Hypoxia Inhibition of Adipocytogenesis in Human Bone Marrow Stromal Cells Requires Transforming Growth Factor-β/Smad3 Signaling*

Received for publication, November 16, 2004, and in revised form, March 25, 2005
Published, JBC Papers in Press, April 20, 2005, DOI 10.1074/jbc.M412953200

Shuanhui Zhou‡, Stanislav Lechpammer‡, Joel S. Greenberger§, and Julie Glowacki¶

From the ‡Department of Orthopedic Surgery, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115 and the §Department of Radiation Oncology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213

Although hypoxia and transforming growth factor-β (TGF-β) inhibit differentiation of adipocytes from pre-adipocytes and bone marrow-derived cells in several species, the relationship between hypoxia and TGF-β signaling in adipocytogenesis is unknown. In this study, we evaluated the mechanisms of inhibition of adipocyte differentiation by hypoxia and TGF-β in human and murine marrow stromal cells (MSCs) and the role of TGF-β/Smad signaling in the inhibition of adipocytogenesis by hypoxia. Both hypoxia-mimetic deferoxamine mesylate (DFO) and TGF-β1 inhibited adipocyte differentiation (1.0% versus the control at 15 μM DFO and 1.4% versus the control at 1 ng/ml TGF-β1) and adipocyte gene expression (peroxisome proliferator-activated receptor-γ2 and lipoprotein lipase) in human MSCs after 21 days of treatment. Hypoxia (2% O2) and DFO (but not TGF-β1) increased hypoxia-inducible factor-1α as shown by Western blotting. Macaroray analyses of Western and Northern blot analyses showed that hypoxia activated the TGF-β2/Smad signaling pathway and that both hypoxia and TGF-β1 modulated adipocyte differentiation pathways such as the insulin-, peroxisome proliferator-activated receptor-γ-, phosphatidylinositol 3-kinase-, and MAPK-associated signaling pathways. Studies with mouse marrow stromal cell lines derived from Smad3+/+ or Smad3−/− mice revealed that the TGF-β type I receptor (ALK-5) and its intracellular signaling molecule Smad3 were necessary for the inhibition of adipocyte differentiation by both TGF-β and hypoxia-mimetic DFO. Thus, the TGF-β/Smad signaling pathway is required for hypoxia-mediated inhibition of adipocyte differentiation in MSCs.

Oxygen homeostasis represents an important organizing principle for human development and physiology. Dysregulation of oxygen homeostasis is found in inflammatory and cardiovascular diseases, cancer, cerebrovascular disease, and chronic obstructive pulmonary disease (1, 2). One of the immediate sequelae of bone fracture is regional hypoxia resulting from vasculature disruption (3). In response to hypoxia or low O2 tension, mammalian tissues show increased expression of a wide variety of genes that stimulate erythropoiesis, angiogenesis, and glycolysis (4). Most of the hypoxia-regulated genes are transcriptionally up-regulated by hypoxia-inducible factor-1α (HIF-1α) (5–7).

Adipocyte differentiation of murine 3T3-L1 preadipocytes is inhibited under hypoxic conditions (0.01–2% O2), and its inhibition is not observed in mouse embryonic fibroblasts deficient in HIF-1α (8). Low levels of O2 reduce in vitro adipocytogenesis of murine skeletal muscle satellite cells compared with 20% O2, a finding that has been corroborated by similar observations in the pluripotent mesenchymal cell line C3H10T1/2 and 3T3 cells (9). A recent report shows that hypoxia-dependent inhibition of adipocyte differentiation of murine 3T3-F442A preadipocytes can be mediated by mitochondrial reactive oxygen species generation (10). Transforming growth factor-β (TGF-β) was reported to strongly inhibit adipocytogenesis and the amount of fat in adipocytes that develops in cultures of human bone marrow-derived stromal cells (11). It has also been observed that TGF-β inhibits adipocytogenesis of the murine 3T3-F442A and NIH3T3 cell lines by signaling through Smad3 (12, 13). In several systems, various effects of hypoxia appear to be mediated by TGF-β signaling. In human peritoneal fibroblasts, hypoxia increases TGF-β1 and TGF-β type I and II receptor mRNA levels, with no effect on TGF-β2 or TGF-β3 (14). In human umbilical vein endothelial cells, hypoxia results in phosphorylation and nuclear transport of Smad2 and Smad3 proteins as well as stimulation of the transcriptional activities of Smad3 and HIF-1α and up-regulation of TGF-β2 and TGF-β type II receptor gene expression (15, 16). In human dermal fibroblasts, up-regulation of COL1A1 mRNA levels by hypoxia is blocked by a TGF-β1 antisense oligonucleotide and fails to occur in fibroblasts from TGF-β1−/− mice (17). It is unknown whether TGF-β signaling has a role in the inhibition of adipocytogenesis by hypoxia.

This study was designed to test the hypothesis that the TGF-β/Smad signaling pathway is required for the inhibition of adipocyte differentiation of human marrow stromal cells (hMSCs) by hypoxia. hMSCs have the potential to differentiate

* This work was supported by Grants AR45570 and AG 025015 from the National Institutes of Health (to J. G.). This work was presented as a plenary poster at the 26th Annual Meeting of the American Society for Bone and Mineral Research, October 1–5, 2004, Seattle, WA. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Orthopedic Surgery, Brigham & Women’s Hospital, Harvard Medical School, 75 Francis St., Boston, MA 02115. Tel.: 617-732-5397; Fax: 617-732-6937; E-mail: jglowacki@rics.bwh.harvard.edu.

