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Angiogenesis by Transplantation of HIF-1α Modified EPCs Into Ischemic Limbs

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Abstract Hypoxia inducible factor-1α (HIF-1α) is a key determinant of oxygen-dependent gene regulation in angiogenesis. HIF-1α overexpression may be beneficial in cell therapy of hypoxia-induced pathophysiological processes, such as ischemic heart disease. To address this issue, human peripheral blood mononuclear cells (PBMNCs) were induced to differentiate into endothelial progenitor cells (EPCs), and then were transfected with either an HIF-1α-expressing or a control vector and cultured under normoxia or hypoxia. Hypoxia-induced HIF-1α mRNA and protein expression was increased after HIF-1α transfection. This was accompanied by VEGF mRNA induction and increased VEGF secretion. Hypoxia-stimulated VEGF mRNA induction was significantly abrogated by HIF-1α-specific siRNA. Functional studies showed that HIF-1α overexpression further promoted hypoxia-induced EPC differentiation, proliferation and migration. The expressions of endothelial cell markers CD31, VEGFR2 (Flk-1) and eNOS as well as VEGF and NO secretions were also increased. Furthermore, in an in vivo model of hindlimb ischemia, HIF-1α-transfected EPCs homed to the site of ischemia. A higher revascularization potential was also demonstrated by increased capillary density at the injury site. Our results revealed that endothelial progenitor cells ex vivo modification by hypoxia inducible factor-1α gene transfection is feasible and may offer significant advantages in terms of EPC expansion and treatment efficacy. J. Cell. Biochem. 103: 321–334, 2008.

Key words: hypoxia inducible factor-1 α; hypoxia; endothelial progenitor cells; endothelial cells; angiogenesis

There has been considerable work aimed at developing novel therapies for ischemic disease. The majority of therapeutic neovascularization research has focused on gene therapy to effect revascularization of ischemic foci. Preclinical studies have raised the prospect of angiogenic growth factors such as vascular endothelial growth factor (VEGF) as a candidate of therapeutic neovascularization. However, VEGF treatment has potential side effects such as increased vascular permeability, interstitial edema and inflammation [Su et al., 2002]. Thus it would be desirable to seek treatment alternatives that augment VEGF action while avoiding its deleterious effects. The discovery of the transcription factor hypoxia-inducible factor-1 (HIF-1) gave fresh insights into the regulation of hypoxia-inducible genes [Semenza and Wang, 1992; Hockel et al., 1996]. A recent paper reported that more than 2% of all human genes are regulated by HIF-1 in endothelial cells (ECs) [Manalo et al., 2005]. HIF-1 regulates genes encoding angiogenic cytokines,

Abbreviations used: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor-1; EC, endothelial cells; PBMNCs, peripheral blood mononuclear cells; SDF-1, stromal-derived factor 1; EPCs, endothelial progenitor cells; BBE, bovine brain extract.

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including VEGF and stromal-derived factor 1 (SDF-1), providing a mechanism by which cells are assured of adequate perfusion. Thus, HIF-1 is viewed as an extrinsic regulator of ECs and their progenitors, which express Flk-1, Flt-1, and CXCR4 and SDF-1, respectively [Semenza, 2006]. These data have demonstrated that HIF-1 serves as an upstream controller of the expression of key angiogenic factors to further activate ECs.

Regulation of HIF-1, which is a dimer of the inducible HIF-1α and constitutive HIF-1β, is controlled by oxygen tension [Wang and Semenza, 1993; Semenza, 2001; Lee et al., 2004]. HIF-1 is specifically activated in hypoxia and induces target genes involved in cell proliferation, vascular development [Lando et al., 2002], vascular tone, and energy metabolism [Flamme et al., 1997]. HIF-1 directly activates the transcription of the VEGF gene by binding to a hypoxia response element located 5′ to the gene [Forsythe et al., 1996].

Asahara et al. [1997] discovered that peripheral blood of adult species contains progenitor ECs (EPCs) that presumably derived from CD34-positive mononuclear cells and that the transfused EPCs participated in neovascular formation in mature immunodeficient animals. The use of EPCs as a tool for cell therapy of diseases involving defects of angiogenesis, such as ischemia, was envisioned as soon as these cells were discovered [Vasa et al., 2001; Strauer et al., 2002; Tateishi-Yuyama et al., 2002]. However, the feasibility of using these cells as a cell therapy product in patients with ischemic diseases was hampered by the rarity of the cells in the peripheral blood. The extent to which these cells could be expanded without losing their vascularization capacities is an important issue. Although HIF-1 is essential in vascular development during embryogenesis [Ryan et al., 1998], its role in the development of adult stem cells and progenitor cells, especially the EPCs has not been examined. The present study would like to explore the feasibility and efficacy of combining HIF-1α and EPCs for therapeutic angiogenesis. We specifically asked whether overexpressing HIF-1α could promote EPC expansion under normoxic and hypoxic conditions, and whether EPCs transduced with HIF-1α could functionally facilitate therapeutic neovascularization in an in vivo model of hindlimb ischemia.

MATERIALS AND METHODS

EPC Culture

EPCs were cultured using an improved method based on the Kalka method [Kalka et al., 2000]. Each PBMNC sample was isolated from 200 ml human peripheral blood by density gradient centrifugation with Ficoll-Paque plus (Amersham Bioscience, Piscataway, NJ). Cells were plated on 6 cm culture dishes coated with human 2 μg/cm^2 fibronectin (Chemicon, Temecula, CA) and cultured in M199 medium (Invitrogen, San Diego, CA). After 1 h in culture, all the non-adherent cells were collected and cultured in M199 medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 12 μg/ml bovine brain extract (BBE, Sigma, St. Louis, MO), 10 ng/ml human VEGF165 (CytoLab/Peprotech, Rehovot, Israel), 2 ng/ml human basic fibroblast growth factor (CytoLab/Peprotech). After 3 days of culture, non-adherent cells were removed by washing with PBS.

Transfection

pcDNA3-HIF-1α expression plasmid was a gift from Prof. H. Franklin Bunn (Division of Hematology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA). It contained a PCR-amplified full length HIF-1α cDNA driven by CMV promoter.

For siRNA studies, an siRNA motif was selected for human HIF-1α cDNA sequence (GenBank Accession U22431) using Ambion software package. The target sequence was localized 145 bp downstream of the start codon. Forward and reverse primers 5′-TGTGAGTTCGCATCTTGATTTCAAGAGAA-TCAAGATGCGAACTCACATTTTTT-3′ and 5′-AATTAAAAAATGTGAGTTCGCATCTTGAT-TCTCTTGAAATCAAGATGCGAACTCACA-GGCC-3′ with 5′ overhangs were annealed according to the Ambion manufacturer's instructions. The insertion was cloned into the pSilencer vector (Ambion, Austin, TX) and the sequence was verified by sequencing.

