The stem cell niche is a unique tissue microenvironment that regulates the self-renewal and differentiation of stem cells. Although several stromal cells and molecular pathways have been identified, the microenvironment of the stem cell niche remains largely unclear. Recent evidence suggests that stem cells are localized in areas with low oxygen. We have hypothesized that hypoxia maintains the undifferentiated phenotype of stem/precursor cells. In this report, we demonstrate that hypoxia reversibly arrests preadipocytes in an undifferentiated state. Consistent with this observation, hypoxia maintains the expression of pref-1, a key stem/precursor cell gene that negatively regulates adipogenic differentiation. We further demonstrate that the hypoxia-inducible factor-1 (HIF-1) constitutes an important mechanism for the inhibition of adipogenic differentiation by hypoxia. Our findings suggest that hypoxia in the stem cell niche is critical for the maintenance of the undifferentiated stem or precursor cell phenotype.

Stem and/or precursor cells exist in a distinct tissue structure called the niche that regulates the self-renewal and differentiation of stem cells (1, 2). As shown recently, the bone marrow microenvironment has lower oxygen concentration than other tissues and stem cells are localized in the hypoxic regions (3), suggesting that hypoxia may be important for stem cell maintenance. However, the role of hypoxia in stem cell maintenance remains to be fully understood.

Hypoxia can regulate cellular differentiation. Under hypoxic conditions, the differentiation of embryonal stem cells, as well as precursor cells is inhibited (4–6). Studies in cancer biology have shown that hypoxia is strongly correlated with an undifferentiated phenotype in solid tumors such as neuroblastoma (7), breast cancer (8), and cervical cancer (9). These observations indicate that hypoxia plays a critical role in the maintenance of the undifferentiated stem cell phenotype.

Cellular response to hypoxia is manifested by the activation of the hypoxia-inducible factor-1 (HIF-1), a transcription factor of the basic helix-loop-helix Per, Arnt, and Sim family (10, 11). HIF-1 consists of the O2-regulated HIF-1α subunit and the O2-independent HIF-1β subunit. Under normoxia, HIF-1α protein becomes hydroxylated at proline-402 and proline-564 in its O2-dependent degradation (ODD) domain and is targeted by the von Hippel-Lindau protein for proteasome-mediated degradation (10, 11). As pO2 decreases to hypoxic levels, HIF-1α is no longer hydroxylated and thus becomes stabilized. Upon nuclear translocation, HIF-1α dimerizes with the O2-independent HIF-1β to initiate gene transcription (10, 11). HIF activation results in increased expression of several key stem cell markers such as CXCR4 (12, 13), SDF-1/CCL12 (3), and OCT4 (14). Conversely, the prodifferentiation gene, peroxisome proliferator-activated receptor γ (PPARγ), is down-regulated as a result of HIF activation (6).

Using the 3T3-L1 preadipocytes as a model, we have investigated the effects of hypoxia on the maintenance of the precursor phenotype. Our data demonstrate that the preadipocytes treated with adipogenic hormones under hypoxia maintain their precursor phenotype and can fully commit to adipogenic differentiation upon returning to normoxia. We have also found that hypoxia is capable of maintaining the preadipocyte phenotype of the adipose-derived primary mesenchymal cells. Based on these findings, we propose that hypoxia plays an essential role in the maintenance of stem and/or precursor cells. Our results underline the importance of hypoxia in the stem cell niche.

**EXPERIMENTAL PROCEDURES**

Plasmids—The siRNA against HIF-1α was cloned into pSI-REN-RetroQ between BamHI and EcoRI (BD Biosciences). The sequences for the siRNA against HIF-1α are: 5′-GATCCGTC-TAGAGATGCAAGAAGATTCAGAGATCTTTGCTGAT-CTCTAGACTTTTTTGG-3′ (sense strand) and 5′-AATCCAA-AAAAAGTCTAGAGATGCAGCAAGATCTCTTGTAGACG-3′ (antisense strand). The sequences for the scrambled siRNA sequence are: 5′-GATCCT-CAGAACGATGACTGAGATTTTTGCTGATCTCTTGTAGACG-3′ (sense strand) and 5′-AATTC-AAAAATCAGAACGATGACTGAGATTTTTGCTGATCTCTTGTAGACG-3′ (antisense strand). The scrambled siRNA sequence did not share homology to any known mammalian genes in the GenBank™ data bank. These constructs were sequence-verified using a primer for the U6 promoter. The constitutively active HIF-1α mutants: ΔODD

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(deletion of the oxygen-dependent degradation domain) and Pro/Mut (P402G/P564A) were described previously (5).

