DIFFERENTIATION ARREST BY HYPOXIA

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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 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 (ES) 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, AhR and Sim (bHLH-PAS) 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 (VHL) 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 pro-differentiation 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 pSIREN-RetroQ between BamHI and EcoRI (BD Biosciences, Palo Alto, CA). Sequence for the siRNA against HIF-1α: 5’-GATCCGTCTAGAGATGAGC-AGATCTGCT-GCATCTCCTAGACTTTTTG-3’ (sense strand) and 5’-AATTCAGAAAAGTGCTAGAGA-TGCACAAAGAATCTTGAATCTTGGCAGCCTCTAGACG-3’ (antisense strand). Sequence for the scrambled siRNA sequence: 5’-GATCC TCAGAACGATGACCTG-ACTTTTTG-3’ (sense strand) and 5’-AATTCAGAAAAGTGCTAGAGA-TGCACAAAGAATCTTGAATCTTGGCAGCCTCTAGACG-3’ (antisense strand). The scrambled siRNA sequence did not share homology to any known mammalian genes in the GenBank database. These constructs were sequence-verified using a primer for the U6 promoter. The constitutively active HIF-1α mutants: ∆ODD (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 (GM): DMEM containing 10% calf serum and 1 mM sodium pyruvate. For adipogenic differentiation (6), confluent 3T3-L1 cells were maintained in GM for 2 days before stimulation for 2 days in the differentiation medium (DM): DMEM containing 10% FBS and IDM (10 μg/ml Insulin, 1 μM Dexamethasone and 0.5 mM Isobutylmethylxanthine). Cells were then maintained in DMEM containing 10% FBS 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 and subjected to differentiation as described above.

For hypoxia treatment, preadipocytes were maintained in a hypoxia chamber (Invivo 400, Biotrace International) 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-Aldrich Co.) 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 previously described (6). For quantitative analysis, the cell-absorbed Oil Red O was extracted in 100% isopropanol 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 minutes at 37°C in a collagenase buffer containing 0.1 M 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 (DM) 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 (Life Technologies). The following plasmids were used for cDNA template preparations: MSV-C/EBPβ, MSV-C/EBPδ and MSV-C/EBPα (S. L. McKnight), pSVsport-PPARγ and pBS-adipsin (B. M. Spiegelman), pTrcHis-adiponectin (H. F. Lodish), pCMV-Sport6.1-pref-1 (IMAGE 6393667), pCMV-Sport6.1-AP2δ (IMAGE 6438317), pBabe-GATA3 (G. S. Hotamisligil).
The cDNA probes were labeled with $\beta^32$P-dCTP. Hybridization was carried out at 65°C for 6 to 12 hr. The radioactive blots were exposed to Kodak Biomax films or alternatively visualized on Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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

Statistical Analysis – Statistical difference between two groups was analyzed by the two-tailed, unpaired Student 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 (C/EBPβ) and C/EBPδ (Group I) were induced within hours of adipogenic stimulation by the IDM cocktail. The adipogenic determination genes PPARγ and C/EBPδ (Group II) were induced between 24 to 48 hr after the IDM treatment. The mature adipocytes were characterized by the expression of αP2, adipin, and adiponectin (Group III). The key event during adipogenesis is the transcriptional induction of PPARγ and/or C/EBPδ (15,16). Hypoxia inhibited adipogenic differentiation of preadipocytes. At the transcriptional level, hypoxia repressed the expression of the essential adipogenic genes PPARγ and C/EBPδ (Lanes 9-14, Fig. 1). The lack of adipocyte-specific genes: αP2, adipin and adiponectin (Group III, Lanes 9-14, Fig. 1), confirmed that no terminal differentiation occurred under hypoxia.

Hypoxia had minimal effect on the expression of C/EBPβ and C/EBPd within the first 24 hr 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 to 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 PPARγ and/or C/EBPδ under hypoxia. Our previous study showed that the hypoxia-induced transcription repressor DEC1/Stra13 repressed PPARγ expression and was sufficient to inhibit adipogenesis (6).