† The abbreviations used are: HIF-1α, hypoxia-inducible factor-1α; TGF-β, transforming growth factor-β; hMSCs, human marrow stromal cells; FBS-HI, heat-inactivated fetal bovine serum; RT, reverse transcription; PPARγ, peroxisome proliferator-activated receptor-γ; LPL, lipoprotein lipase; DFO, deferoxamine mesylate; PBS, phosphate-buffered saline; P30K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; BicTiss, 2-bis(2-hydroxyethyl)aminomethyl-2-hydroxymethyl propane-1,3-diol; PAI-1, plasminogen activator inhibitor-1; VEGF, vascular endothelial growth factor; C/EBP, CAAT/enhancer-binding protein; JNK, c-Jun N-terminal kinase.
**Hypoxia and TGF-β Inhibit Adipocytogenesis in MSCs**

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Adherent hMSCs were prepared from femoral bone marrow that was obtained as discarded material from a 42-year-old woman undergoing total hip replacement for osteoarthritis (21). Low density mononuclear cells were isolated by density centrifugation with Ficoll/Histopaque 1077 (Sigma). This procedure enriches for undifferentiated cells, including a fraction of non-adherent hematopoietic cells and a stromal fraction capable of adherence and differentiation into various connective tissue cells (21). The adherent fraction was expanded in monolayer culture with phenol red-free α-minimal essential medium (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS-HI; Atlanta Biologicals, Inc., Norcross, GA), with 1 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN), or and/or treat obesity and osteopenia/osteoporosis.

**Conditions for Adipocyte Differentiation of hMSCs**—After hMSCs reached confluence, the medium was changed to α-minimal essential medium (phenol red-free), 1% FBS-HI, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Invitrogen). Mouse Smad3++/− and Smad3−/− MSCs were derived from bone marrow cultures of wild-type and Smad3-null mice, respectively (22). The Smad3-null mice were provided by Dr. K. C. Flanders (National Institutes of Health, Bethesda, MD). The murine MSCs were maintained in McCoy’s 5A medium (Invitrogen) with 10% FBS until they reached confluence.

**RNA Isolation and RT-PCR**—Total RNA was isolated from MSCs with TRIzol. For RT-PCR, 2 μg of total RNA was reverse-transcribed into cDNA with SuperScript II (Invitrogen) following the manufacturer’s instructions. One-tenth of the cDNA was used in each 50-μl PCR (30–35 cycles of 94°C for 1 min, 55–89°C for 1 min, and 72°C for 2 min) using gene-specific primers. The human β-actin primer was 5’-GAGATTCTCTGTATGGCAC-3’ and the reverse primer was 5’-CTGCAAAAGAAGCATTTCTTC-3’ (1261–1536 bp; GenBankTM accession number NM_00237.1). Primers for the human PPARγ2 (24) and mouse adipin (25) genes were used for amplification as described previously.

**Gene Macroarray—GEArrayTM**—human TGF-β/bone morphogenetic protein signaling pathway and insulin pathway macrosarrays (SuperArray Bioscience Corp, Bethesda, MD) were performed as described previously (20, 26). In brief, 3 μg of total RNA was reverse-transcribed with [α-32P]dCTP (PerkinElmer Life Sciences) and the reagents provided by the manufacturer. The labeled cDNAs were hybridized overnight to the macroarrays; washed; and exposed to x-ray film for 5, 18–24, 48, and 72 h. Digital images were obtained by scanning autoradiographs with an Epson transparency adapter. Data were extracted from the images with ScanAlyze software as described in detail elsewhere (26). Data were organized by functional category of the genes, and a qualitative measure of relative expression under control conditions is indicated. Genes whose expression levels under control conditions were measured during short film exposures (5–24 h) were classified as high expression (+ + +); 48-h film exposures were classified as moderate expression (+ +); and 72-h film exposures were classified as low expression (+). Undetectable or absent bands (− −) were made for pixel intensity equal to the background level. The expression value for each gene on the array was calculated with GEArrayAnalyzer™ software provided by the manufacturer and was verified by visual comparison with the autoradiographs. The usable linear range of pixel intensity was established as 9,000–65,000 as described previously (26). The quantitative magnitude of change evoked by treatment is expressed as
fold change upon treatment relative to the control. A qualitative increase or decrease or no change evoked by treatment relative to the control is indicated for genes that were expressed at levels too low for quantification or where a neighboring strong spot bled into the feature of interest.