For electroporation, cells were resuspended at 1×10^6 cell/ml in Opti-Mem I (Invitrogen) serum-free medium. All the samples were electroporated only once. Samples of 400 μl each were placed in 4 mm gap cuvettes (Bio-Rad, CA), gently mixed with the pHIF-1α, pSilencer or control vector pGFP (Clontech, CA) to a final concentration of 2 μM, and kept on ice for 5 min

before being electroporated once at 280 mV, 1,000 μF. After electroporation, the cells were washed and resuspended in M199 medium for further culture. The media were supplemented with VEGF and bFGF 24 h later for continuous culture.

Establishment of Hypoxic Culture Condition

After transfection, cells were transferred to two different chambers. The hypoxia chamber contained 1% O_2, 5% CO_2 and 94% N_2 at 37°C. The normoxia chamber contained 21% O_2, 5% CO_2 and 74% N_2 at 37°C.

Real-Time RT-PCR

Human EPC mRNA expression was evaluated by real-time RT-PCR 12 h after transfection under hypoxia. One μg RNA per sample as the template was copied to cDNA using reverse transcriptase (TAKARA, Dalian, China) and random primers. Real-time PCR was performed using the ABI Prism 7500 system (Applied Biosystems, Foster City, CA) with SYBR-Green as the fluorescent dye. The primer pairs were: HIF-1α (NM001530): 5′-AGCCAGACGATCATGCAGCTACTA-3′ (forward) and 5′-TGTGGTAATCCACTTTCATCCATTG-3′ (reverse); VEGF (NM003376): 5′-GAGCCTTGCCTTGCTGCTCTAC -3′ (forward) and 5′-CACCAGGGTCTCGATTGGATG-3′ (reverse); β-actin (NM001101) 5′-ATTGCCGACAGGATGCAGA-3′ (forward); 5′-GAGTACTTGCGCTCAGGAGGA-3′ (reverse).

Flow Cytometry Analysis of Cell Surface Markers

At the end of culture, EPCs were detached with 1 mmol/L EDTA (pH 7.4) for 10 min at 37°C. After centrifugation at 1,000 rpm for 5 min, the cells were resuspended in PBS and then incubated with PE-conjugated anti-human CD133 (Miltenyi Biotec, Auburn, CA), FITC-conjugated anti-human CD34 (Miltenyi Biotec) or FITC-conjugated anti-human CD31 antibody (Caltag Laboratories, Burlingame, CA) for 15 min at 4°C. Samples were analyzed on a Beckman flow cytometer and 10,000 events were counted per sample. Isotype mouse IgG staining served as negative control.

UEA-1 Lectin Binding Sites Labeling and Dil-acLDL Labeling

The cultures were incubated for 4 h in the presence of Dil-acLDL (Molecular Probes Eugene, OR) and FITC-conjugated UEA-1 lectin (Lectin from *Ulex europaeus*, Sigma) for 60 min at 4°C. The cells were then washed twice in PBS, fixed in 4% paraformaldehyde in PBS for 30 min and prepared for confocal microscopy.

Measurement of VEGF, NO and SDF-1α

Secreted VEGF (R&D Systems, Minneapolis, MN) was measured using commercially available sandwich immunoassay kits according to the manufacturer's instructions. NO release in the supernatant was measured using a commercial kit (Jingmei Bioengineering, Beijing, China) according to the manufacturer's instructions. The conditioned media were not changed and new media were added every 3 days until time of assay at day 7. Values were normalized to the final number of cells (1×10^6) in the dish from which the supernatant was harvested. SDF-1α was measured by ELISA. Mouse tissue extracts from both limbs were prepared by homogenization and lysis as indicated [Su et al., 2002]. Recombinant murine SDF-1α supplied with the Quantikine Kit (R&D Systems) were used to generate standard curves. Total protein was quantified by the Bradford assay.

Western Blotting

At the end of culture, cells were homogenized as indicated [Su et al., 2002]. Samples containing 100 μg protein per well were resolved on 8% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes (Pharmacia Biotech). Membranes were blocked in TBS-T for 1 h at room temperature and then incubated with primary antibodies [anti-human HIF-1α mouse monoclonal antibody (Sigma) at a dilution of 1:250, anti-human VEGF mouse monoclonal antibody (Santa Cruz, CA) at a dilution of 1:100, mouse anti-Flk-1 mAb (Santa Cruz) at a dilution of 1:100 or rabbit anti-eNOS at a dilution of 1:250 (Santa Cruz)]. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies, and immunoreactivity was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and captured on X-ray film. For loading control, anti β-actin mAb (Sigma) was used at a dilution of 1:6,000.

Colony Formation, Cell Proliferation and Cell Migration/Invasion Assays

For colony formation, cells were seeded at a density of 10^8 per 100 mm dish in triplicates and were transfected and placed under normoxia or

hypoxia. Seven days after transfection, they were screened for the presence of colonies. The number of colonies (aggregates each with more than 50 cells) was estimated by the total colony counts from 10 randomly selected fields multiplied by the proportional factor for the area of the field relative to that of the dish. For proliferation assays, cells were seeded in M199 medium for 3 days before transfection and hypoxic challenge. Cells were counted by hemacytometer 7 and 12 days after transfection. The number of divisions (i.e. population doubling) was estimated using the formula: ln(number of cells counted/number of cells at the beginning of the assay)/ln 2 [Bompais et al., 2004]. To assess migration from established monolayers, cells (10^5) were dispersed onto filters (Corning Transwell system, 8-μm pore size), after which they were challenged by the addition of 600 μl of a chemo attractant solution composed of conditioned media and 50 ng/ml VEGF, 10 ng/ml bFGF to the lower compartments. Migration was allowed to proceed for 24 h at 37°C in either normoxia or hypoxia. Cells that had migrated to the lower surfaces of the filters were fixed with 10% neutral buffered formalin, stained with 0.1% crystal violet/20% MeOH, and counted. The average number of migrating cells per field was assessed by counting at least four random fields per filter.

Animal Studies

Institutional Animal Care and Use Committee approved all animal protocols. Unilateral hindlimb ischemia was induced by resecting the left femoral arteries of Balb/c nude mice (age 8–10 weeks, body weight 17–22 g) under sodium pentobarbital anesthesia (30 mg/kg i.p.) [Couffinhal et al., 1998]. EPCs on day 7 after transfection were transplanted 24 h after surgery. Specifically, GFP-EPCs (5×10^6 cells), HIF-1α-EPCs (5×10^6), or an equal volume (200 μl) of M-199 medium was given to the animals ($n = 5$ per treatment group) via tail vein injection.

Fluorescent microscopic analysis of GFP-EPCs was conducted with a Berthold Nightowl LB 981 microscope (Germany). Images were analyzed with Winlight 32 software. Blue color excitation light and yellow filters were used for the in vivo real-time imaging.

