Hypoxic Preconditioning Results in Increased Motility and Improved Therapeutic Potential of Human Mesenchymal Stem Cells

Ivana Rosová[a], **Mo Dao**[a], **Ben Capoccia**[a], **Daniel Link**[a], and **Jan A. Nolta**[b]

[a]Division of Oncology, Hematopoietic Development and Malignancy Program, Washington University School of Medicine, St. Louis, Missouri, USA

[b]Department of Internal Medicine, Stem Cell Program, University of California, Davis, Sacramento, California, USA

Abstract

Mesenchymal stem cells (MSC) are adult multipotent cells found in bone marrow, adipose tissue, and other adult tissues. MSC have been shown to improve regeneration of injured tissues in vivo, but the mechanisms remain unclear. Typically, MSC are cultured under ambient, or normoxic, conditions (21% oxygen). However, the physiological niches for MSC in the bone marrow and other sites have much lower oxygen tension. When used as a therapeutic tool to repair tissue injuries, MSC cultured in standard conditions must adapt from 21% oxygen in culture to less than 1% oxygen in the ischemic tissue. We therefore examined the effects of preculturing human bone marrow-derived MSC in hypoxic conditions (1%–3% oxygen) to elucidate the best conditions that enhance their tissue regenerative potential. We demonstrated that MSC cultured in hypoxia activate the Akt signaling pathway while maintaining their viability and cell cycle rates. We also showed that MSC cultured in hypoxia induced expression of cMet, the major receptor for hepatocyte growth factor (HGF), and enhanced cMet signaling. MSC cultured in hypoxic conditions increased their migration rates. Since migration and HGF responsiveness are thought to be key mediators of MSC recruitment and/or activation in vivo, we next examined the tissue regenerative potential of MSC cultured under hypoxic conditions, using a murine hind limb ischemia model. We showed that local expression of HGF is increased in ischemic muscle in this model. Intra-arterial injection of MSC cultured in either normoxic or hypoxic conditions 24 hours after surgical induction of hind limb ischemia enhanced revascularization compared with saline controls. However, restoration of blood flow was observed significantly earlier in mice that had been injected with hypoxic preconditioned MSC. Collectively, these data suggest that preculturing MSC under hypoxic conditions prior to transplantation improves their tissue regenerative potential.

Correspondence: Jan A. Nolta, Ph.D., Stem Cell Program, University of California at Davis, Room 653, 2425 Stockton Boulevard, Sacramento, California 95817, USA. Telephone: 916-453-2335; Fax: 916-453-2173; Jan.nolta@ucdmc.ucdavis.edu.

Keywords

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Introduction

Mesenchymal stem cells (MSC) are adult pluripotent cells isolated from bone marrow and other adult tissues, capable of giving rise to adipogenic, osteogenic, and chondrogenic lineages [1]. Transplantation of bone marrow-derived mesenchymal stem cells has proven to be an effective treatment for tissue injuries, such as cardiac infarction and hind limb ischemia [2–7]. The homing mechanisms of MSC to the site of injury after transplantation are just beginning to be defined [8], and methods to improve the targeted migration from the bloodstream to the site of injury after i.v. injection can now be developed in a rational manner.

The expression of hepatocyte growth factor (HGF) and the expression of its receptor cMet have been previously described in MSC, and the ability of MSC to migrate toward the HGF gradient has been characterized [9,10]. Since HGF was shown to be activated at the sites of ischemic injury [11], we hypothesized that HGF is one of the signals that recruit MSC to the damaged tissue. Once localized to the ischemic tissue, MSC encounter severe hypoxic conditions, ranging from 0.4% to 2.3% O_2 [12], which often result in apoptosis. Hypoxia-induced apoptosis can be circumvented by preconditioning cells in less severe hypoxic conditions (1%–3% O_2) for a period of time before exposing them to the severe ischemia at the site of injury in other cell types [13]. In our report, we test whether hypoxic preconditioning prior to transplantation improves the tissue-regenerating ability of MSC. The culture in hypoxic conditions (1%–3% O_2) may also be beneficial for the MSC, as this oxygen tension is more similar to the physiologic niche for MSC in the bone marrow (2%–7% O_2). Previous reports have observed that in addition to maintaining their viability when cultured in 2%–5% O_2, MSC also increase their proliferation rate after an initial lag phase [14–17]. There is a discrepancy in the field, however, about how hypoxia affects the self-renewal and differentiation potential of MSC. A number of studies have found MSC cultured in hypoxic conditions to be able to differentiate more and to have better self-renewal [14,18], whereas some have observed a decreased differentiation potential and decreased self-renewal [16,19,20]. In this study, we examine the effects of HGF and hypoxia on MSC migration and tissue regenerative potential.

Materials and Methods

Cell Culture

Bone marrow-derived mesenchymal stem cells (BMSC) were cultured from human donor bone marrow aspirates, as we have previously described [21,22]. All studies were done in accordance with university regulatory committees. Aspirates were filtered through 70-μm filters (ref. 352350; BD Biosciences, San Jose, CA, http://www.bdbiosciences.com/home), and the filters were subsequently flushed with MSC medium (Iscove's Modified Dulbecco's Medium, 15% fetal bovine serum, 15% horse serum, 10^{-6} M hydrocortisone, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine) to recover bony spicules. BMSC were left to adhere for 24–48 hours, and then the nonadherent fraction was removed. Cultures were kept under 80% confluence. All experiments were done with cells passaged 3–7 times. Cells released by cell dissociation buffer (Gibco, Grand Island, NY, http://www.invitrogen.com) were phenotyped using a Coulter FC500 flow cytometer (Beckman Coulter, Hialeah, FL, http://www.beckmancoulter.com) and monoclonal antibodies. Antibodies for CD34, CD45,

CD90, and CD73 were purchased from BD Pharmingen (catalog nos. 55824, 34796, 555595, and 550257, respectively; BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml, and the antibody for CD105 was purchased from R&D Systems Inc. (catalog no. 10971A; R&D Systems, Minneapolis, http://www.rndsystems.com). In vitro differentiation assays were performed as per the manufacturer's instructions (BioWhittaker; Cambrex, Walkersville, MD, http://www.cambrex.com).

Hypoxia

The hypoxic condition was generated using a hypoxia chamber (catalog no. 27310; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) according to the manufacturer's instructions. Briefly, the cultures were enclosed in the chamber and flushed with a mixture of gasses (95% N_2 and 5% CO_2) for 3 minutes. At the end of the flushing period, the chamber was closed to prevent free flow of exogenous air into the chamber. The final level of hypoxia was 1%–3%, as specified by the manufacturer.