Northern Blot Analysis—Northern blot analysis was performed with methods as described previously (26). In brief, 10 μg of total RNA was electrophoretically resolved on formaldehyde-containing 1% agarose gel and blotted onto a positively charged nylon membrane (Roche Diagnostics) by downward capillary transfer in 20× SSC. The nylon membrane was prehybridized in formamide prehybridization/hybridization buffer (5× SSC, 5× Denhardt’s solution, 1% SDS, 50% formamide, and 0.1 mg/ml denatured salmon sperm DNA) for 3 h at 42 °C. Hybridization was performed overnight in formamide prehybridization/hybridization buffer with random-labeled probes at 42 °C. The probes for human TGFβ1, plasminogen activator inhibitor-1 (PAI-1), PKM2, and INSR (insulin receptor) were labeled with a random-primer DNA labeling kit (Roche Diagnostics) with 25 ng of RT-PCR products of each gene and purified using the QiAquick nucleotide removal kit (Qiagen Inc.). The primers for human TGFβ1 (26), PAI-1 (27), PKM2 (28), and INSR (29) were described previously. The internal control 18 S oligonucleotide probe (Ambion Inc.) was labeled with a DNA 5′-end labeling kit (Roche Diagnostics). For removal of nonspecifically bound probes, membranes were washed twice with 2× SSC with 0.1% SDS (5 min each) and twice with 0.2× SSC and 0.1% SDS (5 min each) at room temperature and twice with 0.2× SSC and 0.1% SDS (15 min each) at 42 °C. Hybridization was visualized by autoradiography at −80 °C with Eastman Kodak X-Omat Blue XB-1 film.

Statistical Analysis—All experiments were performed two to five times. Data are presented as the means ± S.D. Quantitative data were analyzed by one-way analysis of variance. A p value <0.05 was considered significant.

RESULTS

Hypoxia and Its Mimetic DFO Stabilize HIF-1α and Activate the TGF-β Signal in hMSCs—The effects of hypoxia, hypoxia-mimetic DFO, and TGF-β1 on HIF-1α and TGF-β intracellular signaling molecule Smad proteins in hMSCs (from a 42-year-old woman) were assessed by Western immunoblot analysis. Confluent hMSCs were cultured in α-minimal essential medium containing 1% FBS-HI and supplemented with or without hypoxia (2% O2) or normoxia (19% O2) conditions with or without 15 μM DFO or 1 ng/ml TGF-β1. Hypoxia (2% O2) or its mimetic DFO (15 μM) up-regulated HIF-1α protein (120 kDa) compared with normoxia and the vehicle control (Fig. 1). Under these conditions, TGF-β1 (1 ng/ml) did not modulate HIF-1α protein level in hMSCs. Similar results were obtained in the human bone marrow stromal cell line KM101 (data not shown). In addition, hypoxia, its mimetic DFO, and TGF-β1 activated the TGF-β signal as shown by increased phosphorylated Smad2/3 in hMSCs by Western blotting (Fig. 1).

Hypoxia-mimetic DFO and TGF-β Inhibit Adipocytogenesis and Adipocyte Gene Expression in hMSCs—hMSCs (from a 42-year-old woman) were used to assess the effects of hypoxia and TGF-β on adipocyte differentiation. After 3 weeks of culture in adipocytogenic supplements with or without hypoxia-mimetic DFO (2.5–15 μM) or TGF-β1 (1 ng/ml), development of lipid-containing cells was visualized by staining with oil red O (Fig. 2A). DFO significantly blocked adipocyte differentiation in a dose-dependent manner (38.8, 20.4, and 1.0% versus the control at 2.5, 5.0, and 15.0 μM DFO, respectively) (Fig. 2B). TGF-β1 significantly decreased adipocyte number (1.4% versus the control; *p < 0.001) (Fig. 2B). Both DFO (50 μM) and TGF-β1 (1 ng/ml) blocked expression of the PPAR-γ2 and LPL gene expression in hMSCs as shown by RT-PCR (Fig. 2C).

Hypoxia-mimetic DFO Does Not Alter Cell Viability—The effect of DFO on cell viability was determined with the trypan blue exclusion assay. After 24 h of treatment with 0, 1, 10, 50, and 1000 μM DFO in α-minimal essential medium containing 1% FBS-HI, the viability of hMSCs (from a 42-year-old woman) was 97, 96, 97, 95, 95, and 92%, respectively. Following 3 weeks of treatment with 2.5, 5.0, and 15.0 μM DFO, the viability of hMSCs was similar for DFO-treated and control groups.

Hypoxia and TGF-β Alter Gene Expression in Adipocyte-associated Pathways—Because both hypoxia and TGF-β inhibited adipocyte differentiation in hMSCs, we assessed whether hypoxia and TGF-β have similar effects on adipocyte-associated signaling pathways. We surveyed effects on gene expression with a cDNA macroarray. Many categories of adipocyte-associated genes were coordinately up- or down-regulated upon 48 h of exposure to hypoxia (2% O2) (Table I). Hypoxia down-regulated PPARγ and its target genes, including adipin (43% versus the normoxic control), AEBP1 (70%), and SLC27A4 (38%). Among the insulin receptor-associated genes, hypoxia down-regulated CAP (adenylyl cyclase-associated protein; 53%), INSR (84%), NCK2 (32%), CRK (63%), and PPP1CA (protein phosphatase-1, catalytic subunit, alpha isoform). Ex-
expression of the primary insulin target gene v-jun was decreased by hypoxia (64%). In the PI3K pathways, hypoxia altered gene expression of PI3K pathway components, including E1F4EBP1 (up-regulation), PIK3R1 (12%), PIK3R2 (25%), and GSK3A and GSK3B (down-regulation), and PI3K pathway target genes, including PAI-1 (426%), vascular endothelial growth factor (VEGF; 210%), PKM2 (310%), the low density lipoprotein receptor, and ACACB (acyetyl-coenzyme A carboxylase beta; down-regulation). In the MAPK pathway, hypoxia down-regulated MAPK pathway components, including MAP2K2 (55%), K-ras, and c-raf, and MAPK pathway target genes, including angiogenin (88%) and bcl-x (48%). Under these conditions, there was no effect on insulin-activated transcription factor SREBP1A/DD1 gene expression, but there was up-regulation of the SREBP1 target genes PKM2 (310%) and PCK2 (313%) and down-regulation of ACACB (acyetyl-coenzyme A carboxylase beta) in hMSCs.