Seven and 28 days after cell transplantation, the adductor and semimembranosus muscles were harvested, processed either for paraffin-embedding or for acetone-fixed frozen sections and sliced into 6 μm sections. For HIF-1α and CD31 immunostaining, endogenous peroxidase activity was quenched by 3% H_2O_2 and non-specific binding was blocked by 10% normal horse serum. Sections were incubated with anti-mouse HIF-1α antibody (Sigma) or rat anti-mouse CD31 antibody (Pharmingen, San Diego, CA) followed by incubation with an HRP-conjugated secondary antibody. Capillary density was measured as described by Couffinhal et al. [1998]. Capillary density, as an index of angiogenesis, was examined by counting the number of capillaries in light microscopic sections. The entire leg below the knee from each animal was examined. Serial sections were cut at two different levels approximately 200 μm apart. On each series, CD31 staining was performed. Four fields of CD31 staining were counted from the two levels for each of the five animals per time point, for a total of 20 fields per time point. Capillary density was measured from five randomly selected fields for each of the four sections at 400× magnification. To ensure that capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, capillary/muscle fiber ratio was determined.

Statistical Analysis

Statistical analysis was performed by one-way ANOVA, followed by pairwise contrasts using the Dunnett test. Data (mean ± SD) were considered significant at $P < 0.05$.

RESULTS

Identification of EPCs

At day 1 and day 7, flow cytometry analysis for EPC surface markers CD34 and CD133 was carried out (Fig. 1A). About 30% of the PBMNCs were CD34+ and over 10% were CD133+ at day 7 while no more than 1% positive CD34/CD133 cells were found at day 1. Specific antigen CD31, Dil-acLDL, UEA-1 were EC phenotypic markers (Fig. 1B,C). After 3 days of culture, EPCs were transfected with control (GFP) expressing plasmid and the transfection efficiency was evaluated 48 h later. Nearly 25% of all the cells were GFP positive (Fig. 1D).

Effect of HIF-1α Overexpression and Hypoxia on VEGF Expression

HIF-1α mRNA was analyzed in HIF-1α-transfected EPCs 12 h under hypoxia and

compared with those in untransfected EPCs. We found enhancement in HIF-1α mRNA expression by approximately 1.5-fold, when compared to GFP control (Fig. 2A, $P < 0.05$). In HIF1α-transfected cells under hypoxia, VEGF expression was strongly up-regulated to a level far greater than the control cells (Fig. 2A). These data suggest that HIF-1α levels play a key role in controlling VEGF mRNA expression.

To further demonstrate the contribution of hypoxia and HIF-1α on VEGF induction, HIF-1α-siRNA was introduced to suppress the expression of HIF-1α. As shown in Figure 2A, HIF-1α mRNA was significantly reduced in siRNA-transfected cells. Because of the limited transfection efficiency, reduction of HIF-1α mRNA is expected to be even stronger than has been measured. In parallel, the expression of VEGF mRNA was also significantly abrogated. These results again demonstrate a direct and major role of HIF-1α in VEGF upregulation under hypoxia.

We next investigated HIF-1α and VEGF protein expression under hypoxia by Western

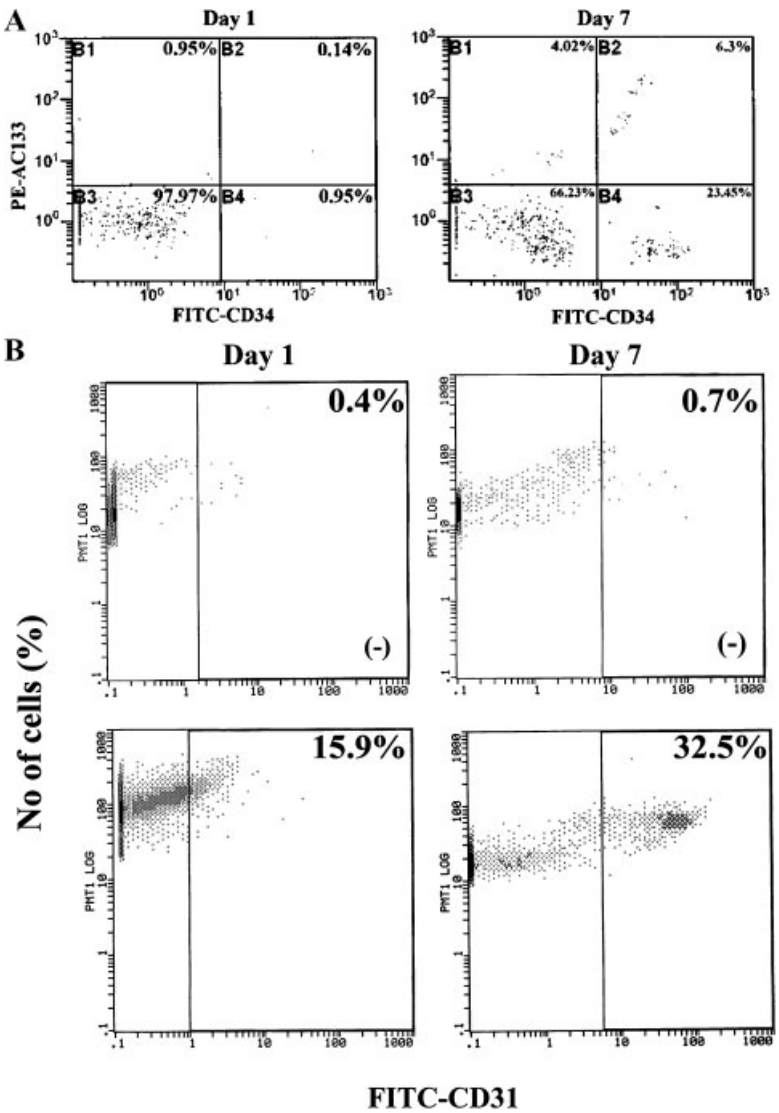

Fig. 1. Identification of EPCs. **A**: The $CD34^+$ and $CD133^+$ fractions of PBMNCs were analyzed by flow cytometry on day 1 and day 7 in culture. $CD34^+$ fraction of PBMNCs was over 30% while $CD133^+$ fraction was much less on day 7. **B**: Expression of endothelial-associated antigen CD31 in day 7 culture (30%) and day 1 culture (16%). **C**: Dil-acLDL uptake and Ulex lectin binding as markers for endothelial lineage (double positive as arrows shown, Dil-acLDL: red, UEA-1: green). Magnification: 200×. **D**: EPCs were transfected with GFP and observed 48 h later. Data represented one of three independent experiments.