Scratch Test Assay

BMSC were grown to 70%–80% confluence in tissue culture-treated plates, as we have described [21,22]. The plates were scratched with a sterile cell scraper (catalog no. 08-773-2; Fisher Scientific International, Hampton, NH, http://www.fisherscientific.com) to generate a 5-mm-wide area free of cells. The scratch border was marked with a fine black line immediately after the scraping. BMSC cultures were then incubated at 37°C, 5% CO_2, in hypoxic or normoxic conditions in the presence or absence of 25 ng/ml HGF (catalog no. 294-HGN; R&D Systems) for 24 hours. The migration of cells was assessed as a function of how far from the scratch line the cells had progressed, and the overall number of cells migrating, over the 24-hour period. Alternatively, the cells were fixed with ice-cold methanol for 5 minutes, stained with Harris hematoxylin (Sigma catalog no. HHS32) for 15 minutes, washed with water, and allowed to air dry. Pictures were taken with a Carl Zeiss camera (AxioCam MRc5; Carl Zeiss, Jena, Germany, http://www.zeiss.com), and the number of nuclei that had migrated over the scratch line was counted using the Bioquant Osteo program (Bioquant Image Analysis Corporation, Nashville, TN, http://www.bioquant.com).

Apoptosis Assay

Apoptosis assays were performed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis antibody (catalog no. 556419; BD Pharmingen) according to the manufacturer's instructions. Briefly, cells were collected and resuspended in binding buffer. Annexin V-FITC and propidium iodide were added, and the reaction was incubated in the dark for 15 minutes. Cells were analyzed by flow cytometry using a FACScan flow cytometer.

Cell Cycle Assay

Cells were collected and fixed with ice-cold 90% ethanol, added dropwise while vortexing. The fixation reaction was allowed to proceed for 1–24 hours while the cells were kept at 4°C. Cells were then collected by centrifugation and resuspended in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 20 μg/ml RNase and incubated for 30 minutes at 37°C. Propidium iodide at a final concentration of 50 μg/ml was added, and cells were analyzed by flow cytometry.

Western Blot Analysis

After washing with cold PBS, lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Hepes, 1 mM Na_3VO_4, and protease inhibitor cocktail [Complete Mini; catalog no. 11836153001;

Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com]) was applied directly to MSC on tissue culture plates. For immunoprecipitation, the protein lysates were incubated with a cMet antibody (sc-10; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) at 4°C for 3 hours and then incubated with protein G-agarose beads (catalog no. 1243233; Roche Diagnostics). Western blotting was performed as previously described [23]. Protein bands were quantified using ImageJ software (Rasband, WS, ImageJ; National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij). The following antibodies were used in this study: phospho-Akt (Ser-473; catalog no. 9271; Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com), Akt (catalog no. sc-8312; Santa Cruz Biotechnology), phospho-p44/p42 mitogen-activated protein kinase (MAPK) (catalog no. 9106; Cell Signaling Technology), Erk2 (catalog no. sc-154; Santa Cruz Biotechnology), phospho-p38 (catalog no. sc-7973; Santa Cruz Biotechnology), p38 (catalog no. sc-535; Santa Cruz Biotechnology), phospho-Jun N-terminal Kinase (JNK) (catalog no. 9255; Cell Signaling Technology), JNK (catalog no. sc-474; Santa Cruz Biotechnology), α-tubulin (catalog no. sc-5286; Santa Cruz Biotechnology), and phosphotyrosine/4G10 (catalog no. 05-321; Upstate, Charlottesville, VA, http://www.upstate.com).

Enzyme-Linked Immunosorbent Assay

Abductor muscles were harvested and homogenized using tissue homogenization buffer (1 M Tris-HCl, pH 7.4, 1 M EDTA, pH 7.4, with the protease inhibitor cocktail Complete Mini). The homogenates were centrifuged, and the supernatants were analyzed using the rat HGF enzyme-linked immunosorbent assay (ELISA) kit (B-Bridge International, Mountain View, CA, http://eng.b-bridge.com).

Animal Surgery and MSC Injection

All animal procedures were approved by the Animal Studies Committee at Washington University in St. Louis. Under anesthesia, NOD/SCID/β-2-microglobulin-deficient mice or NOD/SCID mice were subjected to unilateral hind limb ischemia surgeries as described [24]. The mice were shaved and prepped, then the right femoral artery and vein were exposed and dissected from the femoral nerve, and the proximal portion of the femoral artery and vein were ligated with 6-0 silk sutures. The distal portion of the saphenous artery and vein and the remaining collateral arterial and venous side branches were ligated and completely excised from the hind limb. The overlying skin was closed using Nexaband veterinary glue (Abbott Animal Health, Abbott Park, IL, http://www.abbottanimalhealth.com). MSC (5×10^5 cells per mouse) were injected into the left ventricle 1 day after surgery, as described [25,26].

Laser Doppler Perfusion Imaging

Blood perfusion was monitored by laser Doppler imaging (MoorLDI-2; Moor Instruments, Devon, U.K., http://www.moor.co.uk). Animals were anesthetized and placed on a heating block at 37°C before scanning to minimize temperature variations. The laser Doppler images were analyzed by averaging the perfusion, expressed as the relative unit of flux over the surface of both ischemic and nonischemic foot, as determined by the Doppler imaging. The final data were expressed as the flux ratio of ischemic and nonischemic foot. This provides a healthy contralateral positive control in each image, to minimize light and temperature variations.

Results

Bone Marrow Mesenchymal Stem Cell Characterization

Bone marrow MSC were isolated from human bone marrow aspirates by adhesion to tissue culture-coated plates in complete medium as previously described [21,22]. By the third passage, a homogeneous population of fibroblast-like cells was obtained (data not shown). To prevent hematopoietic cell contamination, which might be present in earlier passages, or the presence of senescent or differentiating MSC in later passages, we used cells between passages 3 and 7 throughout the study. Characterization by flow cytometric analysis confirmed the absence of CD45- and CD34-positive cells in the culture. In addition, MSC expressed CD105 (96.005% ± 1.42%), CD 90 (95.09% ± 2.41%), and CD73 (90.62% ± 3.4%) (Fig. 1A, 1B). Values are reported as mean ± SD. This marker profile is consistent with previous reports [21,22,27,28]. MSC, plated at 5×10^3 to 6×10^3 cells per cm^2, had a population doubling time of 40 hours (data not shown) and were able to differentiate into the adipogenic, osteogenic, and chondrogenic lineages in vitro (Fig. 1C–1E).

