ARTICLE
Journal of Cellular Biochemistry 112:804–817 (2011)

Journal of Cellular Biochemistry

Differential Roles of Hypoxia Inducible Factor Subunits in Multipotential Stromal Cells Under Hypoxic Condition

Kenichi Tamama,[1*] Haruhisa Kawasaki,[1] Svetoslava S. Kerpedjieva,[1] Jianjun Guan,[2] Ramesh K. Ganju,[3] and Chandan K. Sen[4]

[1]*Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261*
[2]*Department of Materials Science and Engineering, The Ohio State University, Columbus, Ohio 43210*
[3]*Department of Pathology, Comprehensive Wound Center, The Ohio State University, Columbus, Ohio 43210*
[4]*Department of Surgery and Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio 43210*

ABSTRACT

Cell therapy with bone marrow multipotential stromal cells (MSCs) represents a promising approach to promote wound healing and tissue regeneration. MSCs expanded in vitro lose early progenitors with differentiation and therapeutic potentials under normoxic condition, whereas hypoxic condition promotes MSC self-renewal through preserving colony forming early progenitors and maintaining undifferentiated phenotypes. Hypoxia inducible factor (HIF) pathway is a crucial signaling pathway activated in hypoxic condition. We evaluated the roles of HIFs in MSC differentiation, colony formation, and paracrine activity under hypoxic condition. Hypoxic condition reversibly decreased osteogenic and adipogenic differentiation. Decrease of osteogenic differentiation depended on HIF pathway; whereas decrease of adipogenic differentiation depended on the activation of unfolded protein response (UPR), but not HIFs. Hypoxia-mediated increase of MSC colony formation was not HIF-dependent also. Hypoxic exposure increased secretion of VEGF, HGF, and basic FGF in a HIF-dependent manner. These findings suggest that HIF has a limited, but pivotal role in enhancing MSC self-renewal and growth factor secretions under hypoxic condition. J. Cell. Biochem. 112: 804–817, 2011.

KEY WORDS: MESENCHYMAL STEM CELLS; HYPOXIA-INDUCIBLE FACTOR; HYPOXIA; SELF-RENEWAL; DIFFERENTIATION; OXYGEN

Adult bone marrow multipotential stromal cells/mesenchymal stem cells (MSCs) are multipotent cells with strong paracrine activities of various growth factors [Friedenstein et al., 1976; Owen and Friedenstein, 1988; Kinnaird et al., 2004; Pittenger and Martin, 2004; Tamama et al., 2006; Phinney and Prockop, 2007; Tamama et al., 2008; Kawasaki et al., 2010; Tamama et al., 2010]. Cell therapy with MSCS represents a promising approach to promote wound healing and tissue regeneration, as summarized recently by Ohishi and Schipani in this journal [Ohishi and Schipani, 2010].

Availability of sufficient number of MSCs that retain their multipotency and paracrine activity is prerequisite for successful MSC-based therapeutics. With low concentrations of MSCs in the adult bone marrow (one in 10^5–10^6 bone marrow mononuclear cells) [Pittenger et al., 1999], which decreases even more in elder donors [Caplan, 2009], MSCs need to be expanded in vitro after harvest. Such expansion, however, poses the threat of loss of differentiation, proliferative, and therapeutic potentials of MSCs [Colter et al., 2000; Crisostomo et al., 2006; Roobrouck et al., 2008]. Thus, preservation of self-renewal and therapeutic potentials of undifferentiated MSCs is critically important for therapeutic purposes.

In 1977, Packer and Fuehr first reported in *Nature* that limiting O_2 concentration prolongs replicative lifespan of cultured human

Abbreviations used: MSC, multipotential stromal cell/mesenchymal stem cell; HIF, hypoxia inducible factor; HRE, HIF response element; pO_2, partial oxygen pressure; TERT, telomerase reverse transcriptase; RLU, relative light unit; RPLPO, ribosomal protein, large, P0; UPR, unfolded protein responses; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor; Osx, osterix; PPARγ, peroxisome proliferation-activated receptor γ.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: American Heart Association; Grant number: 09BGIA2050227; Grant sponsor: National Institute of Health; Grant number: R01CA109527 R01GM077185 R01GM069589.

*Correspondence to: Dr. Kenichi Tamama, MD, PhD, 3550 Terrace Street, S737 Scaife Hall, Pittsburgh, PA 15261, USA. E-mail: tamamakj@upmc.edu

Received 23 April 2010; Accepted 9 November 2010 • DOI 10.1002/jcb.22961 • © 2010 Wiley-Liss, Inc.
Published online 22 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

fibroblasts [Packer and Fuehr, 1977]. Cultured cells are routinely maintained in the laboratory at an ambient O_2 concentration (20%)(in vitro normoxia); however it is much higher than in vivo O_2 tension of peripheral tissues (2–9%)(in vivo normoxia) [Sen et al., 2006; Simon and Keith, 2008]. In the bone marrow, O_2 tension is even lower (1–7%) [Chow et al., 2001; Antoniou et al., 2004] compared to in vitro culture conditions. Thus, ambient O_2 concentration is indeed supraphysiological to cells in culture [Khanna et al., 2006; Sen et al., 2006].

MSCs produce more colonies, have longer replicative lifespan, secrete more growth factors and cytokines, and remain undifferentiated in hypoxic condition more than when cultured in room air condition (20%O_2) [Kinnaird et al., 2004; Grayson et al., 2006; Fehrer et al., 2007; Crisostomo et al., 2008; Rosova et al., 2008]. These studies suggested that hypoxic condition promotes self-renewal of undifferentiated MSCs and enhances therapeutic potentials. However, mechanistic insights remain to be addressed.

Hypoxic exposure activates several signal transduction pathways. Hypoxia inducible factor (HIF) is a master transcription factor that regulates the expression of hundreds of genes to promote cellular adaptation to hypoxic condition [Rocha, 2007; Semenza, 2007]. Both HIF-1α and HIF-2α signaling pathways were shown to control self-renewal and multipotency of stem cells and progenitor cells, suggesting the crucial roles of HIFs in stem cell biology [Csete, 2005; Gustafsson et al., 2005; Covello et al., 2006; Keith and Simon, 2007].

Although HIF-1/2α pathways are key signaling molecules regulating cellular response to hypoxic exposure, other pathways are also activated in response to hypoxic exposure. For example, unfolded protein responses (UPRs) are activated in response to hypoxic exposure [Simon and Keith, 2008].

In this report, we hypothesized that HIF signaling plays a major role to enhance self-renewal of MSCs in hypoxic condition, and we tested this hypothesis. Unexpectedly, we found that HIF signaling pathways play rather limited roles in enhanced self-renewal in hypoxic condition. Among HIF-independent pathways activated in response to hypoxic exposure, UPRs are responsible for hypoxia-initiated decrease of MSC differentiation to adipogenic lineage.

MATERIALS AND METHODS

MATERIALS

Anti HIF-1α (clone H1α67)(ab7), anti HIF-2α (ab81635), and anti TATA binding protein (TBP) (clone 1TBP18)(ab818) antibodies were from Abcam (Cambridge, MA). HIF-1β (H1beta234) antibody was from Novus Biologicals (Littleton, CO). MEMα, DMEM culture media and supplements were all from Invitrogen (Carlsbad, CA). FBS was from Atlanta Biologicals (Lawrenceville, GA). Retrovirus expression vector of the constitutively active (stable) forms of HIF-1α (HA-HIF1alpha P402A/P564A-pBabe-puro) and HIF-2α (HA-HIF2alpha-P405A/P531A-pBabe-puro) (Addgene plasmid 19005&19006) were courtesy of Dr. Kaelin at the Harvard Medical School (Boston, MA) [Yan et al., 2007]. Retrovirus expression vector of pBABE-puro (Negative control) (Addgene plasmid 1764) was courtesy of Dr. Weinburg at MIT (Cambridge, MA). Lentivirus Mission® shRNA vectors and all of the reagents were from Sigma (Saint Louis, MO). These studies were approved by the IBC committees of the Ohio State University and the University of Pittsburgh for recombinant DNA research and biohazard agents in obedience to National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

CELL CULTURE

Cultured human primary MSCs were purchased from Lonza (Basal, Switzerland) and were cultured in MEMα supplemented with 17% FBS, 2 mM L-glutamine, 1 mM pyruvate, and 100 μM non-essential amino acids. Human telomerase reverse transcriptase—immortalized human MSCs (immortalized-MSCs) were the kind gift from Dr. Junya Toguchida (Kyoto University, Kyoto, Japan) [Okamoto et al., 2002], and were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM pyruvate, and 100 μM non-essential amino acid. The multipotency of these cells was confirmed previously [Tamama et al., 2006]. All of the experiments were conducted in ambient O_2 condition (20%) unless otherwise specified.

RETROVIRAL EXPRESSION VECTORS

Retroviral vector was prepared according to the manufacture's instruction (Cell Biolabs, San Diego, CA) [Morita et al., 2000]. In brief, PLAT-GP 293 packaging cells (Cell Biolabs, San Diego, CA) were plated at 4×10^6 cells per 10 cm dish and incubated overnight. Next day, the cells were transfected with 9 μg of pBABE-puro, HA-HIF1alpha P402A/P564A-pBABE-puro or HA-HIF2alpha-P405A/P531A-pBABE-puro vectors with FuGENE®6 transfection reagent (Roche, Basel, Switzerland). Virus-containing cell culture supernatant was collected after 48 h and transfection, and was filtered with 0.45 μm pore-size filter (Millipore, Bedford, MA). The titer was measured by QuickTiter™ Retrovirus Quantitation Kit (Cell Biolabs, San Diego, CA) according to manufacturer's instruction. Retroviral vectors were aliquoted and stored in −80°C freezer until used. Immortalized-MSCs were infected with these retroviruses at MOI of 100 in the presence of 8 μg/ml polybrene.

