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IFATS Collection: Adipose Stromal Cells Adopt a Proangiogenic Phenotype Under the Influence of Hypoxia

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ABSTRACT

Evolving evidence suggests a possible role for adipose stromal cells (ASCs) in adult neovascularization, although the specific cues that stimulate their angiogenic behavior are poorly understood. We evaluated the effect of hypoxia, a central mediator of new blood vessel development within ischemic tissue, on proneovascular ASC functions. Murine ASCs were exposed to normoxia (21% oxygen) or hypoxia (5%, 1% oxygen) for varying lengths of time. Vascular endothelial growth factor (VEGF) secretion by ASCs increased as an inverse function of oxygen tension, with progressively higher VEGF expression at 21%, 5%, and 1% oxygen, respectively. Greater VEGF levels were also associated with longer periods in culture. ASCs were able to migrate towards stromal cell-derived factor (SDF)-1, a chemokine expressed by ischemic tissue, with hypoxia augmenting ASC expression of the SDF-1 receptor (CXCR4) and potentiating ASC migration. In vivo, ASCs demonstrated the capacity to proliferate in response to a hypoxic insult remote from their resident niche, and this was supported by in vitro studies showing increasing ASC proliferation with greater degrees of hypoxia. Hypoxia did not significantly alter the expression of endothelial surface markers by ASCs. However, these cells did assume an endothelial phenotype as evidenced by their ability to tubularize when seeded with differentiated endothelial cells on Matrigel. Taken together, these data suggest that ASCs upregulate their proneovascular activity in response to hypoxia, and may harbor the capacity to home to ischemic tissue and function cooperatively with existing vasculature to promote angiogenesis. STEM CELLS *2009;27:266–274*

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

INTRODUCTION

Use of autologous stem or progenitor cells for the treatment of vascular disease is emerging as a promising new therapy [1, 2]. Some initial clinical trials suggest that cell-based induction of neovascularization may be a viable therapeutic option [3, 4]. The utility of both endothelial progenitor cells (EPCs) and bone marrow-derived mesenchymal stromal cells (BMMSCs) in augmenting ischemic neovascularization has been investigated with some rigor, although the limited prevalence of the former and the invasive means required to isolate the latter represent potential obstacles to the widespread clinical adoption of these cell types [3–5]. In contrast, adipose stromal cells (ASCs) are easily and abundantly harvested from lipoaspirate, are capable of differentiating into multiple tissue lineages, and may have a potential role in neovascularization and tissue engineering [6]. Existing evidence suggests that these cells can ameliorate ischemic tissue injury, produce the appropriate cytokine milieu to promote angiogenesis, and possibly also differentiate into endothelial cells [7–11]. The specific environmental cues that serve to induce the proangiogenic behavior of these cells remain poorly understood.

We and others have identified hypoxia as playing a fundamental role in ischemic neovascularization [12, 13]. Both EPCs and BMMSCs are capable of responding to ischemic injury in a manner proportional to the hypoxic stress [12, 14–16]. At the cellular level, the response to hypoxia is mediated by the master regulator, hypoxia inducible factor (HIF)-1 [13]. The α-subunit of this transcription factor (HIF-1α) is stabilized under low oxygen tension, and regulates expression of several genes, most notably vascular endothelial growth factor (*VEGF*), that are critical to the neovascular process [13]. Hypoxia also facilitates recruitment of progenitor cells to sites of new blood vessel development, and promotes cellular adoption of the functions and characteristics necessary for angiogenesis [12, 17].

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Given the central role of hypoxia in ischemic neovascularization, we evaluated its influence on the angiogenic properties of ASCs in order to determine whether hypoxia directly enhances their proangiogenic behavior. Our study specifically examined the ability of ASCs to secrete angiogenic cytokines and proliferate under hypoxic stress, migrate towards a chemokine known to be expressed in ischemic tissue, and assume endothelial features when subjected to a hypoxic environment.

MATERIALS AND METHODS

ASC Harvest and Culture

Wild-type C57/BL6 and transgenic green fluorescent protein (GFP) mice (β-actin/GFP) were purchased from Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). Inguinal fat pads were excised from 6-week-old mice and washed in serial dilutions of Betadine (Purdue Frederick Co., Norwalk, CT, http://www.pharma.com). Fat pads were finely minced and the resulting tissue was digested with 0.075% type II collagenase (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) dissolved in Dulbecco's Modified Eagle's Medium (DMEM) (4.5 g/ml glucose; Gibco, Carlsbad, CA, http://www.invitrogen.com) for 30 minutes at 37°C. Collagenase was inactivated by adding two volumes of cell culture media (DMEM, 10% fetal bovine serum [FBS], 1% penicillin/streptomycin; Gibco) and the solution was subsequently pelleted to separate mature adipocytes from the remaining stromal-vascular fraction (SVF). The supernatant was discarded, and the pellet was resuspended and filtered through a 100-μm cell strainer to remove undigested tissue fragments. Cells were pelleted, resuspended in cell culture medium, and plated at 3×10^6 cells per 100 cm^2 culture plate. Early passage cells (P3–P4) were used in all experiments, as specified below.

Animal care was provided in accordance with the Stanford University School of Medicine guidelines and policies for the use of laboratory animals. All protocols were approved by the Administrative Panel on Laboratory Animal Care at the Stanford University School of Medicine.