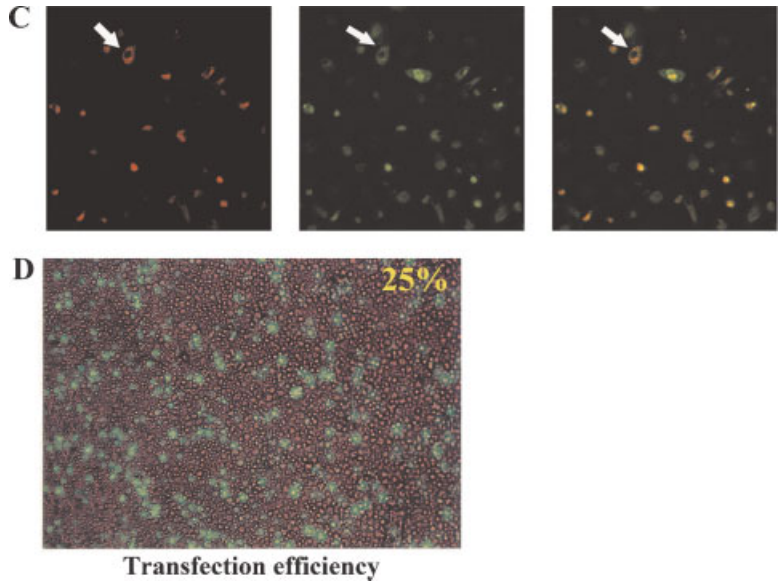

Fig. 1. (*Continued*)

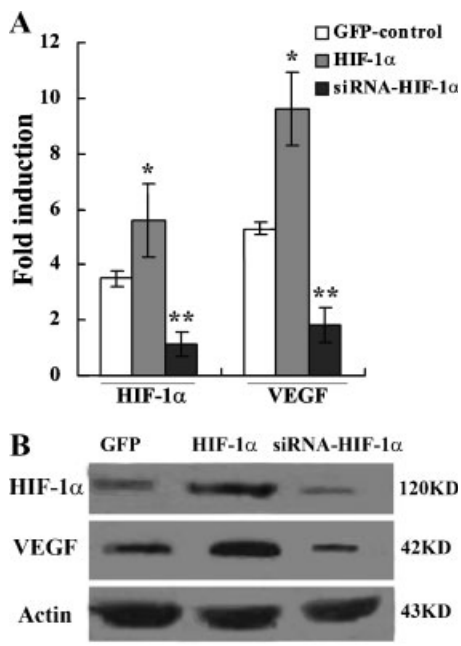

Fig. 2. Effect of HIF-1α transfection on HIF-1α and VEGF expression. EPCs were transfected with either control (GFP), HIF-1α expression plasmid or HIF-1α-siRNA vector and placed under hypoxic conditions. **A**: The expression of HIF-1α and VEGF mRNA over a period of 12 h was quantified by real-time PCR and expressed as fold induction relative to those of β-actin. Statistical significance ($P < 0.05$) for difference in means is noted by symbols: * for HIF-1α overexpression group compared to GFP group; ** for siRNA-HIF-1α group compared to GFP group. Similar results were obtained under normoxia (data not shown). **B**: Corresponding protein expression of HIF-1α and VEGF. All values were means ± SD from three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

blot analysis (Fig. 2B). A significant increase of HIF-1α protein expression was detected 12 h after HIF1α transfection (Fig. 2B). This induction was markedly reduced in siRNA-HIF-1α treated cells. Meanwhile, similar changes in VEGF were detected.

Effect of HIF-1α Overexpression and Hypoxia on EPC Differentiation, Proliferation and Migration

To explore whether HIF-1α and hypoxia promoted EC differentiation, we evaluated the expression of EC surface marker CD31 by flow cytometry. CD31 expression was assessed on days 3 and 10 after transfection (Fig. 3). Results showed that for normoxic cultures on day 3, more than half of the cells were positive for CD31 in HIF-1α-transfected group compared to one-third in the control group ($58.36 \pm 2.98\%$ vs. $34.18 \pm 4.40\%$, $P < 0.05$). Hypoxia significantly increased the proportion of $CD31^+$ cells in both control and HIF-1α transfected cells ($60.13 \pm 8.22\%$ and $73.53 \pm 6.82\%$). After 10 days of culture, the effect of HIF-1α and hypoxia remained significant: in normoxic culture, $CD31^+$ cells increased to $69.54 \pm 3.12\%$ in the HIF-1α group versus $51.8 \pm 3.47\%$ in the control group; and in hypoxic culture, they reached $86.1 \pm 1.91\%$ and $59.0 \pm 3.47\%$, respectively. These data suggest that HIF-1α and hypoxia work in concert to promote EC differentiation.

Consistent with previous studies, hypoxia led to a marked increase of VEGF secretion.