LENTIVIRAL shRNA VECTORS

Lentiviral vector was prepared according to the manufacture's instruction (Sigma–Aldrich, St. Louis, MO). In brief, 293FT cells (Invitrogen, Carlsbad, CA) were plated at 1×10^6 cells per 10 cm dish and incubated overnight. Next day, the cells were transfected with 2.6 μg of shRNA transfer vector and 26 μl of Mission Lentiviral Packaging Mix (SHP001, Sigma–Aldrich, St. Louis, MO) with Fugene 6 transfection reagent (Roche). Virus-containing cell culture supernatant was collected twice in 48 and 72 h after transfection, and was filtered with 0.45 μm pore-size filter (Millipore, Bedford, MA). Viral particle-associated p24 antigen was quantitated by HIV p24 ELISA kit (Cell Biolabs, San Diego, CA) and converted to functional viral titer. Lentiviral vectors were aliquoted and stored in −80°C freezer until used.

HYPOXIC CONDITION

Hypoxic condition was produced by culturing cells in premixed gas (1%O_2, 5%CO_2, balance N_2) (Praxair, Danbury, CT) in a hypoxia chamber (Stemcell Technologies, Vancouver, Canada). In brief, cell culture dishes or multiwell plates were placed in the chamber

equipped with airtight seal. Then, the chamber was flushed for 5 min or more with premixed gas (20 L/min) according to the manufacture's instruction. Partial oxygen pressure (pO_2) of cell culture medium (20 ml in 10 cm culture dish) within the chamber was traced by Oxylite 2000E with BF/O/E pO2 Bare-Fibre sensor (Oxford Optronix Ltd, Oxford, UK) after mixed gas flushing, which indicated that pO_2 reaches equilibrium within 4 h for 1%O_2 mixed gas. The chamber was flushed with premixed gas every 3 days or less, as pO_2 was maintained up to day 4 after flushing (data not shown). Cell culture media, trypsin/PBS and PBS were all pre-equilibrated with 1%O_2 mixed gas for overnight prior to medium change or any manipulations to minimize the exposure of cells to room air (20%O_2) during procedures such as medium change unless stated otherwise.

COLONY FORMATION ASSAY

Total of 500 cells was seeded in 100 mm culture dish and culture in 14 days as indicated. To exclude the possibility that hypoxic exposure affects MSC colony formation through altering adherence efficiency of MSC to plastic [Ginis et al., 1995], MSCs were allowed to adhere to plastic under 20%O_2 condition after seeding for 24 h before initiation of hypoxic treatment. Culture medium was changed at day 7. Upon completion of the indicated treatment, the formed colonies were rinsed with PBS twice to dislodge the dead cells and debris, then fixed and stained by 5% crystal violet in methanol. The number of colony formation (>2 mm diameter) was enumerated manually by three independent researchers. The result was given as a colony count per 1000 seeded cells.

OSTEOGENIC DIFFERENTIATION

Osteogenic differentiation was induced as previously described [Tamama et al., 2006]. In brief, MSCs were seeded at 5×10^4 cells per 24-well and cultured in Osteogenic differentiation medium containing dexamethasone, ascorbate, and β-glycerophosphate (Lonza, Basal, Switzerland) for 7 days. Induction of alkaline phosphatase expression is an early stage of osteogenic differentiation of MSC [Jaiswal et al., 1997], and intracellular alkaline phosphatase enzyme activity was measured by quantitative colorimetric assay kit (BioAssay Systems, Hayward, CA).

ADIPOGENIC DIFFERENTIATION

Adipogenic differentiation was induced as previously described after slight modifications [Tamama et al., 2006]. In brief, MSCs were seeded at 1×10^6 cells per 6-well and cultured in Adipogenic differentiation induction medium for 2–3 days followed by Adipogenic differentiation maintenance medium for 2 days in 1–2 cycles, as manufacture's instruction (Lonza, Basal, Switzerland). Adipogenic differentiation was evaluated by staining intracellular lipid droplets by Oil Red O and by induction of adipogenic marker peroxisome proliferation-activated receptor γ (PPARγ) expression [Pittenger et al., 1999; Tamama et al., 2006].

QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (QRT-PCR)

The level of mRNA transcripts was assessed by two step qRT-PCR. In brief, total RNA was prepared with TRIzol plus (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. TURBO DNA-free kit (Applied Biosystems, Foster City, CA) was used for the removal of possible contaminated genomic DNA and subsequent removal of DNase itself from the total RNA preparation. Then, cDNA was synthesized from total RNA with High Capacity RNA-to-DNA kit (Applied Biosystems). Then, cDNA was mixed with Taqman® Universal Master Mix and each Taqman® gene-specific probe/primers (Applied Biosystems) and subject to the following PCR reaction according to the Taqman® PCR universal thermal cycling conditions defined by Applied Biosystems: 95°C for 10 min and then, 40 cycles of two-temperature PCR at 95°C for 15 s for denaturing, 60°C for 1 min for annealing and extension. Mx3000P (Stratagene, La Jolla, CA) was used as a quantitative real-time PCR thermal cycler.

Taqman® probe/primers were used for HIF-1α (Hs00936368_m1), HIF-2α (Hs01026142_m1), HIF-1β or aryl hydrocarbon receptor nuclear translocator (Hs01121918_m1), $p16^{INK4A}$ or cyclin-dependent kinase inhibitor 2A (Hs00923894_m1), Osterix (Osx) (Hs01866874_s1), and PPARγ (Hs01115513_m1) (Applied Biosystems). Taqman® probe/primers for human ribosomal protein, large, P0 (RPLP0) (4333761F) were used as an endogenous invariant control (housekeeping gene) (Applied Biosystems). All PCR primers except for Osx were designed to span intron(s) to discriminate cDNA amplicons from genomic amplicons according to the manufacture. Amplification efficacy of each PCR reaction was assessed initially with serial dilution of control samples; those fell into 95–105%. Comparative Ct method was utilized to assess the levels of each mRNA transcript relative to that level of RPLP0 mRNA transcript.

IMMUNOBLOTTING

After the indicated treatments, cell lysates were generated as instructed by Abcam. Briefly, cells were lysed on ice in a buffer containing 10 mM Tris-HCl (Ph 8.0), 1 mM EDTA, 150 mM NaCl, 0.5% NP-40, 5 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM PMSF. Lysates were centrifuged at 500 g at 4°C for 5 min to precipitate nuclei. Then, these nuclei were further lysed in a buffer containing 20 mM Hepes, 400 mM NaCl, 1 mM EDTA, 1 mM Dithiothreitol, 20 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM PMSF. The protein content was determined by using BCA protein reagents (Thermo Fisher Scientific, Rockford, IL). Protein electrophoresis was performed by using NuPAGE electrophoresis system (Invitrogen, Carlsbad, CA). In brief, after adding NuPAGE LDS Sample Buffer (Invitrogen) and NuPAGE Sample Reducing Agent (Invitrogen), protein lysates were incubated at 70°C for 10 min, then, 5 μg of nuclear lysate was loaded, separated by NuPAGE® Novex Bis-Tris Gels (Invitrogen) with MOPS SDS Running Buffer (Invitrogen), and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Blots were probed by primary antibodies before visualizing with HRP conjugated secondary antibodies included in Pierce Fast Western Blot kit (Thermo Fisher Scientific, Waltham, MA).

HRE REPORTER ASSAY

The activity of HIF signaling was measured by using an adenovirus HIF response element (HRE) luciferase promoter–reporter construct (Ad-HRE-Luc), as described previously [Khanna et al., 2006; Roy

et al., 2007]. In brief, the Ad-HRE-Luc vector was infected at MOI of 50 in the presence of polybrene (2 μg/ml) at 48 h before analyzing HRE activity. Then, cell lysate was obtained and cell luciferase activity was determined by Luciferase Assay kit according to the manufacture's protocol (Stratagene, La Jolla, CA). HRE-luc-derived luminescence was measured using a LB 9507 luminometer (EG and G Berthold, Bad Wildbad, Germany), and the results were expressed in relative light unit (RLU) per second after adjusted against total protein concentration in the samples.

GENE KNOCKDOWN WITH shRNA

The HIF-1α shRNA (MISSION shRNA lentivirus, TRCN0000003810: CCGGGTGATGAAAGAATTACC GAATCTCGAGATTCGGTAATTCTTT-CATCACTTTTT), HIF-2α shRNA (MISSION shRNA lentivirus, TRCN0000003804: CCGGCGACCTGAAGATTGAAGTGATCTCGAGAT-CACTTCAATCTTCAGGTCGTTTTT), and HIF-1β shRNA (MISSION shRNA lentivirus, TRCN0000003819: CCGGGAGAAGTCAGATGGTT-TATTTCTCGAGAAATAAACCATCTGACTTCTCTTTTT) and Non-target shRNA control vector (MISSION Non-target shRNA control lentivirus, CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATC-TTGTTGTTTTT) were purchased from Sigma–Aldrich (Saint Louis, MO). Immortalized-MSCs plated on 12-well plate at 5×10^4 cells were infected with shRNA lentivirus at MOI of 100 in the presence of polybrene (8 μg/ml).

PROLIFERATION ASSAY

Primary MSCs were seeded in 6-well plate at the density of 50 cells/cm^2 (480 cells/well) in regular medium containing 17%FBS and cultured in 20%O_2 condition or 1%O_2 condition until reaching 70% confluence. Cell culture medium was changed every 3–4 days. Then, the total cell count was determined by actual enumeration by Coulter Cell Counter Z2 (Beckman Coulter, Inc. Fullerton, CA) in the indicated timing and those cells were re-plated and repeat the same process. Cell plating was done twice after initial cell seeding during 52-day experiment (Fig. 1A). Population doubling was calculated based upon those cell counts.