Flow Cytometric Analysis of ASCs

ASCs were incubated with fluorescently conjugated antibodies against CD45 (fluorescein isothiocyanate [FITC]), stem cell antigen-1 (Sca-1, phycoerythrin [PE]), CD44 (allophycocyanin), lineage (Lin) markers (TER119, B220, CD4, CD8, CD11b, Gr-1; PE-Cy5), CD31 (FITC), Flk-1 (PE), and CXCR4 (PE) for 30 minutes at 4°C in 2% FBS in phosphate-buffered saline (PBS) (1 μl antibody per 1×10^6 cells). Cells were also incubated with an unconjugated antibody against CD90 and subsequently labeled with PE-Cy5 anti-rat secondary antibody under the same conditions. Cells not stained with these antibodies were incubated with the appropriate isotype controls or left unstained. Passage 3 cells were trypsinized, washed, and then run on a Becton Dickinson-LSR Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Data were analyzed using CellQuest digital fluorescence-activated cell sorting software (Becton, Dickinson and Company). Lineage antibodies were purchased from eBiosciences (San Diego, CA, http://www.ebioscience.com). PE-Cy5 anti-rat secondary antibody was purchased from Caltag Laboratories (Burlingame, CA, http://www.caltag.com). All other antibodies were purchased from BD Biosciences (San Diego, CA, http://www.bdbiosciences.com).

In Vitro Hypoxia

In vitro hypoxia experiments were performed with a customized hypoxic incubator (Biospherix X Vivo Hypoxia Chamber; BioSpherix, Lacona, NY, http://www.biospherix.com) that continuously infuses a calibrated gas mixture (95% N_2, 5% CO_2). Experiments were performed at oxygen concentrations of 21%, 5%, and 1%.

VEGF Enzyme-Linked Immunosorbent Assay

Conditioned medium was obtained from passage 3 ASCs cultured at various oxygen tensions. VEGF levels were assayed using a Quantikine murine VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) in accordance with the manufacturer's protocol. Cells were harvested at the same time that the medium was removed, and cell counts were performed with a hemacytometer. VEGF levels were normalized to the number of cells at the time of harvest, expressed as pg/ml per 10^6 cells. Experiments were performed with $n = 3$, in quadruplicate.

Western Blot

Cultured ASCs (passage 3) were placed in normoxia or hypoxia (1% oxygen) for 24 hours. Nuclear protein was subsequently obtained using Pierce Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, http://www.piercenet.com) according to manufacturer's instructions. Protein content was standardized using the BCA Protein Assay kit (Pierce). Twenty micrograms of nuclear protein was separated by SDS-PAGE on 7.5% gels, transferred to polyvinylidene difluoride membranes, and blocked with 5% milk in tris-buffered saline with Tween. Protein detection was performed with primary antibodies for HIF-1α (Novus Biologicals, Littleton, CO, http://www.novusbio.com), with β-actin serving as an internal loading control (Lab Vision, Fremont, CA, http://www.labvision.com). Blots were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com). Blots were developed with ECL reagent (Amersham) and exposed for 1–10 minutes on Kodak Biomax-MS film (Sigma-Aldrich). Band densities were quantified with ImageJ software (National Institutes of Health, Bethesda, MD, http://www.nih.gov). Experiments were performed with $n = 3$.

Migration Assay

ASCs (2×10^4, passage 3) suspended in DMEM were seeded on the upper chamber of 8-μm pore size Transwell plates (Corning Costar, Corning, NY, http://www.corning.com). For the hypoxia group, ASCs were incubated in 1% oxygen for 24 hours prior to commencement of the assay. For the anti-CXCR4 antibody group, cells were pretreated with 1 μl of CXCR4 antibody (BD Biosciences) for 30 minutes prior to beginning the assay, and 1 μl of antibody was also added to the upper chamber of the Transwell plate following cell seeding. Recombinant murine stromal cell-derived factor (SDF)-1 (100 nM; R&D Systems) was added to the bottom chamber. Plates were placed in 21% oxygen and the cells were allowed to migrate for 6 hours at 37°C. Nonmigrating cells were removed from the top surface of the upper chamber using a cotton tip applicator. Migrating cells adhering to the undersurface of the upper chamber were measured by 4′, 6′-diamidino-2-phenylindole (Vector Corp., Marion, IA, http://www.vectorcorporation.com) staining. Experiments were performed with $n = 3$. In total, five random fields of each membrane were analyzed using a Zeiss Axioplan two microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com) at 200× magnification.

ASC Proliferation Assay

ASCs (2×10^3, passage 3) were seeded in 96-well plates and serum starved in DMEM containing 1% FBS for 24 hours to induce quiescence. The medium was then changed to fully supplemented medium (10% FBS) and cultured at varying oxygen tensions. At 18 hours, 5-bromo-2′-deoxyuridine (BrdU) (Cell Proliferation ELISA, BrdU; Roche Bioproducts, Basel, Switzerland, http://www.roche-applied-science.com) was added to the medium, and following an additional 6 hours, cell proliferation was assayed according to the manufacturer's instructions. Experiments were performed with $n = 3$, in quadruplicate.

