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Inhibition of *PPARγ2* Gene Expression by the HIF-1-Regulated Gene *DEC1/Stra13*: A Mechanism for Regulation of Adipogenesis by Hypoxia

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Summary

Cellular differentiation involves transcriptional responses to environmental stimuli. Adipocyte differentiation is inhibited under hypoxic conditions, indicating that oxygen (O_2) is an important physiological regulator of adipogenesis. Hypoxia inhibits *PPARγ2* nuclear hormone receptor transcription, and overexpression of *PPARγ2* or *C/EBPβ* stimulates adipogenesis under hypoxia. Mouse embryonic fibroblasts deficient in hypoxia-inducible transcription factor 1α (*HIF-1α*) are refractory to hypoxia-mediated inhibition of adipogenesis. The HIF-1-regulated gene *DEC1/Stra13*, a member of the *Drosophila hairy/Enhancer of split* transcription repressor family, represses *PPARγ2* promoter activation and functions as an effector of hypoxia-mediated inhibition of adipogenesis. These data indicate that an O_2-sensitive signaling mechanism regulates adipogenesis. Thus, agents that regulate HIF-1 activity or O_2 sensing may be used to inhibit adipogenesis and control obesity.

Introduction

Molecular oxygen (O_2) is vital to nearly all forms of life on earth, perhaps via its role in energy homeostasis, embryogenesis, and differentiation. In response to hypoxia or low O_2 tensions, mammals increase the expression of a wide variety of genes, including erythropoietin, vascular endothelial growth factor (VEGF), and glycolytic enzymes, to stimulate erythropoiesis, angiogenesis, and glycolysis (Bunn and Poyton, 1996). Most of these hypoxia-regulated genes are transcriptionally induced by the hypoxia-inducible factor 1 (HIF-1), a member of the basic helix-loop-helix *Per*, *AhR*, and *Sim* (bHLH-PAS) family (Semenza and Wang, 1992; Wang et al., 1995a). Under normoxia, HIF-1α protein becomes hydroxylated at proline 564 in its O_2-dependent degradation domain (Ivan et al., 2001; Jaakkola et al., 2001) and is targeted by the von Hippel-Lindau (VHL) protein for proteosome-mediated degradation (Maxwell et al., 1999; Ohh et al., 2000). Under hypoxia, HIF-1α becomes stabilized, translocates to the nucleus, and dimerizes with the O_2-independent HIF-1β to initiate gene expression (Jewell et al., 2001; Kallio et al., 1997). The importance of cellular responses to hypoxia in development and differentiation is demonstrated in mouse models in which homozygous deletion of either *HIF-1α* or *HIF-1β* is embryonically lethal. The *HIF-1α*$^{-/-}$ embryos succumb, between 9 and 10 days postcoitum (dpc), to loss of mesenchymal cells and impaired vascular development (Iyer et al., 1998; Ryan et al., 1998). The *HIF-1β*$^{-/-}$ embryos die by 10.5 dpc, due to vascular deficiencies in the yolk sac and/or placenta (Kozak et al., 1997; Maltepe et al., 1997). Interestingly, mice heterozygous for *HIF-1α* exhibit increased weight loss when subjected to chronic hypoxia (Yu et al., 1999), reinforcing the essential and complex role *HIF-1α* plays in cellular homeostasis in a low-O_2 environment.

During the first trimester, a human embryo is located in a low-O_2 environment (3% O_2) (Rodesch et al., 1992). In rat embryos, O_2 tensions are low before 9.5 dpc (Mitchell and Yochim, 1968). The establishment of uteroplacental circulation relies on cytotrophoblast invasion into the uterine spiral arterioles. Studies indicate that cytotrophoblasts proliferate with a poorly differentiated phenotype at low O_2 tensions and differentiate into a highly invasive phenotype at high O_2 tensions (Caniggia et al., 2000; Genbacev et al., 1997). High O_2 tensions also favor terminal differentiation of megakaryocytes into platelets (Mostafa et al., 2000). In contrast, differentiation of other cell types seems to prevail at lower O_2 tensions. At 3% O_2, rat mesencephalic precursor cells exhibit higher growth rates and higher levels of differentiation into a dopaminergic phenotype than at 20% O_2 (Studer et al., 2000). Low O_2 tensions have been found to promote osteochondrogenesis. Mesenchymal stem cells from rat bone marrow display enhanced colony-forming capability and increased proliferation at 5% O_2 compared to those at 20% O_2, and they produce more osteocytes when implanted in vivo (Lennon et al., 2001). These observations suggest that the effect of O_2 on cell differentiation is extensive and cell-type specific.