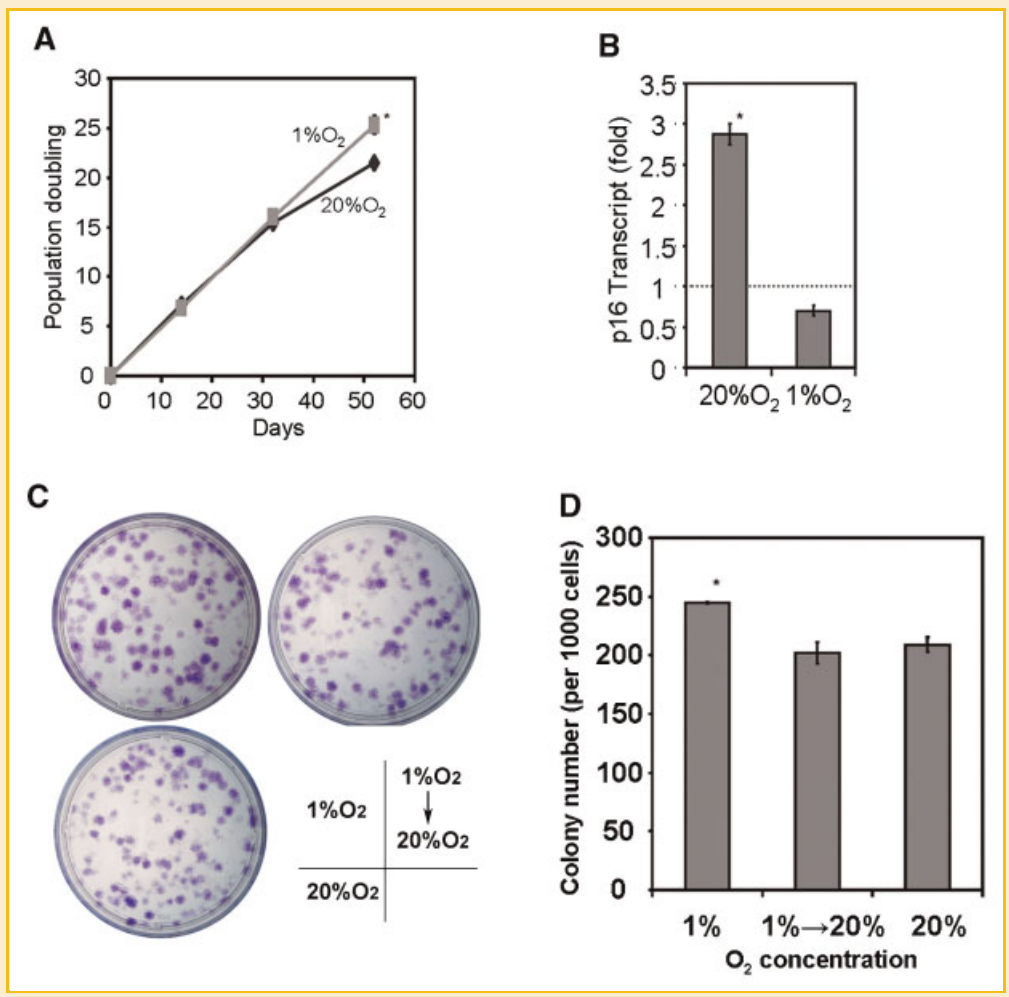

Fig. 1. (A) Population doubling of MSCs up to 52 days by actual enumeration (*<0.05 to 20%O_2). Cell counts were expressed as a population doubling of the original cell number seeded in each well. (B) The level of $p16^{INK4A}$ mRNA transcript in day 52 by quantitative RT-PCR. (*$P < 0.05$ to 20%O_2 in day 0). The level of $p16^{INK4A}$ transcript in day 0 was normalized to 1.0. (C and D) Effects of hypoxic exposure on primary MSC colony formation. Five hundred cells were seeded in 10 cm dish and the number of formed colonies was counted in day 14. (*$P < 0.05$ to 20%O_2 condition in D). Part C shows representative images of MSC colony formation under 1%O_2, 20%O_2, and 20%O_2 after pre-treatment with 1%O2 for 4 days.

ELISA

Human immortalized-MSCs were cultured in serum reduced medium (0.1%FBS) for 24 h during the indicated treatments. The conditioned media were collected and the secreted vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) were measured by quantitative sandwich ELISA kits according to each manufacture's instruction (R&D Systems, Minneapolis, MN). The readouts were adjusted by the total amount of cellular protein contents.

STATISTICAL ANALYSIS

All experiments were performed in duplicate or triplicate. Data were analyzed using student *t*-tests. Significance was set at $P < 0.05$ or more stringent as noted in the text and figure legends. The data of multiple observations were provided as mean ± SEM for at least three separate experiments unless stated otherwise.

RESULTS

The oxygen tension within bone marrow varies spatially and it is estimated to be 1–7% [Ishikawa and Ito, 1988; Chow et al., 2001; Harrison et al., 2002]. Oxygen tension is an essential regulator of wound healing and tissue regeneration [Sen, 2009]. Indeed, tissue ischemia is one of the main contributing factors arresting tissue regeneration and wound healing processes [Schaffer et al., 2002], and such a microenvironment is the one which benefits from MSC

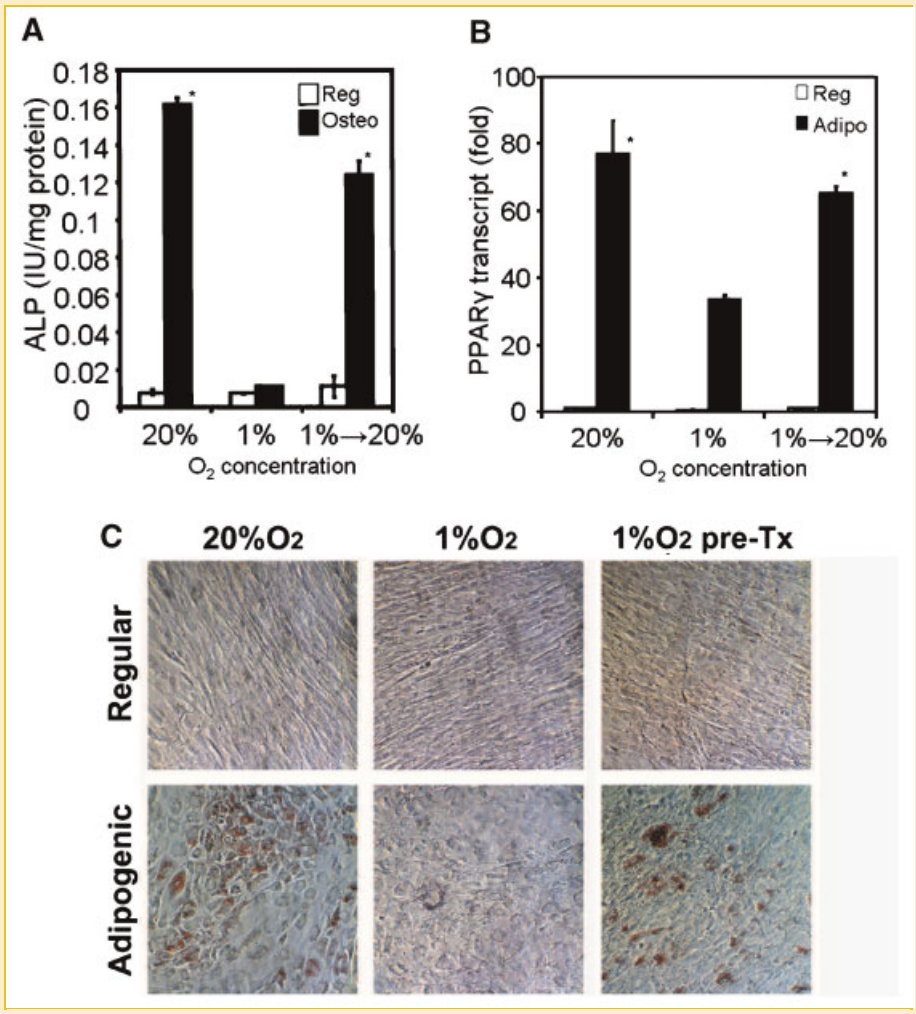

Fig. 2. Effects of hypoxic exposure on the osteogenic and adipogenic differentiation of human primary multipotential stromal cells (MSCs). (A) Effects of hypoxic exposure on osteogenic differentiation of MSCs. Cells were cultured in regular medium (Reg) or osteogenic medium (Osteo) under 20%O_2 (with and without 1%O_2 pre-treatment for 4 days) or 1%O_2 conditions for 7 days, and an early phase of osteogenic differentiation was evaluated by quantitation of alkaline phosphatase (ALP) activity in cell lysates. ($^*P < 0.0005$ to regular medium control of each respective condition). (B and C) Effects of hypoxic exposure on adipogenic differentiation of primary MSCs. Cells were cultured in regular medium (Reg) or two cycles of adipogenic stimulations (Adipo) under 20%O_2 (with and without 1%O_2 pretreatment for 4 days) or 1%O_2 conditions for 8–10 days, and an adipogenic differentiation was evaluated by mRNA transcript levels of peroxisome proliferation-activated receptor γ (PPARγ) expression relative to RPLPO transcripts (housekeeping gene) by quantitative RT-PCR (B) or by microscopic observation of intracellular lipid droplets with Oil Red O staining (C) ($^*P < 0.005$ to adipogenic condition under 1%O_2 condition). Each photograph is 550 μm^2.

transplantation to facilitate tissue regenerating process. Hence, we chose 1%O_2 to represent in vivo O_2 tension of bone marrow as well as ischemic tissues. By using hypoxic flush chamber, hypoxic culture condition was produced successfully within 4 h after flushing with hypoxic gas. Conversely, it took approximately 30 min from 1%O_2 condition to re-equilibrate pO_2 to 20%O_2 (data not shown).

HYPOXIC CONDITION ENHANCES MSC SELF-RENEWAL

Hypoxic condition was shown to enhance self-renewal of certain stem cells through promoting proliferation, reducing apoptosis, and maintaining undifferentiated state [Csete, 2005; Keith and Simon, 2007]. We first evaluated the effects of hypoxic exposure to proliferation, colony formation, and adipogenic and osteogenic differentiation of human primary MSCs.

Cell proliferation rate did not differ significantly between 1%O_2 and 20%O_2 conditions up to 33 days (Fig. 1A); however, accumulated population doubling was 21.45 under 20%O_2 and 25.29 under 1%O_2 at day 52 (Fig. 1A), along with the elevation of senescent marker $p16^{INK4A}$ (or cyclin-dependent kinase inhibitor 2A) transcript (Fig. 1B) only under 20%O_2 condition [Krishnamurthy et al., 2004; Janzen et al., 2006; Shibata et al., 2007]. These data suggest that 1%O_2 condition prolongs replicative lifespan of MSC as compared to 20%O_2 condition, consistent with previous studies [Fehrer et al., 2007; Jin et al., 2010].

