

Suppression of Hepatocyte Growth Factor Production Impairs the Ability of Adipose-Derived Stem Cells to Promote Ischemic Tissue Revascularization

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Key Words. Adipose-derived stem cells • RNA interference • Paracrine • Hepatocyte growth factor

ABSTRACT

The use of adipose-derived stem/stromal cells (ASCs) for promoting repair of tissues is a promising potential therapy, but the mechanisms of their action are not fully understood. We and others previously demonstrated accelerated reperfusion and tissue salvage by ASCs in peripheral ischemia models and have shown that ASCs secrete physiologically relevant levels of hepatocyte growth factor (HGF) and vascular endothelial growth factor. The specific contribution of HGF to ASC potency was determined by silencing HGF expression. RNA interference was used to downregulate HGF expression. A dual-cassette lentiviral construct expressing green fluorescent protein (GFP) and either a small hairpin RNA specifically targeted to HGF mRNA (shHGF) or an inactive control sequence (shCtrl) were used to stably transduce ASCs (ASC-shHGF and ASC-shCtrl, respectively). Transduced ASC-shHGF secreted

>80% less HGF, which led to a reduced ability to promote survival, proliferation, and migration of mature and progenitor endothelial cells in vitro. ASC-shHGF were also significantly impaired, compared with ASC-shCtrl, in their ability to promote reperfusion in a mouse hind-limb ischemia model. The diminished ability of ASCs with silenced HGF to promote reperfusion of ischemic tissues was reflected by reduced densities of capillaries in reperfused tissues. In addition, fewer GFP⁺ cells were detected at 3 weeks in ischemic limbs of mice treated with ASC-shHGF compared with those treated with ASC-shCtrl. These results indicate that production of HGF is important for the potency of ASCs. This finding directly supports the emerging concept that local factor secretion by donor cells is a key element of cell-based therapies. STEM CELLS 2007;25:3234–3243

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The use of stem cells for promoting repair of tissues has gained much interest as a potential new therapeutic approach. Clinical studies indicate modest but significant benefits from treatment of ischemic heart and peripheral tissue diseases with autologous stem cells [1–7]. However, controversy exists as to whether the benefits observed in humans, as well as in preclinical animal disease models, are due to differentiation of the cells or alternatively result from other indirect mechanisms such as paracrine support of ischemic tissues [8–11].

Recently it was discovered that pluripotent cells, which exhibit stem cell properties, reside in the stromal compartment of adipose tissues [12]. These adipose stromal (stem) cells (ASCs) not only possess the ability to differentiate into multiple mesenchymal cell types [12–15] but also secrete significant levels of many potent growth factors and cytokines, including vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [8, 16]. Based on these studies demon-

strating the ability of ASCs to promote endothelial cell (EC) survival as well as to enhance vascular supply in vivo, we have suggested that paracrine factor secretion is a primary mechanism of the effects of ASCs, much as was described for endothelial progenitors [17, 18].

We are presently systematically elucidating the contribution of specific secreted factors to the potency of ASCs. Initially we have focused on HGF because of its many potentially beneficial functions and recent findings that HGF may be the dominant angiogenic and protective factor secreted by ASCs [16]. HGF is a growth and motogenic factor for diverse cell types, including endothelial and smooth muscle cells, which also possesses potent angiogenic effects [19, 20]. Previously, HGF has been studied for “therapeutic angiogenesis” both via direct gene transfer [21–25] as well as approaches using cells transduced with vectors expressing these genes [26, 27]. It recently has been reported that treatment with bone marrow-derived mesenchymal stem cells overexpressing HGF could improve functional parameters of ischemic myocardium [26]. The secretion of HGF by both ASCs and adipose tissue has been described as

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particularly important in vasculature [28]. Thus, the multiplicity of cellular targets suggests that HGF plays a particularly significant role in the paracrine function of ASCs.

To specifically evaluate the importance of HGF for ASC-mediated contributions to revascularization both *in vitro* and *in vivo*, we employed RNA interference knockdown of HGF using lentiviral delivery of a small hairpin RNA (shRNA), based on a small interfering RNA sequence [29] that had been shown previously to selectively target HGF and downregulate its expression [30]. Our studies have demonstrated a requirement for HGF secretion in mediating the effects of human ASCs on microvascular EC growth and viability as well as promoting their angiogenic sprout formation. Extension of this approach, employing an integrating lentiviral-shRNA HGF vector for *in vivo* testing in the hindlimb ischemia model in nude mice, revealed a marked reduction in the angiogenic effects of ASCs with silenced HGF secretion when compared with ASCs transduced with control constructs. This study demonstrates that the proangiogenic, prosurvival, and repair-promotion activities of ASC in tissue ischemia are significantly mediated by HGF.

MATERIALS AND METHODS

Isolation and Culture of Human ASCs

Human subcutaneous adipose tissue samples were obtained following routine, elective lipoaspiration of a 50-year-old healthy female patient under approval from the Indiana University Institutional Review Board. The lipoaspirate was digested in collagenase type I solution (Worthington Biochemical, Lakewood, NJ, <http://www.worthington-biochem.com>) under gentle agitation for 1 hour at 37°C, filtered sequentially with 500- μ m and 250- μ m Nitex filters, and centrifuged at 200g for 5 minutes to separate the stromal cell fraction (pellet) from adipocytes. The ASC fraction was treated with red blood cell lysis buffer for 5 minutes at 37°C and then centrifuged at 300g for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in endothelial growth medium-2 MV (EGM-2MV; Cambrex, Walkersville, MD, <http://www.cambrex.com>), which consists of endothelial basal medium-2 (EBM-2), 5% fetal bovine serum (FBS), and the supplemental growth factors VEGF, basic fibroblast growth factor, epidermal growth factor, and insulin-like growth factor.

Construction of Lentiviral RNA Interference Vectors

The double-stranded hairpin loop-containing sequence of the shRNA used in this study was 5'-GTATCCTCACGAGCATGACAAGGCC TCAGTCATGCTCGTGAGGATAC-3', where the non-hairpin region corresponds to human HGF mRNA [30] (as italicized). The shRNA was cloned into the expression vector pND-776 (E. Rosen, unpublished manuscript) using the *Apa*I 5'/EcoRI 3' cloning sites in the polycloning region. This vector also contains sequences encoding green fluorescence protein (GFP) downstream of the multiple cloning sites. Expression of the shRNA was driven by promoter U6, whereas GFP expression derived from a separate cytomegalovirus promoter. The parent vector, which expresses a nonspecific RNA derived from the multicloning sequence, served as the negative control.

The expression cassettes were cloned between the *Asp*198 and *Xho*I sites of pENTR-1 (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>). The resulting plasmid was cloned into pcDNA-HIV-CS-CGW (a gift from Phil Zoltick, Children's Hospital, Philadelphia).