Peripheral evidence in the literature supports a role of HIF-1 in adipogenesis. Using a subtraction cloning approach, Imagawa et al. (1999) found that *HIF-1α* mRNA is transiently induced in 3T3-L1 (L1) preadipocytes upon treatment with the adipogenic hormone cocktail containing insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IDM). However, the consequence of such transient *HIF-1α* expression was never investigated. Interestingly, adipogenesis can be inhibited by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which requires the aryl-hydrocarbon receptor (AhR), also a member of the bHLH-PAS family (Alexander et al., 1998; Phillips et al., 1995). Since AhR activates gene transcription by dimerization with HIF-1β (Probst et al., 1993), it is reasonable to hypothesize that the inhibition of adipogenesis may be a function shared by some members of the bHLH-PAS family, such as HIF-1α/β and AhR/HIF-1β.

Adipocyte differentiation results from sequential induction of transcription factors *C/EBPβ*, *C/EBPδ*, *PPARγ*, and *C/EBPα* (Rangwala and Lazar, 2000; Rosen and Spiegelman, 2000). *C/EBPβ* and *C/EBPδ* are induced immediately but transiently upon IDM treatment

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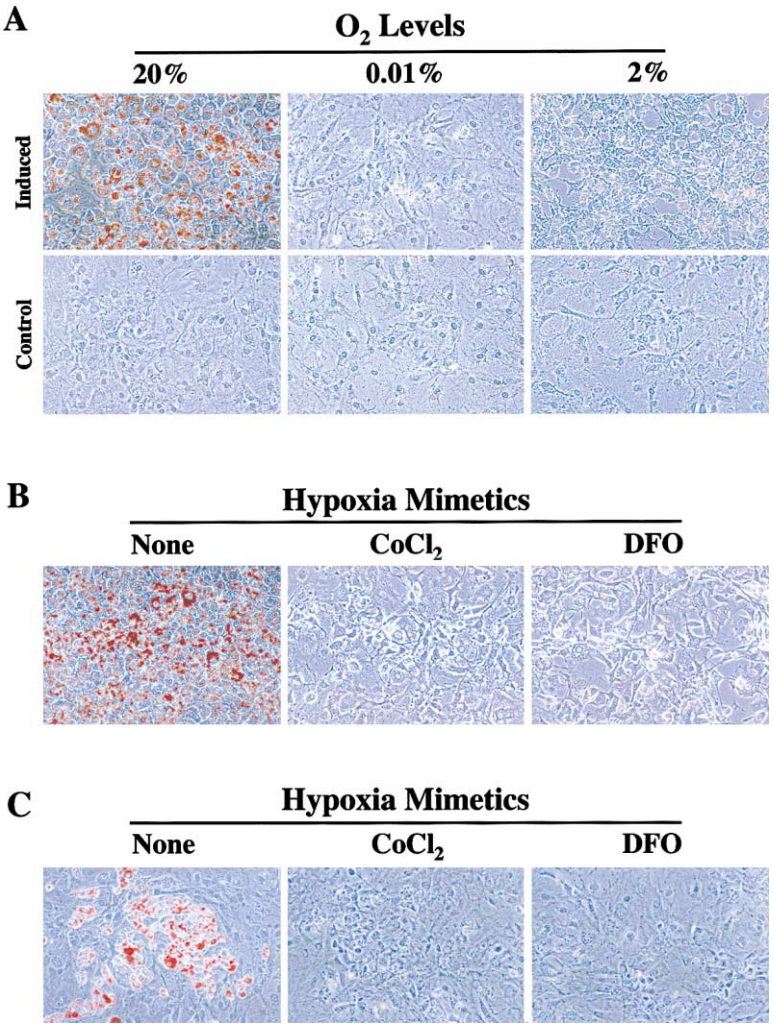

Figure 1. Hypoxia Inhibits Adipogenesis

(A) L1 cells were induced to differentiate (Induced) or left uninduced (Control) at indicated O_2 tensions. Cells were stained on day 6 with Oil Red O and photographed (20×).

(B) L1 cells were induced to differentiate in the presence or absence of $CoCl_2$ (50 μM) or DFO (100 μM) for the first 2 days together with IDM and then maintained in media without $CoCl_2$ or DFO. Cells were stained on day 6 and photographed (20×).