One of the prominent characteristics of MSC is their ability to produce colonies after seeded at low density [Friedenstein et al., 1976; Pochampally, 2008]. All MSC populations analyzed by clonal assays were shown to be heterogeneous, containing both early progenitors or rapidly self-renewing (RS) cells and mature/senescent

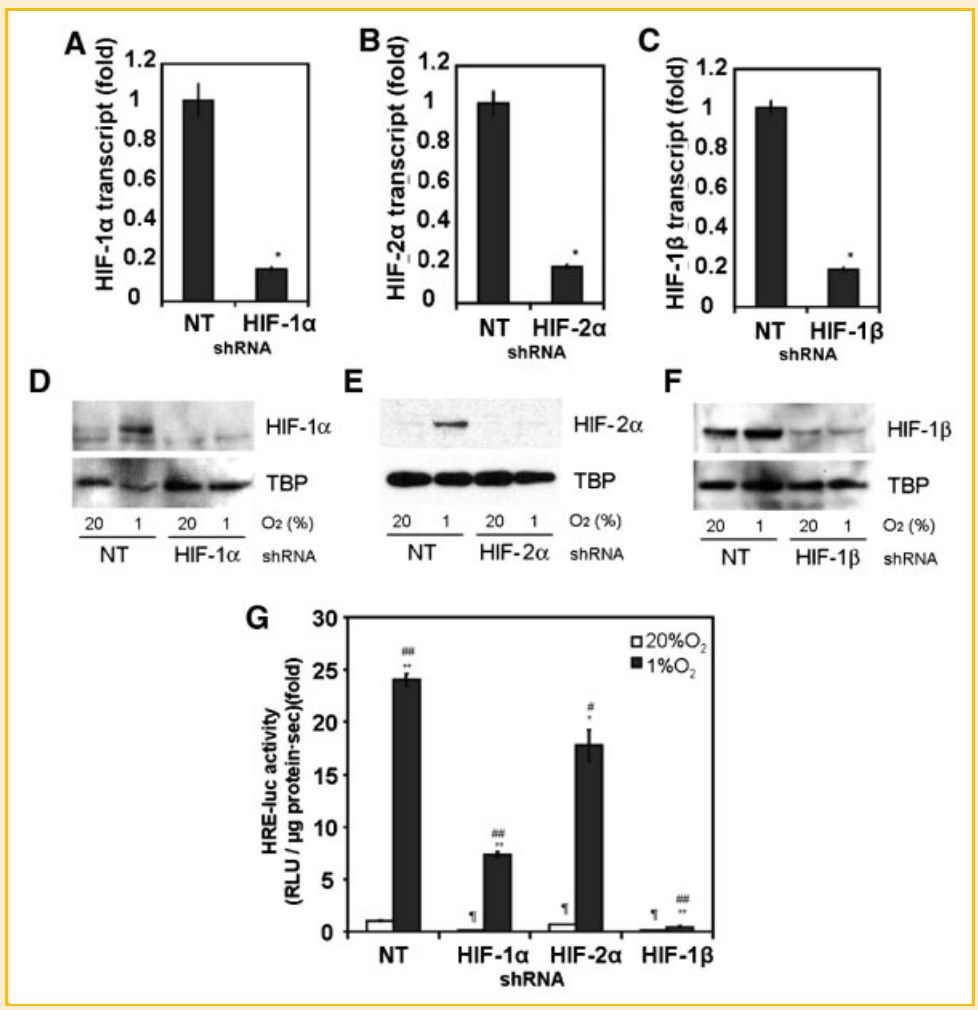

Fig. 3. shRNA-mediated knockdown of HIF-1α (A and D), HIF-2α (B and E), and HIF-1β (C and F) of immortalized- multipotential stromal cell (MSC) evaluated by quantitative RT-PCR (A–C) or by immunoblotting (D–F). (A–C) The levels of HIF-1α (A), HIF-2α (B), and HIF-1β (C) mRNA transcripts were quantitated relative to RPLPO transcript expression (housekeeping gene) by using quantitative RT-PCR ($^{*}P < 0.05$ to Non-target (NT) control). (D–F) Nuclear protein lysates (5 μg each) were loaded in each lane and the protein levels of HIF-1α (D), HIF-2α (E), and HIF-1β (F) were assessed by immunoblotting. NT shRNA was used as a negative control. TATA binding protein (TBP) immunoblots were used as loading controls. (G) HIF reporter element (HRE) reporter activities in immortalized-MSCs with shRNA against HIF-1α, HIF-2α, and HIF-1β under 20%O_2 and 1%O_2 conditions. ($^{*}P < 0.001$, $^{**}P < 0.0001$ to Ctrl under 20%O_2 counterpart in each shRNA condition; ¶ $P < 0.001$ to NT under 20%O_2 condition; #$P < 0.01$, ##$P < P < 0.0001$ to NT under 1%O_2 condition).

cells [Colter et al., 2000; Colter et al., 2001; Sekiya et al., 2002]. A convenient way to evaluate the proportion of early progenitors in a MSC population is colony forming assay, as early progenitors are colony forming cells [Colter et al., 2000; Smith et al., 2004; Pochampally, 2008]. Hypoxic condition (1%O_2) increased colony formation of primary MSCs as compared to 20%O_2 condition (Fig. 1C and D), even 1%O_2 condition did not increase cell proliferation in the first 14 days (Fig. 1A). This discrepancy between colony formation and cell proliferation clearly shows that colony formation is not a mere reflection of cell proliferation; it is rather a reflection of the presence of colony forming cells, which were shown to be early progenitors before [Colter et al., 2000; Smith et al., 2004; Pochampally, 2008]. Pre-treatment of MSC with 1%O_2 did not increase the colony formation as compared with 20%O_2 (Fig. 1C and D). These data suggest that early progenitor fraction of MSCs is better preserved under hypoxic condition in a reversible manner.

Another characteristic feature of MSC is multidifferentiation potential; however, differentiation process itself interferes with self-renewing of undifferentiated MSCs. The default differentiation pathway of MSC is osteogenic differentiation [Phinney and Prockop, 2007; Wagner et al., 2008], and we evaluated the effects of hypoxic exposure on MSC osteogenic differentiation. Hypoxic condition decreased MSC differentiation into osteogenic lineage (Fig. 2A). Although hypoxic condition itself decreases ongoing differentiation, it is reversible as MSCs retained osteogenic differentiation potential after pre-treatment with hypoxic exposure (Fig. 2A). Hypoxic condition also decreased MSC differentiation into adipogenic lineage in a reversible manner (Fig. 2B and C). These results suggest that hypoxic culture condition promote self-renewal of MSCs through preserving early progenitor fraction and maintaining undifferentiated state, in agreement with previous studies with MSCs [Grayson et al., 2006; Fehrer et al., 2007].

shRNA SUCCESSFULLY KNOCKS DOWN THE EXPRESSION OF HIF-2α, AND HIF-1*B*, WHEREAS STABLE FORMS OF HIF-1α AND HIF-2α REPRODUCE HRE-DEPENDENT TRANSACTIVATION EVEN UNDER 20%O_2 CONDITION

HIF is a master transcription factor that regulates the expression of hundreds of genes through binding to HRE in response to hypoxia [Rocha, 2007; Semenza, 2007]. Since HIF signaling pathways were shown to be critical in maintaining stemness of embryonic stem cells [Csete, 2005; Gustafsson et al., 2005; Covello et al., 2006; Keith and Simon, 2007], we hypothesized that HIF signaling pathways play pivotal roles in MSC self-renewal under hypoxic culture condition.

HIF consists of the constitutively expressed β-subunit (HIF-1β or ARNT) and the regulatory α-subunit (HIF-1α and HIF-2α), which is stabilized mainly in response to hypoxia [Rocha, 2007; Semenza, 2007]. HIF-1α is most widely expressed in almost all tissues and the best studied and characterized to date, while the expression of HIF-2α is restricted to certain cell types, such as vascular endothelial cells and less characterized [Tian et al., 1997; Wiesener et al., 2003].

We utilized both loss of function and gain of function approaches to dissect the role of HIF signaling pathways in MSCs in response to hypoxic exposure. As for loss of function study, we utilized lentivirus-vector shRNAs against HIF-1α and HIF-2α, which successfully decreased the levels of mRNA transcripts less than 20% of non-target (NT) control (Fig. 3A and B), and these were further validated in protein level by immunoblot (Fig. 3D and E). But HRE-dependent transactivation under 1%O_2 condition decreased only to 30.6% of NT with HIF-1α shRNA and 73.9% of NT with HIF-2α shRNA, presumably due to the presence of the other HIF-α in each condition (Fig. 3G). It was confirmed by utilizing lentivirus-vector shRNA against HIF-1β, as HIF-1β is a shared partner for heterodimer formation for both HIF-1α and HIF-2α. The transcript level of HIF-1β was less than 20% as compared with NT control (Fig. 3C), and it was confirmed in protein level by immunoblot (Fig. 3F). HRE-dependent transactivation was also reduced under 20%O_2 condition (6.9% of NT) as well as under 1%O_2 condition (1.8% of NT) (Fig. 3G), further confirming the effective blockade of HRE-dependent transactivation by both HIF-1α and HIF-2α.