Lentiviral Vector Transductions

Lentiviral stocks were produced by transient transfection of HEK293T cells (American Type Culture Collection, Manassas, VA, <http://www.atcc.org>) as described previously [31–37]. A total of 5×10^6 cells were seeded in 75-cm² flasks 24 hours before transfection. Transfection was performed by calcium phosphate

precipitation method (Profection kit; Promega, Madison, WI, <http://www.promega.com>) according to the manufacturer's instructions. Plasmids used were the pcDNA-HIV-CS-CGW derived vectors (18 μ g), pMDLg (6.6 μ g), pRSV/REV (3.3 μ g; Cell Genesys, Foster City, CA, <http://www.cellgenesys.com>), and pMD.G (4.6 μ g). The vector-containing supernatants were harvested 48 hours after transfection, filtered through a 0.45- μ m syringe filter, and then stored at -80°C. To transduce ASCs with lentivirus constructs, passage 1 cells were seeded at a density of 10^4 cells per cm² in 6-well plates. Various volumes (50–400 μ l) of lentivirus suspension and 8 μ g/ml polybrene were added to EBM-2 (Cambrex) with 5% FBS in a total volume of 1 ml. Cells were allowed to incubate at 37°C for 12 hours before removing the medium and replacing it with 2 ml of fresh EGM-2MV for expansion of transductants.

Flow Cytometric Characterization of Human ASCs

Transduced GFP-expressing ASCs were detached with 0.05% Trypsin-EDTA and washed with phosphate-buffered saline (PBS), and then 2×10^4 cells were fixed in 2% paraformaldehyde (Tousimis, Rockville, MD, <http://tousimis.com>) and analyzed by flow cytometry on a FACSCalibur instrument (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>) to evaluate transduction efficiency based on GFP-positive signal. Populations of transduced ASCs resulting from the use of the least volume of lentivirus stock yielding >99% GFP⁺ cells were used in the studies described below.

Expression of the surface marker CD31 was also analyzed in some experiments. Cells were incubated for 20 minutes with CD31-PE (BD Biosciences, San Diego, <http://wwwbdbiosciences.com>) or matching isotype controls at a final concentration of 5 mg/ml. The antibody-labeled cells were subsequently washed with 2% FBS in PBS and fixed with 2% paraformaldehyde for analysis using a Calibur flow cytometer analyzer and Cell Quest Pro software (Becton Dickinson).

Enzyme-Linked Immunosorbent Assay Analysis of HGF Level in Human ASC Supernatants

After the fourth passage, ASCs transduced with either of the lentivirus vectors or untransduced ASCs were seeded at a density of 10^4 cells per cm² in a 6-well culture dish and grown in EBM-2 with 5% FBS (EBM-2/5% FBS) at 37°C in 5% CO₂. After overnight attachment, the medium was changed and the cells were incubated an additional 24 hours before the media were collected and cells removed by centrifugation at 300g for 5 minutes for enzyme-linked immunosorbent assay (ELISA) analysis. Each well of a 96-well ELISA plate (catalog number 3585; Corning Costar, Acton, MA, <http://www.corning.com/lifesciences>) was coated with 0.1 μ g of a mouse monoclonal HGF capture antibody (MAB 694; R&D Systems Inc., Minneapolis, <http://www.rndsystems.com>) and incubated for 1 hour at room temperature. Nonspecific binding sites were blocked with 300 μ l of PBS containing 1% bovine serum albumin, 5% sucrose, and 0.05% NaN₃. Supernatants or purified HGF (294HG; R&D Systems) standard (100 μ l) at the appropriate dilutions were added to wells in triplicate and incubated for 2 hours before adding 100 μ l of the biotinylated detection antibody (anti-mouse IgG; R&D Systems; BAF 294, 200 ng/ml) for 2 hours. After washing, 100 microliters per well streptavidin conjugated horseradish peroxidase (HRP; DY998; R&D Systems), diluted 1:200, was added, and the plate was incubated for 20 minutes, followed by three washes, after which 100 microliters per well Substrate Solution (H202 and DY999; R&D Systems) was added, and the plate was incubated for 20–30 minutes before adding 50 microliters per well Stop Solution (1 M H₂SO₄). The optical density of each well was assayed at 450 nm and corrected for the background light absorption. VEGF in cell supernatants was determined using commercial ELISA kits (human VEGF Quantikine; R&D Systems).

The Effect of Serum Deprivation on the Survival of Transduced ASCs

Human adipose stromal cell-small hairpin control (ASC-shCtrl) and adipose stromal cell-small hairpin hepatocyte growth factor (ASC-shHGF) were seeded in EGM-2MV at 5,000 cells per well in

96-well plates and incubated overnight. The medium was replaced with EBM-2 with or without 5% FBS, and the cells were incubated for 12 hours. Cell viability was determined using the CellTiter 96 AQueous One Solution Reagent (number G358; Promega) according to the manufacturer's protocol. Data are expressed as relative fluorescence per 1,000 cells.

Human Microvascular Endothelial Cell Proliferation Assays

Human microvascular endothelial cells (HMVECs) were purchased from Cambrex (cc-7030) and cultured in EGM-2MV medium. Cells were used within three passages of initial thawing. To assay the effects of conditioned media on growth, HMVECs were seeded at a density of 5,000 cells per cm² in 12-well plates (Techno Plastic Products AG, Trasadingen, Switzerland, <http://www.tpp.ch>). HMVECs were incubated for 24 hours with 2 ml of fresh EBM-2/5% FBS medium, which supports viability but not growth or migration of these cells. For cell migration and proliferation studies, an equal volume of EBM-2/5% FBS medium harvested from ASCs was added to the medium and the cells were cultured. For some experiments, the ASC-conditioned medium was preincubated for 1 hour with 1 µg/ml anti-human HGF neutralizing antibody (AF-294-NA; R&D Systems). Cells were detached after 72 hours and cell numbers counted using 0.4% trypan blue exclusion to monitor viability. Individual experiments were performed in triplicate wells and repeated at least three times.

Quantitation of In Vitro Sprout Formation by HMVECs

Angiogenesis in vitro was tested as described previously [34]. Briefly, the HMVECs were grown to confluence on cytodex-3 microcarrier beads (C-3275; Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) whose diameter is 133–215 µm, and approximately 100 beads were placed into each well of a 12-well culture plate coated with 0.5 ml of 2.5 mg/ml fibrinogen (F-4883; Sigma) in PBS that had been polymerized by adding 0.48 U/ml thrombin (T-7009; Sigma). Approximately 1 ml of either the conditioned medium, EGM-2MV (positive control), or EBM-2/5% FBS (negative control) was added to each well, and the chambers were then incubated at 37°C, 5% CO₂. After 1 hour, the medium was exchanged and the plates were incubated for an additional 48 hours. For quantitation, only the sprouts that were longer than the diameter of the average bead size were counted using a phase contrast microscope. Experiments were performed in triplicate and repeated at least three times.