Hypoxia and HGF Treatment Cause Akt Activation

The PI3K/Akt signaling pathway has been shown to be an important effector pathway when cells are presented with either HGF stimulation or hypoxic conditions, whereas inhibiting this pathway leads to abrogation of cells' improved migratory ability and their angiogenic factor secretion [10,29,30]. AKT activation is also associated with overcoming anoikis, death by integrin detachment, in adhesion-dependent cells such as MSC [31–34]. To determine whether Akt is activated in MSC at reduced oxygen and/or HGF stimulation, cells were cultured for 16 hours in 1%–3% oxygen (hereafter defined as hypoxic conditions) or ambient oxygen (21%) with or without 25 ng/ml HGF. Activation of Akt, as measured by phosphorylation on Ser-473, was increased in MSC treated with hypoxia, HGF, or the combination of the two factors (Fig. 2A, 2B); no change in total Akt was observed in any of the conditions. These data suggest that preincubation of MSC in hypoxic conditions and in the presence of HGF can maintain the cells at an elevated level of signaling through the Akt pathway, possibly enabling downstream effects of these stimuli, such as improved migratory ability and activation of prosurvival factors, even when detached from the plate for injection.

MSC Viability Is Maintained Under Hypoxic Conditions

Since previous reports suggested that prolonged hypoxia and serum deprivation can lead to an increase in MSC apoptosis [35], we next examined cell viability in our different culture conditions. Cell apoptosis was assessed using a standard flow cytometry-based Annexin V/ propidium iodide apoptosis assay. In this assay, the cells undergoing apoptosis are Annexin V-positive and propidium iodide-negative; propidium iodide staining designates dead cells, and conversely, a lack of Annexin V staining and propidium iodide uptake is observed on live cells. After culture for 16 hours in serum-free hypoxic conditions, the percentage of viable (Annexin V$^-$ and propidium iodide$^-$) cells was comparable to that of MSC cultured under normoxic conditions (Fig. 2C, 2D). The addition of HGF to the culture had no effect on cell viability. These data show that cell viability is maintained after culture for 16 hours in serum-free hypoxic conditions.

HGF Stimulation Activates Erk Signaling Pathway but Not JNK and p38 Pathways

In addition to the Akt pathway, both HGF and hypoxia are known to trigger a complex signaling cascade through multiple other pathways. In particular, MAPK have been implicated to be essential for migratory response to HGF stimulation in cortical neurons and kidney epithelial cells [36,37]. To investigate whether any of the MAPK, Erk, p38, or JNK are activated after a treatment with HGF or hypoxia, we incubated MSC for 16 hours in

hypoxic or normoxic conditions, with and without addition of HGF. Western blot analyses revealed that levels of JNK and p38 remained unchanged under these different culture conditions, as did their phosphorylation states (Fig. 3A–3C), suggesting that there is no persistent signaling through these pathways after HGF stimulation for 16 hours. Erk phosphorylation levels, however, were observed to be increased when MSC were cultured with HGF, in both hypoxic and normoxic conditions. Long-term hypoxia did not cause Erk activation on its own, as the level of phosphorylated Erk in these MSC was equivalent to that of cells cultured in normoxia. These data demonstrate that in response to HGF, MSC can maintain elevated levels of signaling through the Erk pathway over a relatively long (16-hour) stimulation period, a condition that might be encountered in certain in vivo situations, such as transplantation of MSC into an ischemic tissue.

Hypoxia and HGF Do Not Alter MSC Proliferation

Proliferation is one of the cellular processes regulated by the Erk signaling pathway. Since we demonstrated that Erk is activated in MSC after a 16-hour stimulation with HGF, we further investigated whether HGF or hypoxia would alter the proliferation status of MSC. Similar to previous experiments, cells were cultured in hypoxic or normoxic conditions, with or without HGF, and analyzed for cell cycle status. A similar percentage of cells in the $S/G_2/M$ phases of the cell cycle was observed in all culture conditions. (Fig. 4A, 4B). Thus, despite Erk activation, no increase in cell proliferation was observed in HGF-treated MSC. Exposure to hypoxia for 16 hours had no effect on MSC proliferation.

cMet Expression and Signaling Are Increased in MSC Cultured in Hypoxic Conditions

Having determined that MSC can respond to HGF and hypoxia through Akt and Erk signaling pathways, we further examined the effects of these stimuli on the expression of the HGF receptor cMet. cMet is a receptor tyrosine kinase composed of two subunits, a 45-kDa α-subunit and a 145-kDa β-subunit, both of which are products of a proteolytic cleavage of a 170-kDa precursor protein. Consistent with previous reports, Western blot analysis showed that both the precursor and mature forms of cMet are expressed in MSC cultured in normoxic conditions (Fig. 5A, 5B). Interestingly, expression of the precursor and mature forms of cMet was increased in MSC cultured in hypoxic conditions for 16 hours.

To determine whether the hypoxia-induced cMet upregulation resulted in an increased level of cMet activation upon HGF treatment, the level of cMet phosphorylation was examined after a short-term HGF stimulation of MSC precultured in hypoxic or normoxic conditions for 24 hours. As expected, HGF stimulation of MSC cultured in normoxic conditions resulted in the phosphorylation of cMet with maximal activation at 15 minutes (Fig. 5C, 5D). The cells cultured in hypoxic conditions had a higher level of cMet phosphorylation than those cultured in normoxic conditions after a 15-minute HGF treatment. A 30-minute HGF stimulation of MSC resulted in a decrease or maintenance of cMet phosphorylation levels in hypoxic- and normoxic-preconditioned MSC, respectively, compared with the 15-minute time point (Fig. 4C, 4D). The cMet receptor activation in hypoxic MSC at the 30-minute time point, however, was equivalent to its activation in normoxic MSC after HGF stimulation, suggesting that hypoxic preconditioned cells responded more robustly to the HGF stimulation at an early time point, after which this response is downregulated, yet still maintained at a level comparable to that of normoxic MSC. Together, these data suggest that hypoxic conditioning of MSC results in enhanced early responsiveness to HGF.