As for gain of function study, we utilized retrovirus-based constitutively active (stable) HIF-1α and HIF-2α expression vectors to specifically enhance either HIF-1α or HIF-2α signaling pathway. These expression vectors carry both prolyl hydroxylation sites (proline 402 and 564 in HIF-1α and proline 405 and 531 in HIF-2α) replaced by alanine in the oxygen-dependent degradation domain,

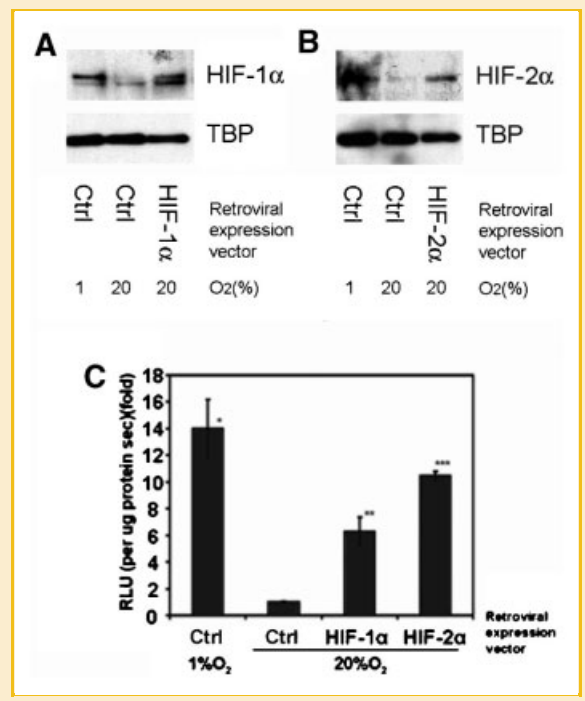

Fig. 4. Forced expression of stable form of HIF-1α and HIF-2α in immortalized-multipotential stromal cell (MSC). (A and B) Immunoblot analysis of HIF-1α (A) or HIF-2α (B) in immortalized-MSC with stable HIF-1α or HIF-2α under indicated O_2 condition. Nuclear protein lysates were loaded in 5 μg each. Puromycin-resistant blank expression vector (Ctrl) served as a control. TATA binding protein (TBP) immunoblots served as loading controls. (C) HIF reporter element (HRE) reporter activities in immortalized-MSCs with stable HIF-1α or HIF-2α expression vector under 20%O_2 condition. Puromycin-resistant blank expression vector (Ctrl) was used as a control. ($^{*}P < 0.005$, $^{**}P < 0.001$ to Ctrl under 20%O_2 condition).

thus escaping subsequent von Hippel-Lindau tumor-suppressor protein (VHL)-mediated degradation even in the presence of 20%O_2 [Yan et al., 2007] (Fig. 4A and B). Transfection of these expression vectors induced HRE-dependent transactivation in 20%O_2 condition in a comparable degree to 1%O_2 exposure, confirming the physiological activation of either HIF-1α or HIF-2α signaling pathway in immortalized-MSCs (Fig. 4C).

HYPOXIC CONDITION DECREASES OSTEOGENIC DIFFERENTIATION IN A HIF-DEPENDENT MANNER

Osteogenic differentiation, the default pathway for MSCs, could antagonize self-renewing of undifferentiated MSCs. Hypoxic condition decreased MSC osteogenic differentiation (Fig. 2A), and we queried whether hypoxia-dependent inhibition of osteogenic differentiation is HIF-dependent.

We first introduced stable HIF-1α or HIF-2α into immortalized-MSCs to delineate the role of HIF-1α and HIF-2α signal pathways on osteogenic differentiation. Both stable HIF-1α and HIF-2α decreased mRNA transcript of osteogenic transcription factor Osx (Suppl Fig. 1) and alkaline phosphatase activity in immortalized-MSCs (Fig. 5A), suggesting that both HIF-1α and HIF-2α help maintain undifferentiated state of MSCs through decreasing osteogenic differentiation under hypoxic condition.

We also utilized shRNAs against HIF-1α and HIF-2α to further elucidate the role of HIF in hypoxia-mediated decrease of osteogenic differentiation. shRNA against HIF-1α or HIF-2α alone failed to reverse the decrease of osteogenic differentiation induced by hypoxic exposure, presumably due to residual HIF signaling by the other form of HIF-α (Fig. 5B–D). On the other hand, shRNA against HIF-1β reversed the decrease of osteogenic differentiation induced by hypoxia (Fig. 5E), as shRNA against HIF-1β was able to decrease HRE-transactivation by both HIF-1α and HIF-2α (Fig. 3G).

HYPOXIC CONDITION DECREASES ADIPOGENIC DIFFERENTIATION INDEPENDENT OF HIF SIGNALING

Adipogenic differentiation is another key differentiation pathway for MSCs. Hypoxic condition also decreased MSC adipogenic differentiation (Fig. 2B and C); however, stable forms of HIF-1α as well as HIF-2α failed to decrease adipogenic differentiation (Fig. 6A). MSCs were able to differentiate into adipogenic lineage in the presence of HIF-α stabilizer dimethyloxaloylglycine (DMOG) under normoxic condition (Suppl Fig. 2A–C). Moreover, shRNAs against HIF-1α, -2α, and -1β all failed to reverse the decrease of adipogenic differentiation induced by hypoxia. These data suggest that hypoxic exposure decreases adipogenic differentiation in a HIF-independent manner.

Besides HIF signaling, hypoxic exposure causes ER stress, which activates UPR [Wouters and Koritzinsky, 2008]. UPR has been suggested to modulate adipogenic differentiation of 3T3-L1 pre-adipocytes [Basseri et al., 2009; Bobrovnikova-Marjon et al.,

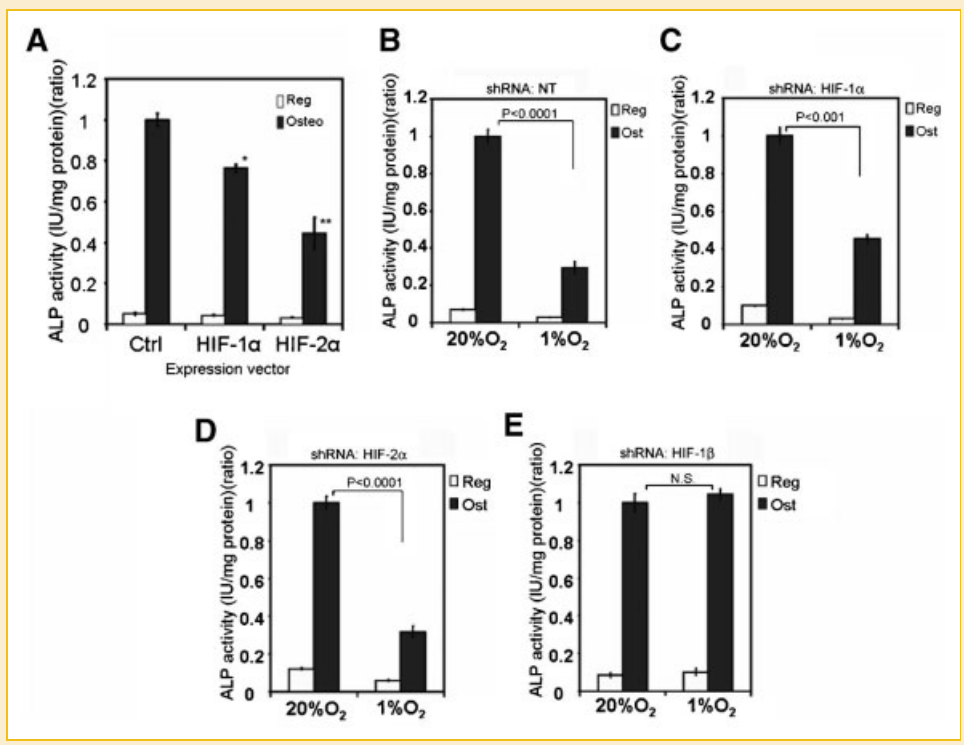

Fig. 5. Hypoxic condition decreases osteogenic differentiation of multipotential stromal cell (MSC) in a HIF-dependent manner. Immortalized-MSCs were cultured in regular medium (Reg) or osteogenic medium (Osteo) for 7 days, and an early phase of osteogenic differentiation was evaluated by quantitation of alkaline phosphatase (ALP) activity in cell lysates. (A) Effects of stable HIF-1α or HIF-2α on osteogenic differentiation under 20%O_2 condition. Puromycin-resistant blank expression vector (Ctrl) was used as a control ($^{*}P < 0.05$, $^{**}P < 0.0005$ to osteogenic medium of Ctrl vector condition). (B–E) Effects of HIF-1α shRNA (C), HIF-2α shRNA (D), and HIF-1β shRNA (E) treatments on osteogenic differentiation of immortalized-MSCs under 20%O_2 and 1%O_2 conditions. NT shRNA (B) was used as a control. The level of ALP activity in osteogenic condition under 20%O_2 was normalized to 1.0 in each figure. *P*-values between 20%O_2 and 1%O_2 conditions in osteogenic condition were given in each figure. (N.S. = not significant).