(C) MEFs were treated in DM containing 5 μM rosiglitazone with or without $CoCl_2$ (25 μM) or DFO (25 μM). Cells were stained on day 6 and photographed (20×).

to mediate the expression of *PPARγ* and *C/EBPα* (Christy et al., 1991; Wu et al., 1995; Yeh et al., 1995). In contrast to *C/EBPδ*, *C/EBPβ* is able to induce spontaneous differentiation in L1 cells and enhance the adipogenic potential in NIH-3T3 fibroblasts (Wu et al., 1995; Yeh et al., 1995). Highly specific for adipose tissues, *PPARγ* plays a critical role in the expression of most adipocyte-specific genes (Tontonoz et al., 1995) and is able to convert nonadipogenic mesenchymal cells, such as fibroblasts and myoblasts, to adipocytes (Hu et al., 1995; Tontonoz et al., 1994). Although developmentally necessary for adipogenesis (Wang et al., 1995b), *C/EBPα* is not always expressed during adipocyte differentiation, especially in cells that already express *C/EBPβ*. For example, *C/EBPα* is not involved in the expression of *GLUT-4*, the insulin-responsive glucose transporter, in 3T3 cells ectopically expressing *C/EBPβ* and *C/EBPδ* (Wu et al., 1998). These data suggest the *PPARγ* and *C/EBPβ* may be potential targets for adipogenic intervention.

The effects of hypoxia are manifested by HIF-1-regulated genes. Others and we have identified the hypoxia-induced gene *DEC1/Stra13*, a member of the *Drosophila hairy/Enhancer of split* (HES) family of bHLH transcription factors (Ivanova et al., 2001). The HES proteins play important roles in cell differentiation by repressing gene expression (Kageyama and Ohtsuka, 1999; Staal et al., 2001). During embryonic development, *DEC1/Stra13* is expressed in neuroectoderm and in some mesoderm- and endoderm-derived structures (Boudjelal et al., 1997). In P19 embryonal carcinoma cells, overexpression of *DEC1/Stra13* promotes neuronal differentiation and inhibits mesodermal and endodermal differentiation (Boudjelal et al., 1997). In differentiating L1 cells, *DEC1/Stra13* expression is increased approximately 2-fold within 1 hr of IDM treatment followed by a rapid decrease within 24 hr (Inuzuka et al., 1999). At present, the role of *DEC1/Stra13* during adipogenesis is ill defined.

Given the importance of O_2 sensing as well as energy homeostasis and cell differentiation in embryonic development, we hypothesize that O_2 tensions may control adipose tissue function by regulating adipogenesis. Since fatty acid metabolism requires mitochondrial respiration, hypoxia prevents the use of fatty acids and thus may obviate the need for more adipose tissue. Therefore, we have investigated whether hypoxia inhibits adipogenesis through the HIF-1-dependent induction of the *DEC1/Stra13* gene expression. As mentioned above, adipocyte differentiation in vitro is determined by precisely orchestrated expression of the *C/EBP*s and

PPARγ. We have thus determined whether the *C/EBP* family or *PPARγ* is the critical target of DEC1/Stra13 and whether overexpression of *DEC1/Stra13* is sufficient to inhibit adipocyte differentiation. The regulation of adipogenesis by hypoxia opens new directions for research in understanding how the microenvironment regulates cell differentiation both under physiological settings as well as during the malignant progression of tumors.

Results

Hypoxia Inhibits Adipogenesis via HIF-1

To evaluate the effect of hypoxia on adipogenesis, we treated L1 cells at different O_2 tensions with the standard cocktail of adipogenic hormones, IDM (Figure 1A). Under normoxia (20% O_2), L1 cells differentiate into mature adipocytes loaded with fat droplets, as indicated by Oil Red O staining. However, adipocyte differentiation is completely inhibited when L1 cells are induced to differentiate under hypoxia (0.01% or 2% O_2). No significant changes in cell death were detected in hypoxia-treated cells by the trypan blue exclusion assay when compared to the normoxic control. Hypoxic effects can be mimicked by iron chelators (deferoxamine or DFO) or divalent transition-metal ions (cobalt). Inhibition of L1 cell differentiation is observed when $CoCl_2$ or DFO is added to the treatment media throughout the course of induction (6 days) (data not shown). However, treatment with $CoCl_2$ or DFO for the first 2 days of induction with IDM is sufficient to prevent L1 cell differentiation (Figure 1B). Mouse embryonic fibroblast (MEF) cells were used to evaluate whether the inhibition of adipogenesis by hypoxia was a general phenomenon. MEFs are induced to differentiate into adipocytes by IDM supplemented with 5 μM rosiglitazone, a synthetic PPARγ2 ligand (Lehmann et al., 1995). Consistent with the literature, about 15%–20% of the MEF cells differentiate into adipocytes (Alexander et al., 1998). Treatment of MEFs with $CoCl_2$ or DFO suppresses adipogenesis (Figure 1C). Similar observations are made when MEF cells are maintained at 2% O_2 (data not shown).