**Cell Culture and Adipogenic Differentiation—**3T3-L1 preadipocytes (ATCC) were maintained in growth medium: DMEM containing 10% calf serum and 1 mM sodium pyruvate. For adipogenic differentiation (6), confluent 3T3-L1 cells were maintained in growth medium for 2 days before stimulation for 2 days in the differentiation medium: DMEM containing 10% fetal bovine serum and IDM (10 μg/ml insulin, 1 mM dexamethasone, and 0.5 mM isobutylmethylxanthine). Cells were then maintained in DMEM containing 10% fetal bovine serum and 1 μg/ml insulin, and the medium was replaced every other day. For retroviral infection, 3T3-L1 cells were infected at 30–50% confluence as described previously (5, 6). The infected cells were allowed to reach confluence and subjected to differentiation as described above.

For hypoxia treatment, preadipocytes were maintained in a hypoxia chamber (Invivo2, 400, Ruskin Inc.), and the media were replaced every other day inside the chamber. In this study, normoxia was considered as the ambient atmosphere containing 21% O2 and hypoxia, 1% O2. Deferoxamine mesylate (DFO, Sigma) was used to mimic the hypoxic effects at 21% O2 (6).

Mature adipocytes were visualized by staining with 60% of the Oil Red O solution, as described previously (6). For quantitative analysis, the cell-absorbed Oil Red O was extracted in 100% isopropyl alcohol, and optical density was measured at 510 nm.

**Isolation and Differentiation of Adipose-derived Vascular-Mesenchymal (ADVM) Cells—** Epididymal fat pads were aseptically excised from four 5–6-week-old BALB/c mice. Fat pads were minced with scissors and incubated for 45 min at 37 °C in a collagenase buffer containing 0.1M HEPES at pH 7.4, 120 mM NaCl, 5.2 mM KCl, 1.3 mM CaCl2, 0.09%D-glucose, 1.5% bovine serum albumin, and 1% Type I collagenase (Worthington Biochemical Co., Lakewood, NJ). The undigested tissue was removed by filtration through a nylon mesh. Adipocytes were removed by centrifugation. Red blood cells were eliminated by resuspension of cell pellets in red blood cell lysis buffer containing 155 mM NH₄Cl, 5.7 mM K₂HPO₄, and 0.1 mM EDTA at pH 7.3. After washing, ADVM cells were plated in preadipocyte growth medium and expanded for 1–2 additional passages. For the adipogenesis assay, ADVM cells were plated in triplicates into 24-well plates. The confluent monolayer culture was differentiated using the standard IDM protocol.

**Northern and Western Blotting—** Total cellular RNA was isolated with TRIzol reagent (Invitrogen). The following plasmids were used for cDNA template preparations: MSV-C/EBPα, MSV-C/EBPβ, and MSV-C/EBP8 (S. L. McKnight), pSVsport-PPARγ2 and pBS-adipsin (B. M. Spiegelman), pTrcHis-adiponectin (H. F. Lodish), pCMV-Sport6.1-pref-1 (IMAGE 6393667), pCMV-Sport6.1-AP2α (IMAGE 6438317), pBabeGATA3 (G. S. Hotamisligil). The cDNA probes were labeled with [α-32P]dCTP. Hybridization was carried out at 65 °C for 6–12 h. The radioactive blots were exposed to Kodak Biomax films or alternatively visualized on Storm 860 PhosphorImager (GE Healthcare).

For Western blotting analysis, cell lysates were prepared on ice using 25 mM HEPES buffer, pH 7.4, containing 1% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, and a protease inhibitor mixture (Complete™, Roche Diagnostics). Equal amounts of proteins were analyzed with the following primary antibodies: polyclonal rabbit anti-pref-1 (Chemicon International, Temecula, CA), anti-β-tubulin (CRP, Inc.), according to the chemiluminescence method. For detection of HIF-1α, HIF-1β, and HIF-2α, nuclear extracts were prepared using the high salt extraction method as described previously (5). Polyclonal antibody against HIF-1α, HIF-1β, and HIF-2α were purchased from Novus Biologicals, Inc. (Littleton, CO).