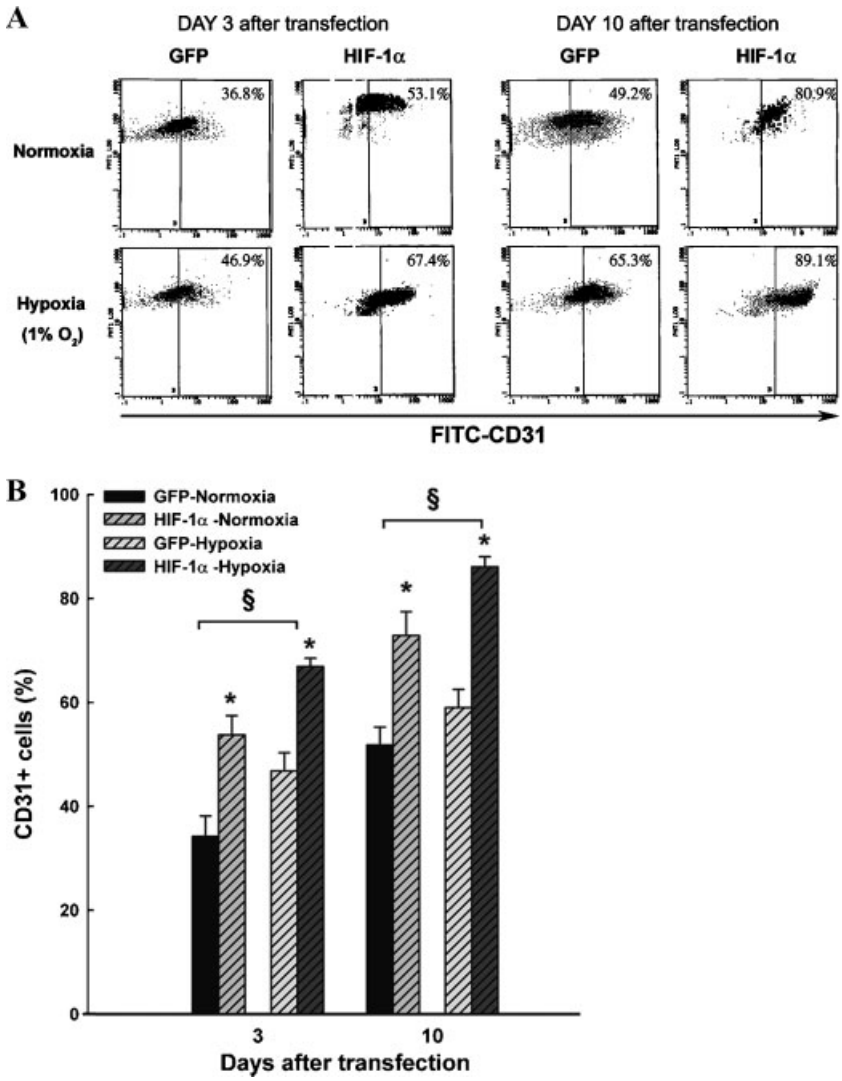

Fig. 3. Ex vivo expansion of endothelial progenitor cells. EPCs were cultured as in Figure 2 except they were cultured for up to 10 days. Cells were labeled with a FITC-CD31 antibody and were analyzed by flow cytometry. **A**: One representative experiment out of 3 is shown. **B**: The fraction of $CD31^+$ cells was expressed as mean % ± SD over three independent experiments. Hypoxia: the cells were cultured in hopoxia after electroporation. Asterisks (*) indicate significant difference ($P < 0.05$) between HIF-1α transfected and control cells and § indicates significant difference ($P < 0.05$) between cells under hypoxia and normoxia.

Moreover, when HIF-1α was overexpressed, there was a 50% increase of VEGF secretion from 584 to 901 pg/ml under normoxia ($P < 0.05$, Fig. 4A). Furthermore, HIF-1α overexpression was still able to produce an additional 35% increase in VEGF release from 1753 to 2374 pg/ml under hypoxia. Therefore, HIF-1α represents an important mechanism in supporting VEGF release regardless of oxygen levels. In addition, hypoxia and HIF-1α overexpression each enhanced Flk-1 and eNOS protein expression over the control levels (Fig. 4B).

VEGF-stimulated eNOS expression and resultant NO production are hallmarks of functional vascular endothelial cells. We measured nitrate levels in the culture supernatants as an indicator of total NO production after 2 days of VEGF stimulation. In control cells, there was a trend towards increased NO production as VEGF increased from 1 to 50 ng/ml, although the results did not reach statistical significance (Fig. 4C). There was also no difference between cells under hypoxia or normoxia. Overexpression of HIF-1α led to

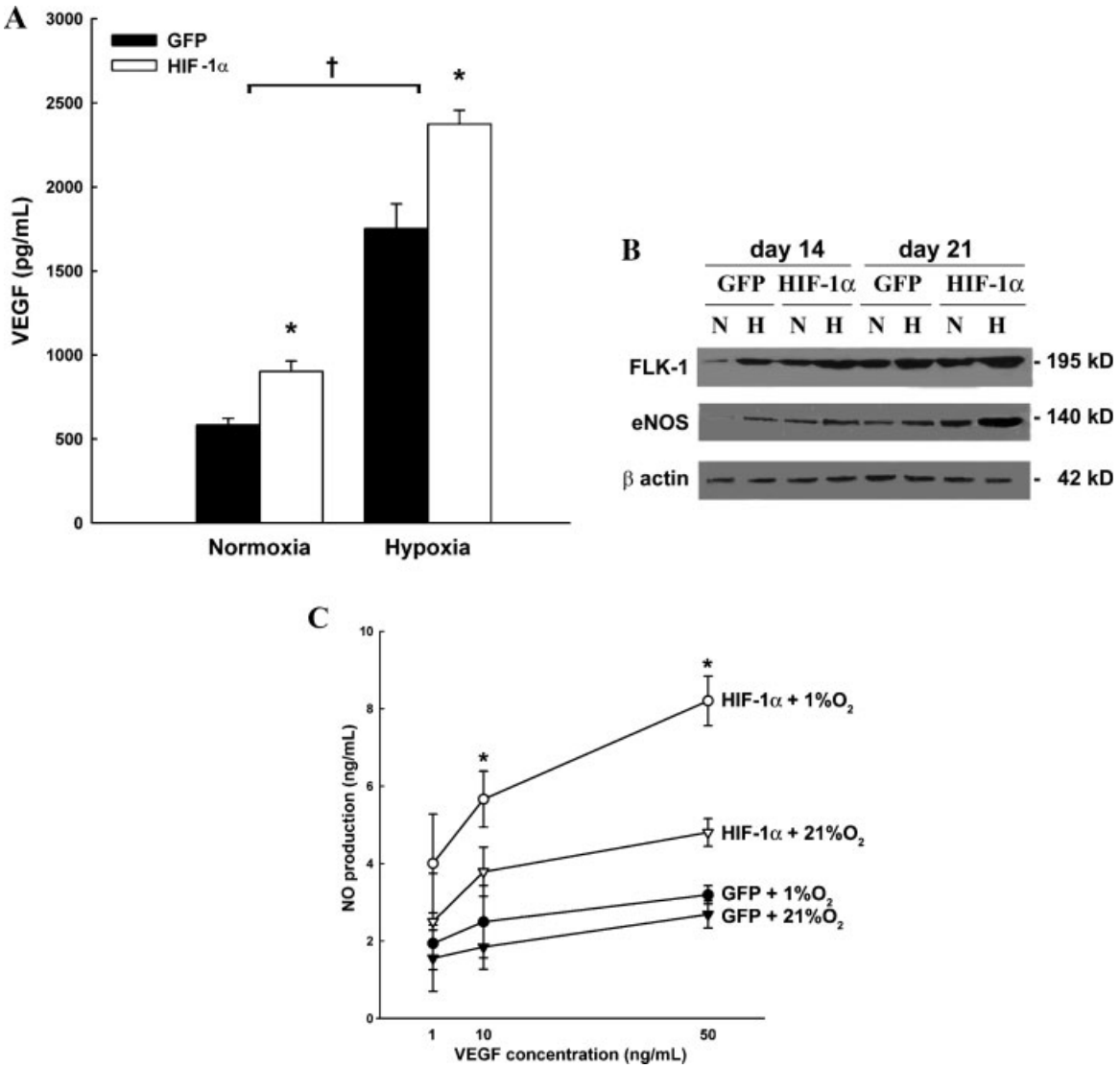

Fig. 4. Effect of HIF-1α and hypoxia on VEGF secretion (**A**), FLK-1 and eNOS expression (**B**), NO production (**C**). A: EPCs were treated as in Figure 2. After 10 days of culture, VEGF secretion per 10^6 cells was measured by ELISA. Data, from three independent experiments, were expressed as mean ± SD. Asterisks (*) indicate statistically significant difference ($P < 0.05$) between HIF-1α transfected and control cells and § indicates significant difference ($P < 0.05$) between cells under hypoxia and normoxia. B: Cells were treated as indicated. N, normoxia; H, hypoxia. Cell lysates were collected at the indicated times for immunoblotting of FLK-1, eNOS, and β-actin. C: EPCs were treated as in Figure 2 except VEGF was given in escalating concentrations to stimulate EC differentiation. VEGF at 1 ng/ml could be viewed as basal levels and 10 ng/ml VEGF was required to stimulate EC differentiation. NO production was quantitated by ELISA using supernatants from day 2 cultures (n = 3). Asterisks (*) indicate statistical significant difference ($P < 0.05$) compared to control normoxic levels.

increased basal level (1 ng/ml VEGF) and stimulated levels of NO production, regardless of oxygen tension, with the effect most pronounced at 50 ng/ml VEGF (Fig. 4C). Hypoxia further potentiated NO release at all VEGF levels. These results support the idea that overexpression of HIF-1α for even a short period of time may be significant in driving EPCs along their differentiation path.