Hypoxia and HGF Promote MSC Migration

HGF, also known as "scatter factor," has been shown to induce MSC migration [8,9]. Since our previous experiments had demonstrated that the HGF receptor cMet is upregulated on MSC in hypoxic conditions, we further investigated whether hypoxia increased HGF-

induced MSC migration. Confluent plates of MSC were denuded in a straight line with a cell scraper to create an artificial wound, using the standard "scratch test" technique [38]. The cultures were then incubated in hypoxic or normoxic conditions, with or without HGF for 24 hours. Plates were imaged by phase-contrast microscopy at the 24 hour endpoint, to assess the degree of cell migration from the original line (Fig. 6A). The quantitation of the migrated cells revealed that cells did not migrate when cultured in the "standard" conditions, ambient oxygen and minimal medium. A significant increase in the migratory capacity of MSC was observed when cells were stimulated with hypoxia alone (Fig. 6B). Addition of HGF induced MSC migration (Fig. 6B), and this increase was independent of culture in hypoxic versus normoxic conditions. These data suggest that HGF and hypoxia act as independent stimuli to increase MSC migration.

Hind Limb Ischemia Injury Induces Local Secretion of HGF

HGF has previously been reported to be secreted at sites of ischemic injury [11]. During regeneration, expression of HGF from the damaged tissue is upregulated and is highest at 48 hours [39,40]. The role of HGF in repair has been well documented in the liver [41,42] heart [11,43], and muscle [44]. To confirm that HGF is one of the factors activated after hind limb ischemia injury, we analyzed muscle lysates from NOD/SCID mice that were treated with either sham surgery or hind limb ischemia surgery. Abductor muscles were harvested at 6, 12, 24, or 48 hours postsurgery, and muscle lysates were analyzed by ELISA for the presence of HGF. The results show a slight increase in HGF levels in the muscles of sham-operated animals starting at 12 hours postsurgery (Fig. 6C); however, the tissue ischemia caused a significant increase in the HGF levels in the injured muscle, which steadily increased up to 48 hours after surgery (two-way analysis of variance, $p < .001$; Fig. 6C). These results indicate that hind limb ischemia is a good model by which to test the therapeutic potential of MSC transplantation, the contribution of HGF to the injury repair process, and the HGF-mediated recruitment of MSC to sites of hypoxic damage and tissue ischemia.

Hypoxia-Pretreated MSC Accelerate Restoration of Blood Flow After Surgical Induction of Hind Limb Ischemia

In the previous in vitro experiments, we demonstrated that culture in hypoxic conditions elevated the levels of the cMet receptor, activated a prosurvival Akt signaling pathway in MSC, and increased their migratory potential. We further tested the hypothesis that hypoxic pretreatment of MSC would be beneficial, by preparing the cells to better migrate to the site of ischemic injury and to repair the damaged tissue in the hind limb ischemia injury model. Bone marrow-derived MSC cultured in normoxic or hypoxic conditions were injected into the left ventricles of NOD/SCID β-2-microglobulin$^{-/-}$ mice (5×10^5 cells per mouse) 24 hours after surgical induction of hind limb ischemia and compared with saline-injected controls, as described [25,26]. Laser Doppler perfusion imaging showed that mice transplanted with MSC recovered faster and to a higher degree compared with saline controls (Fig. 6D). Of note, although blood flow recovery was similar by day 14 after surgery, mice injected with hypoxic MSC displayed a significantly earlier (day 5) recovery in blood flow. There was a significantly better improvement in blood perfusion in the experimental group transplanted with normoxic MSC, as well as hypoxic MSC, by the time points from days 8–15. The hypoxic preconditioning caused a statistically better improvement at the early, day 5 time point. These data demonstrate that human bone marrow-derived MSC are an efficient therapeutic tool to enhance recovery in a hind limb ischemia injury and that hypoxia-preconditioned MSC promoted an earlier recovery from ischemic injury.

Discussion

Consistent with other reports [8–10], in current study we confirm that HGF acts as a motogenic factor for MSC, increasing their migration potential, whereas the cells exposed to hypoxia alone demonstrate an even higher migratory ability, suggesting that high levels of HGF might also act as a retention signal to maintain MSC at the site of tissue injury. Once they have migrated to the site of injury, the cells encounter elevated HGF levels and severe hypoxia. Our in vitro experiments show that even after a relatively long-term exposure to hypoxia and HGF, MSC are able to maintain Akt and Erk signaling, which regulate important cellular functions such as survival, migration, and proliferation. In this study, we demonstrated that MSC cultured with HGF in hypoxic conditions maintain their viability and proliferation status. To summarize, this is, to our knowledge, the first report showing that improvement of MSC tissue regeneration potential by hypoxic preconditioning might be also due to the HGF-cMet signaling pathway.

The current studies suggest that pretreatment of MSC under hypoxic conditions not only increases cMet, which could enhance HGF-mediated chemotactic recruitment to sites of tissue damage, but might also enhance the survival of these stem cells upon arrival at the damaged site, through increasing the levels of phosphorylation of the prosurvival protein AKT. Phosphorylation of Akt on Ser-473 stimulates its activity and plays a major role in the suppression of anoikis [47], or "death by detachment." This is especially important for MSC, which begin to undergo apoptosis within hours after their integrin detachment from the substrate. AKT has been used to modify MSC for injection to circumvent this problem. Gnecchi et al. showed that, in a mouse model, conditioned medium from AKT-transduced MSC had the same protective effect as the injected cells in acute myocardial infarction [48]. All improvements were initiated during the first 72 hours postinfarct; later injection of medium or cells had no effect. These data suggest that a paracrine effect from the MSC is more important than direct differentiation or fusion. Our data show that preculture of the cells in hypoxia prior to injection naturally increases AKT activity, without artificial overexpression, which could be tumorigenic. This effect of hypoxic preconditioning was likely an important factor in causing improved survival and retention of the cells in the damaged tissues and faster functional improvement.