**Statistical Analysis—** The statistical difference between two groups was analyzed by the two-tailed, unequal Student’s t test using Prizm 3.0cx (GraphPad Software Inc.). Significant difference between two groups was declared if p < 0.05.

**RESULTS AND DISCUSSION**

**Hypoxia Maintains Precursor Phenotype—** Adipogenic differentiation is controlled by sequential expression of adipocyte-related genes (15, 16). During the normal differentiation of preadipocytes (lanes 2–8, Fig. 1), CAAT enhancer binding protein β (C/EBPβ) and C/EBPα (Group I) were induced within hours of adipogenic stimulation by the IDM mixture. The adipogenic determination genes PPARγ2 and C/EBPα (Group II) were induced between 24 and 48 h after the IDM treatment. The mature adipocytes were characterized by the expression of 4P2, adipin, and adiponectin (Group III). The key event during adipogenesis is the transcriptional induction of PPARγ2 and/or C/EBPα (15, 16). Hypoxia inhibited adipogenic differentiation of preadipocytes. At the transcriptional level, hypoxia repressed the expression of the essential adipogenic genes PPARγ2 and C/EBPα (lanes 9–14, Fig. 1). The lack of adipocyte-specific genes: aP2, adipin, and adiponectin (Group III, lanes 9–14, Fig. 1), confirmed that no terminal differentiation occurred under hypoxia.
Differentiation Arrest by Hypoxia

A: Experimental Scheme

3T3-L1 Cells

+ or - IDM

1% O2 or DFO

Pretreatment

4 days

21% O2

Recovery

2 days

+ or - IDM

6 days

21% O2

Re-stimulation

B: Controls (4-day)

- + + - +

IDM

IDM

1% O2

DFO

Re-stimulation with IDM

C: Pretreatment: hypoxia without IDM

- + + - +

1% O2

DFO

Re-stimulation with IDM

D: Pretreatment: hypoxia with IDM

- + + - +

1% O2

DFO

Re-stimulation with IDM

E: Quantitative adipo-differentiation

O.D. 510 nm

B: Control

C: Hypoxia

D: Hypoxia/IDM

F: Controls (4-day)

- - + - +

IDM

1% O2

DFO

Re-stimulation with IDM

G: Re-differentiation of hypoxia-treated cells

Pretreatment: hypoxia - IDM

1% O2

DFO

Pretreatment: hypoxia + IDM

1% O2

DFO

FIGURE 2. Hypoxia arrests preadipocytes in the progenitor state. A, the overall experimental scheme is illustrated. B, control experiments include unstimulated 3T3-L1 cells, as well as the cells stimulated with IDM. Cells were stained with Oil Red O after the 4-day treatment. C, 3T3-L1 cells were first treated under hypoxic conditions without IDM, allowed to recover for 2 days at 21% O2, and then restimulated with IDM (+) or left untreated (-). D, 3T3-L1 cells were first treated under hypoxic conditions with IDM, allowed to recover for 2 days at 21% O2, and then restimulated with IDM (+) or left untreated (-). E, quantification of adipo-differentiation by Oil Red O staining in the control experiments (“B: Control”). 3T3-L1 cells were treated with or without IDM in the presence of absence of hypoxia for 4 days. For the recovery experiments, 3T3-L1 cells were pretreated for 4 days with hypoxia alone (“C: Hypoxia”) or hypoxia + IDM (“D: Hypoxia/IDM”), allowed to recover for 2 days under normoxia, and then re-stimulated with the IDM mixture (“+IDM”) or left alone (“-IDM”) under normoxia. Data shown are mean ± S.D. of a triplicate experiment. *, p < 0.003 versus the undifferentiated control cells at 21% O2 (without IDM, normoxia, open bar). Values below the horizontal line are not statistically different from the undifferentiated control cells at 21% O2 (p > 0.05). F, the ADVM cells were treated for 4 days at the indicated conditions. G, ADVM cells were pretreated and then restimulated with IDM as indicated in A. Differentiated adipocytes were visualized by Oil Red O staining. All experiments were independently performed three times.