Transwell Chamber Migration Assays

A Falcon cell culture insert system (Becton Dickinson) along with a compatible 24-well Falcon tissue culture plate were used for migration assays. The polyethylene terephthalate membrane pore size of 8 µm was selected to allow passage of high proliferative potential human umbilical vein-derived endothelial progenitor cells (EPCs) [35]. The membrane was precoated with 60 µl of a 50 µg/ml solution of type I collagen. The bottom chamber contained 600 µl of conditioned medium, in some cases with purified HGF or the HGF neutralizing antibody. The top chamber was seeded with 3 × 10⁴ EPCs at the beginning of the experiment. Migration assays were terminated after 4 hours, and EPCs that had migrated through the membrane were then stained (after removal of cells remaining on top with a wet Q-tip) using Diff-Quik staining kit (Dade Behring, Newark, DE, <http://www.dadebehring.com>) according to manufacturer's instructions. Stained cells in 3–5 micrograph fields, obtained at ×400 magnification, were counted manually. Results presented are from three independent experiments with duplicate wells for each condition.

Mouse Hindlimb Ischemia Model

The animal studies were approved by the Indiana University School of Medicine Animal Use and Care Committee. Unilateral hindlimb ischemia was created in 8-week-old male nude mice as described previously [36, 37]. The animals were anesthetized by isoflurane

inhalation. An incision was made at the midline of the left hindlimb. The femoral artery and its branches were ligated, beginning from the inguinal ligament to the bifurcation of saphenous and popliteal arteries. The region between the ligatures was excised. The incision was closed with 6–0 silk sutures (Ethicon, Somerville, NJ, <http://www.ethicon.com>).

After surgery, the mice were randomly separated into three groups (*n* = 10 per group). One day after creation of unilateral hindlimb ischemia, 10⁶ ASC-shHGF or ASC-shCtrl in 100 µl saline or saline only were injected through the tail vein using a 0.3-cc insulin syringe and a 28-gauge needle.

Blood flow restoration in ischemic limbs after treatment was evaluated by laser Doppler perfusion imaging (Moor Instruments, Devon, U.K., <http://www.moor.co.uk>) as described previously [8]. Briefly, animals were anesthetized by isoflurane inhalation and placed on a heating pad set at 37°C. Data were collected from the plantar surfaces of both limbs. Measurements were performed on days 1, 5, 10, 15, and 20. The results are expressed as the ratio of perfusion in ischemic (left) to nonischemic (right) limbs.

Histological Analysis

Three mice from each group were sacrificed at 1 day and the rest at 20 days after cell infusion. Gastrocnemius muscles of both limbs were removed, fixed with 10% neutrally buffered zinc formalin, and embedded in paraffin. Thin sections (5 µm) were probed with biotinylated antibodies to CD31 (550274; BD Pharmingen, San Diego, http://wwwbdbiosciences.com/index_us.shtml) and GFP (632380; Clontech, Palo Alto, CA, <http://www.clontech.com>). Visualization of binding was accomplished by streptavidin-HRP complex formation and followed by color development with 3,3'-diaminobenzidine (Sigma). Images of sections were obtained on a Nikon (Tokyo, <http://www.nikon.com>) microscope (model TE2000-S) and were analyzed with Image J software (National Institutes of Health, Bethesda, MD, <http://www.nih.gov>). Five fields from each muscle section were randomly selected for quantification of CD31 (×400 magnification).

Fluorescence Imaging of Tissue Sections

Slide-mounted, fixed, and paraffin-embedded muscle thin sections (5 µm) were preincubated with M.O.M. (BMK-2202; Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>) mouse Ig blocking reagent before incubating overnight with the mouse monoclonal GFP antibody (diluted 1:300) and rabbit anti-smooth muscle α-actin (RB-9010-P0; diluted 1:50 Lab Vision Corp., Fremont, CA, <http://www.labvision.com>). Slides were washed and then incubated with fluorescein-conjugated chicken anti-mouse IgG (A21200; Invitrogen) and goat anti-rabbit IgG (T2769; Invitrogen) for 30 minutes. Finally, sections were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (D8417; Sigma) for 1 minute. Images of sections were obtained at ×400 magnification and were analyzed with Image J software.

An extensive quantitation of GFP⁺ ASCs present in muscle tissues at 1 and 20 days was also performed. The entire muscle was sectioned into 10–15 thin sections, each separated by 100-µm intervals, and then 10 randomly selected fields (×200 magnification) from each section were quantitated.

Statistical Analysis

Data are expressed as mean ± standard deviation or standard error of the mean, as noted in the text and figures. Statistical comparisons between groups were performed with a two-tailed Student's *t* test. Comparisons of multiple groups were done with analysis of variance with corrections for multiple comparisons; *p* < .05 was considered significant.