The effects of reduced oxygen tension have previously been studied to understand how cells react to a hypoxic environment in injured ischemic tissues or to understand the cellular processes in their natural niches, which have a much lower oxygen tension than the 21% O_2 used in tissue culture systems. In this report, we show that culturing MSC in hypoxic conditions improves their tissue regenerative potential in the mouse model of hind limb ischemia, whereas this effect might be caused by several factors that are altered by hypoxic culture conditions. We and others have demonstrated that MSC have different characteristics when cultured in hypoxic environment (2%–5% O_2), and in particular the effects of hypoxia on MSC survival, proliferation, and stem cell potential have been noted. Although one study has found that hypoxic conditioning and serum deprivation cause apoptosis in rat bone marrow-derived MSC [35], the consensus in the field is that either short- or long-term hypoxic culture does not adversely affect MSC survival, consistent with our results [14–17]. In fact, after the MSC overcome a lag phase in proliferation upon the initiation of hypoxic culture conditions, they have been shown to proliferate faster than MSC cultured at 21% O_2 [15]. Although it is likely that severe ischemia (<0.5% O_2) present at the site of injury might have a negative effect on MSC survival and proliferation, a hypoxic preconditioning regiment such as that used in the current study might enable the cells to resist the apoptotic stimuli, possibly through increased Akt prosurvival signaling, since conditioning is too short to negatively affect the cells' proliferation status. It is also not known whether enhanced levels of cell division are actually beneficial in MSC culture or are causing cells to

differentiate, as has been shown for the hematopoietic stem and progenitor cells, which remain more primitive in the nonproliferating state.

The expression of cMet and HGF has previously been observed to be increased in a tumor setting, which is naturally ischemic, and it has been demonstrated to correlate with tumor angiogenesis [49–54]. Furthermore, MSC have been shown to express cMet and to migrate toward HGF [9,10]. In this study we hypothesized that HGF and its receptor cMet are important factors in directing the ability of MSC to repair the ischemic tissue. We demonstrated that hypoxia caused an increase in cMet levels on MSC, and thus preconditioned MSC were then able to respond more robustly to HGF, although elevated levels of HGF are present at the site of ischemic muscle. Because MSC are known to express other growth factor receptors that regulate directional MSC migration, it is unlikely that HGF-cMet axis is the only pathway sufficient for MSC homing to the site of injury. Therefore, additional in vivo studies are being performed to understand the importance of HGF and cMet signaling in MSC-mediated repair of hind limb ischemia.

MSC and their therapeutic capabilities have previously been explored in the literature. Even though MSC have been shown to differentiate into adipogenic, chondrogenic, and osteogenic lineages in vitro, in addition to smooth muscle, endothelial, or myocardial differentiation in vivo, the leading theory in the field is that MSC mediate tissue repair through their secretion of angiogenic factors. There is some evidence suggesting that hypoxia might enhance the secretion of angiogenic factors from MSC, thus revealing another possible mechanism by which the hypoxic preconditioning enhances the tissue repair potential of MSC. It has been demonstrated that MSC secrete multiple proangiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, HGF, and placental growth factor in response to hypoxic stimulus [2,17,55–57]. In a recent study, a conditioned medium from hypoxic MSC prevented endothelial cell apoptosis and enhanced tube formation in vitro [57]. In addition, when ischemic muscles were treated with conditioned medium harvested from MSC cultured in hypoxic conditions, the experimental animals recovered faster than the control groups, suggesting that the release of angiogenic factors by MSC is sufficient to enhance the revascularization of the injured tissue [55,56]. In all studies, however, the conditioned media or MSC were injected directly into the injured muscle, which eliminated the necessity of MSC to home to the site of injury. In this report, we show that hypoxic MSC are able to enhance the repair of the ischemic muscle even if injected into the arterial system of the mouse, suggesting that these cells enhance the blood flow recovery either through secreting angiogenic factors into the bloodstream or through more efficient homing and subsequent tissue repair compared with normoxic MSC.

We addressed the question of whether hypoxia enhances the migratory/homing potential of MSC by an in vitro scratch test assay, observing a significant increase in MSC migration. In addition, a previously published study demonstrated a mobilization of rat MSC into the peripheral blood under chronically hypoxic conditions [58], confirming that hypoxia acts as a potent motogenic factor for MSC in vivo. Since ischemic injury triggers a secretion of various cytokines, including HGF, SDF-1, MCP-1, and VEGF, it is interesting to examine whether hypoxia can also enhance a directional migration toward a growth factor gradient. Indeed, a few published reports have demonstrated that hypoxia increases the migration of MSC toward various growth factors, such as VEGF, SDF-1, and CX3CR1 in vitro, whereas this process is dependent on Hif1α-mediated elevation of receptors for particular growth factors [16,59]. The data that we present in this study show that although hypoxia increases the levels of the HGF receptor cMet on MSC, the long-term exposure to HGF and hypoxia actually decreases the migratory ability of MSC compared with hypoxia alone, possibly suggesting that higher levels of HGF allow the maintenance of the MSC in the region of hypoxic injury. Notably, we detected a low level of human MSC retained in the injured

muscle 2 weeks after the transplantation, suggesting that MSC are able to home and survive in the injury area (data not shown).

In the current studies, we sought pretransplantation culture conditions for human MSC that will prepare the cells to have the most robust cell migration and to promote the most rapid functional repair, in the hind limb ischemia hypoxic injury model. The techniques and strategies developed here will be useful in determining the requirements of transplanted human stem cells for in vivo survival, recruitment to areas of hypoxic damage, and initiation of cascades of endogenous repair. As a result of these studies, we will gain a better understanding of how injected stem cells will be recruited to the damaged tissue to enhance revascularization by endogenous cells. HGF expressed locally at the area of damage, activated by hypoxia, can act as a chemotactic factor to recruit injected multipotent MSC from the bloodstream, into the site of injury, and to extend their survival to participate in tissue repair and to promote revascularization. The current study provides a practical and clinically relevant model for pretreating cells to potentially enhance blood flow to human limbs that have critical ischemia. Peripheral vascular disease and critical limb ischemia present painful and costly clinical problems in the U.S. and worldwide, especially in diabetic populations. Our report provides methods to culture and treat the cells in conditions that are more physiological, prior to transplantation. Of interest, Hu et al. have just reported that rodent MSC precultured in hypoxia promote enhanced revascularization in a cardiac injury model [60]. Our report is, to our knowledge, the first time that hypoxia-preconditioned *human* MSC have been shown to better promote recovery from vascular injury.

We measured the percentage of human cells remaining at the site of injury at the 2-week time point, using quantitative polymerase chain reaction as we have previously described [61]. Cells were present in an average of approximately 0.2% of the tissue (data not shown). However, the function of the cells is not to recreate new vessels or muscle, but to release cascades of trophic factors to enhance the endogenous revascularization process. Therefore, from a clinical and regulatory agency point of view, it is actually preferable that the cells do not remain in the local area for months after the injury has been repaired. We have shown in the current study that hypoxic preconditioned MSC have the best migratory potential and the most rapid tissue repair potential. This finding could enhance therapeutic approaches for enhancing local tissue repair by injected human mesenchymal stem cells.