Murine Ischemia Model and In Vivo ASC Analysis

A reproducible model of graded soft tissue ischemia was created on the dorsum of C57/BL6 mice as previously described [12]. Briefly,

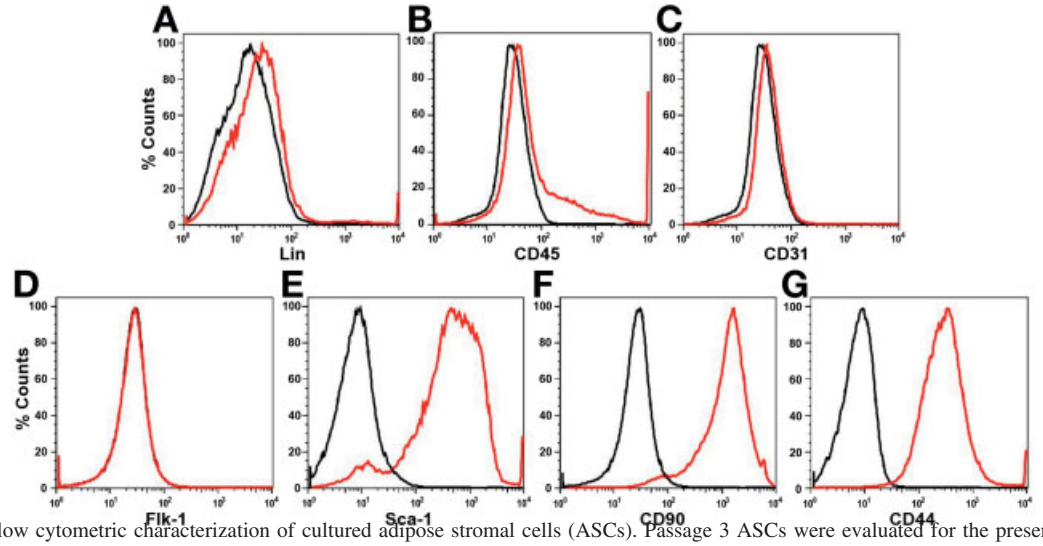

Figure 1. Flow cytometric characterization of cultured adipose stromal cells (ASCs). Passage 3 ASCs were evaluated for the presence of lineage (Lin) markers **(A)**, CD45 **(B)**, CD31 **(C)**, Flk-1 **(D)**, Sca-1 **(E)**, CD90 **(F)**, and CD44 **(G)**. Red curves represent cells incubated with antibody against the noted marker, whereas black curves show cells labeled with the appropriate isotype control.

a U-shaped peninsular incision (1.25 cm × 2.5 cm) was made penetrating the skin, dermis, and underlying adipose tissue. This tissue was elevated from the underlying muscular bed and a 0.13-mm thick silicone sheet was inserted to separate the skin from the underlying tissue bed. The skin flap was sutured back into place with interrupted 6–0 nylon. Ischemia is graded from proximal to distal and this was confirmed by tissue oximetry and color laser Doppler. Oxygen tensions are highest in the intact/cranial portion of the raised peninsular tissue bed, and decrease in a gradient moving more caudally (reaching a nadir of 2 mmHg in the most ischemic area of skin). Tissue oxygen levels typically recover to their baseline values 14–21 days postsurgery.

At designated time points (0, 7, 14, and 21 days), mouse inguinal fat pads were harvested and the SVF was isolated as described above. Undigested fragments were removed by filtration through a 100-μm cell strainer, and the cells were subsequently pelleted and resuspended in 2% FBS in PBS. Cells were stained with fluorescently conjugated antibodies against CD45 (PE; BD Biosciences) and Sca-1 (FITC; BD Biosciences). Cells were washed and then incubated with unconjugated antibodies against Lin markers (BD Biosciences) and labeled with a PE-Cy5 anti-rat secondary antibody (Caltag Laboratories). Staining conditions, flow cytometry, and data analysis were as described above. Experiments were performed with $n = 5$ mice per timepoint.

Endothelial Differentiation of ASCs

ASCs (2×10^4, passage 3) were seeded in six-well plates in medium consisting of DMEM plus 10% FBS. Plates were placed under 21% or 1% oxygen. Recombinant mouse VEGF (50 ng/ml; R&D Systems) was added to appropriate wells after initial seeding and during every-other-day medium changes. Following 5 days of such treatment, cells were harvested and suspended in 2% FBS in PBS. Cells were stained with fluorescently conjugated antibodies to CD31 (FITC) and Flk-1 (PE). Staining conditions, flow cytometry, and data analysis were as described above. Experiments were performed with $n = 3$.

Matrigel Tubule Assay

Matrigel (BD Biosciences) was thawed and placed in four-well chamber slides at 37°C for 30 minutes to allow solidification. One $\times 10^5$ GFP$^+$ ASCs (passage 3) were plated alone on Matrigel and incubated at 37°C under 21% or 1% oxygen for 18 hours. Five $\times 10^4$ GFP$^+$ ASCs were also coplated with 5×10^4 PKH26-labeled murine bEND.3 endothelial cells (American Type Culture Collection, Manassas, VA, http://www.atcc.org) on Matrigel and incubated under the same conditions. This assay was repeated using 1×10^5 PKH26-labeled murine bEND.3 endothelial cells plated alone, as above. Tubule formation was defined as a structure exhibiting a length four times its width. Experiments were performed with $n = 6$. Tubule counts were determined in 10 random fields per well using an inverted Leica DMIL light microscope (Leica Microsystems GmbH, Wetzlar, Germany, http://www.leica-microsystems.com) at 100× magnification. Confocal microscopy was performed using a Leica TCS SP2 AOBS confocal system. Three-dimensional confocal images were generated from individual slices using high-resolution opacity rendering. Fluorescence microscopy was performed with a Leica DMI4000 B microscope.

Statistical Analysis

Results are presented as mean ± standard error of the mean. Data analysis was performed using the Student's *t*-test (Matrigel, migration, endothelial differentiation, CXCR4 expression, and HIF-1α quantitation assays) or one-way analysis of variance (VEGF ELISA and proliferation studies). Results were considered significant for $p < .05$. Data were analyzed with Prism software (GraphPad Software Inc., La Jolla, CA, http://www.graphpad.com).