RNA obtained from hMSCs treated for 48 h with TGF-β1 (1 ng/ml) or the vehicle control was also evaluated using the signaling pathway macroarray (Table I). TGF-β1 decreased expression of PPARγ and its target genes adipsin (36% versus the control) and AEBP1 down-regulated (38%), and increased expression of PAI-1 (426%), vascular endothelial growth factor (VEGF; 210%), PKM2 (310%), the low density lipoprotein receptor, and ACACB (acyetyl-coenzyme A carboxylase beta; down-regulation). In the MAPK pathway, hypoxia down-regulated MAPK pathway components, including MAP2K2 (55%), K-ras, and c-raf, and MAPK pathway target genes, including angiogenin (88%) and bcl-x (48%). Under these conditions, there was no effect on insulin-activated transcription factor SREBP1A/DD1 gene expression, but there was up-regulation of the SREBP1 target genes PKM2 (310%) and PCK2 (313%) and down-regulation of ACACB (acyetyl-coenzyme A carboxylase beta) in hMSCs.

TABLE I
Adipocyte-associated signaling and target gene expression profiles of hMSCs after exposure to hypoxia (2% O2) or treatment with TGF-β1 (1 ng/ml) for 48 h

| Category and gene | GenBank™ accession no. | Level of expression (control) | Fold change by |
|-------------------|------------------------|--------------------------------|---------------|
|                   |                        |                                | Hypoxia       | TGF-β1       |
| PPARγ and its target genes |                       |                                |               |
| PPARγ             | NM_015869              | ++                             | ↓↓            | ↓↓           |
| Adipsin           | NM_001928              | +                              | ←→           | ←→          |
| AEBP1             | NM_001299              | +                              | 0.43          | 0.36         |
| C/EBP-α           | NM_004384              | +                              | 0.70          | 3.46         |
| C/EBP-β           | NM_005194              | +                              | ↓↓            | ←→          |
| SLC27A4           | NM_005094              | +                              | 0.38          | 0.76         |
| SREBP1 and its target genes |                       |                                |               |
| SREBP1            | NM_004176              | +                              | ←→           | ←→          |
| PKM2              | NM_002654              | +                              | 3.10          | 1.89         |
| PCK2              | NM_004563              | +                              | 3.13          | 1.22         |
| ACACB             | NM_001093              | +                              | 0.96          | 1.64         |
| Insulin receptor-associated genes |                       |                                |               |
| CAP               | BC017196               | ++                             | 0.53          | 2.04         |
| IRS2              | NM_000208              | ++                             | 0.84          | 0.49         |
| NCK2              | NM_003581              | ++                             | 0.32          | 0.67         |
| PTPRF (LAR)       | NM_002840              | +                              | 1.13          | 1.49         |
| CRK               | NM_016823              | +                              | 0.63          | 0.41         |
| SHC1              | U73377                 | +                              | 1.06          | 1.63         |
| PTPN11            | NM_002834              | +                              | 0.96          | 1.64         |
| PIP5CA            | AB028949               | +                              | 0.64          | 0.33         |
| Insulin signaling target gene |                       |                                |               |
| v-jun             | NM_002228              | +                              | 1.63          | 1.63         |
| PI3K pathway      |                        |                                |               |
| AKT (PKBβ)        | M77198                 | ++                             | 4.26          | 4.51         |
| E1F4EBP1          | NM_004095              | ++                             | 2.52          |               |
| PIK3R1            | M61906                 | +                              | 0.12          | 27           |
| PIK3R2            | NM_005027              | +                              | 0.25          | ←→           |
| GLUT1             | NM_006516              | +                              | ←→           | 1.71         |
| GSK3B             | NM_002093              | +                              | 0.53          | 2.04         |
| GSK3A             | NM_019884              | +                              | 1.71          | 0.63         |
| Target genes      |                        |                                |               |
| PAI-1             | M16006                 | +                              | 4.26          | 4.51         |
| VEGF              | NM_000376              | +                              | 2.10          | 1.22         |
| LDLR              | NM_005027              | +                              | ←→           |               |
| MAPK pathway      |                        |                                |               |
| RRA5              | NM_006270              | +                              | 1.35          | 0.82         |
| MAP2K1            | NM_002755              | +                              | 0.55          | 0.31         |
| MAP2K2            | L11285                 | +                              | 1.07          | 1.18         |
| SOS2              | L13858                 | +                              | 0.84          | 0.51         |
| K-ras             | M54968                 | +                              | 0.43          | 0.47         |
| c-ras             | X03484                 | +                              | 0.43          | 0.47         |
| Target genes      |                        |                                |               |
| Angiogenin        | M11567                 | +                              | 0.88          | 0.64         |
| bcl-x             | Z31115                 | +                              | 0.48          | 0.47         |
| TIEG              | U21847                 | +                              | 0.88          | 1.10         |
| ERCC1             | M286650                | +                              | 1.10          | 1.10         |

* Symbols indicate the relative level of expression in controls: +++, high expression, normalized to the housekeeping gene peptidylprolyl isomerase A or β-actin (16–24-h exposure); + +, low expression, normalized to the housekeeping gene peptidylprolyl isomerase A (48-h exposure); +, very low, normalized to the housekeeping gene peptidylprolyl isomerase A (3-day exposure); −, undetectable, no normalized data (3-day exposure).