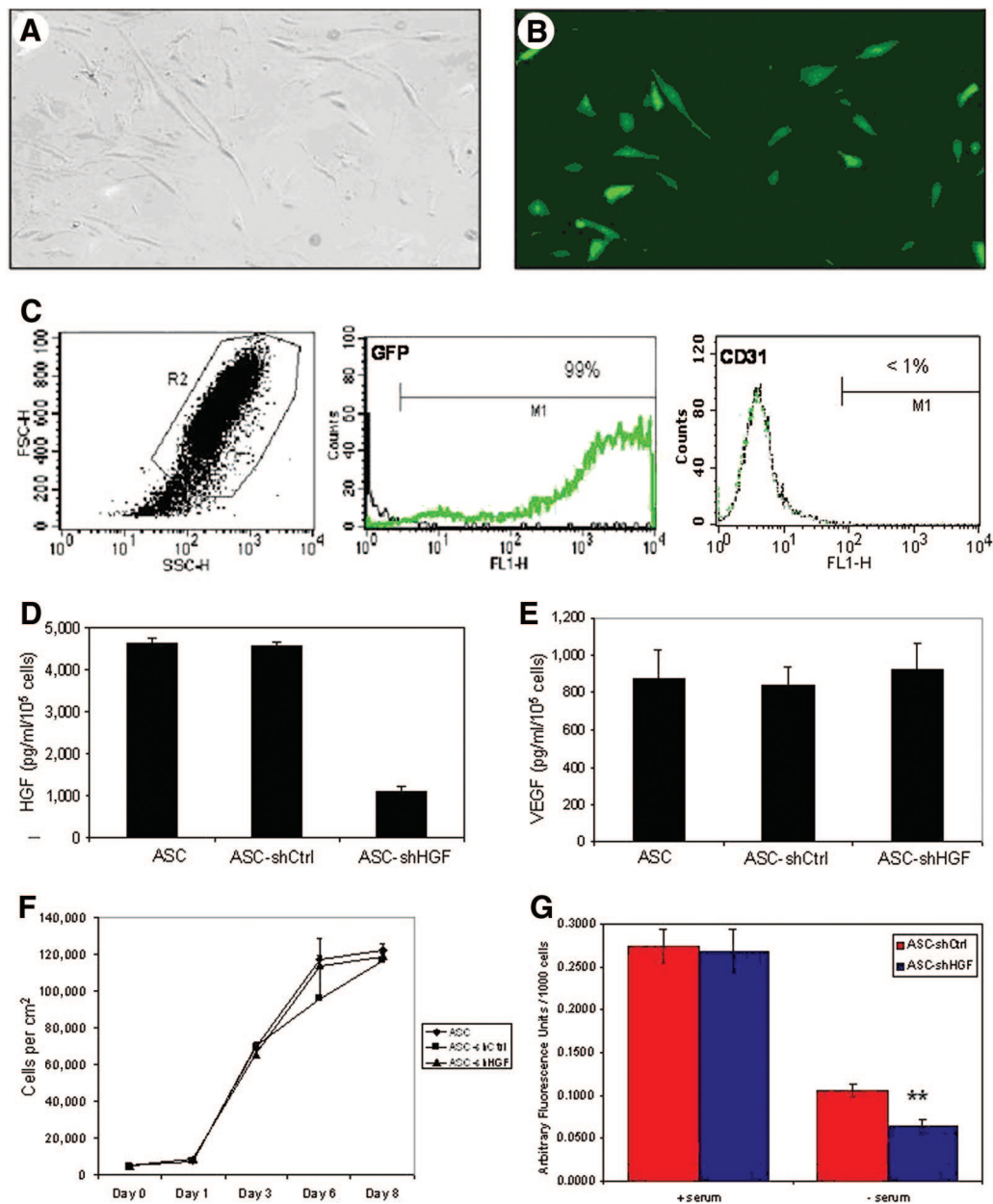


Figure 1. Characterization of ASC-shHGF and ASC-shCtrl transductants. Phase contrast (A) and fluorescent (B) micrographs of ASC-shHGF or -shCtrl transductants (100 \times). Flow cytometry demonstrating GFP⁺ fluorescence (C) and absence of CD31⁺. ASC-shHGF reduced HGF secretion by >80% without affecting VEGF expression (D, E) or cell growth (F). Serum starvation had a greater impact on viability of ASC-shHGF than ASC-shCtrl by MTS assay (G) (**, $p < .01$). Abbreviations: ASC, adipose stromal cell; FSC, forward scatter; GFP, green fluorescent protein; HGF, hepatocyte growth factor; shCtrl, small hairpin RNA targeted to an inactive control sequence; shHGF, small hairpin RNA targeted to hepatocyte growth factor mRNA; SSC, side scatter; VEGF, vascular endothelial growth factor.

RESULTS

Knockdown of HGF Expression in Primary Human ASCs by Stable shRNA Expression

The level of HGF secreted by human ASCs was selectively reduced by introducing a lentiviral construct that expressed a shRNA targeted to HGF mRNA (shHGF). The interfering sequence used was described in an earlier study of HGF function in human adenocarcinoma cells [30]. In addition, the vector coexpressed GFP to aid in identifying transduced cells (Fig. 1). The vector-containing shHGF, as well as a similar vector containing an shRNA targeted to an inactive control sequence (shCtrl), yielded >99% ASC trans-

ductants (ASC-shHGF and ASC-shCtrl, respectively), as determined by flow cytometry (Fig. 1C). Transductants were cultured for at least four passages before use. These GFP⁺ populations were devoid of cells containing the endothelial cell marker CD31⁺ (Fig. 1C). The resulting ASC-shHGF transductants secreted nearly fivefold less HGF during culture compared with both ASC-shCtrl and untransduced ASCs (Fig. 1D). The levels of HGF and VEGF in the growth medium were at undetectable levels (data not shown). Transduction with either lentiviral construct did not affect expressed levels of unrelated proteins, including VEGF (Fig. 1E), or cell growth (Fig. 1F), although ASC-shHGF cells were significantly less able to withstand the stress of serum withdrawal (Fig. 1G).

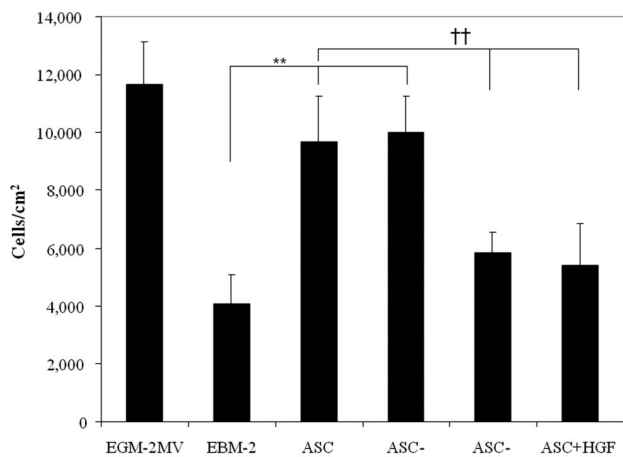


Figure 2. Silencing HGF expression reduces ASC ability to promote human microvascular endothelial cell (HMVEC) proliferation. HMVEC proliferation increased by 2.4-fold in EBM-2 when supplemented with untransduced ASC or ASC-shCtrl conditioned medium (CM) 1:1 (**, $p < .01$ vs. EBM-2). This growth stimulation was absent when ASC-shHGF CM or ASC CM pretreated with HGF neutralizing antibody was added (††, $p < .01$ vs. ASC). Abbreviations: Ab, antibody; ASC, adipose stromal cell; EBM-2, endothelial basal medium-2; EGM-2MV, endothelial growth medium-2 MV; HGF, hepatocyte growth factor; shCtrl, small hairpin RNA targeted to an inactive control sequence; shHGF, small hairpin RNA targeted to hepatocyte growth factor mRNA.

Silencing HGF Expression Reduces the Ability of ASCs to Promote EC Proliferation

The effects of silenced HGF expression on the potential of ASCs to provide paracrine support were examined by testing the potency of conditioned medium (CM) from the various cells for the ability to promote proliferation of HMVEC in the growth factor-depleted basal medium (EBM-2/5% FBS) (Fig. 2). The

levels of HGF and VEGF in this basal medium were less than the assay detection limit (approximately 5 pg/ml). As a positive control for the in vitro experiments, complete EGM-2MV medium (EBM-2 basal medium with the addition of epidermal growth factor, VEGF, basic fibroblast growth factor, and insulin-like growth factor-1) was used. In all experiments, EGM-2MV induced stronger responses than a 1:1 mixture of EBM-2 and ASC-conditioned medium (Figs. 2–4). Although ASCs cultured in EBM-2 produce all of the supplemented protein factors present in EGM-2MV, most of these accumulate in the medium to substantially lower levels than what is added to EGM-2MV [38]. In addition, ASCs may also secrete factors that negatively influence the performance of endothelial cells in the assays employed in this study.