Conclusion

Our studies show that culturing MSC in hypoxic conditions improves the rapidity of their tissue regenerative potential, as demonstrated by our in vivo experiments. Using a hind limb ischemia injury model, we showed that mice that had received hypoxic preconditioned MSC recovered faster than the control groups that had received normoxic MSC or saline. In addition, we demonstrated that HGF, previously shown to be secreted in response to liver and heart injury [43,45,46], is also secreted in a hind limb ischemia injury model in immunodeficient mice. On the basis of our in vitro experiments showing that MSC cultured in hypoxic conditions have elevated levels of cMet compared with those cultured in normoxia, we further suggest that HGF and its receptor cMet might be important factors in the mechanism by which hypoxia enhances the homing and tissue-repairing capacity of MSC. This theory is supported by immunoblotting experiments that show that hypoxic preconditioned MSC respond more robustly to HGF stimulation than those cultured in normoxic conditions, therefore suggesting that MSC that are expressing higher levels of cMet might respond faster to the HGF present at the site of tissue injury.

Acknowledgments

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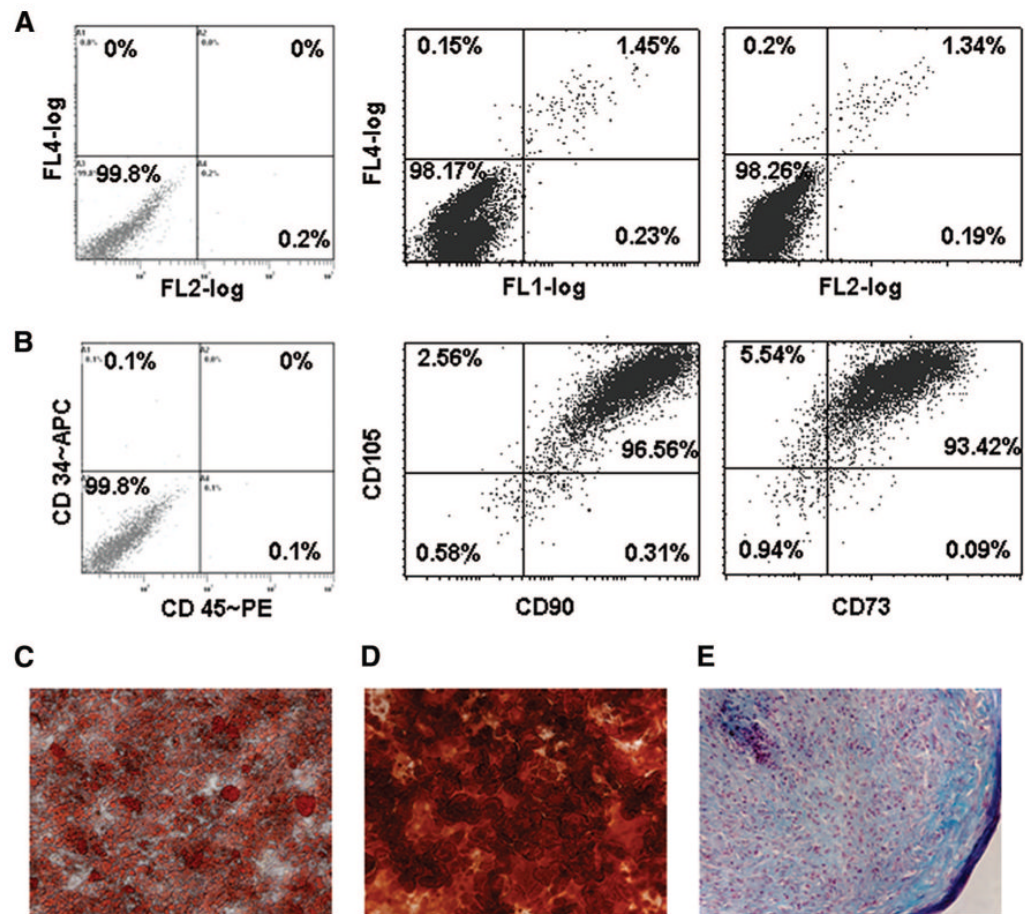
Figure 1.
Characterization of mesenchymal stem cells (MSC). Human MSC were cultured from bone marrow aspirates in complete medium. They were selected by adherence to plastic while hematopoietic contamination was excluded by fluorescent-conjugated antibody staining of the MSC for CD45 and CD34. The phenotypic markers CD105, CD90, and CD73 were assessed by flow cytometry. **(A):** Representative plots of the fluorescence-activated cell sorting analysis of unlabeled MSC. **(B):** Antibody-labeled samples. Assessment of the lack of CD34 and CD45 was performed with all cell cultures used for Western blot experiments, whereas three MSC cultures derived from three separate donors were analyzed for CD105, CD90, and CD73. When cultured in defined differentiation medium, MSC differentiated into the adipogenic lineage, shown here by oil red O stain **(C)**, the osteogenic lineage, shown by alizarin red stain **(D)**, and the chondrogenic lineage, shown by Alcian Blue stain **(E)**. Abbreviations: APC, allophycocyanin; FL1, fluorescence channel 1, detecting FITC; FL2, fluorescence channel 2, detecting PE; FL4, fluorescence channel 4, detecting APC; PE, phycoerythrin.