RESULTS

Flow Cytometric Characterization of ASCs

Early passage (P3) ASCs expressed negligible or low levels of Lin markers and the panhematopoietic marker CD45 (Fig. 1). Also largely absent were the endothelial surface markers CD31 and Flk-1. Greater than 85% of ASCs expressed Sca-1, and 96% of cells expressed CD90. Virtually all cells expressed CD44, a cell surface glycoprotein involved in cell–cell and cell–matrix interactions. Such an expression profile is consistent with previous reports for murine ASCs [9, 18].

Hypoxia Upregulates VEGF and HIF-1α Expression by ASCs

Prior studies have demonstrated that ASCs secrete greater levels of VEGF than control cell types (e.g., vascular smooth muscle) and also upregulate VEGF expression under hypoxia [8, 9]. We sought to clarify the impact of hypoxia on VEGF expression by ASCs, specifically examining the influence of varying oxygen levels and time (i.e., days in culture) on VEGF secretion (Fig. 2A). ASCs demonstrated an oxygen tension-dependent increase in VEGF expression, with levels of the cytokine increasing progressively under 21%, 5%, and 1% oxygen after 1 day in

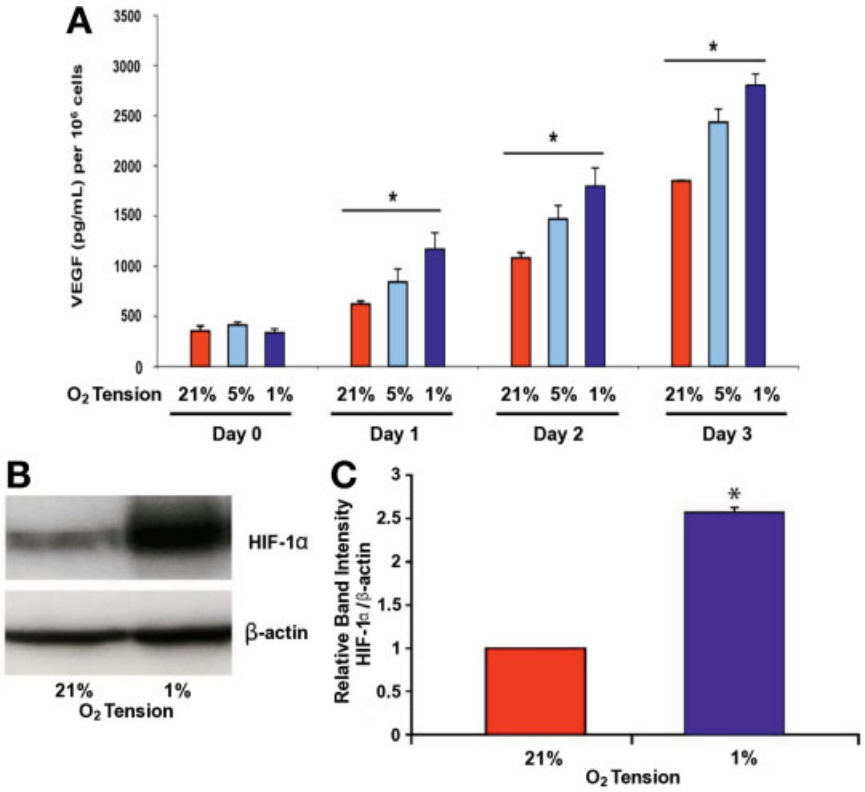

Figure 2. Hypoxia upregulates ASC expression of VEGF and HIF-1α. **(A):** VEGF secretion by ASCs incubated at 21%, 5%, and 1% oxygen. Day 0 values represent levels of VEGF shortly after medium change. VEGF levels were normalized to the number of cells at the time of harvest, expressed as pg/ml per 10^6 cells. *$p < .01$. **(B):** Western blot showing HIF-1α expression by ASCs grown in normoxia (21% oxygen) and hypoxia (1% oxygen). **(C):** Western blot densitometric analysis of HIF-1α expression by ASCs grown in normoxia and hypoxia. *$p < .05$. Abbreviations: ASC, adipose stromal cell; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor.

culture (623, 843, and 1,169 pg/ml, respectively; $p < .01$). ASCs maintained for longer periods in culture (2 and 3 days) showed two- to threefold higher levels of VEGF expression at each oxygen tension (21% O_2: 623–1,082–1,851 pg/ml, 5% O_2: 843–1,471–2,435 pg/ml, 1% O_2: 1,169–1,796–2,802 pg/ml, for days 1, 2, and 3, respectively; $p < .05$). Thus, these data suggest an oxygen- and culture-dependent increase in VEGF secretion by ASCs.

Increased HIF-1α expression leads to transcriptional activation of several genes including *VEGF*, and represents an important initial step in the cascade of events that mediate ischemic neovascularization [19]. Given its fundamental role in the cellular response to hypoxia, we evaluated HIF-1α expression in ASCs subjected to an acute (24-hour) hypoxic exposure (Fig. 2B, 2C). As expected, HIF-1α expression was greater than twofold higher in ASCs cultured in hypoxia (1% oxygen) compared with cells grown in normoxia ($p < .05$).

Hypoxia Augments ASC Migration

Previous work has suggested that ASCs can enhance the migratory ability of endothelial cells [9]. We examined the intrinsic potential of ASCs to migrate towards the chemokine SDF-1 (Fig. 3A). SDF-1 is critical to the recruitment of stem cells and vascular progenitors to sites of neovascularization and tissue regeneration [20, 21]. For ASCs cultured under normoxic conditions, approximately fourfold more cells migrated towards a recombinant SDF-1 stimulus as compared with control groups, demonstrating that ASCs are indeed responsive to this chemokine ($p < .005$). When the same experiment was performed with ASCs pretreated in hypoxia (1% oxygen) for 24 hours, approximately 2.5-fold more cells migrated towards SDF-1 relative to the normoxia group, suggesting that hypoxia significantly augments the migratory capacity of these cells ($p < .005$).