* Arrows indicate qualitative change by hypoxia or TGF-β1 in genes for which quantification was not possible: ↑, increase; ↓, decrease; ←→, no change.

* PKBβ, protein kinase Bβ; LDLR, low density lipoprotein receptor.
addition, both hypoxia and TGF-β inhibit adipocytogenesis in MSCs. Among the PI3K target genes, TGF-β1 up-regulated expression of PAI-1 (451%) and VEGF (122%), down-regulated ACAB, and had no effect on SREBP1 and the low density lipoprotein receptor. In the MAPK pathway, TGF-β1 down-regulated MAPK pathway components, including RRAS (82%), MAP2K1 (28%), and MAP2K2 (31%); decreased MAPK target genes, including angiogenin (64%) and bcl-x (47%); and increased MAPK target genes, including TIEG (110%) and ERCC1. Under these experimental conditions, TGF-β1 had no effect on insulin-activated transcription factor SREBP1/ADD1 gene expression, but up-regulated the SREBP1 target genes PKM2 (189%) and PCK2 (122%) and down-regulated ACAB.

Changes in adipocyte-associated signaling and target genes induced by hypoxia were compared with changes induced by TGF-β (Table I). First, there were very similar effects on most genes, such as decreased expression of PPARγ and its target genes adipin and SLC27A4; the insulin receptor-associated genes INSR, NCK2, CRK, and PPP1CA; the insulin target gene v-jun; PI3K pathway-associated genes such as PIK3R1 and GSK3A; MAPK pathway-associated genes such as MAP2K2, angiogenin; and bcl-x; and the SREBP1 target gene ACAB. In addition, both hypoxia and TGF-β1 increased expression of the SREBP1 target genes PKM2 and PCK2 and the PI3K component gene E1F4EBP1 and its PAI-1 and VEGF target genes. Second, there were different effects on some genes, including the PPARγ target gene AEBP1; the PI3K pathway component genes PIK3R2, GLUT1, and GSK3B; the PI3K low density lipoprotein receptor target gene; the MAPK pathway component genes RRAS, MAP2K1, K-ras, and c-ras; and the MAPK target gene ERCC1.

Inhibition of PPARγ and LPL Gene Expression in hMSCs by Hypoxia-mimetic DFO, TGF-β1, a PI3K Inhibitor, or MAPK Inhibitors—Specific inhibitors were used to determine the effects of hypoxia, TGF-β, PI3K, and MAPK signaling on PPARγ adipocyte master gene and LPL adipocyte marker gene expression. After confluence, hMSCs were cultured with or without IDM and treated with the vehicle control, hypoxia-mimetic DFO (50 μM), TGF-β1 (1 ng/ml), the PI3K inhibitor LY294002 (40 μM), the p38 MAPK inhibitor SB203580 (10 μM), or the p42/44 MAPK inhibitor PD98059 (50 μM). Analysis by RT-PCR showed that hMSCs expressed the PPARγ and LPL genes when cultured with IDM for 7 days (Fig. 3A). Hypoxia-mimetic DFO, TGF-β1, the PI3K inhibitor LY294002, and the p38 MAPK inhibitor SB203580 significantly down-regulated PPARγ and LPL gene expression compared with the IDM control (n = 3; p < 0.001) (Fig. 3A, A and B). In contrast, the p42/44 MAPK inhibitor PD98059 appeared to up-regulate the PPARγ gene (p < 0.05, n = 3) and did not modulate LPL gene expression compared with the IDM control (Fig. 3A, A and B).

Hypoxia Alters Gene Expression in the TGF-β/Smad Signaling Pathway—A specific macroarray was used to assess the effects of hypoxia on TGF-β/Smad signaling in hMSCs cultured with IDM for 48 h (Table II). Under control normoxic conditions (19% O2), hMSCs expressed TGF-β type II and III receptors Smad2, Smad5, and Smad9; their expression was not modulated by hypoxia (2% O2). Hypoxia up-regulated TGF-β1 and its intracellular signaling molecule Smad3 and target genes, including TGFBI, PAI-1, TIMP1, COLIA2, p21/WAF1/CIP1, etc. The genes altered most by hypoxia in hMSCs were TGFBI (310% versus the normoxic control), PAI-1 (361%), and inhibin-α (12%). Hypoxia moderately up-regulated COLIA2 (142%), TIMP1 (185%), p21, ALK-1, ALK-6, and RUNX1/AML1 and down-regulated STAT1, RUNX2/CBFA1 (71%), endoglin (70%), and v-jun (83%) gene expression. These data demonstrate that hypoxia activates the TGF-β/Smad signaling pathway.