To assess the role of HIF-1 in the inhibition of adipogenesis, we used MEFs in which each allele of *HIF-1α* was flanked by *lox*P sites (Ryan et al., 2000). The *HIF-1α* gene is efficiently excised when MEFs are transduced with adenovirus containing the cre recombinase gene (Seagroves et al., 2001). Both cre-treated and control MEF cells differentiate into adipocytes upon hormonal stimulation in the absence of hypoxia mimetics (Figure 2). When the *HIF-1α* gene is deleted from the genome by cre, the *HIF-1α*-deficient MEF cells continue to undergo significant adipocyte differentiation in the presence of either $CoCl_2$ (up to 50 μM) or DFO (up to 25 μM) (Figure 2). In contrast, $CoCl_2$ or DFO significantly represses differentiation of the control-treated MEF cells under the same conditions (Figure 2). This result indicates that HIF-1 is involved in inhibition of adipogenesis by hypoxia.

Hypoxia Inhibits Induction of *PPARγ2* Expression

We investigated the effect of hypoxia on the expression of three key transcription factors, *C/EBPβ*, *C/EBPδ*, and *PPARγ*, during L1 differentiation (Rangwala and Lazar, 2000; Rosen and Spiegelman, 2000). At 20% O_2, both

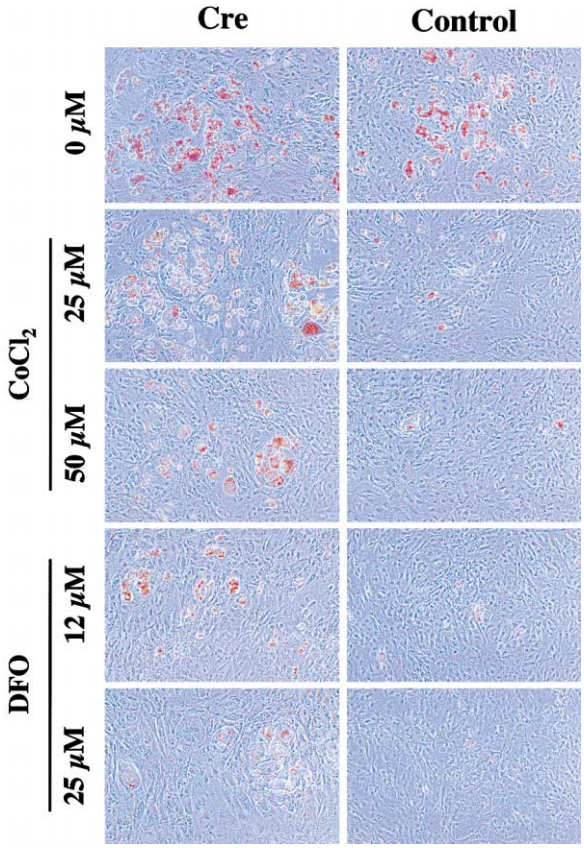

Figure 2. HIF-1 Is Required for Hypoxia-Mediated Inhibition of Adipogenesis

MEFs with *HIF-1α* alleles flanked by *lox*P sites were incubated with cre adenovirus (Cre) or control adenovirus (Control) and induced to differentiate as described. $CoCl_2$ or DFO was added at the indicated final concentrations for the entire course of treatment. Cells were stained on day 7 and photographed (20×).

C/EBPβ and *C/EBPδ* are significantly induced within 2 hr of IDM treatment followed by their gradual decrease (Figures 3A and 3B). *PPARγ2* mRNA is induced by day 2 post-IDM treatment following the expression of *C/EBPβ* and *C/EBPδ* and remains elevated throughout the rest of the differentiation process (Figures 3A and 3B). Under hypoxia, the induction of *PPARγ2* expression is completely abolished, and that of *C/EBPβ* is reduced (Figures 3A and 3B). The *PPARγ2* expression is also repressed in L1 cells treated with $CoCl_2$ or DFO (Figure 3C). Unexpectedly, *C/EBPδ* expression becomes dysregulated, and its mRNA remains elevated under hypoxia (Figures 3A and 3B). To assess the role of HIF-1 in *PPARγ2* inhibition, we analyzed the *PPARγ2* mRNA in MEFs that were treated with cre to excise the *HIF-1α* alleles. As shown in Figure 3D, *PPARγ2* induction is protected in cre-treated MEFs but not in mock-treated cells, indicating that HIF-1 is required for the inhibition of *PPARγ2* expression. These results suggest that negative regulation of *PPARγ2* and/or *C/EBPβ* gene expression is a key mechanism for hypoxia-mediated inhibition of adipogenesis.