Binding of SDF-1 to its receptor, CXCR4, mediates the homing of circulating stem cells to sites of neovascularization and tissue repair [12]. Subjecting ASCs pretreated in hypoxia to anti-CXCR4 antibody attenuated the migration of these cells towards SDF-1 ($p < .001$). Flow cytometric analysis showed approximately 0.5% of ASCs expressing CXCR4 under normoxic conditions, with exposure to hypoxia for 48 hours quadrupling the prevalence of CXCR4 on these cells (Fig. 3B, 3C) ($p < .05$). Taken together, these data suggest that the observed increase in ASC migration under hypoxia may be due in part to upregulation of this receptor.

Hypoxia Stimulates ASC Proliferation In Vitro and In Vivo

ASCs have been shown to promote the proliferation and viability of endothelial cells [8, 9]. As the ability of ASCs to enhance their own proliferative capacity under conditions of low oxygen tension would likely contribute to their functional significance in the setting of ischemic neovascularization, we evaluated the influence of hypoxia on ASC proliferation both in vitro and in vivo.

ASCs subjected to 18 hours of hypoxia in vitro showed increasing rates of proliferation under progressively lower oxygen tensions (Fig. 4A) (0.43, 0.59, and 0.70 [BrdU incorporation, arbitrary units] at 21%, 5%, and 1% oxygen, respectively; $p < .001$), further demonstrating the capacity of these cells to exhibit a proportionate response to hypoxic stress.

For in vivo studies, C57/BL6 mice were subjected to a dorsal soft tissue ischemia model, in which a reproducible ischemic gradient (confirmed by tissue oximetry and color laser Doppler evaluation) is achieved through elevation of a peninsular flap (Fig. 4B) [12]. Oxygen tensions are highest in the intact/cranial portion of the raised peninsular tissue bed, and decrease in a gradient moving more caudally. The SVF of mouse inguinal fat pads was subsequently harvested at the designated postoperative days (0, 7, 14, and 21).

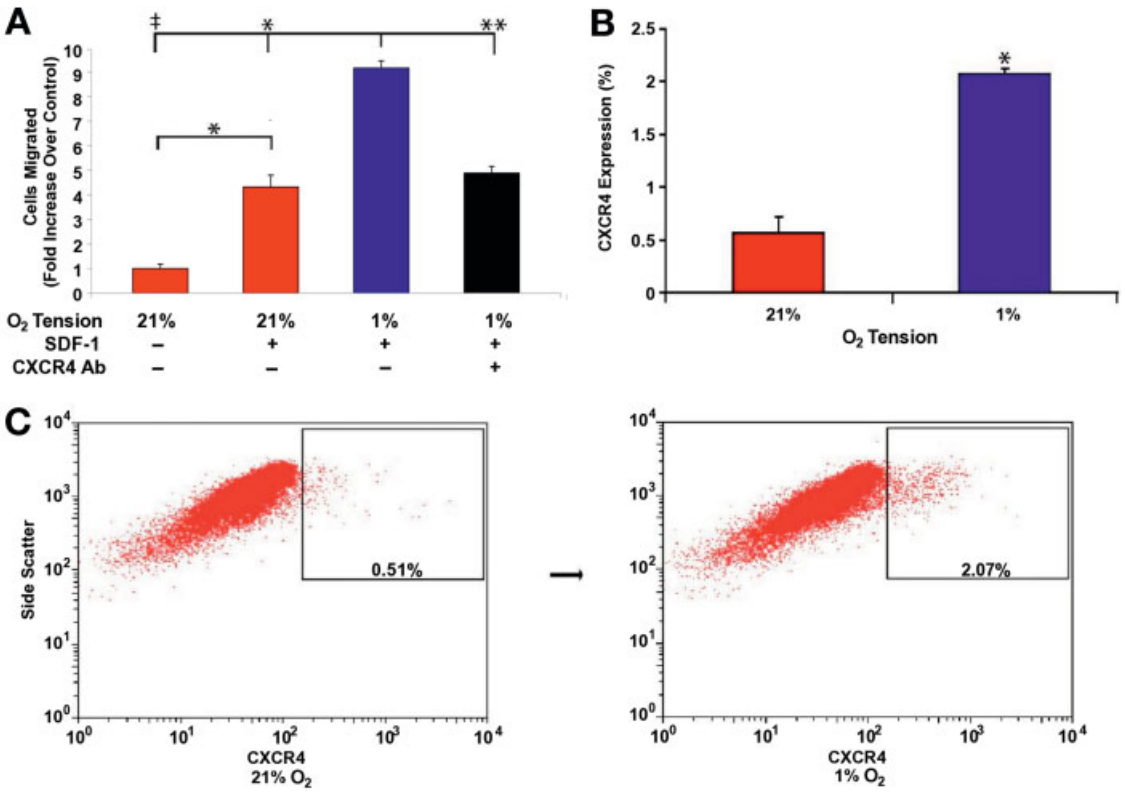

Figure 3. Hypoxia augments ASC migration to SDF-1. **(A):** Migration of ASCs to SDF-1 in normoxia (21% oxygen), hypoxia (1% oxygen), and hypoxia with addition of anti-CXCR4 antibody. *$p < .01$, **$p < .001$, ‡$p < .00001$. **(B):** CXCR4 expression by ASCs after 48-hour exposure to normoxia or hypoxia. *$p < .05$. **(C):** Representative flow cytometry plot of CXCR4 expression by ASCs after 48-hour exposure to normoxia or hypoxia. Abbreviations: Ab, antibody; ASC, adipose stromal cell; SDF, stromal cell-derived factor.