Confirmation of Macroarray Data by Northern Blot Analysis—The expression levels of selected genes that were shown by macroarrays to be affected by hypoxia and/or TGF-β were evaluated by Northern blotting (Fig. 4A). This showed changes similar to those in the arrays, but with different magnitudes (Fig. 4B). Hypoxia (2% O2 for 48 h) up-regulated TGFBI/IGH3 (383% versus the normoxic control), PAI-1 (866%), and PKM2 (135%) and down-regulated INSR (35%) gene expression. TGF-β1 (1 ng/ml for 48 h) had similar effects compared with hypoxia on expression of these genes. TGF-β1 up-regulated TGFBI/IGH3 (353% versus the control), PAI-1 (134%), and PKM2 (66%) and down-regulated INSR (44%) gene expression.

Smad3 Is One of the Mediators of Inhibition of Adipocyte Differentiation in Murine MSCs by Hypoxia or TGF-β—Murine MSCs were used to assess the role of Smad3 in mediating the inhibition of adipocytogenesis. Smad3−/− MSCs were derived from bone marrow cultures of neonatal Smad3-null mice; Smad3+/− MSCs were derived from wild-type mice. There were two notable differences in adipocyte development in bone marrow cultures from Smad3−/− mice and Smad3+/− mice. First, there was more extensive (37-fold) adipocyte differentiation in bone marrow cultures from Smad3−/− mice than from Smad3+/− mice cultured under adipocytogenic conditions (Fig. 5, A and B). This finding suggests that Smad3 is a critical inhibitor of adipocyte differentiation. Second, there was a 7-fold attenuation of TGF-β inhibition and a 2-fold attenuation of DFO inhibition of adipocytogenesis in cells lacking Smad3 (Fig. 5B). Hypoxia-mimetic DFO (15 μM) decreased adipocyte number in Smad3−/− MSCs (32.9% versus the control; p < 0.05) compared with Smad3+/− MSCs (67.7% versus the control; p < 0.05). TGF-β1 (1 ng/ml) decreased adipocyte number in Smad3+/− cells (5.5% versus the control; p < 0.05) compared with Smad3−/− cells (39.2% versus the control; p < 0.05). These data support the conclusion that Smad3 is required for the inhibition of adipocyte differentiation by either TGF-β or hypoxia.
effect of Smad3 on lipid accumulation was reflected in the expression pattern of the adipocyte marker gene adipsin in Smad3+/− and Smad3−/− MSCs. After 7 days of treatment, 15 μM DFO or 1 ng/ml TGF-β1 down-regulated adipsin gene expression in Smad3+/− MSCs, but not in Smad3−/− MSCs (Fig. 5C). SB431542 (10 μM), an specific inhibitor of the TGF-β type I receptor (ALK-5), antagonized the inhibitory effects of DFO and TGF-β1 on adipsin gene expression in Smad3+/− MSCs (Fig. 5C). This is confirmation that TGF-β/Smad is required for the inhibition of adipocyte differentiation by either TGF-β or hypoxia.

**DISCUSSION**

Although hypoxia and TGF-β both inhibit adipocyte differentiation, the relationship between their signaling pathways in adipocytogenesis was unknown. In this study, we investigated the mechanisms of inhibition of adipocyte differentiation of MSCs by hypoxia and TGF-β as well as the role of the TGF-β/Smad signaling pathway in this process.

Hypoxia-induced gene expression is mediated by HIF-1α. Hypoxia stabilizes the HIF-1α protein, which is otherwise (under normoxic conditions) degraded by a ubiquitin-dependent proteasome (5–7). Our results show that hypoxia and its mimetic DFO stabilized the HIF-1α protein in hMSCs and that continued treatment with DFO inhibited adipocyte differentiation and PPARγ-2 and LPL adipocyte gene expression in hMSCs. It has been reported that many growth factors, cytokines, and circulatory factors, including insulin, insulin-like growth factor-1 and -2, platelet-derived growth factor, epidermal growth factor, epidermal growth factor-2, TGF-β1, hepatocyte growth factor, tumor necrosis factor-1, interleukin-1β, etc., stimulate HIF-1α through pathways distinct from that employed by the classical hypoxic pathway (30). Our results show that 1 ng/ml TGF-β1 blocked adipocyte differentiation and down-regulated PPARγ2 and LPL adipocyte gene expression, but did not change HIF-1α protein levels in hMSCs. These data indicate that hypoxia may inhibit adipocytogenesis of hMSCs by stabilizing HIF-1α, but that, under these conditions, TGF-β does not require the stabilization of HIF-1α for its inhibition of adipocytogenesis of hMSCs.

To compare the effects of hypoxia and TGF-β on adipocyte-associated signaling, we used a targeted macroarray that included several insulin receptor-associated genes and insulin signaling target genes, PI3K pathway components and target genes, MAPK pathway components and target genes, insulin-activated transcription factor SREBP1 target genes, and PPARα target genes. The macroarray data were confirmed by Northern blot analysis of several highly expressed genes. PPARα appears to function as both a direct regulator of many fat-specific genes and as a master regulator that can trigger the program of adipocytogenesis (31). Hypoxia inhibits PPARγ2 expression in murine 3T3-L1 preadipocytes (8). TGF-β3Smad3 inhibits induction of C/EBP-α and PPARγ in murine 3T3-F442A preadipocytes (12). Our RT-PCR and macroarray results show that both hypoxia and TGF-β1 inhibited PPARγ2 expression in hMSCs and down-regulated several PPARγ target genes. Another transcription factor that promotes adipocy-
togenesis is the insulin-activated transcription factor SREBP1c (sterol regulatory element-binding protein-1c)/ADD1 (32, 33). It is a basic helix-loop-helix protein expressed abundantly in adipose tissue (34) and has been shown to increase fatty acid and fat synthesis; this has been attributed in part to its proposed influence on PPAR activity (35). Expression of SREBP1/ADD1 is also increased during osteoblast differentiation; however, this indicates that increased expression of this protein is not specific to adipocyte differentiation (36). Our results show that hypoxia and TGF-β had no effect on SREBP1 gene expression in hMSCs.