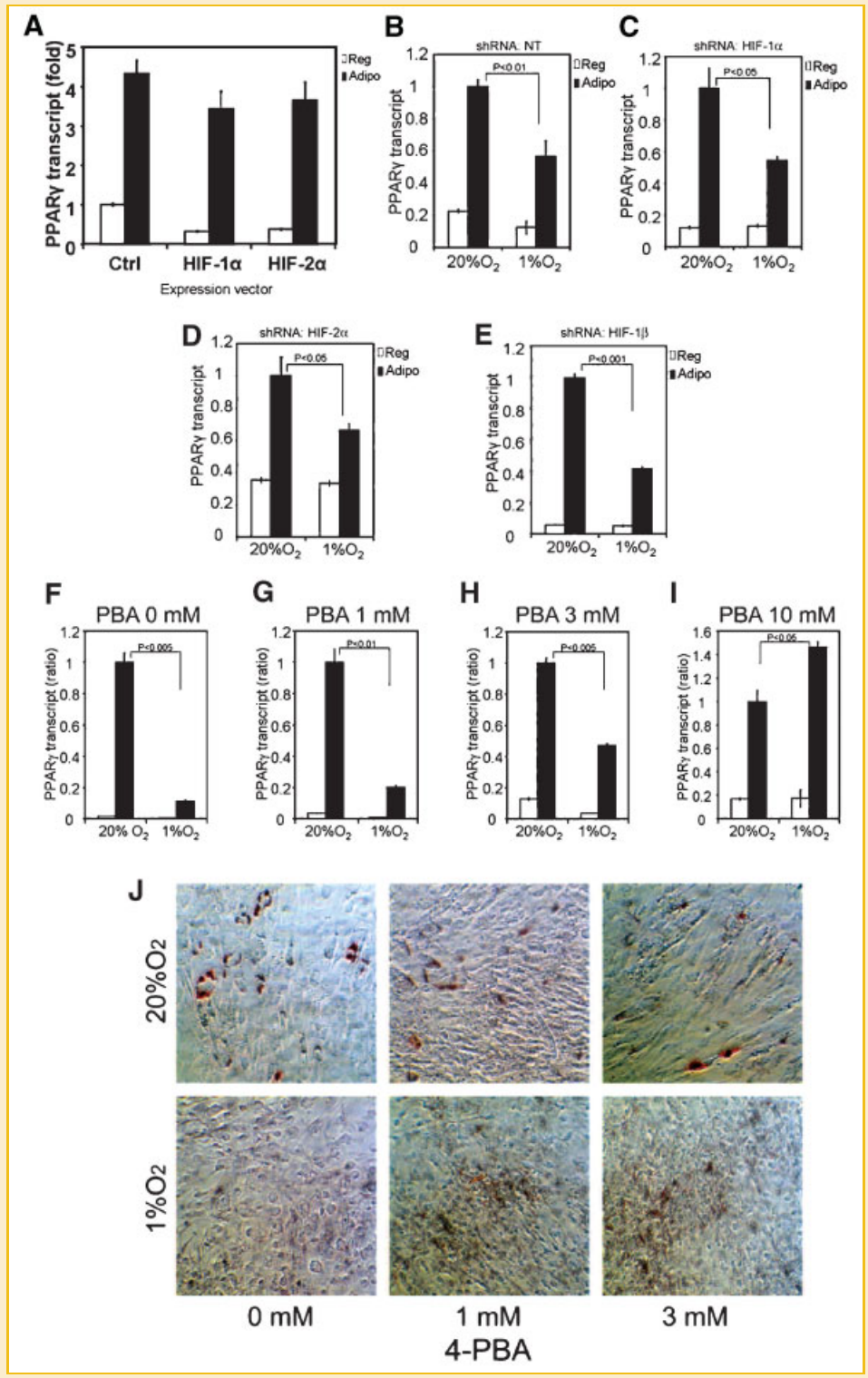

Fig. 6. Hypoxic condition decreases adipogenic differentiation of multipotential stromal cell (MSC) in an unfolded protein response (UPR)-dependent, but not a HIF-dependent manner. MSCs were cultured in regular medium (Reg) or stimulated with two cycles of adipogenic induction/maintenance media (Adipo) for 8–10 days, and adipogenic differentiation was evaluated by mRNA transcript levels of peroxisome proliferation-activated receptor γ (PPARγ) by quantitative RT-PCR (A–I) or by staining intracellular lipid droplets by Oil Red O (J). (A) Effects of stable HIF-1α or HIF-2α on adipogenic differentiation of immortalized MSCs under 20%O_2 condition. Puromycin-resistant blank expression vector (Ctrl) was used as a control. (B–E) Effects of HIF-1α shRNA (C), HIF-2α shRNA (D), and HIF-1β shRNA (E) treatments on adipogenic differentiation of immortalized-MSCs under 20%O_2 and 1%O_2 conditions. NT shRNA (B) was used as a control. The level of PPARγ transcript in adipogenic condition under 20%O_2 was normalized to 1.0 in each figure. *P*-values between 20%O2 and 1%O2 conditions in osteogenic condition were given in each figure. (F–J) Effects of chemical chaperon 4-phenyl butyric acid (PBA) on adipogenic differentiation of primary MSCs under 20%O_2 and 1%O_2 conditions. The level of PPARγ transcript in adipogenic condition under 20%O_2 was normalized to 1.0 in each figure. *P*-values between 20%O2 and 1%O2 conditions in adipogenic condition were given in each figure. (J) Representative images of Oil Red O staining after adipogenic treatments in the presence of PBA. Each photograph is 550 μm^2.

2008; Sha et al., 2009]. By using 4-phenyl butyric acid (PBA), a chemical chaperon which eases ER stress and decreases UPR activation [Ozcan et al., 2006], we queried the possible involvement of UPR pathway in the hypoxia-mediated decrease of adipogenic differentiation. PBA dose-dependently reversed the decrease of hypoxia-mediated adipogenic differentiation, suggesting that UPR activation is responsible for decreasing adipogenic differentiation under hypoxic condition (Fig. 6F–J).

HYPOXIC CONDITION ENHANCES MSC COLONY FORMATION INDEPENDENT OF HIF SIGNALING

MSC colony formation unit reflect the proportion of early progenitor cells within MSC preparation [Colter et al., 2000; Smith et al., 2004; Pochampally, 2008]. Although 1%O_2 condition increased colony formation of primary MSCs as compared to 20%O_2 condition (Fig. 1C and D), it was not reversed by shRNA against HIF-1α, HIF-2α, and HIF-1β under 1%O_2 condition, suggesting that HIF signaling is not responsible for hypoxia-mediated increase of colony formation (Fig. 7A and B).

HYPOXIC CONDITION INCREASES VEGF, HGF AND bFGF SECRETIONS FROM MSCS IN A HIF-DEPENDENT MANNER

MSCs were shown to have a strong paracrine capability of various growth factors such as VEGF or HGF, which promote angiogenesis and tissue regeneration after MSC transplantation [Kinnaird et al., 2004; Crisostomo et al., 2008]. Hypoxic exposure augments paracrine secretion from MSCs, and we tested whether activation of HIF signaling is responsible for hypoxia-induced increase of growth factor secretion.

Stable HIF-1α and HIF-2α increased VEGF secretion, whereas only stable HIF-1α increased HGF secretion from MSCs (Fig. 8A and C). shRNA against HIF-1β reversed the increased secretion of VEGF and HGF under hypoxic condition (Fig. 8B and D). Unexpectedly, bFGF secretion was not altered by HIF-1β shRNA treatment (Fig. 8F), even constitutively active HIF-1α increased bFGF secretion (Fig. 8E), suggesting that HIF-1α enhances bFGF secretion without associating with HIF-1β. In sum, HIF pathway is responsible for hypoxia-mediated increase of VEGF, HGF, and bFGF secretion from MSC.

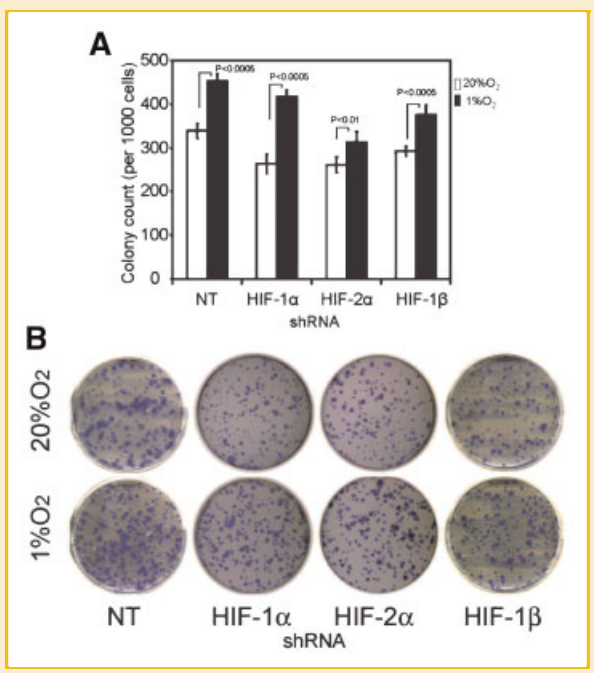

Fig. 7. Hypoxic exposure enhances multipotential stromal cell (MSC) colony formation in a HIF independent manner. (A) Effects of HIF-1β shRNA against HIF-1α, HIF-2α, and HIF-1β on the colony formation of immortalized-MSCs under 1%O_2 and 20%O_2 conditions. *P*-values are given within the figure. (B) Representative images of colony formation of immortalized-MSCs with shRNA against HIF-1α, HIF-2α, and HIF-1b shRNA or HIF-1β. Non-target shRNA (NT) served as a control.

DISCUSSION

Hypoxic condition enhances self-renewal of stem cells through promoting proliferation, reducing apoptosis, and maintaining undifferentiated state of these cells [Csete, 2005; Keith and Simon, 2007]. Cell differentiation is an antagonizing process against self-renewal of undifferentiated early progenitors in MSC populations, and thus it should be suppressed during in vitro expansion [Cellot and Sauvageau, 2007]. HIF signaling pathways are thought to be critical in those processes [Csete, 2005; Gustafsson et al., 2005; Covello et al., 2006; Keith and Simon, 2007], but the significance of HIF signaling pathways in these processes has not been demonstrated in MSCs. Hypoxic condition increased colony formation (Fig. 1C and D) and decreased osteogenic and adipogenic differentiation in a reversible manner (Fig. 2); however, our study revealed that HIF signaling pathways have rather limited roles in MSC self-renewal in hypoxic condition.

Our study showed that hypoxic condition decreased osteogenic differentiation (Fig. 2A), which was reproduced by stable HIF-1α and HIF-2α under 20%O_2 condition and was reversed by shRNA against HIF-1β under 1%O_2 condition (Fig. 5). Core binding factor/runt-related gene 2 α1 (Cbfa1/Runx 2) and Osx are main transcriptional factors promoting osteogenic differentiation [Franceschi et al., 2007]. Interestingly, both stable HIF-1α and HIF-2α decreased the levels of Osx transcripts (Suppl Fig. 1) in a similar way that they decreased alkaline phosphatase activity (Fig. 5A), whereas they did not alter the levels of Runx2 transcripts (data not shown). These data demonstrate that hypoxic condition decreases osteogenic differentiation through activation of HIF signaling pathway, presumably through decreasing Osx expression, without diminishing osteogenic differentiation potentials.