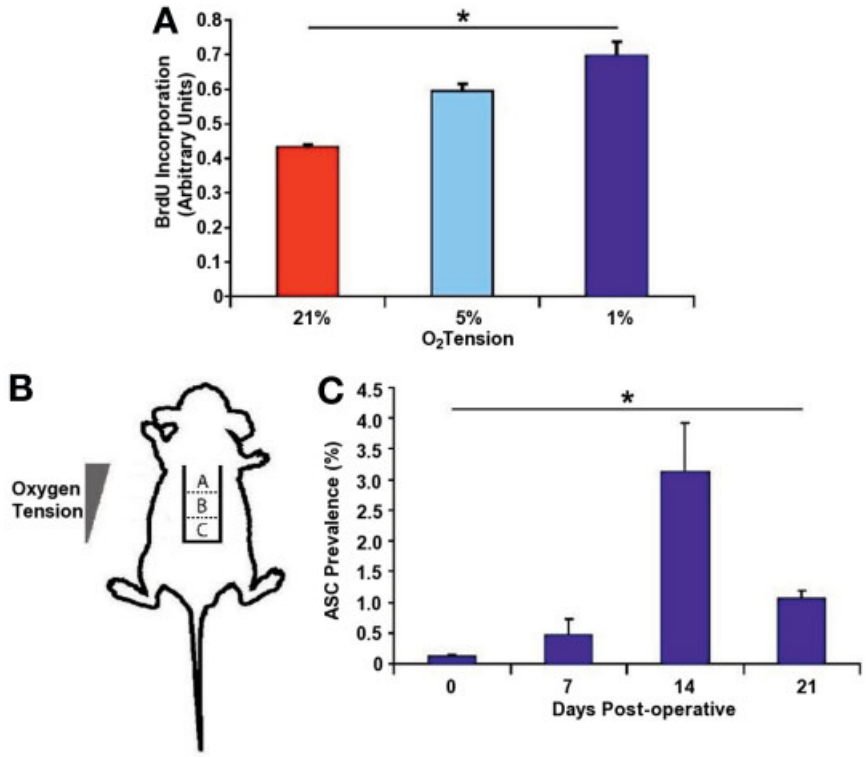

Figure 4. Hypoxia induces ASC proliferation in vitro and in vivo. **(A):** In vitro ASC proliferation, as measured by BrdU incorporation, at 21%, 5%, and 1% oxygen. *$p < .001$. **(B):** Schematic of murine dorsal soft tissue ischemia model. Blood supply is derived only from the superior skin pedicle, resulting in a reproducible gradient of tissue ischemia. The tissue areas are labeled from least to most ischemic (i.e., $A_{O2} > B_{O2} > C_{O2}$). Tissue oxygen levels within the most distal portion of the flap can drop to <5 mmHg, with oxygen levels typically recovering to baseline values 14–21 days postsurgery. **(C):** Prevalence of ASCs in mouse inguinal fat pads following ischemic surgery as a function of postoperative day. *$p < .01$. Abbreviations: ASC, adipose stromal cell; BrdU, 5-bromo-2′-deoxyuridine.

The cell surface marker phenotype Sca-1^+Lin$^-$CD45$^{-/lo}$ was used to prospectively identify a representative subset of the ASC population. We observed a progressive rise in the prevalence of these cells within the inguinal fat pads following

creation of the ischemic flap, with a peak at postoperative day 14 and a subsequent decrease towards baseline values (Fig. 4C) (prevalence: 0.12%, 0.47%, 3.1%, and 1.06% at days 0, 7, 14, and 21, respectively; $p < .01$). These data indicate that ASCs are able to proliferate in response to a peripheral hypoxic insult in vivo, and provide circumstantial evidence to suggest the potential endogenous involvement of this population in ischemic tissue repair.

ASCs Partly Adopt an Endothelial Phenotype Under Hypoxia

We have previously demonstrated that ASCs are capable of assuming endothelial features when subjected to supraphysiologic levels of VEGF in the setting of hypoxia [15]. Here, we evaluated the role of hypoxia alone in inducing an endothelial phenotype in ASCs as evidenced by upregulation of endothelial surface markers, and also by their ability to tubularize when placed on Matrigel.

ASCs were cultured under conditions of normoxia, hypoxia (1% oxygen), or hypoxia plus VEGF (50 ng/ml) for 5 days. Cells were subsequently harvested and evaluated by flow cytometry for concurrent expression of the endothelial markers Flk-1 and CD31 (Fig. 5A). Hypoxia did not induce a significant increase in the number of Flk-1$^+$CD31$^+$ cells when compared with cells grown in normoxia (0.86 versus 0.49%; $p = .36$). When VEGF was added to cells cultured in hypoxia, the prevalence of cells doubly positive for Flk-1 and CD31 increased markedly, paralleling results from our prior studies (3.69%; $p < .01$ versus cells cultured in hypoxia alone).