Hypoxia had minimal effect on the expression of C/EBPβ and C/EBPδ within the first 24 h of the adipogenic stimulation (lanes 9–11 versus lanes 3–5, Fig. 1). However, the effect of hypoxia on the expression of C/EBPβ and C/EBPδ became more pronounced after 2 days of hypoxia treatment (lanes 12–14, Fig. 1), as compared with the normoxia control (lanes 6–8, Fig. 1). The latter phenomenon may indicate cellular adaptation to the chronic exposure to hypoxia. Further analysis is warranted for mechanistic understanding of the transcriptional regulation of C/EBPβ and C/EBPδ under chronic hypoxia. Nevertheless, this result suggests that the endogenously induced C/EBPβ and C/EBPδ are not sufficient to facilitate the transcription of PPARγ2 and/or C/EBPα under hypoxia. Our previous study showed that the hypoxia-induced transcription repressor DEC1/Str13 repressed PPARγ2 expression and was sufficient to inhibit adipogenesis (6).

As shown by our data, the transcriptional induction of PPARγ2 is repressed by hypoxia in the IDM-treated preadipocytes. In contrast, hypoxia does not seem to affect the steady-state transcription of PPARγ2 in mature adipocytes (17). Such discrepancy may suggest the transcriptional regulation of PPARγ2 in mature adipocytes is different from that in preadipocytes.

Three possibilities exist for the fate of the preadipocytes that have been stimulated by the adipogenic IDM mixture under hypoxia. First, the hypoxia-treated preadipocytes remain undifferentiated despite adipogenic stimulation. Second, these preadipocytes are committed to, but are blocked from, terminal differentiation under hypoxia. Third, the precursor phenotype is altered by hypoxia and thus unable to undergo adipogenesis. To test these hypotheses, we treated 3T3-L1 cells for 4 days under hypoxia (1% O2 or the hypoxia-mimetic compound DFO) with or without IDM, allowed the cells to recover for 2 days at 21% O2, and then restimulated with IDM or left untreated for 6 days (Fig. 2A). As a control, 3T3-L1 cells differentiated into Oil Red O-positive adipocytes at 21% O2 after IDM treatment but not under hypoxic conditions (Fig. 2B). Quantitative differences were shown in Fig. 2E (“B: Control”). 3T3-L1 cells pretreated by hypoxia alone retained the full differentiation ability upon restimulation by IDM (Fig. 2C and “C: Hypoxia” in Fig. 2E), indicating that hypoxia per se does not affect the adipogenic potential of preadipocytes. Interestingly, preadipocytes pretreated with IDM under hypoxia remained undifferentiated.
the transcription of C/EBPα (20). The transcription factor GATA-3 can repress the transcription of PPARγ2 (21). Nevertheless, it is not clear how these preadipocyte genes potentially interact to maintain the preadipocyte phenotype.

Under the normal differentiation condition of 21% O₂, the decrease of pref-1 mRNA after adipogenic stimulation (lanes 5 and 6, Fig. 3A) coincided with the robust induction of both PPARγ2 and C/EBPα (lanes 5 and 6, Fig. 1) within first 2 days of IDM treatment. In contrast, pref-1 mRNA remained elevated for the entire 6 days at 1% O₂ despite adipogenic stimulation by IDM (lanes 9–14, Fig. 3A). The expression of GATA-3 rapidly decreased within 2 h following IDM treatment at either 21 or 1% O₂ (lanes 3 and 9, Fig. 3A), suggesting that hypoxia does not affect transcriptional regulation of GATA-3. The expression of AP-2α was also rapidly repressed within 2 h of adipogenic stimulation at 21% O₂ (lane 3, Fig. 3A). Hypoxia only partially prevented the down-regulation of AP-2α by IDM (lane 9, 21% O₂).

Even after returning to normoxia (Fig. 2D and “D: Hypoxia/IDM” in Fig. 2E), indicating that these preadipocytes were not committed to terminal differentiation. Nevertheless, they were able to fully differentiate into adipocytes upon restimulation with IDM (Fig. 2D and “D: Hypoxia/IDM” in Fig. 2E), proving that hypoxia arrested the preadipocytes in their precursor stage without reducing their adipogenic potential. We obtained the same results when preadipocytes were pretreated for 6 days under hypoxia in the presence of IDM before returning to normoxia (data not shown).