Previous reports about the effects of hypoxia/HIF on osteogenic differentiation were controversial. For example, hypoxic condition was shown to inhibit osteogenic differentiation of MSCs and bone marrow-isolated adult multilineage inducible (MIAMI) cells [D'Ippolito et al., 2006; Fehrer et al., 2007]. Other groups also showed that hypoxic exposure decreased Runx2 expression and osteogenic differentiation [Park et al., 2002; Ontiveros et al., 2004]. On the otherhands, some reports demonstrated that HIF-1α and/or HIF-2α promote osteogenesis [Tamiya et al., 2008; Riddle et al., 2009; Shomento et al., 2010; Wan et al., 2010]. The apparent reasons of these discrepancies are unclear; but osteogenic differentiation is

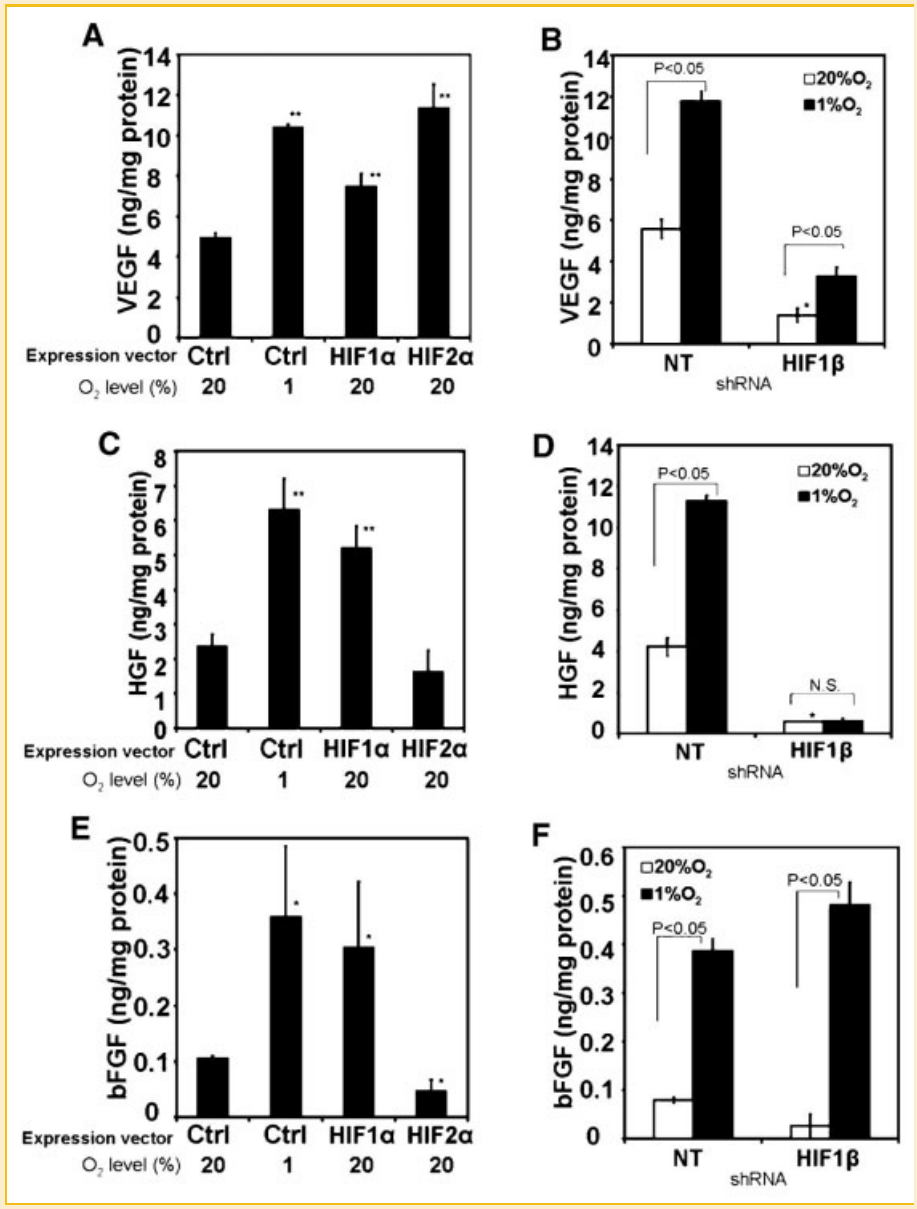

Fig. 8. Effects of stable form of HIF-1α and HIF-2α (A, C, and E) or HIF-1β shRNA (B, D, and F) on the secretion of VEGF (A and B), HGF (C and D), and bFGF (E and F) from immortalized multipotential stromal cells (MSCs). Cells were incubated in the serum-reduced medium for 24 h under indicated O_2 conditions, and the levels of growth factors in the conditioned media were determined by ELISA. Results were normalized by the amount of cellular proteins. ($^{*}P < 0.05$ and $^{**}P < 0.01$ to the control under 20%O_2 condition in each figure for A, C, and E, whereas *P*-values were given in each figure for B, D, and F).

known to have multiple steps [Franceschi et al., 2007], and the effects of hypoxia or HIF-1/2α on osteogenesis might be stage-dependent.

We also showed that hypoxic condition decreased adipogenic differentiation (Fig. 2B and C), but it was not reproduced by stable HIF-1α, stable HIF-2α, and HIF stabilizer DMOG under 20%O_2 condition (Fig. 6 and Suppl Fig. 2). shRNAs against HIF-1α, HIF-2α, and HIF-1β did not reverse it under 1%O_2 condition either (Fig. 6B–E), but PBA, a chemical chaperon easing ER stress, reversed it under 1%O_2 condition (Fig. 6F–J). These data suggest that hypoxic condition decreases adipogenic differentiation, at least partially in an UPR-dependent manner, but not in a HIF-dependent manner. Previous studies showed rather inconsistent results about the effect of hypoxic treatment on adipogenic differentiation. For example, hypoxic condition was shown to reversibly decrease adipogenic differentiation of 3T3 mouse pre-adipocytes, but in a HIF-1α dependent mechanism [Lin et al., 2006; Floyd et al., 2007]. Others showed that hypoxic condition decreased PPARγ2 expression and adipogenic differentiation of mouse embryonic fibroblasts via HIF-1α [Yun et al., 2002]. In contrast, another report showed that hypoxic treatment as well as DMOG treatment (HIF stabilizer) increases adipogenic differentiation of mouse MC3T3-E1 osteoblasts [Irwin et al., 2007]. HIF-2α was shown to rather stimulate adipogenic differentiation of mouse 3T3 pre-adipocytes [Shimba

et al., 2004]. Moreover, another study showed that knockdown of HIF-1α did not reverse the decrease of PPARγ induced by hypoxia [Li et al., 2007], consistent with our data. The apparent reason of these discrepancies is unknown.

UPR has been shown to enhance adipogenic differentiation [Bobrovnikova-Marjon et al., 2008; Basseri et al., 2009; Sha et al., 2009], but UPR-mediated decrease of adipogenic differentiation was also reported [Shimada et al., 2007]. Indeed PBA treatment decreased adipogenic differentiation under 20%O_2 condition; however, at the same time, PBA treatment also increased adipogenic differentiation under hypoxic condition and reverses hypoxia-induced decrease of adipogenic differentiation (Fig. 6). Taken together, we speculate that UPR has dual effects on adipogenic differentiation; it enhances adipogenic differentiation under normoxic condition, but hypoxic condition over-activates UPR and rather inhibits adipogenic differentiation.

It is very important to gain mechanistic insights of hypoxia-mediated increase of MSC colony formation, as colony forming early progenitor cells should be preserved as much as possible during in vitro MSC expansion. Our data showed that hypoxic condition increases MSC colony formation, but it was not reversed by shRNAs against HIF-1α, HIF-2α, and HIF-1β under 1%O2 condition (Fig. 7), suggesting that this hypoxic condition increases MSC colony formation in a HIF-independent manner. The underlying mechanism of hypoxia-mediated increase of MSC colony formation remains unknown. Though 1%O_2 condition decreases intracellular reactive oxygen (ROS) levels, MSC treatment with N-acetylcystein, an antioxidant, failed to increase the colony formation, even intracellular ROS levels were decreased (data not shown). PBA, at least up to 3 mM, also failed to reverse the hypoxia-mediated increase of colony formation (data not shown).

Besides differentiation and direct incorporation of MSC into host tissues, paracrine secretion of growth factors and cytokines plays a pivotal role in MSC-based wound healing and tissue regeneration in vivo [Kinnaird et al., 2004; Phinney and Prockop, 2007; Caplan, 2009]. These wounded tissues are mostly hypoxic in vivo due to impaired blood supplies and thus vascular support through angiogenesis is critical for wound healing and regeneration of these tissues. Our data showed that hypoxic condition further MSCs secrete both VEGF, HGF, and bFGF, and the secretions of these growth factors are further enhanced by hypoxic condition in a HIF-dependent manner (Fig. 8), consistent with previous reports [Rankin et al., 2005; Keith and Simon, 2007; Simon and Keith, 2008].

In summary, we demonstrate that hypoxic condition promotes self-renewal through enhancing preservation of colony forming early progenitor cells and maintaining undifferentiated phenotype of MSCs. HIF signaling pathways are responsible for hypoxia-mediated decrease of osteogenic differentiation and increase of growth factor secretions, but both hypoxia-mediated decrease of adipogenic differentiation and enhanced colony formation are HIF-independent.

ACKNOWLEDGMENTS

We would like to thank Dr. J. Toguchida (Kyoto University, Kyoto, Japan) for providing the immortalized hTERT-human MSCs, Dr. W. Kaolin (Harvard Medical School, Boston, MA) for providing constitutively active HIF-1*α* and HIF-2*α* expression vectors, Dr. Weinberg (MIT, Cambridge, MA) for providing pBABE-puro expression vector, Dr. J. Van Brooklyn (OSU, Columbus, OH) for his generous instrumental support and Ms Miwa Tamama for their technical assistance.

REFERENCES

Antoniou ES, Sund S, Homsi EN, Challenger LF, Rameshwar P. 2004. A theoretical simulation of hematopoietic stem cells during oxygen fluctuations: prediction of bone marrow responses during hemorrhagic shock. Shock 22:415–422.

Basseri S, Lhotak S, Sharma AM, Austin RC. 2009. The chemical chaperone 4-phenylbutyrate inhibits adipogenesis by modulating the unfolded protein response. J Lipid Res 50:2486–2501.

Bobrovnikova-Marjon E, Hatzivassiliou G, Grigoriadou C, Romero M, Cavener DR, Thompson CB, Diehl JA. 2008. PERK-dependent regulation of lipogenesis during mouse mammary gland development and adipocyte differentiation. Proc Natl Acad Sci USA 105:16314–16319.

Caplan AI. 2009. Why are MSCs therapeutic? New data: new insight. J Pathol 217:318–324.

Cellot S, Sauvageau G. 2007. Zfx: at the crossroads of survival and self-renewal. Cell 129:239–241.

Chow DC, Wenning LA, Miller WM, Papoutsakis ET. 2001. Modeling pO(2) distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. Biophys J 81:685–696.

Colter DC, Class R, DiGirolamo CM, Prockop DJ. 2000. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci USA 97:3213–3218.

Colter DC, Sekiya I, Prockop DJ. 2001. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 98:7841–7845.