ASCs are able to promote tubule formation by endothelial cells, and existing evidence suggests that BMMSCs show improved tubularization under hypoxic conditions [9, 17]. We consequently evaluated the influence of hypoxia on the induction of tubulogenesis by ASCs. ASCs were seeded on Matrigel alone or in the presence of murine bEND.3 endothelial cells, and subjected to normoxia or hypoxia (1% oxygen) for 18 hours (Fig. 5B, 5C). ASCs demonstrated relatively poor tubule formation under both normoxic and hypoxic conditions (0.38 and 0.52 tubules per high power field [HPF], respectively; $p = .45$). Under normoxic conditions, the addition of bEND.3 cells resulted in greater tubule formation than when the assay was performed in either normoxia or hypoxia for ASCs alone (4.37 tubules per HPF; $p < .001$). Tubularization was most marked when bEND.3 cells and ASCs were placed in hypoxia (7.33 tubules per HPF; $p < .001$ versus all other conditions). We also evaluated the formation of tubules from bEND.3 cells seeded alone on Matrigel, and noted poor tubulogenesis in normoxia (0.37 tubules per HPF), a finding described previously [22]. The ability of these cells to tubularize in hypoxia was similarly limited (0.13 tubules per HPF), suggesting that the enhanced tubularization observed with coseeding of ASCs and bEND.3 cells occurs through cooperative interaction between the two cell types. This was qualitatively confirmed by confocal and fluorescence microscopy, with tubules from GFP$^+$ ASCs coseeded with PKH26-labeled bEND.3 cells on Matrigel showing both cell types organizing in concert (Fig. 5D and supporting information Fig. 1).

In aggregate, these data demonstrate that ASCs are capable of assuming certain endothelial features under the influence of hypoxia, with significant adoption of endothelial character occurring in the presence of endothelial cells or exogenous VEGF.

DISCUSSION

In the present study, we provide evidence in support of the notion that, within the proper setting, ASCs are able to assume functions that would promote angiogenesis. We have specifically focused on the contextual role of hypoxia in mediating the proneovascular behavior of these cells. Previous reports have identified the capacity of murine and human ASCs to express high levels of angiogenic cytokines such as VEGF and hepatocyte growth factor, with evidence to suggest that VEGF is selectively upregulated by hypoxia [8, 9]. Our findings are of particular significance because they demonstrate the ability of ASCs to secrete VEGF (and proliferate) in a manner directly proportional to the hypoxic insult. This capacity to adapt commensurately to external hypoxic stress would be especially important at sites of ischemia, where tissue injury is likely nonuniform, with resultant gradients of hypoxia. Evidence suggests, however, that a threshold level of hypoxic exposure probably exists for these cells, beyond which they may become more quiescent and limit their progrowth activity [23].

The ability of ASCs to enhance vascular growth would be of limited consequence if these cells were unable to home to ischemic/regenerating tissue. Our demonstration that ASCs are able to migrate towards SDF-1 in vitro, and further, that hypoxic exposure increases their expression of CXCR4 and augments this migratory ability, lends additional support to the potential functional significance of these cells during ischemic neovascularization. Strategies aimed at increasing CXCR4 expression by ASCs (e.g., hypoxic pretreatment) or enhancing SDF-1 expression at sites of vascular remodeling may prove to be effective in exploiting the angiogenic properties of these cells. Our in vivo experiments were able to identify a proliferative response by resident ASCs to a remote hypoxic stimulus, although our model did not define the ultimate fate of these cells or directly establish their capacity for long distance migration. Future experiments will seek to characterize the precise role of endogenous ASCs in hypoxia-induced neovascularization, and will also explore the in vivo significance of the SDF-1:CXCR4 axis in ASC recruitment and homing.

Prior work has suggested the capacity for human ASCs to differentiate into endothelial cells when used in mouse models of hindlimb ischemia [10, 11]. We did not observe a dramatic adoption of endothelial phenotypic features by ASCs exposed only to hypoxia, although it is likely that differentiation of ASCs into endothelial cells requires the presence of multiple factors not accounted for in our in vitro experiments. Our finding of greater tubule formation and cooperative intercellular interaction when ASCs were seeded with endothelial cells on Matrigel under hypoxia suggests that these cells may be capable of situating themselves within the neovascular environment in a manner that enhances their paracrine, proangiogenic effects. Although we cannot exclude the possibility that these results may be influenced by contaminating endothelial cells within the primary cell population, the marker profiles described in Figure 1 provide circumstantial evidence against a committed endothelial population at this stage.

Hypoxia does not universally induce a progrowth phenotype in ASCs, as evidenced by previous reports documenting the inhibition of their differentiation capacity in the setting of low oxygen tension. Impaired adipogenesis in the murine 3T3-L1 cell line has been linked to increased HIF-1α expression, and studies using primary ASCs with multilineage potential have also demonstrated attenuated differentiation into specific mesodermal lineages (e.g., muscle, bone, fat) in the hypoxic environment [24–26]. Cartilage formation is known to occur within a hypoxic niche, and there is evidence to suggest that low oxygen tension enhances chondrogenesis, and further, that targeted deletion of HIF-1α in ASCs substantially inhibits the chondrogenic pathway [27–29]. Based on these data and the results of our experiments, we speculate that low oxygen tension may directly influence the "flux" of ASCs along their potential