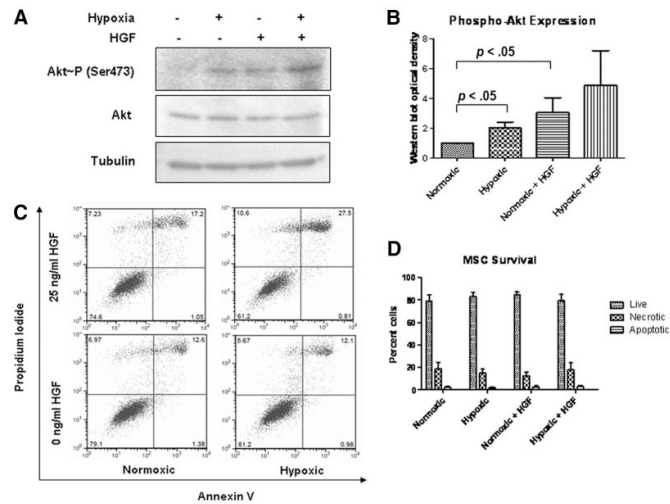
Figure 2.
Hypoxia and HGF treatment activate the Akt signaling pathway and maintain MSC viability. Bone marrow mesenchymal stem cells were incubated for 16 hours in serum-free medium exposed to hypoxic or normoxic conditions in the presence or absence of 25 ng/ml human recombinant HGF. MSC were tested for expression of Akt and its activation by Western blotting **(A)** and for viability by flow cytometry with Annexin V and propidium iodide staining **(C)**. Western blot densitometry **(B)** showed a significant increase in Akt phosphorylation when cells were stimulated with hypoxia and HGF, whereas quantitation of flow cytometry data **(D)** demonstrated no significant difference between the different treatment groups, using the Student's *t* test. Shown are representative data of four **(A)** and five **(C)** separate experiments. Abbreviations: HGF, hepatocyte growth factor; MSC, mesenchymal stem cells.
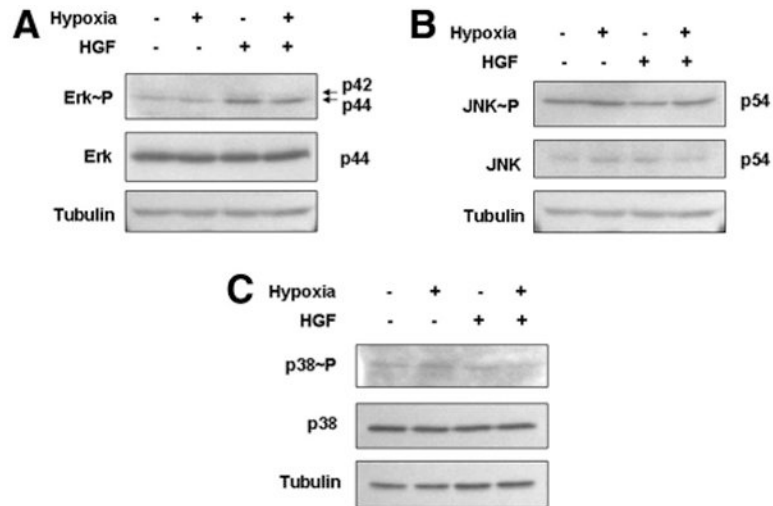
Figure 3.
HGF treatment stimulates Erk but not p38 or JNK signaling pathways. Mesenchymal stem cells were cultured in serum-free medium in hypoxia or normoxia in the presence or absence of 25 ng/ml HGF for 16 hours. Cell lysates were collected and separated on SDS-polyacrylamide gel electrophoresis gel and probed for Erk~P, Erk **(A)**, JNK~P, JNK **(B)**, and p38~P, p38 **(C)** antibodies. Pictured are representative results of five **(A)** and four **(B, C)** separate experiments. Abbreviation: HGF, hepatocyte growth factor; JNK, Jun N-terminal Kinase.
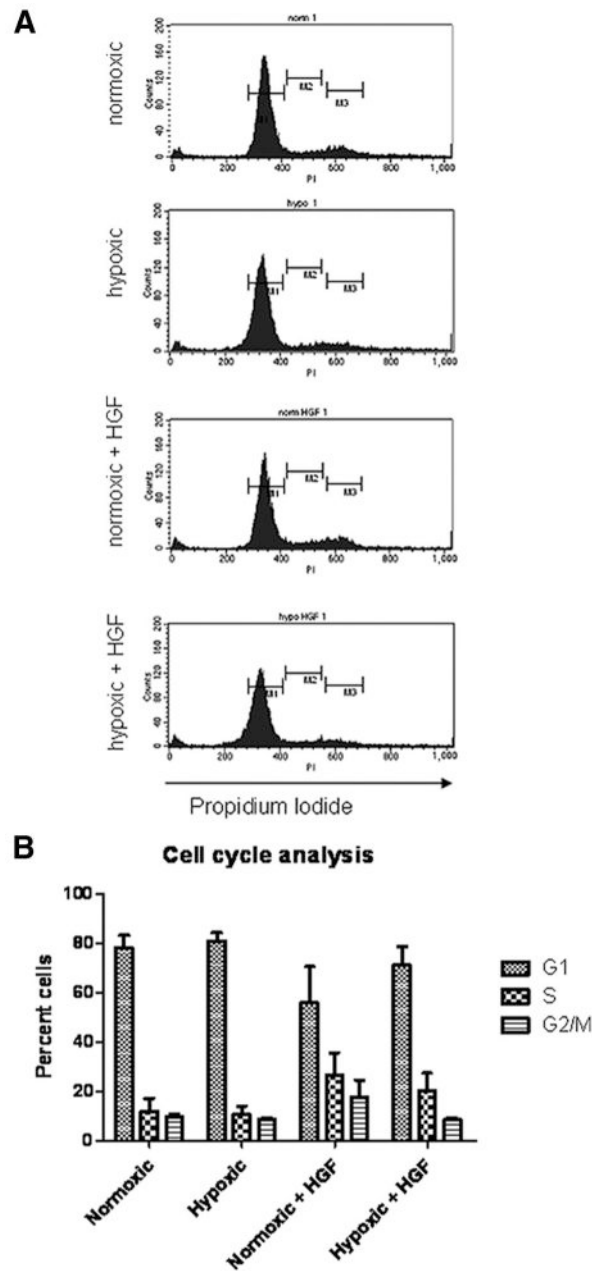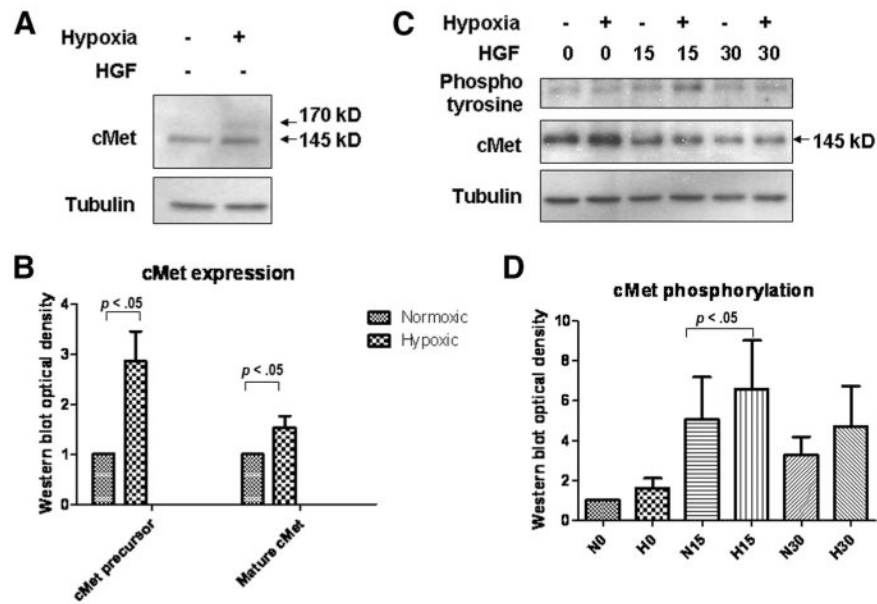
Figure 4.
Hypoxia and HGF treatment do not significantly change the proliferation status of mesenchymal stem cells (MSC). MSC were cultured in serum-free medium in hypoxic versus normoxic conditions in the presence or absence of 25 ng/ml HGF. Cells were collected, fixed, stained with PI, and analyzed with flow cytometry for cell cycle status. Fluorescence-activated cell sorting plots are representative results of five separate experiments **(A)**. The bar graph shows quantitation of flow cytometry data **(B)**. Differences were not significant, according to Student's *t*-test. Abbreviations: HGF, hepatocyte growth factor; PI, propidium iodide.