Insulin, the major anabolic hormone, promotes in vivo accumulation of adipose tissue. Insulin is a potent inducer of adipocytogenesis, and differentiation of adipocytes requires many components of the insulin signaling pathway, including the insulin receptor, insulin receptor substrate-1, and PI3K (37). There is growing interest in the effects of age on redistribution of body fat deposits, on insulin actions on site-specific adipocytes and their progenitors, and on dysdifferentiation of mesenchymal precursors into mesenchymal adipocyte-like default cells (38). Our macroarray results show strikingly similar effects of hypoxia and TGF-β in decreasing expression of the insulin receptor-associated genes INSR, NCK2, CRK, and PPP1CA and the insulin target gene v-jun. These findings suggest that both treatments block insulin signaling as part of their inhibition of adipocyte differentiation in MSCs.

PI3K is important in a wide variety of cellular processes, including intracellular trafficking; organization of the cytoskeleton; cell growth, differentiation, and transformation; and prevention of apoptosis (39, 40). Pharmaceutical inhibition of PI3K or overexpression of dominant-negative mutant subunits abrogates differentiation of adipocytes in vitro (41, 42); this suggests that the insulin/PI3K/AKT signaling pathway is required for adipocytogenesis. Our RT-PCR results show that the PI3K inhibitor LY294002 down-regulated PPARY2 and LPL adipocyte gene expression; this demonstrates that the PI3K pathway promotes adipocyte differentiation in hMSCs. Our macroarray results show that hypoxia down-regulated PI3K pathway-associated genes and increased expression of PI3K target genes such as PAI-1 and VEGF. TGF-β1 had similar effects on PI3K signaling gene expression compared with hypoxia. Although...
PAI-1 and VEGF were named as PI3K pathway target genes in this commercial macroarray, the induction of the PAI-1 (43, 44) and VEGF (45) genes by hypoxia involves HIF-1, hypoxia response elements in the PAI-1 and VEGF gene promoters, and PI3K/AKT (protein kinase B). Therefore, we cannot conclude whether hypoxia activates the PI3K pathway through up-regulation of the PAI-1 and VEGF genes by hypoxia. The effects of hypoxia and TGF-β on the PI3K pathway in adipocyte differentiation in hMSCs need to be further evaluated. Down-regulation of PI3K pathway components, including PIK3R1, PIK3R2, GS3K3A, and GS3K3B, may contribute to the inhibition of adipogenesis in hMSCs by hypoxia and TGF-β because the PI3K inhibitor down-regulated PPAR-γ and LPL gene expression.

Both hypoxia and TGF-β inhibited the p42/44 MAPK signaling pathway during adipocyte differentiation of hMSCs. Reports in the literature concerning the role of the p42/44 MAPK pathways in adipocyte differentiation have been contradictory (46). p42/44 MAPK is required (47, 48) or has no effect or inhibits (49) adipocyte differentiation of preadipocytes under different conditions. Under our experimental conditions (1% FBS-III, IDM), the p42/44 MAPK inhibitor PD98059 up-regulated the PPAR-γ2 gene and had no effect on LPL gene expression in hMSCs; this indicates that p42/44 MAPK may inhibit adipocyte differentiation in hMSCs. Thus, the inhibition of the p42/44 MAPK signaling pathway by both hypoxia and TGF-β is unlikely to mediate their inhibition of adipocyte differentiation in hMSCs. It has been reported that JNK suppresses the process of adipocyte differentiation (50), and p38 MAPK has been shown to promote adipocyte differentiation (51). It was unknown whether p38 MAPK and JNK play roles in the effects of hypoxia or TGF-β on adipocyte differentiation. It has been reported that hypoxia activates JNK and p38 MAPK in carcinoma cells (52). Like hypoxia, TGF-β has also been reported to activate the JNK pathway in skeletal muscle cells (53) and the p38 MAPK signaling pathway in murine mesangial cells (54). The effects of hypoxia and TGF-β on JNK and p38 MAPK need to be evaluated further in our system. Nevertheless, our RT-PCR data show that the p38 MAPK inhibitor blocked PPAR-γ and LPL gene expression in hMSCs and imply that p38 MAPK is an activator of adipogenesis in hMSCs. Thus, the inhibition of adipocyte differentiation is not through activation of p38 MAPK by hypoxia or TGF-β in hMSCs.

Based on these new data, we propose that hypoxia inhibition of adipogenesis in hMSCs requires TGF-β/Smad signaling. Hypoxia up-regulated TGF-β1, its intracellular signaling molecule Smad3, and many of its target genes such as TGFBI, PAI-1, TIMP1, COL1A2, and p21WAF1/CIP1 in hMSCs. The experiments with mouse Smad3 cell lines showed that Smad3 was required for inhibition of adipogenesis by either DFO or TGF-β1. In addition, SB431542 abrogated the inhibitory effects of DFO on adipocyte gene expression. SB431542 has been identified as an inhibitor of ALK-5 (TGF-β type I receptor) (55). These data demonstrate that hypoxia inhibition of adipocyte differentiation in MSCs requires activation of the TGF-β/Smad signaling pathway. There is no information about bone marrow or body fat in Smad3−/− mice or about the mechanism of their substantial perinatal mortality. Recently, however, we reported that long-term bone marrow cultures established from neonatal Smad3−/− mice show 16.5-fold more adipocytes in the adherent layer and prolonged hematopoiesis (>20 weeks) compared with Smad3+/− mice (56). Those data confirm the negative regulation by endogenous TGF-β of adipogenesis and long-term hematopoiesis in vitro.