During 72-hour culture, HMVECs failed to proliferate in EBM-2 basal medium. Proliferation increased by 2.4-fold in EBM-2 that had been supplemented with a 1:1 mixture of ASC or ASC-shCtrl CM ($p < .01$). This growth stimulation was absent when conditioned medium from ASC-shHGF was added. For comparison of the effect, ASC CM was also treated with a neutralizing HGF antibody. Together these data indicate that HGF may be a particularly important component of the mixture of factors secreted by ASC in regard to supporting survival and proliferation of HMVECs when cultured under growth-limiting conditions.

Silencing HGF Compromises the Ability of ASC to Support Angiogenesis In Vitro

The consequence of reduced HGF expression was further functionally analyzed by determining the ability of the various conditioned media to stimulate angiogenic sprout formation by HMVECs in vitro (Fig. 3; representative image of HMVEC forming sprouts is shown in 3A). Sprout formation was 2.1-fold higher when cultured in the CM from untransduced ASCs (1.82 ± 0.12 sprouts per bead) and ASC-shCtrl transduced with control shRNA (1.82 ± 0.26 sprouts per bead) compared with

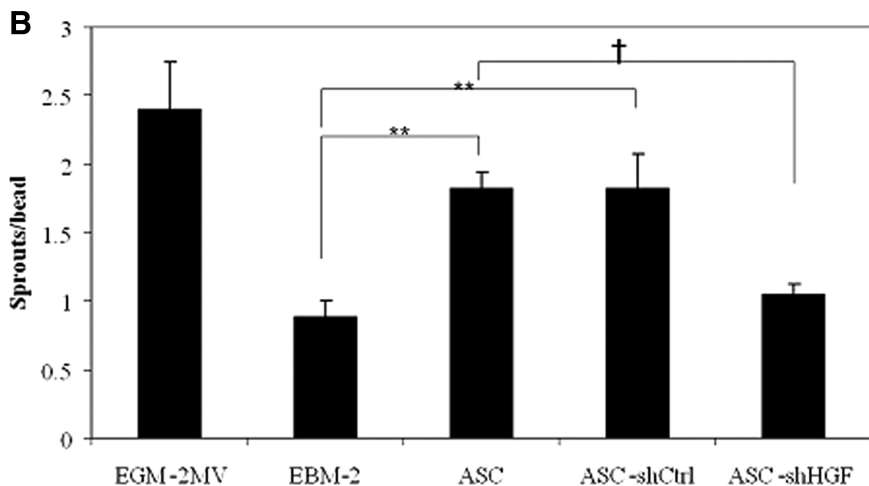
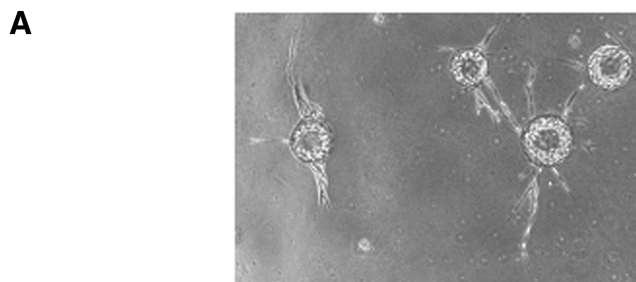


Figure 3. Silencing hepatocyte growth factor compromises ASC ability to support angiogenesis in vitro. A representative phase contrast micrograph of human microvascular endothelial cell (HMVEC)-coated beads cultured in ASC conditioned medium (CM) (A). Sprout formation by HMVECs was 2.1-fold higher in the CM from untransduced ASCs and ASC-shCtrl (**, $p < .01$ vs. EBM-2). This stimulation was abolished when ASC-shHGF CM was used (†, $p < .05$ vs. ASC) (B). Abbreviations: ASC, adipose stromal cell; EBM-2, endothelial basal medium-2; EGM-2MV, endothelial growth medium-2 MV; shCtrl, small hairpin RNA targeted to an inactive control sequence; shHGF, small hairpin RNA targeted to hepatocyte growth factor mRNA.

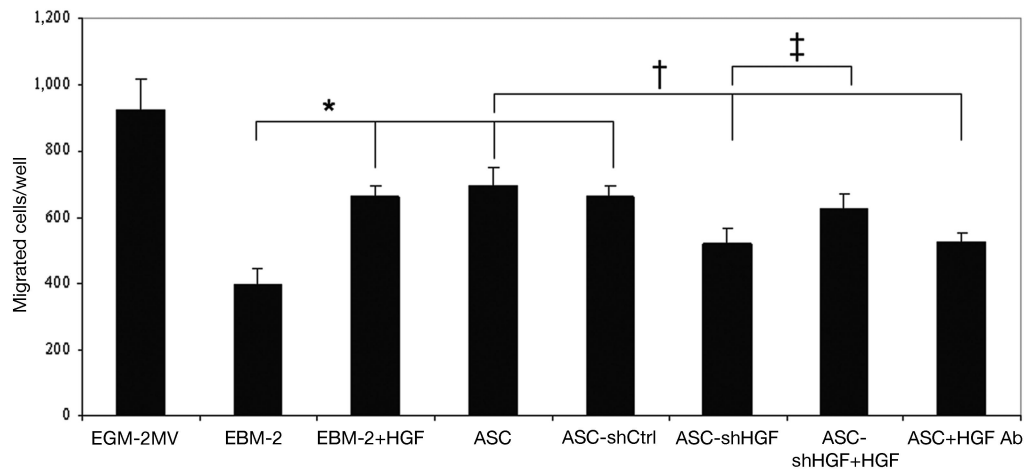


Figure 4. HGF expression by ASCs is essential for promoting endothelial progenitor cell (EPC) migration. EPC migration was enhanced by 1.75-fold with untransfected ASC or ASC-shCtrl conditioned medium (CM) added 1:1 to EBM-2/5% fetal bovine serum (*, $p < .05$ vs. EBM-2); migration was inhibited when CM was added from either ASC-shHGF or ASC CM + HGF inactivating antibody (†, $p < .05$ vs. ASC-shCtrl), which was reversed when HGF was added back (‡, $p < .05$ vs. ASC-shHGF). Abbreviations: Ab, antibody; ASC, adipose stromal cell; EBM-2, endothelial basal medium-2; EGM-2MV, endothelial growth medium-2 MV; HGF, hepatocyte growth factor; shCtrl, small hairpin RNA targeted to an inactive control sequence; shHGF, small hairpin RNA targeted to hepatocyte growth factor mRNA.