Covello KL, Kehler J, Yu H, Gordan JD, Arsham AM, Hu CJ, Labosky PA, Simon MC, Keith B. 2006. HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev 20:557–570.

Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR. 2008. Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. Am J Physiol Cell Physiol 294:C675–C682.

Crisostomo PR, Wang M, Wairiuko GM, Morrell ED, Terrell AM, Seshadri P, Nam UH, Meldrum DR. 2006. High passage number of stem cells adversely affects stem cell activation and myocardial protection. Shock 26:575–580.

Csete M. 2005. Oxygen in the cultivation of stem cells. Ann N Y Acad Sci 1049:1–8.

D'Ippolito G, Diabira S, Howard GA, Roos BA, Schiller PC. 2006. Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone 39:513–522.

Fehrer C, Brunauer R, Laschober G, Unterluggauer H, Reitinger S, Kloss F, Gully C, Gassner R, Lepperdinger G. 2007. Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. Aging Cell 6:745–757.

Floyd ZE, Kilroy G, Wu X, Gimble JM. 2007. Effects of prolyl hydroxylase inhibitors on adipogenesis and hypoxia inducible factor 1 alpha levels under normoxic conditions. J Cell Biochem 101:1545–1557.

Franceschi RT, Ge C, Xiao G, Roca H, Jiang D. 2007. Transcriptional regulation of osteoblasts. Ann N Y Acad Sci 1116:196–207.

Friedenstein AJ, Gorskaja JF, Kulagina NN. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267–274.

Ginis I, Mentzer SJ, Li X, Faller DV. 1995. Characterization of a hypoxia-responsive adhesion molecule for leukocytes on human endothelial cells. J Immunol 155:802–810.

Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. 2006. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. J Cell Physiol 207:331–339.

Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, Ruas JL, Poellinger L, Lendahl U, Bondesson M. 2005. Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell 9:617–628.

Harrison JS, Rameshwar P, Chang V, Bandari P. 2002. Oxygen saturation in the bone marrow of healthy volunteers. Blood 99:394.

Irwin R, LaPres JJ, Kinser S, McCabe LR. 2007. Prolyl-hydroxylase inhibition and HIF activation in osteoblasts promotes an adipocytic phenotype. J Cell Biochem 100:762–772.

Ishikawa Y, Ito T. 1988. Kinetics of hemopoietic stem cells in a hypoxic culture. Eur J Haematol 40:126–129.

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312.

Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT. 2006. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443:421–426.

Jin Y, Kato T, Furu M, Nasu A, Kajita Y, Mitsui H, Ueda M, Aoyama T, Nakayama T, Nakamura T, Toguchida J. 2010. Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase. Biochem Biophys Res Commun 391:1471–1476.

Kawasaki H, Guan J, Tamama K. 2010. Hydrogen gas treatment prolongs replicative lifespan of bone marrow multipotential stromal cells in vitro while preserving differentiation and paracrine potentials. Biochem Biophys Res Commun 397:608–613.

Keith B, Simon MC. 2007. Hypoxia-inducible factors, stem cells, and cancer. Cell 129:465–472.

Khanna S, Roy S, Maurer M, Ratan RR, Sen CK. 2006. Oxygen-sensitive reset of hypoxia-inducible factor transactivation response: prolyl hydroxylases tune the biological normoxic set point. Free Radic Biol Med 40:2147–2154.

Kinnaird T, Stabile E, Burnett MS, Epstein SE. 2004. Bone marrow-derived cells for enhancing collateral development: mechanisms, animal data, and initial clinical experiences. Circ Res 95:354–363.

Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, Sharpless NE. 2004. Ink4a/Arf expression is a biomarker of aging. J Clin Invest 114:1299–1307.

Li X, Kimura H, Hirota K, Sugimoto H, Kimura N, Takahashi N, Fujii H, Yoshida H. 2007. Hypoxia reduces the expression and anti-inflammatory effects of peroxisome proliferator-activated receptor-gamma in human proximal renal tubular cells. Nephrol Dial Transplant 22:1041–1051.

Lin Q, Lee YJ, Yun Z. 2006. Differentiation arrest by hypoxia. J Biol Chem 281:30678–30683.

Morita S, Kojima T, Kitamura T. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 7:1063–1066.

Ohishi M, Schipani E. 2010. Bone marrow mesenchymal stem cells. J Cell Biochem 109:277–282.

Okamoto T, Aoyama T, Nakayama T, Nakamata T, Hosaka T, Nishijo K, Nakamura T, Kiyono T, Toguchida J. 2002. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. Biochem Biophys Res Commun 295:354–361.

Ontiveros C, Irwin R, Wiseman RW, McCabe LR. 2004. Hypoxia suppresses runx2 independent of modeled microgravity. J Cell Physiol 200:169–176.

Owen M, Friedenstein AJ. 1988. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 136:42–60.

Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS. 2006. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 313:1137–1140.

Packer L, Fuehr K. 1977. Low oxygen concentration extends the lifespan of cultured human diploid cells. Nature 267:423–425.

Park JH, Park BH, Kim HK, Park TS, Baek HS. 2002. Hypoxia decreases Runx2/Cbfa1 expression in human osteoblast-like cells. Mol Cell Endocrinol 192:197–203.

Phinney DG, Prockop DJ. 2007. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair–current views. Stem Cells 25:2896–2902.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Pittenger MF, Martin BJ. 2004. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 95:9–20.

Pochampally R. 2008. Colony forming unit assays for MSCs. Methods Mol Biol 449:83–91.

Rankin EB, Higgins DF, Walisser JA, Johnson RS, Bradfield CA, Haase VH. 2005. Inactivation of the arylhydrocarbon receptor nuclear translocator (Arnt) suppresses von Hippel-Lindau disease-associated vascular tumors in mice. Mol Cell Biol 25:3163–3172.

Riddle RC, Khatri R, Schipani E, Clemens TL. 2009. Role of hypoxia-inducible factor-1alpha in angiogenic-osteogenic coupling. J Mol Med 87:583–590.

Rocha S. 2007. Gene regulation under low oxygen: holding your breath for transcription. Trends Biochem Sci 32:389–397.

Roobrouck VD, Ulloa-Montoya F, Verfaillie CM. 2008. Self-renewal and differentiation capacity of young and aged stem cells. Exp Cell Res 314:1937–1944.

Rosova I, Dao M, Capoccia B, Link D, Nolta JA. 2008. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells 26:2173–2182.

Roy S, Shah H, Rink C, Khanna S, Bagchi D, Bagchi M, Sen CK. 2007. Transcriptome of primary adipocytes from obese women in response to a novel hydroxycitric acid-based dietary supplement. DNA Cell Biol 26:627–639.

Schaffer M, Witte M, Becker HD. 2002. Models to study ischemia in chronic wounds. Int J Low Extrem Wounds 1:104–111.

Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. 2002. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20:530–541.

Semenza GL. 2007. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. Biochem J 405:1–9.

Sen CK. 2009. Wound healing essentials: let there be oxygen. Wound Repair Regen 17:1–18.

Sen CK, Khanna S, Roy S. 2006. Perceived hyperoxia: oxygen-induced remodeling of the reoxygenated heart. Cardiovasc Res 71:280–288.

Sha H, He Y, Chen H, Wang C, Zenno A, Shi H, Yang X, Zhang X, Qi L. 2009. The IRE1alpha-XBP1 pathway of the unfolded protein response is required for adipogenesis. Cell Metab 9:556–564.

Shibata KR, Aoyama T, Shima Y, Fukiage K, Otsuka S, Furu M, Kohno Y, Ito K, Fujibayashi S, Neo M, Nakayama T, Nakamura T, Toguchida J. 2007. Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. Stem Cells 25:2371–2382.

Shimada T, Hiramatsu N, Okamura M, Hayakawa K, Kasai A, Yao J, Kitamura M. 2007. Unexpected blockade of adipocyte differentiation by K-7174: implication for endoplasmic reticulum stress. Biochem Biophys Res Commun 363:355–360.

Shimba S, Wada T, Hara S, Tezuka M. 2004. EPAS1 promotes adipose differentiation in 3T3-L1 cells. J Biol Chem 279:40946–40953.

Shomento SH, Wan C, Cao X, Faugere MC, Bouxsein ML, Clemens TL, Riddle RC. 2010. Hypoxia-inducible factors 1alpha and 2alpha exert both distinct and overlapping functions in long bone development. J Cell Biochem 109:196–204.

Simon MC, Keith B. 2008. The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol 9:285–296.

Smith JR, Pochampally R, Perry A, Hsu SC, Prockop DJ. 2004. Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. Stem Cells 22:823–831.

Tamama K, Fan VH, Griffith LG, Blair HC, Wells A. 2006. Epidermal growth factor as a candidate for ex vivo expansion of bone marrow-derived mesenchymal stem cells. Stem Cells 24:686–695.

Tamama K, Kawasaki H, Wells A. 2010. Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC. J Biomed Biotechnol 2010:795385.

Tamama K, Sen CK, Wells A. 2008. Differentiation of bone marrow mesenchymal stem cells into the smooth muscle lineage by blocking ERK/MAPK signaling pathway. Stem Cells Dev 17:897–908.

Tamiya H, Ikeda T, Jeong JH, Saito T, Yano F, Jung YK, Ohba S, Kawaguchi H, Chung UI, Choi JY. 2008. Analysis of the Runx2 promoter in osseous and non-osseous cells and identification of HIF2A as a potent transcription activator. Gene 416:53–60.

Tian H, McKnight SL, Russell DW. 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev 11:72–82.

Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD. 2008. Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS ONE 3:e2213.

Wan C, Shao J, Gilbert SR, Riddle RC, Long F, Johnson RS, Schipani E, Clemens TL. 2010. Role of HIF-1alpha in skeletal development. Ann N Y Acad Sci 1192:322–326.

Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU. 2003. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J 17:271–273.

Wouters BG, Koritzinsky M. 2008. Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nat Rev Cancer 8:851–864.

Yan Q, Bartz S, Mao M, Li L, Kaelin WG Jr. 2007. The hypoxia-inducible factor 2alpha N-terminal and C-terminal transactivation domains cooperate to promote renal tumorigenesis in vivo. Mol Cell Biol 27:2092–2102.

Yun Z, Maecker HL, Johnson RS, Giaccia AJ. 2002. Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Dev Cell 2:331–341.