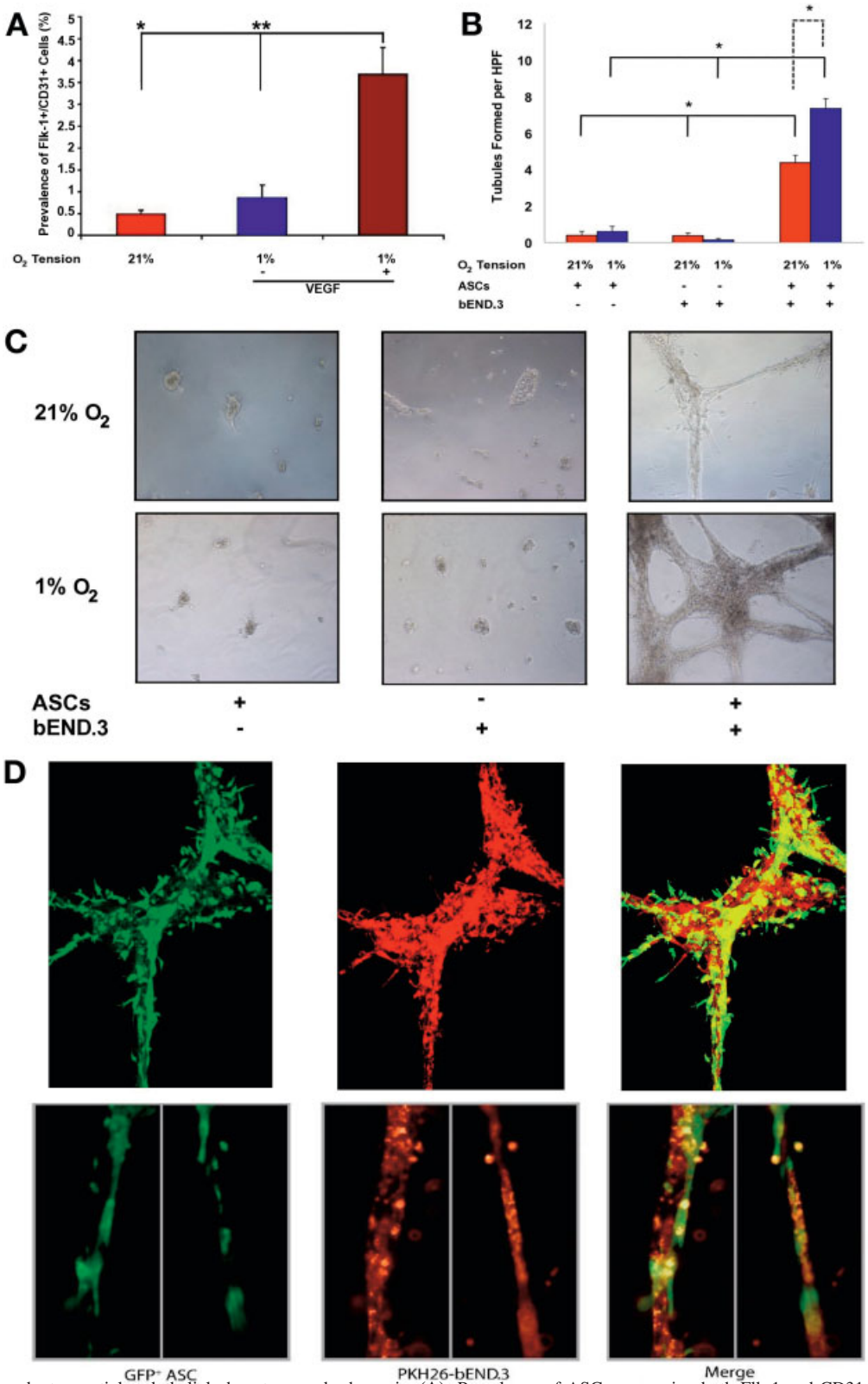

Figure 5. ASCs adopt a partial endothelial phenotype under hypoxia. **(A):** Prevalence of ASCs expressing both Flk-1 and CD31 after 5 days of exposure to normoxia (21% oxygen), hypoxia (1% oxygen), or hypoxia plus 50 ng/ml of VEGF. $*p < .05$, $**p < .01$. **(B):** Tubule formation of murine ASCs and bEND.3 endothelial cells on Matrigel, under normoxia and hypoxia. $*p < .0001$. **(C):** Representative light microscopy images of the experiment described in **(B)**. **(D):** Confocal microscopy (top) and fluorescence microscopy (bottom) images of GFP^+ ASCs (green) coseeded with PKH26-labeled bEND.3 cells (red) on Matrigel under hypoxic conditions. Merged images show ASCs and bEND.3 cells working cooperatively in the formation of tubules. All images were taken at 100× magnification. Abbreviations: ASC, adipose stromal cell; GFP, green fluorescent protein; HPF, high power field; VEGF, vascular endothelial growth factor.

differentiation pathways such that certain mesodermal lineages are inhibited, whereas others (angiogenic precursors and possibly chondrogenic progenitors) are promoted. This effect is likely heavily dependent upon the local tissue milieu, with certain conditions selectively enhancing either neovascularization or chondrogenesis.

It is notable that BMMSCs demonstrate responses to hypoxia analogous to their adipose-derived counterparts. When subjected to low oxygen tension, BMMSCs have been shown to secrete angiogenic cytokines such as VEGF, increase their rates of proliferation, and exhibit improved migration and tubularization [17, 30, 31]. Indeed, as with ASCs, some evidence exists to suggest that hypoxia may impair the osteogenic differentiation capacity of BMMSCs [32]. Such commonalities suggest a conserved response program to hypoxia for stromal progenitor cells regardless of tissue derivation, and provide circumstantial support for the notion that ASCs may harbor an efficacy similar to BMMSCs for the treatment of diseases characterized by ischemia and impaired neovascularization. Should future investigation prove this to be true, the relative abundance and accessibility of ASCs would make them the more attractive therapeutic choice.

SUMMARY

Our studies demonstrate the ability of ASCs to adopt certain proneovascular functions, such as angiogenic growth factor secretion and increased proliferation, in a manner commensurate with the surrounding hypoxic stress. Hypoxia also promoted the migratory ability of these cells, and enhanced their capacity for tubule formation in the presence of endothelial cells. Harnessing the full therapeutic potential of ASCs will require a more rigorous understanding of the hypoxia-induced alterations in their function.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors have indicated no potential conflicts of interest.

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