EPC maturation was also observed by cell morphology. EPCs were cultured for 3 days before gene transfer and were further cultured in hypoxic or normoxic conditions for 3 days. As shown in Figure 5A, HIF-1α-transfected cells under normoxia sprouted more rapidly from the EPC colonies than the untransfected cells or cells transfected with a GFP vector, which essentially maintained the original

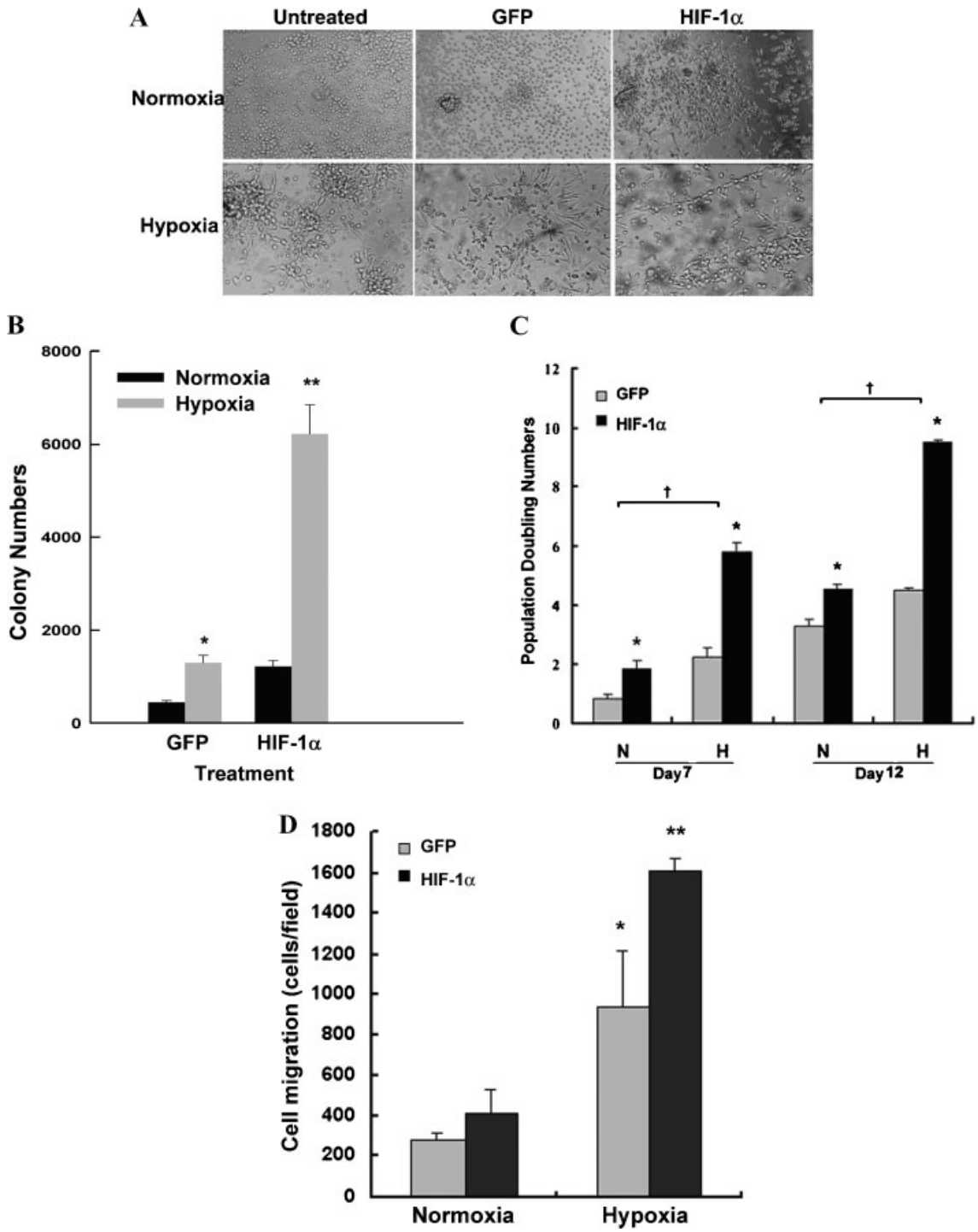

Fig. 5. Effect of HIF-1α and hypoxia on cell morphology, colony formation, proliferation and migration. **A**: Cell morphology was observed by light microscopy. **B**: Colony formation assay. Values were expressed as mean ± SD. Asterisks (both * and **) indicate statistical significant difference ($P < 0.05$) compared to respective normoxic levels. **C**: Cells were counted by hemacytometer 7 and 12 days after transfection. Values were expressed as mean ± SD from three independent experiments. **D**: Migration was allowed to proceed for 24 h under normoxic or hypoxic conditions in the presence of VEGF and bFGF. Four different fields were counted. * indicates statistically significant differences ($P < 0.05$) compared with GFP. ** indicates statistically significant differences ($P < 0.05$) compared with control (serum-free conditioned media) in hypoxic conditions.

colony formation. When cultured in hypoxia, control EPC colonies sprouted spindle-shaped cells from their periphery in a manner similar to HIF-1α-transfected cells under normoxia. Therefore, in contrast to the well-organized colony formation in normoxic conditions, they appeared more disseminated and overlapping. Moreover, HIF-1α-transfected cells took on an array-like arrangement rather than random dispersal, suggesting that they were in an advanced state of differentiation. Figure 5B indicates that while hypoxia treatment or HIF-1α overexpression had a modest positive effect, together they markedly increased the number of colonies by nearly 10 folds. Consistent with this, HIF-1α-transfected cells also proliferated at a faster rate as estimated by the number of cell doublings (Fig. 5C).

The effect of HIF-1α and hypoxia on EPC migration was investigated in a Transwell system. For control cells transfected with GFP, there was a threefold increase in cell migration when cells were placed under hypoxia versus under normoxia (Fig. 5D). In contrast, while there was no difference in cell migration between control cells and cells transfected with HIF-1α under normoxic conditions likely due to HIF-1α degradation, there was a fourfold increase for cells overexpressing HIF-1α under hypoxia. The additional 60% increase seen in cells overexpressing HIF-1α compared to the control cells both under hypoxia again suggested an additive or synergistic effect.