As shown by our data, the transcriptional induction of PPARγ is repressed by hypoxia in the IDM-treated preadipocytes. In contrast, hypoxia does not seem to affect the steady-state transcription of PPARγ in mature adipocytes (17). Such discrepancy may suggest the transcriptional regulation of PPARγ 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 cocktail 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. 1E (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 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), 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 PPARγ (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 5-6, Fig. 3A) coincided with the robust induction of both PPARγ and C/EBPα (Lanes 5-6, 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 hr following IDM treatment at either 21% or 1% O2 (Lanes 3 & 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 hr of adipogenic stimulation at 21% O2 (Lane 3, Fig. 3A). Hypoxia only partially prevented the down-regulation of AP-2α by IDM (Lane 9, Fig. 3A). Taken together, these findings suggest that the maintenance of pref-1 mRNA by hypoxia may be a critical mechanism by which hypoxia regulates the preadipocyte phenotype.

Consistent with its mRNA, pref-1 protein was also maintained at elevated levels under hypoxia (Lanes 7-11, Fig. 3C) in IDM-treated preadipocytes. We further found that hypoxia did not affect the half-life of either pref-1 mRNA or protein (data not shown). These observations suggest that the persistent expression of pref-1 protein under hypoxia in IDM-treated preadipocytes results from the sustained transcription of pref-1 mRNA under hypoxia.

We next investigated the role of HIF in the regulation of pref-1 expression. HIF-1α mRNA is constitutively expressed from preadipocytes (Lanes 1-2, Supplemental Fig. 1A) to mature adipocytes (Lanes 7-8, Supplemental Fig. 1A). The expression of HIF-1α protein is controlled by its posttranslational hydroxylation of proline residues (10,11). The levels of HIF-1α protein changed only slightly during adipogenic differentiation at 21% O2, but was robustly induced by hypoxia (Lanes 7-8, Fig. 3D) before returning to the basal level after 2 days (Lanes 9-11, Fig. 3D). On the other hand, the O2-insensitive HIF-1α subunit was not affected by hypoxia, nor did its expression change during adipogenic differentiation (Fig. 3D).
HIF-2α, the structurally and functionally related homologue of HIF-1α (10,11), showed a distinct profile of expression during adipogenesis. HIF-2α mRNA was not found in preadipocytes (Lanes 1-2, Supplemental Fig. 1B), but was expressed in differentiating adipocytes after IDM stimulation (Lanes 3-7, Supplemental Fig. 1B). Similarly, HIF-2α protein was not detected in preadipocytes, but only in differentiated adipocytes after 4 days of adipogenesis at 21% O2 (Lanes 5-6, Fig. 3E). Consistent with our findings, Shimba et al. also reported that HIF-2α protein was only found in mature adipocytes, but not in preadipocytes (24). Our data also revealed that HIF-2α protein was mainly detected in whole cell lysates (WCL) of adipocytes (Lanes 5-6, Fig. 3E). These results indicate that HIF-2α protein is primarily expressed in mature adipocytes and is likely to be regulated by a hypoxia-independent pathway. In contrast, HIF-1α is the predominant hypoxia-sensing pathway in preadipocytes.

However, we found that neither HIF-1 nor hypoxia affected the transcription activity of a 10-kb rat pref-1 promoter in preadipocytes using the luciferase reporter gene assay (data not shown). In addition, ectopic expression of constitutively active 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 (HDAC) activity in stem cells (25). It is possible that hypoxia may regulate pref-1 expression in preadipocyte via modulation of HDAC activities. It has also been shown that dexamethasone in the IDM cocktail can cause down regulation of pref-1 transcription (26). It is therefore likely that hypoxia may block the dexamethasone-dependent repression of pref-1 transcription. We further investigated whether knocking-down pref-1 protein by siRNA could rescue adipogenic 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 5-6 vs. Lanes 2-3 and 8-9, 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 to 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. 4A & B). However, knocking-down HIF-1α protein by siRNA was not sufficient to restore adipogenic differentiation under the low pO2 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 pO2 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.
# REFERENCES

FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Effects of hypoxia on the expression of key genes during adipogenesis. 3T3-L1 cells were stimulated with IDM at 21% or 1% O2. RNA was prepared from the unstimulated cells just before confluence (day -2) or from the 2-day old confluent monolayer culture (day 0). After IDM-stimulation, RNA was isolated at the indicated time points from 2 hr to day 6. Northern Blotting was performed using [32P]-dCTP labeled cDNA probes specific for each of the genes indicated on the left. The 18S RNA was used as loading control. Experiments were performed three times.

Fig. 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 adipogenesis 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 cocktail (+IDM) or left alone (-) under normoxia. Data shown are mean ± standard deviation (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 adipose-derived vascular-mesenchymal (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.

Fig. 3. Effects of hypoxia on the expression of preadipocyte genes. A, RNA was prepared from 3T3-L1 cells after IDM-stimulation at 21% or 1% O2. Northern Blotting was performed using [32P]-dCTP labeled cDNA probes specific for pref-1, AP-2γ or GATA-3, with 18S RNA as loading control. B, Temporal changes in the levels of mRNA were analyzed using NIH Image 1.63. C, Whole cell lysates (1% NP-40) were prepared at the indicated time points after IDM-stimulation at 21% or 1% O2 and were subjected to Western Blotting for pref-1 with β-tubulin as loading control. D, HIF-1α and HIF-1β were analyzed by Western Blotting in nuclear extracts prepared under the same conditions as in (C). E, HIF-2α was analyzed both in nuclear extracts (Nuc. Ext.) and in whole cell lysates (WCL). Three experiments were performed for each condition.
Fig. 4. A direct role of HIF-1α in the inhibition of adipogenic differentiation. A, 3T3-L1 cells were infected with retrovirus expressing siRNA against HIF-1α, scrambled siRNA or the empty vector. Adipogenic differentiation was carried out in the presence or absence of 50 µM DFO. Adipocytes were identified by Oil Red O staining. B, Adipogenic differentiation was quantified by spectrometry at 510 nm. Data shown are mean ± s.d. *: p > 0.05 for siRNA versus Vector and siRNA versus Scrambled siRNA under normoxia (open bars); **: p < 0.01 for siRNA versus Vector and siRNA versus Scrambled siRNA under hypoxia (closed bars). C, The siRNA-mediated knock-down of HIF-1α expression was confirmed by Western Blotting. D, The siRNA-mediated inhibition of HIF-1α transcription activity was confirmed using the 5xHRE-luciferase construct as a reporter. Data shown are mean ± s.d. *: p < 0.003 for siRNA versus Vector and siRNA versus Scrambled siRNA in the presence of DFO (closed bar). **: p < 0.0005 for siRNA versus Vector and siRNA versus Scrambled siRNA at 1% O₂ (hatched bar). E & F, 3T3-L1 cells were infected with retrovirus expressing constitutively active HIF-1α mutants (ΔODD or Pro/Mut), DEC/Stra13 or lacZ control. The expression of ΔODD and Pro/Mut was verified by Western Blotting (E). The infected cells were stained with Oil Red O on day 6 after IDM-stimulation (F). All experiments were independently performed three times.
A: Experimental Scheme

3T3-L1 Cells → 4 days → 2 days → 6 days

1% O₂ or DFO

Pretreatment

Recovery

Restimulation

B: Controls (4-day)

C: Pretreatment: hypoxia without IDM

D: Pretreatment: hypoxia with IDM

E:

F: Controls (4-day)

G: Re-differentiation of hypoxia-treated cells
Differentiation arrest by hypoxia
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