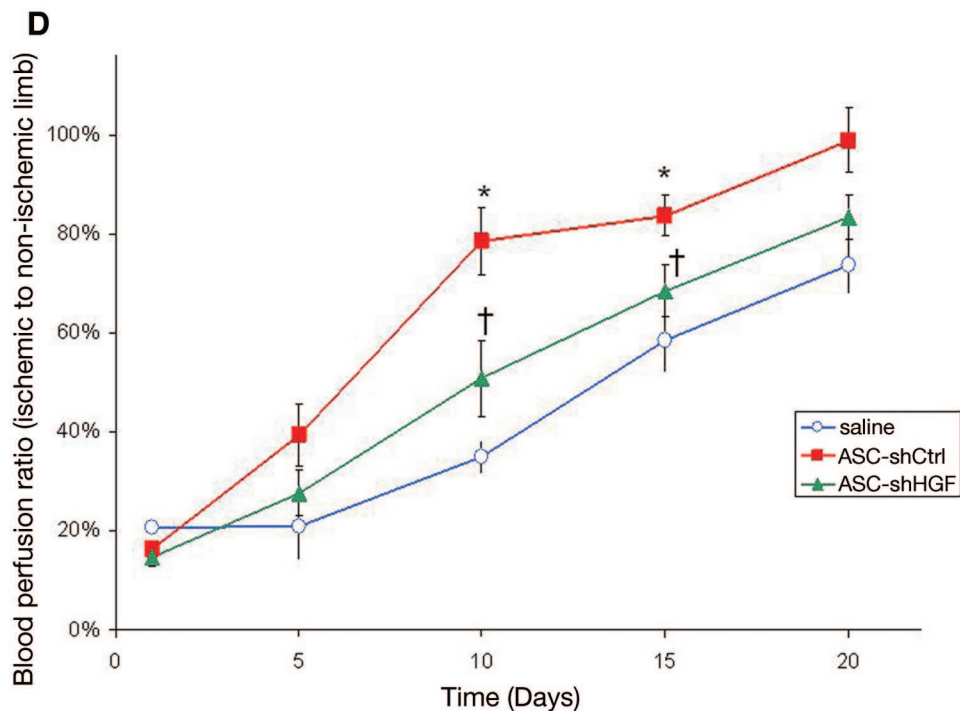
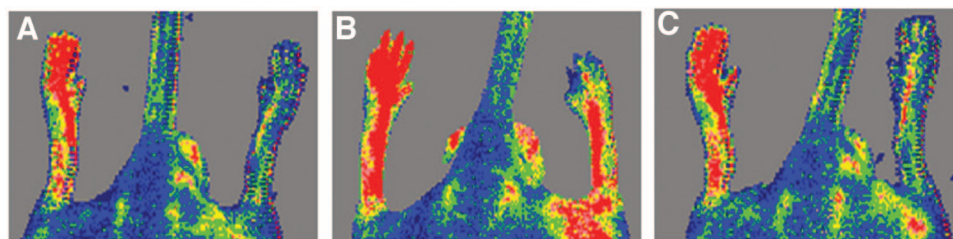


Figure 5. Hepatocyte growth factor expression by ASCs is essential for promoting reperfusion of ischemic tissues. Blood flow images of mouse hindlimbs treated with saline (A), ASC-shCtrl (B), and ASC-shHGF (C). Relative perfusion (ischemic/nonischemic limb) over time in mice treated with ASC-shCtrl cells was greater than both saline and ASC-shHGF groups (*, $p < .05$ vs. saline; †, $p < .05$ vs. ASC-shCtrl). Abbreviations: ASC, adipose stromal cell; shCtrl, small hairpin RNA targeted to an inactive control sequence; shHGF, small hairpin RNA targeted to hepatocyte growth factor mRNA.

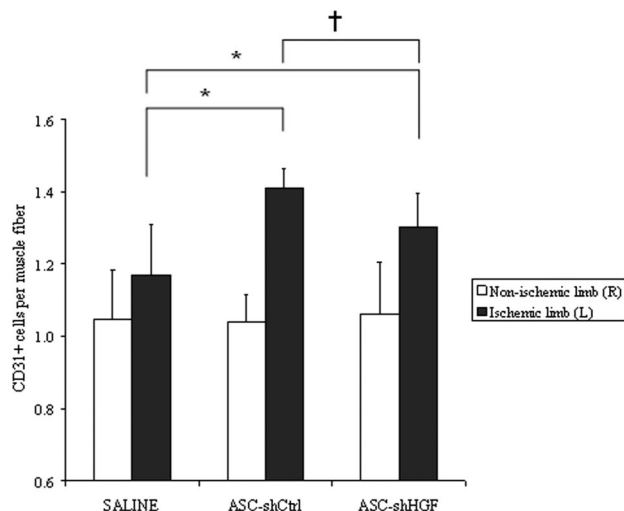


Figure 6. Microvessel densities in reperfused tissues correlated with perfusion status. Capillary (von Willebrand factor-positive structures) densities in gastrocnemius muscle were higher in both cell-treated groups compared with the saline group (*, $p < .05$). The capillary density was reduced in the ASC-shHGF group compared with ASC-shCtrl (†, $p < .05$ vs. ASC-shCtrl). Abbreviations: ASC, adipose stromal cell; L, left; R, right; shCtrl, small hairpin RNA targeted to an inactive control sequence; shHGF, small hairpin RNA targeted to hepatocyte growth factor mRNA.

EBM-2/5% FBS (0.88 ± 0.13 sprouts per bead, $p < .01$). Stimulation was nearly absent when an equivalent amount of ASC-shHGF CM was used (1.04 ± 0.09). Thus, angiogenic stimulation by ASCs is also greatly compromised by silencing HGF expression.

HGF Expression by ASCs Is Essential for Promoting EPC Migration

Angiogenesis in tissues occurs through stimulating nearby ECs to proliferate as well as by attracting circulating progenitor cells to the site of neovascularization. As discussed above, it has been shown previously that HGF potently promotes mobilization of many cell types; therefore, we next determined whether silencing HGF expression by ASCs would affect the ability of CM to promote EPC migration (Fig. 4). EPC migration was enhanced by 1.8-fold when untransfected ASC CM (693 ± 55 migrated cells per well) and ASC-shCtrl CM (661 ± 34 migrated cells per well) were added to EBM-2/5% FBS (396 ± 50 migrated cells per well, $p < .05$), whereas migration was inhibited in CM in which HGF levels were reduced by either shHGF transduction (521 ± 45 migrated cells per well) or by preincubating ASC CM with an inactivating HGF antibody (524 ± 26 migrated cells per well) compared with control ASC CM ($p < .05$). This inhibition was reversed when purified HGF was added to ASC-shHGF CM (625 ± 45 migrated cells per well). Thus, the levels of HGF secreted by ASCs are active in promoting EPC migration, and specific reduction of HGF significantly reduces this effect.