Homing of EPCs

To assess the in vivo effect of HIF-1α, HIF-1α-over-expressing EPCs were transplanted into nude mice whose hindlimb had been surgically injured to cause ischemia. The reporter gene GFP allowed for identification and tracking of the exogenous EPCs in real time images. As we expected, fluorescence microscopic examination showed that in non-ischemic mice, we did not see the GFP tagged cells in hindlimb but found them spread through the tail vein and mainly accumulated in bursula tastium. Strikingly, high levels of fluorescence were detected in ischemic foci (arrow) but negligible levels of fluorescence could be seen in the nonischemic limb. These data suggest that human-derived EPCs maintained good viability in nude mice and could selectively home to the injury site (Fig. 6A). The increased HIF-1α protein expression lining the vessels in the HIF-1α group at 7 days after transplantation supports the involvement of HIF-1α in EPC homing (Fig. 6B). It is known that SDF-1 plays a role in stem cell recruitment during ischemia but is not sufficient to induce homing in the absence of injury. Transplantation of GFP-EPC resulted in significantly increased, SDF-1 expression by ELISA when compared to the untreated group ($P < 0.05$, Fig. 6C, shown as *). Moreover, transplantation of HIF-1α-EPCs further increased SDF-1 in ischemic hindlimb ($P < 0.05$, shown as **). These data suggest that HIF-1α may somehow promote endogenous SDF-1 expression, which may function to further recruit more EPCs to the injury site.

New Vessel Formation by EPCs

Revascularization of the ischemic tissue was evaluated by immunostaining of sections taken from the ischemic and nonischemic limbs over the course of 28 days postoperatively. There was a significant increase in capillary density over the control, as measured by capillary/muscle fiber ratio, in all treatment groups as early as day 6 (Fig. 6D). When the EPC groups were compared with the ischemia alone group, capillary density was increased by 25% and 50% for GFP-EPCs and HIF-1α-EPCs, respectively ($P < 0.05$). The effect was sustained and increased even at day 28.

DISCUSSION

The main findings of the present study are: (1) hypoxic preconditioning enhanced the differentiation of PBMNCs into EPCs and exhibited greater angiogenic properties compared with normoxia-conditioned EPCs; (2) HIF-1α upregulation could account for much of hypoxia-induced VEGF induction and subsequent activation of EPC proliferation/differentiation; and (3) SDF-1 was involved in injury-induced homing and HIF-1α enhanced SDF-1 secretion which augmented EPC recruitment. (4) Last but not least, overexpression of HIF-1α by gene transfer further accelerated EC maturation and demonstrated increased revascularization efficacy in an in vivo model of ischemia.

With the aim of improving functioning of cells under hypoxia and increased angiogenesis, we used EPCs transfected with HIF-1α to examine the effect of HIF-1α and hypoxia on EPC

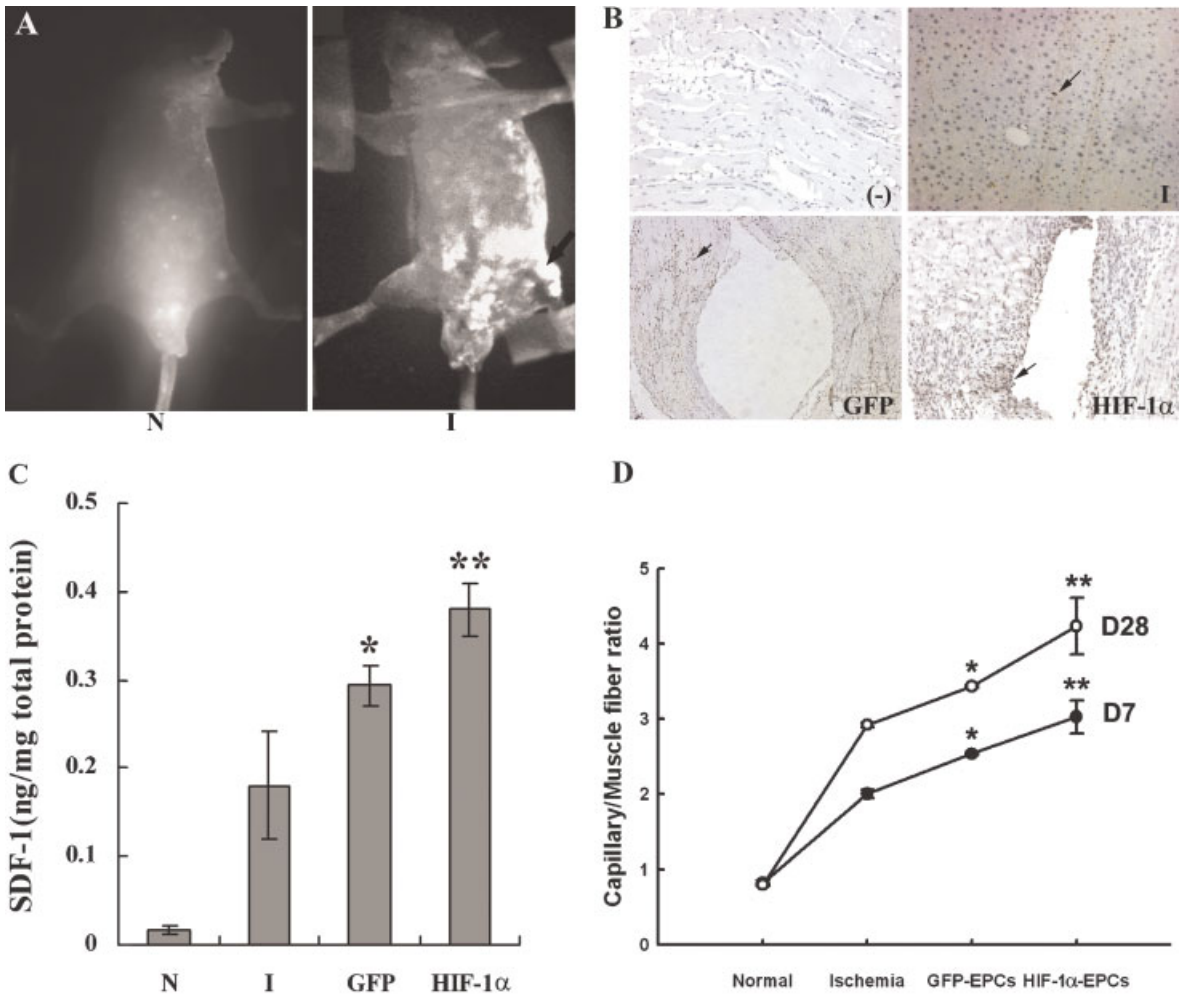

Fig. 6. Effect of EPCs on revascularization of ischemic injury. **A**: Real-time image was done to analyze the homing of GFP-EPCs 2 days after transplantation. N: non-ischemic control. I: ischemic mouse. Note the fluorescent intensity (arrow) at ischemic hindlimb. **B**: Seven days after transplantation, HIF-1α immunochemistry was detected. Arrows showed HIF-1α positive protein. (−) PBS was used instead of the primary antibody. **C**: ELISA results of murine SDF-1α at 12 h after transfection. * indicates statistically significant differences ($P < 0.05$) between GFP group and I (ischemic limb). ** indicates statistically significant differences ($P < 0.05$) between HIF-1α group and I. **D**: Capillary density as measured by capillary/muscle fiber ratio. Sections were processed for CD31 immunostaining. The number of vessels was counted. Capillary density is shown as the capillary/muscle fiber ratio. N = 5. Asterisks (* and **) indicate significant difference compared to untreated ischemic limb group ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

