal micrograph of calf muscle sections from an hADSC-treated ischemic limb. Scale bar = 20 μm.

Discussion

In this study, we demonstrate that unsorted hADSCs with relatively short-term *in vitro* culture in endothelial-specific medium have sufficient efficacy for improvement of tissue repair after hypoxic insult in a mouse model of hind limb ischemia. We further demonstrated that the protective effect of hADSCs is potentially mediated by angiogenesis as well as myogenesis in the injured muscle tissues. To our knowledge, this is the first report to show myogenic potential of unselected hADSCs in ischemic injury-induced tissue regeneration even though the overall effect of myogenesis in tissue recovery is not fully determined.

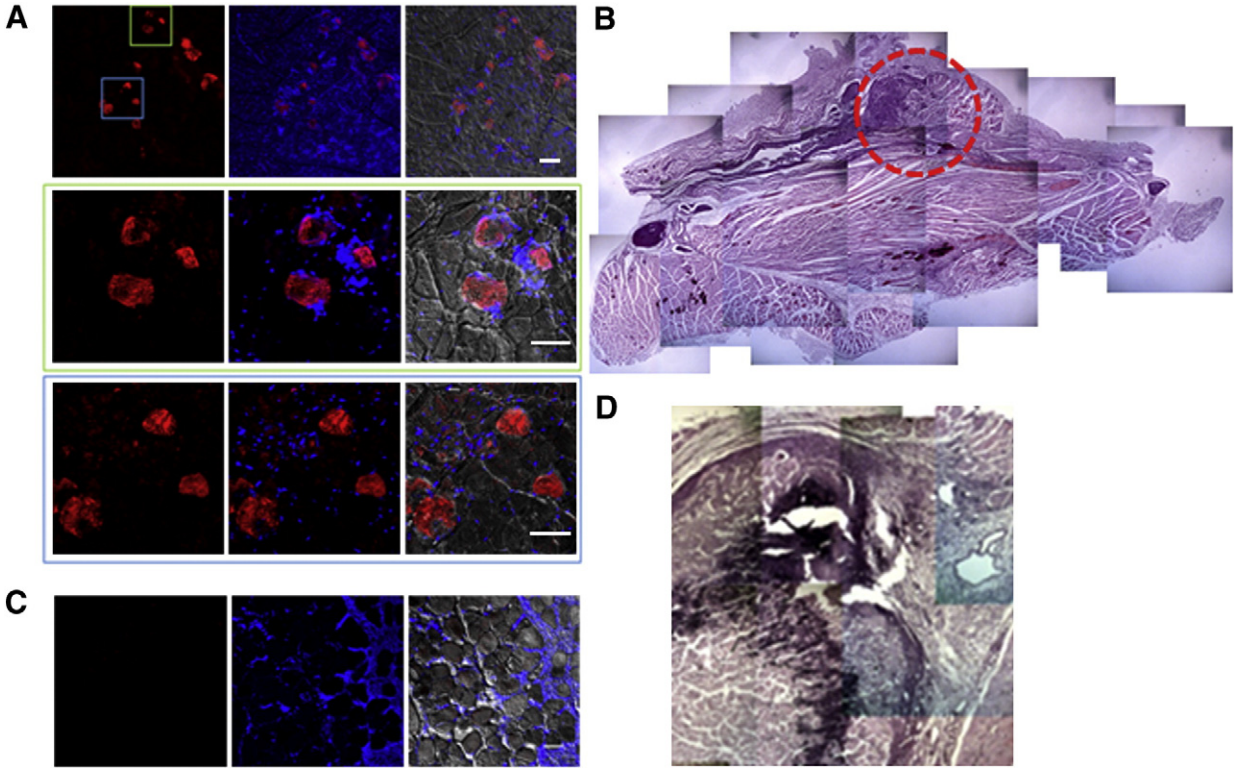

Fig. 5. Myogenic differentiation of hADSCs. (A) Confocal micrographs of calf muscle section from hADSC-transplanted ischemic limb. Upper panel, scale bar = 100 μm. Enlarged area, second, and third panel indicated by green and blue boxes in the upper panel, respectively. Scale bar = 50 μm. (B) Hematoxylin- and eosin-stained whole cross-section of the calf muscle of the same limb as A. The red circle indicates the region of A. (C) Confocal micrographs of the calf muscle sections from ischemic limbs treated with medium. (D) Hematoxylin- and eosin-stained calf muscle sections of representative medium-treated ischemic limb, magnified in and around the ligated vessel.

There are growing numbers of preclinical studies examining the potential of adipose-derived cells for improving ischemic repair. Majority of the preclinical studies has utilized cell sorting techniques to enrich the endothelial precursor cells from freshly isolated ADSCs (Cao et al., 2005; Moon et al., 2006). Since complex steps involving cell sorting might cause lower viability of stem cells, higher contamination rates and increasing cost, it will be favorable to achieve similar protective effect using unsorted and short-term cultured hADSCs from a clinical perspective. Therefore, our result along with similar previous reports on the promising effect of unsorted hADSCs have provided a rationale to test the clinical efficacy of short-term cultured hADSCs for therapeutic angiogenesis in ischemic diseases.