Hypoxia activation through TGF-β/Smad signaling is known in other systems. It has been reported that hypoxia stimulates collagen synthesis and COL1A1 transcription through the action of TGF-β1 in human dermal fibroblast (17). Cooperation between the hypoxia and TGF-β pathways is required for regulation of several genes, including endoglin (57), erythropoietin (58), VEGF (59), etc. Hypoxia results in a significant increase in TGF-β1 and TGF-β type I and II receptor mRNA levels in human peritoneal fibroblasts (14). Exposure of human umbilical vein endothelial cells to hypoxia results in phosphorylation and nuclear transportation of Smad2 and Smad3 proteins as well as stimulation of the transcriptional activities of Smad3 and HIF-1α and culminates in up-regulation of TGF-β2 gene expression (16). Thus, Smad proteins may play an important role in vascular responses to hypoxia and ischemia (15). It has been reported that reactive oxygen species mediate TGF-β-induced expression of PAI-1 in rat mesangial cells (60) and TIMP3 in human and bovine primary articular chondrocytes (61) and apoptosis in rat fetal hepatocytes (62). It is unknown whether reactive oxygen species have a role in the TGF-β/Smad-mediated inhibition of adipogenesis by hypoxia in hMSCs.

Understanding the balance between positive and negative regulators of adipogenesis has important health-related implications for anti-obesity medical therapy and lipodystrophy (63). TGF-β has been shown to be increased in adipose tissue in obese mice (64). However, overexpression of TGF-β1 in adipose tissue in transgenic mice results in a dramatic reduction in total body fat (65). Adipose tissue growth is angiogenesis-dependent (66). Hypoxia has been reported to increase the expression of pro-angiogenic factors in adipocytes and fat tissues (67, 68). Our results show that hypoxia and TGF-β modulated expression of pro-angiogenic genes such as VEGF and angiogenin and inhibited adipocyte differentiation in hMSCs. The relationship between the effects of hypoxia and TGF-β on adipocyte differentiation of MSCs and adipose tissue in obesity merits further investigation. Because of the relationship between bone marrow fat and skeletal aging, these results may also have implications for the regulation of MSC differentiation into adipocytes and osteoblasts.

In summary, hypoxia and TGF-β inhibited adipocyte differentiation and modulated adipocyte-associated signaling pathways in MSCs. Hypoxia activated the TGF-β/Smad signaling pathway, and the TGF-β intracellular signaling molecule Smad3 was necessary for the inhibition of adipogenesis by both TGF-β and hypoxia. These findings indicate that TGF-β/Smad signaling is required for the inhibition of adipocyte differentiation by hypoxia.

Acknowledgments—We greatly appreciate help from Drs. K. C. Flanders, M. Kikuchi, S. Mizuno, X. Wang, C. Wykoff, K. E. Yates, and M. Epperly with different aspects of these experiments.

REFERENCES

1. Semenza, G. L. (2001) Trends Mol. Med. 7, 345–350
2. Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S. K., Doni, A., Rapisarda, A., Bernascono, S., Saccani, S., Nebuloni, M., Vago, L., Mantovani, A., Mellilo, G., and Sica, A. (2000) J. Exp. Med. 198, 1391–1402
3. Komatsu, D. E., and Hadjiargyrou, M. (2004) Bone 34, 680–688
4. Bunn, H. F., and Poyton, R. O. (1996) Physiol. Rev. 76, 839–885
5. Semenza, G. L., and Wang, G. L. (1992) Mol. Cell. Biol. 12, 5447–5454
6. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5510–5514
7. Semenza, G. L. (2001) Cell 107, 1–3
8. Yun, Z., Maecke, H. L., Johnson, R. S., and Giaccia, A. J. (2002) Dec. Cell 2, 331–341
9. Csete, M., Walkonik, J., Slawny, N., Wei, Y., Kornsnes, S., Doyle, J. C., and Wold, B. (2001) J. Cell. Physiol. 189, 189–196
10. Carriere, A., Carmona, M. C., Fernandez, Y., Rigual, M., Weng, R. H., Penicaud, L., and Castella, L. (2004) J. Biol. Chem. 279, 40462–40469
11. Locklin, R. M., Oreffo, R. O., and Triffitt, J. T. (1999) Cell Biol. Int. 23, 185–194
12. Choy, L., Skillington, J., and Derynck, R. (2000) J. Cell Biol. 149, 667–682
13. Choy, L., and Derynck, R. (2003) J. Biol. Chem. 278, 9689–9691
14. Saed, G. M., Collins, R. L., and Diamond, M. P. (2002) Ann. J. Reprod. Immunol. 48, 387–393
15. Akman, H. O., Zhang, H., Siddiqui, M. A., Solomon, W. Smith, R. L., and...