HGF Expression by ASCs Is Essential for Promoting Reperfusion of Ischemic Tissues

The significantly reduced angiogenic potential of HGF-silenced ASCs in vitro suggested that paracrine mechanism of ASC-mediated reperfusion of ischemic tissues may be compromised in cells expressing the shHGF construct. A mouse hindlimb ischemia model was used to compare the effect of ASC-shHGF to ASC-shCtrl. Systemic venous infusion of cells was performed at 24 hours following femoral artery occlusion/excision to allow

targeting of cells to injured tissues and promote dispersion of cells throughout the ischemic region. Blood perfusion of hindlimbs was monitored over time by laser Doppler perfusion imaging (Fig. 5). By day 10, the degree of relative perfusion (ischemic vs. nonischemic limbs) was significantly greater in ASC-shCtrl treated mice ($78.5\% \pm 6.7\%$) compared with both the ASC-shHGF ($50.8\% \pm 7.7\%$) and saline ($35\% \pm 3.3\%$) treated groups ($p < .05$). This difference in perfusion among the groups was evident throughout the entire experiment (20 days).

Microvessel Densities in Reperfused Tissues Correlate with Perfusion Status

Capillary densities in lower leg ischemic gastrocnemius muscles were determined at 20 days following ischemia (Fig. 6). This time point represented the end of the experiment, and it was noted that the difference in perfusion between the groups had narrowed (Fig. 5D). An analysis of the perfusion plots indicated that blood flow in the ASC-shCtrl group had approached an asymptote between days 15 and 20, whereas the other two groups were still in the linear phase of reperfusion at day 20.

The area density of CD31⁺ capillaries in muscle fibers of the ischemic limb in the group treated with ASC-shCtrl was significantly higher ($p < .05$) compared with the ASC-shHGF group (1.41 ± 0.05 and 1.30 ± 0.09 CD31⁺ cells per muscle fiber, respectively). Both ASC-shHGF and ASC-shCtrl treated muscles had a greater capillary density compared with the saline group (1.17 ± 0.14 CD31⁺ cells per muscle fiber).

The Persistence of ASCs in Ischemic Tissues Is Influenced by HGF Levels

The influence of HGF expression on the ability of systemically delivered ASCs to home to ischemic tissues and persist during repair and reperfusion of tissues was determined by quantitating GFP⁺ cells in the lower legs at 1 and 20 days after delivery. Green fluorescing ASCs could be detected in both normal and ischemic tissues of the lower hindlimb (Fig. 7A); however, at 1 day after infusion, both ASC-shHGF and ASC-shCtrl accumulated to a fivefold greater density in ischemic tissues compared with normal tissues. These data indicate that the secretion of HGF did not influence homing of ASCs to sites of ischemic injury.

Conversely, HGF secretion greatly influenced the ability of ASCs to persist over the duration of the experiments in ischemic environments. Although the equivalent numbers of both cell types were present in ischemic tissues at 1 day, by 20 days after infusion the number of detectable GFP⁺ ASC-shHGF cells had declined to levels similar to those observed in normal tissues (Fig. 7A), which presumably represents background levels, whereas approximately one half of the ASC-shCtrl cells detected at 1 day were still present at 20 days. The approximately threefold-higher density ($p < .05$) of GFP⁺ cells present in the repaired ischemic limbs of the ASC-shCtrl group compared with those treated with ASC-shHGF correlates well with the difference in relative blood reperfusion observed in animals of each group (Fig. 5).

Stably Engrafted ASCs Are Often Located in Proximity to Regenerating Myofibers

The spatial location of GFP-expressing ASCs within thin sections of ischemic tissues harvested at 20 days after cell infusion was further explored by fluorescence microscopy. Accurate quantitation of fluorescence in situ can be difficult due to autofluorescence of connective tissues; therefore, we also

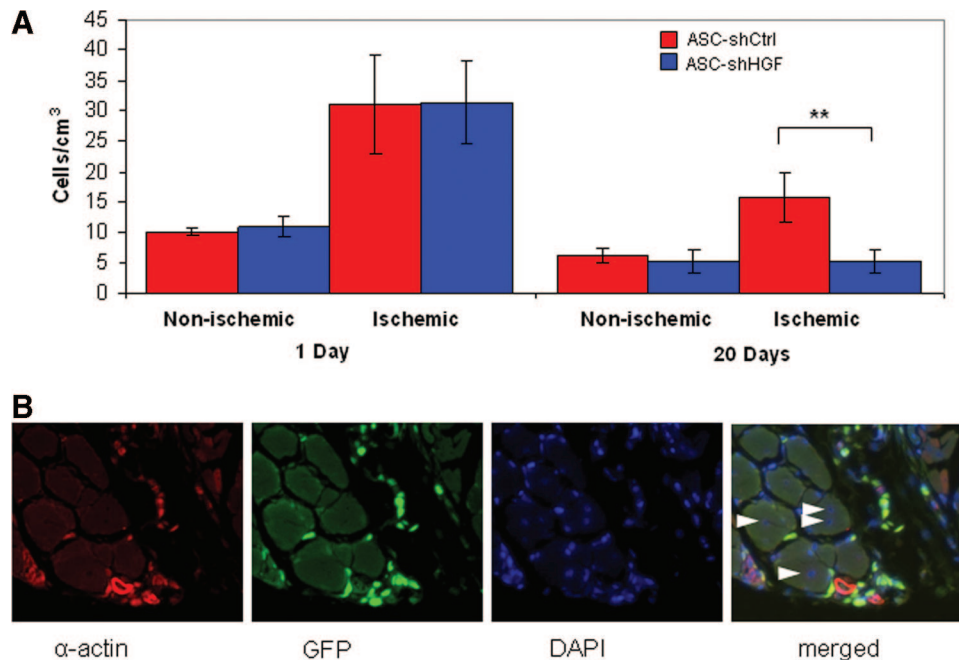


Figure 7. ASC persistence in ischemic tissues is influenced by hepatocyte growth factor. The persistence of ASC-shCtrl in ischemic limbs was significantly higher than ASC-shHGF by quantitation of GFP⁺ cells in gastrocnemius muscles on days 1 and 20 after infusion (**, $p < .01$) (A). Immunofluorescence micrograph ($\times 400$) with α -actin (red), GFP (green), and nuclei (blue) staining. Arrowheads denote centrally located nuclei within regenerating myofibers (B). Abbreviations: ASC, adipose stromal cell; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; shCtrl, small hairpin RNA targeted to an inactive control sequence; shHGF, small hairpin RNA targeted to hepatocyte growth factor mRNA.

probed the tissues with smooth muscle α -actin, which is expressed by ASCs [12], in addition to arterioles (Fig. 7B). It was often observed that the ASCs had engrafted adjacent to skeletal myofibers containing centrally located, uncondensed nuclei, which is a hallmark of regenerating myofibers [38]. Groupings of ASCs were also visible around mature, α -actin-positive vessels (presumably arterioles).