The unique aspects of the present study are several folds. As mentioned above, most of the other related preclinical studies utilized sorted hADSCs to promote therapeutic angiogenesis or myogenesis (Cao et al., 2005; Liu et al., 2007; Moon et al., 2006); while we have shown an equivalent efficacy of unsorted hADSCs by introducing short-term *in vitro* culture. Although similar protective effect of unsorted hADSCs was recently reported (Iwashima et al., 2009), the authors used nude rats for the ischemic hindlimb model and, thereby they did not measure the tissue necrosis as a final endpoint of the ischemic limbs. In the current study, we have measured tissue perfusion by a novel optical imaging technique and compared the correlation with final limb outcomes. Since we have utilized very sensitive ICG perfusion imaging, we could demonstrate a protective effect of hADSCs as early as 3 d after surgery; while most of the previous studies reported delayed protective effect confirmed by laser Doppler imaging (LDI) (Iwashima et al., 2009; Moon et al., 2006).

LDI technology has been widely used to measure the blood flow in the ischemic tissues (Cao et al., 2005; Moon et al., 2006; Nakagami et al., 2005). However, this method does not provide sufficient sensitivity to measure blood flow quantitatively, especially in the ischemic limbs with perfusion less than 50% of the normal rate (Kang et al., 2009a; Kang et al., 2009b). In the present study, we used a novel ICG perfusion imaging for the quantitative measurement of tissue perfusion from early time points after surgery. This novel method enabled us to exclude mice with the perfusion higher than 80%/min in the ischemic limbs immediately after surgery and to divide mice into the control and hADSC-treated group according to the initial perfusion rates, major determinants of tissue damage after surgery. We could detect a significant improvement in tissue perfusion in the hADSC-transplanted ischemic limbs as early as 3 d after surgery, indicating that early recovery of tissue perfusion might be needed to protect against permanent tissue damage after ischemia induction.

It has been reported that mesenchymal stem cells isolated from umbilical cord blood and bone marrow have the potential for skeletal myogenic differentiation *in vitro* (Gang et al., 2004; Shang et al., 2007). The *in vivo* potential of myogenic differentiation of MSCs has also been proposed in an ischemic heart model (Tomita et al., 1999). Likewise, CD105(+)/CD31(−)/flk-1(−) cells from human umbilical cord blood are able to differentiate *in vivo* toward the myogenic lineage and contribute to the muscle regenerative process (Conconi et al., 2006). Our data suggest a possibility that transplanted hADSCs could differentiate into myocytes in the ischemic muscle tissues even though the possibility of fusion of hADSCs with preexisting murine myocytes cannot be excluded at this point. Hypoxic condition of the ischemic tissue seems to act as a major stimulant toward the myogenic differentiation of transplanted hADSCs (Kook et al., 2008).

Even though cultured hADSCs were not able to induce tube formation in the Matrigel *in vitro* (see Supplementary data online, Fig. S2 and Supplementary methods), they may serve as good support for endothelial regeneration to form new vessels. The unselected hADSCs we used in the present study contained small populations of differentiated CD31(+) or vWF(+) cells and a large population of undifferentiated cells still positive for MSC surface markers, such as CD105 and CD44. The unselected hADSCs may have effects on

ischemic tissue recovery in the hind limb ischemic model because undifferentiated MSCs promote tissue repair by direct engraftment and secondary expression of various secretory proangiogenic growth factors such as VEGF especially under hypoxic conditions (Moon et al., 2006; Nakagami et al., 2005; Rehman et al., 2004). In the present study, most of the salvaged regions corresponded to the regions that received the cell injection, supporting the idea that only those regions directly receiving the cells benefit from the cells' presence. However, there were some protective effects on the distal parts (especially tip and toe areas) by hADSC treatment, indicating the possibility of either direct migration or paracrine-like activity of injected stem cells.

Although it is still premature to conclude the applicability of hADSCs, clinical trials of ASCs have begun to show early safety results and promising possibility of efficacy in patients with a range of diseases, including acute myocardial infarction, peripheral vascular disease, and soft and bony tissue defects including cranial bone loss, Crohn's-related fistula, and skin wounds (Hong et al., 2010). Among stem cell-based therapeutic modalities, use of hADSCs seems to be ultimately valuable in the clinical perspective due to their ready availability, pro-angiogenesis and anti-apoptotic factor secretion, immunomodulatory effects, and capacity for multi-lineage differentiation and ready expansion. In the present study, we have shown that unsorted hADSCs transplanted into ischemic tissue can become involved in the early recovery of tissue perfusion by introducing a short-term *in vitro* culture procedure. Collectively, based on our observation and previous reports, we propose that this short-term culture procedure might serve as a useful option for clinical application of hADSCs in various ischemic disorders.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mvr.2010.05.006.

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Glossary

EGM: endothelium growth medium
FITC: fluorescein isothiocyanate
hADSCs: human adipose tissue-derived stem cells
HUVEC: human umbilical vein endothelial cell
ICG: indocyanine green
MSCs: mesenchymal stem cells
PE: phycoerythrin
PECAM: platelet/endothelial cell adhesion molecule
VEGF: vascular endothelial growth factor