differentiation into mature ECs. In our experience, EPC displays its progenitor property accompanied by its EC maturation [Meng et al., 2005a,b]. To exclude the possibility of contamination by vector DNA, pilot trial was done by semi-quantitative PCR without reverse transcriptase. No HIF-1α band was observed (data not shown). Although most cells undergo growth arrest during hypoxia, EPCs proliferate in response to low O_2. Ramirez-Bergeron and Simon [2001] demonstrated that proliferation of embryonic multilineage hematopoietic progenitors was regulated by a hypoxia-mediated signaling pathway. Our findings suggest HIF-1α mRNA overexpression is induced in EPCs, and the subsequent increase of HIF-1α protein expression was detected as expected.

One of the key target genes of HIF-1α is VEGF. Indeed, both VEGF mRNA and protein levels were up-regulated by HIF-1α transfection that closely paralleled that of HIF-1α expression. Our results were consistent with those of Manalo et al. [2005], who showed a more than 1.5-fold VEGF mRNA increase after only 8 h of hypoxic exposure. The level of induction appeared to be higher than that by HIF-1α transfection. This may in part due to

transfection efficiency, which was around 25% using the GFP plasmid as a reporter. Thus the extent of VEGF up-regulation by HIF-1α overexpression and down-regulation by siRNA-HIF-1α is expected to be greater if transfection efficiency is improved.

Over-expression of HIF-1α had functional consequences in terms of EPC differentiation into ECs, including proliferation index, expression of EC markers like CD31, and VEGF/Flk-1/eNOS axis expression [Iwaguro et al., 2002; Ferrari et al., 2003; Herder et al., 2003; Ackah et al., 2005]. Flk-1 signaling upon VEGF binding acts as an early event to induce progenitor cells to proliferate and migrate [Zippo et al., 2004]. In the presence of HIF-1α over-expression, all of these phenotypic and functional properties were increased. This may be why the transient transfection of HIF-1α would likely have long and significant effect in driving EPCs along their differentiation path. Enhanced VEGF/Flk-1 signaling in response to HIF-1α over-expression implies that HIF-1α transfection did not alter the properties of immature cells, rather it might contribute to their higher proliferative capacities. Our results also show that colony formation and cell proliferation after HIF-1α transfection more than offset cell loss due to the transfection procedure. In addition, the morphology of HIF-1α-EPCs was typical of endothelial cells. Therefore, overexpression of HIF-1α under non-hypoxic conditions was sufficient to induce changes in EC biology that were remarkably similar to those induced by hypoxia.

Moreover, exogenous VEGF administration significantly enhanced NO release and the differentiation of PBMNCs into EPCs. Thus, hypoxia-induced VEGF secretion may at least in part account for the enhanced differentiation of PBMNCs into EPCs under hypoxia. Meanwhile, as shown above, HIF-1α overexpression (Fig. 4B) promoted VEGF secretion. Furthermore, Flk-1 and CD31 expression in EPC-like attached cells was enhanced by hypoxia. Because VEGF stimulates EC migration and angiogenesis mainly through Flk-1 [Tateishi-Yuyama et al., 2002], the increased Flk-1 expression may also account for the enhanced angiogenic properties of hypoxia-conditioned EPCs. Our data are consistent with other reports of hypoxia-stimulated Flk-1 expression in ECs. Interestingly, a recent study showed that VEGF-Flk-1 signaling pathway induced Flk-1 expression via a positive feedback mechanism [Talks et al., 2000]. Thus, augmented VEGF release may enhance the expression of Flk-1 on EPCs [Talks et al., 2000].

Apart from VEGF, other target genes of HIF-1 such as SDF [Daniel and Geoffrey, 2005] also came under our investigation. SDF is typically expressed to indicate the presence of tissue ischemia, however, the regulation of SDF-1 and its physiological role in peripheral tissue repair remain incompletely understood. In ischemic hindlimbs, exogenous SDF-1 did not promote limb salvage in the absence of delivered endothelial precursor cells but did decrease endothelial cell apoptosis [Yamaguchi et al., 2003]. Interestingly, in the present data, both SDF-1 and HIF-1 are present in the ischemic tissue, suggesting that hypoxia may be a fundamental requirement for progenitor cell trafficking and function. As such, ischemic tissue may represent a conditional stem cell niche, with recruitment and retention of circulating progenitors regulated by hypoxia through differential expression of SDF-1. In support of this, EPCs overexpressing HIF-1α had increased levels of SDF-1 and enhanced homing. Other studies have indicated that SDF-1 is a potent factor in mobilizing primitive progenitors from the bone marrow niche [Sweeney and Papayannopoulou, 2001; Sweeney et al., 2002] and has been found to be selectively elevated in the serum during periods of ischemia [De Falco et al., 2004]. It is likely that SDF-1 is not the only gene mediating local chemotaxis of EPCs. Others have reported a over 20-fold increase in MMP-9 in the murine heart after myocardial infarction [Abbott et al., 2004]. Meanwhile, SDF receptor CXCR4, VCAM, ICAM, and VEGF may also be associated with increased homing of EPCs. This is our first study to show that SDF-1 plays a role in the accumulation of EPCs after limb ischemia, and more details are under investigation.

In a preliminary experiment, we transplanted transiently overexpressed HIF-1α to EPCs in hindlimb ischemic mouse. Previous studies have raised the prospect of HIF-1α gene therapy or EPC-based cell therapy [Shyu et al., 2002] as novel strategies for the treatment of ischemic disease, but studies combining the two have yet not been reported. Our ex vivo results indicate that HIF-1α cDNA transfection and hypoxia condition can work in concert. We all know there is a hypoxia condition at the

ischemic locus in vivo, so we were able to show HIF-1α-transduced EPCs had superior treatment efficacy than normal EPCs in terms of revascularization as evidenced by increased capillary density.

In summary, although HIF-1 is regulated physiologically mainly by oxygen tension through the oxygen-dependent degradation of its α subunit, in vitro it can also be modulated by gene transfer. When compared to control EPCs, HIF-1α-overexpressing EPCs displayed a higher proliferative potential and sensitivity towards angiogenic factors. Use of HIF-1 inducers or agents that mimic HIF-1 action may be beneficial to the recruitment and differentiation of EPCs. Future use of peripheral blood-derived in vitro modified HIF-1α-EPCs may constitute an innovative strategy for cell therapy.

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