DISCUSSION

This study is the first to directly demonstrate, using cells in which protein production has been modulated rather than conditioned medium or other indirect methods, that paracrine support accounts for a substantial portion of the *in vivo* beneficial effects produced by a therapeutic cell type. In particular, expression of HGF, a protein characterized as having many beneficial properties in a wide variety of different cells, is fundamentally important to the potency of ASCs in promoting reperfusion of ischemic skeletal tissues. Previous studies, including our own, have suggested that paracrine effects resulting from secretion of endogenous cytoprotective and angiogenic factors from stem and progenitor cells may be important [8, 10, 11]; however, this study clearly establishes the paracrine principle *in vivo*.

Identifying functional mechanisms of cell therapy is important in that it defines the boundaries which govern potential therapeutic applications. The complementary, but not mutually exclusive, concepts of therapeutic cells having “manager” or “software” functions versus providing “building blocks” or “hardware” [39, 40] are important in that they define the technical limits for therapeutic applications of cells. The near-term prospects for translating cell therapies to the clinic would appear to be much greater if the therapeutic effect is primarily through paracrine signaling to enhance endogenous repair mechanisms. This concept directly builds on earlier attempts to develop gene

and protein therapeutic modalities but has the potential for enhanced benefit over monotherapies due to the ability of viable cells to deliver a milieu of beneficial growth factors and cytokines to the site of injury. Specific targeting may occur through chemoattraction to the injury site as a result of injury-enhanced cytokine release combined with cell receptor-mediated anchoring to exposed ligands. Selective engraftment could confer additional advantages over other delivery vehicles by potentially reducing stimulation of unwanted cell growth resulting from nonselective exposure to nontarget tissues, which would be particularly important for broad-acting molecules such as HGF.

Although the potential medical benefits of regenerative cellular therapy are enormous, it is evident that achieving productive and directed differentiation of pluripotent cells for repair of tissues will certainly require much more research into the exact mechanisms controlling cellular plasticity in order to adequately address issues of both safety and efficacy. On the other hand, delivery of “mini-drug factories” in the form of cells is comparatively free of technical hurdles and is associated with fewer safety concerns for treating a wide variety of acute and chronic disorders. This would be especially true for cells such as ASCs that can be isolated in high abundance and used autologously with minimal manipulation.

ASCs secrete a plethora of cytokines and growth factors [8], yet the ability of the cells to promote angiogenesis when exposed to acute ischemia is substantially abolished by suppressing levels of a single gene product (HGF). Many of the other factors secreted by ASCs also have well-defined angiogenic effects, and certainly silencing factors, such as VEGF, would result in reduced angiogenic potential of ASCs. It is also possible that enhancing levels of VEGF or other potent growth factors could even overcome the defect caused by HGF silencing. We are presently testing many of these possibilities; presently, however, we chose to specifically silence HGF because it is one of the most abundant proteins secreted by ASCs, and there is a substantial body of literature demonstrating clear and consistent

broad spectrum benefits of exogenously added HGF in many models [41–43]. It is also clearly evident that HGF has critical functions in the endogenous pathophysiological processes inherent to diseased or injured tissues [44, 45].

Secretion of HGF in response to injury or disease appears to promote survival of cells in affected tissues as well as promote repair through attracting stem and progenitor cells. There is substantial evidence that HGF is a key molecule signaling the mobilization, migration, and homing of endogenous stem and progenitor cells originating in various tissue depots [46, 47]. Pathologically induced upregulation of HGF may recruit endogenous progenitor and stem cells to replace damaged tissues. Pluripotent cells from the bone marrow and blood constitutively express functional HGF receptor (c-Met) and are induced to migrate and differentiate in response to HGF gradients [46–49]. Populations of these cells possess the ability to differentiate into vascular and mesenchymal cell lineages [46, 50–52].

Numerous groups have demonstrated the plasticity of ASCs *in vitro*; thus, it is possible that, in addition to providing paracrine support, ASCs also contribute directly to repair through transdifferentiation and incorporation into regenerating skeletal muscle tissues. Although we cannot conclusively rule out differentiation of ASCs into myocytes and vascular cells, it appears that the predominant effect of ASCs, at least when provided systemically, is to support endogenous repair or limitation of ischemic damage. Immunofluorescence micrographs of thin sections revealed that ASCs within ischemic tissues were often localized in juxtaposition to arterioles and myofibers, of which the latter were undergoing active regeneration, as indicated by centrally located, uncondensed nuclei (Fig. 7B). To determine the temporal relationship between ASC engraftment and tissue regeneration, it will be necessary to conduct a more extensive time course to determine whether ASCs specifically home to the region of damaged myocytes and accelerate repair or whether chance localization of these cells promotes repair. These studies, as well as studies addressing transdifferentiation (or even fusion) events, are currently in progress.

Another intriguing hypothesis is that an autocrine loop exists in c-Met-expressing ASCs [53]. Given the antiapoptotic

effects of HGF, it is possible that silencing its expression disrupts this intracellular survival mechanism, making the cells expressing shRNA targeting HGF less fit for survival in hostile ischemic environments, as suggested by the *in vitro* experiments with serum deprivation (Fig. 1G). In fact, although equivalent numbers of both cell types are present in ischemic muscles immediately after infusion, reduced HGF expression by ASC-shHGF causes these cells to be cleared more rapidly during prolonged ischemia compared with ASC-shCtrl cells (Fig. 7). We are presently conducting experiments to determine whether a functional autocrine loop exists in these cells and whether reduced or enhanced signaling through HGF influences the survival of ASCs administered *in vivo*.

In conclusion, we and others have demonstrated the therapeutic potential of ASCs for promoting revascularization of ischemic tissues. These cells appear to have unique properties that make them attractive candidates for autologous cell therapy. Precisely because of these properties, it is possible to anticipate positive outcomes in imminent clinical trials in patients with peripheral arterial disease.

ACKNOWLEDGMENTS

We thank Jonathan Marsh, Shekhar Gangaraju, and Stephanie Merfeld-Clauss for providing excellent technical assistance with lentiviral vector constructions, MVEC sprouting assays, and EPC migration assays, respectively. This work was supported by American Heart Association fellowship number 0610049Z (to L.C.) and National Institutes of Health Grant R01 HL77688-01 (to K.L.M.). K.C. is supported in part by the Indiana Genomic Initiative (INGEN).

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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