To further test this hypothesis, we used primary ADVM cells isolated from mouse epididymal fat pads. The ADVM cells contained a subpopulation of precursor cells capable of adipogenic differentiation in response to IDM (Fig. 2F). Consistent with the observations using 3T3-L1 cells, hypoxia inhibited the adipogenic differentiation of the ADVM cells (Fig. 2F). Our data further showed that the ADVM cells pretreated under hypoxia were still capable of adipogenic differentiation upon returning to normoxia (Fig. 2G). These results suggest that hypoxia has the potential to arrest the primary adipose precursor cells in the undifferentiated state in vivo.

**Hypoxia Maintains the Expression of pref-1—Preadipocytes express a distinct set of precursor cell genes including the transmembrane protein pref-1 (18, 19) and the transcription factors AP-2α (20) and GATA-3 (21). Genetic deletion of pref-1 enhances adiposity (22) and ectopic expression of pref-1 in adipose tissues inhibits adipocyte development (23). On the other hand, the transcription factor AP-2α has the potential to inhibit the transcription of C/EBPα (20). The transcription factor GATA-3 can repress the transcription of PPARγ2 (21). Nevertheless, it is not clear how these preadipocyte genes potentially interact to maintain the preadipocyte phenotype.**

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HIF-1α did not have significant effect on the expression of pref-1 mRNA or its protein (supplemental Fig. 2). As shown recently, hypoxia regulates histone deacetylase activity in stem cells (25). It is possible that hypoxia may regulate pref-1 expression in preadipocyte via modulation of histone deacetylase activities. It has also been shown that dexamethasone in the IDM mixture can cause down-regulation of pref-1 transcription (26). It is therefore likely that hypoxia may block the dexamethasone-dependent differentiation under hypoxia. Down-regulation of pref-1 alone did not restore adipogenesis under hypoxia (data not shown), suggesting that maintenance of precursor phenotype requires multiple preadipocyte genes.

**HIF-1 Is Involved in Maintenance of Preadipocytes**—Because HIF-1 is the predominant hypoxia-signal transduction pathway in preadipocytes, we investigated the role of HIF-1 in the regulation of the preadipocyte phenotype. We used siRNA to specifically repress the expression of HIF-1α protein. We found that HIF-1α protein was no longer induced by hypoxia in the siRNA-expressing cells (lanes 5 and 6 versus lanes 2 and 3 and lanes 8 and 9, Fig. 4C). Consistent with the knocking down of HIF-1α protein, the HIF-dependent transcription was also repressed in cells treated with the siRNA as compared with those treated with vector control or the scrambled siRNA (Fig. 4D).

Importantly, when HIF-1α was repressed by siRNA, the preadipocytes were able to undergo adipogenic differentiation in the presence of the hypoxic mimetic compound DFO (Fig. 4, A and B). However, knocking down HIF-1α protein by siRNA was not sufficient to restore adipogenic differentiation under the low pO_2 condition (data not shown). This discrepancy could potentially be explained by the fact that pref-1 expression was not directly affected by HIF-1 but was rather maintained under the low pO_2 condition. Nevertheless, ectopic expression of the constitutively active HIF-1α protein mutants completely prevented preadipocytes from undergoing adipogenic differentiation (Fig. 4F). Under the same condition, the preadipocytes infected with a control retroviral vector differentiated normally into mature adipocytes (Oil Red O-positive). Consistent with
our previous findings (6), adipogenic differentiation was completely blocked in preadipocytes infected with retrovirus containing DEC1/Stra13, a transcription repressor for PPARγ expression and a direct target of HIF-1 (Fig. 4F). These results indicate that HIF-1 plays a direct role in regulation of preadipocyte differentiation.

**Significance**—Recent evidence suggests that stem cells may reside in a hypoxic microenvironment (3). Our study demonstrated that hypoxia was able to maintain preadipocytes in their undifferentiated state without decreasing their differentiation potential. Our observations suggest that hypoxia in the stem cell niche may be important for the maintenance of the undifferentiated stem cell phenotype. The HIF-1 pathway potentially constitutes an important mechanism in the maintenance of stem/precursor cells.

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