Figure 5.
Hypoxia sensitizes human mesenchymal stem cells (MSC) by elevating the levels of HGF receptor cMet, thus increasing the ability of MSC to respond to HGF stimulation. MSC were cultured in serum-free medium for 16 hours in hypoxic or normoxic conditions and then collected to generate protein lysates, which were resolved on SDS-polyacrylamide gel electrophoresis (PAGE) gel and probed with cMet antibody **(A)**. The bar graph represents Western blot densitometry results, demonstrating significant increases in the levels of mature cMet and its precursor in cells cultured in hypoxia **(B)**. MSC were incubated in hypoxic or normoxic conditions in serum-free medium for 24 hours. Cultures were stimulated for 0, 15, or 30 minutes with 25 ng/ml HGF, and protein lysates were collected and immunoprecipitated with cMet antibody. Protein was resolved on SDS-PAGE gel and probed for anti-phosphotyrosine and cMet antibodies **(C)**. The bar graph represents Western blot densitometry results of phosphorylated cMet **(D)**. Shown are representative results of three **(A, B)** or four **(C, D)** separate experiments. Abbreviations: HGF, hepatocyte growth factor; kD, kilodaltons.
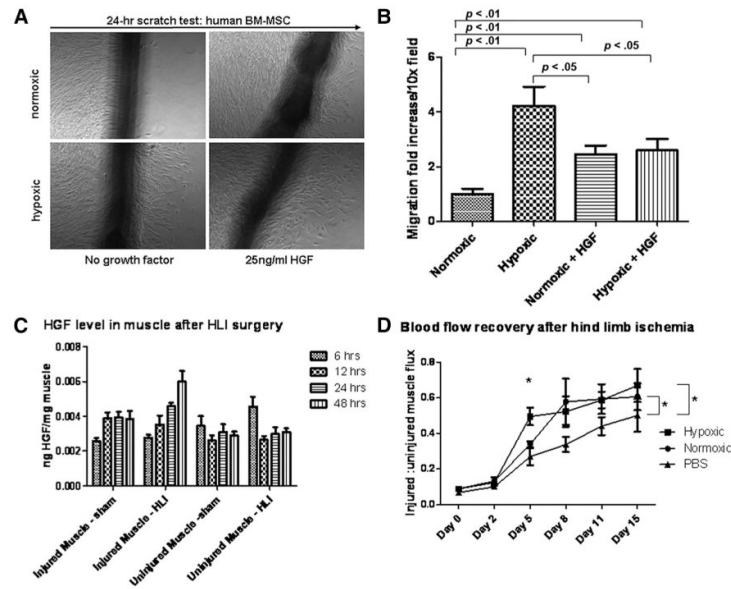
Figure 6.
Hypoxia increases mesenchymal stem cell (MSC) migration and enhances functional recovery after hind limb ischemia. The growth area of MSC plated on tissue culture-treated plates was scratched with a sterile pipette tip to create a wound. The borderline of the scratch was immediately marked with a fine-tip marker, and the cultures were incubated in serum-free medium for 24 hr in hypoxia versus normoxia in the presence or absence of 25 ng/ml HGF. Photographs of cell migration were captured with bright-field microscopy at the endpoint. **(A):** Representative image of three separate experiments. The quantitation of migrated cells demonstrated significant fold increases in MSC motility in samples treated with HGF or combination of hypoxia and HGF, whereas cells cultured in hypoxia alone migrated the most **(B)**. The bar graph **(B)** summarizes the results of three separate experiments. Hind limb ischemia and sham surgeries were performed on NOD/SCID mice ($n = 4$). Abductor muscles were harvested at 6, 12, 24, or 48 hr postsurgery, and muscle lysates were analyzed by enzyme-linked immunosorbent assay for the presence of HGF **(C)**. The results show a slight increase in HGF levels in muscles of sham-operated animals starting at 12 hr postsurgery; however, the tissue ischemia caused a significant increase in the HGF levels in the injured muscle, which steadily increased up to 48 hr after surgery (two-way analysis of variance [ANOVA], $p < .001$). Alternatively, MSC cultured in complete medium were incubated overnight (16–24 hr) in hypoxic or normoxic conditions. Hind limb ischemia was performed on β-2-microglobulin knockout mice, which were then transplanted 1 day after surgery with hypoxic MSC, normoxic MSC, or PBS as a control. The functional recovery was measured by laser Doppler perfusion imaging immediately after the surgery and twice a week for 2 weeks after the transplantation and quantified by Moor LDI image software **(D)**. The *y*-axis of the graph represents flux (measured in arbitrary units) ratio of injured to uninjured leg, and the three curves are average values for each treatment group (seven animals per group). The results demonstrate a significant improvement (ANOVA, $p < .005$) in mice transplanted with MSC versus saline control group, whereas the mice transplanted with hypoxic MSC improved significantly faster, as is shown at day 5 (*, $p = .011$; Student's *t*-test). Abbreviations: BM-MSC, bone marrow-derived mesenchymal stem cells; HGF, hepatocyte growth factor; hr, hours; PBS, phosphate-buffered saline.