We next investigated whether overexpression of *C/EBPβ* gene was sufficient to restore L1 cell differentiation under hypoxia. Consistent with the literature (Yeh

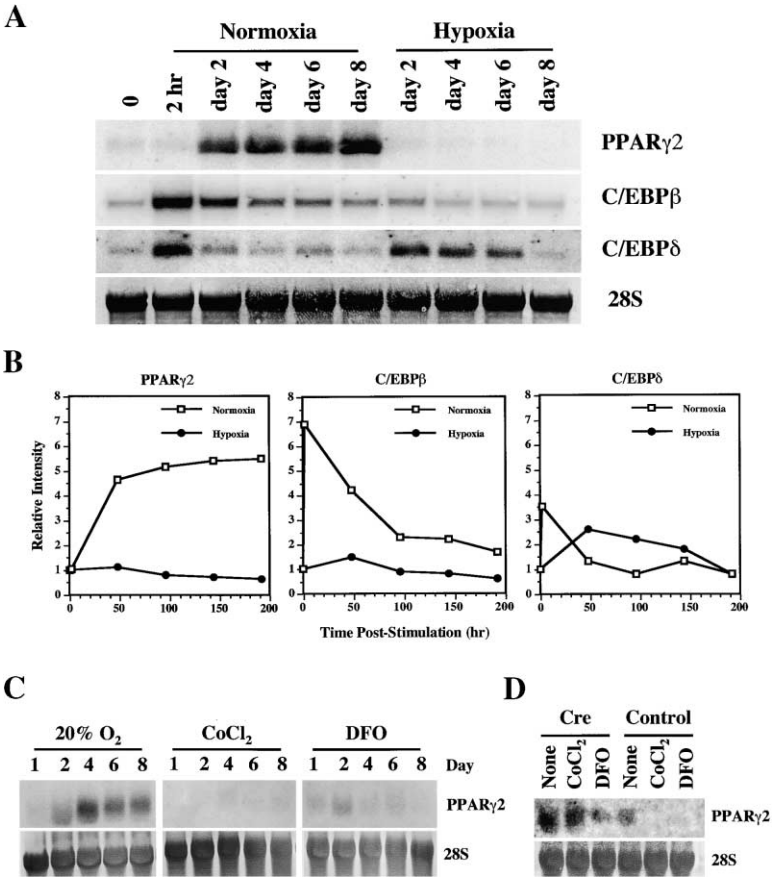

Figure 3. Hypoxia Modulates the Expression of *PPARγ2*, *C/EBPβ*, and *C/EBPδ*

(A and B) L1 cells were induced to differentiate either under normoxia or hypoxia (0.01% O_2). Total cellular RNA was prepared at indicated times after induction. Equal amounts (10 μg/lane) of total RNA were subjected to Northern blotting (A) using ^{32}P-labeled *PPARγ2*, *C/EBPβ*, or *C/EBPδ* cDNA as probes. The relative levels of expression were analyzed by densitometry (B).

(C) L1 cells were induced to differentiate under the following conditions: 20% O_2, 50 μM $CoCl_2$, or 100 μM DFO. Total RNA (5 μg/lane) was analyzed as in (A).

(D) MEFs were treated as in Figure 2. Total RNA was prepared 3 days after hormonal stimulation with or without 25 μM $CoCl_2$ or 12.5 μM DFO. Northern blotting analysis (10 μg/lane RNA) was done as in (A).

et al., 1995), *C/EBPβ*-expressing L1 cells display much higher levels of differentiation than the vector-infected cells with or without stimulation by IDM under normoxic conditions (Figure 4A). At 2% O_2, the *C/EBPβ*-expressing cells continue to differentiate into adipocytes, independent of IDM treatment (Figure 4A). However, the differentiation of the vector-infected cells is completely inhibited at 2% O_2. When hypoxia mimetics are used, we find that continuous presence of $CoCl_2$ or DFO is necessary for significantly reducing differentiation and/or fat accumulation of the *C/EBPβ*-expressing L1 cells (Figure 4B). If $CoCl_2$ or DFO is present for the first 2 days of adipogenic induction, the *C/EBPβ*-expressing L1 cells are still able to differentiate into fat-laden adipocytes (Figure 4B). Thus, while overexpression of *C/EBPβ* can make L1 cells refractory to hypoxia or hypoxia mimetics, the level of adipocyte differentiation induced by IDM in these same cells is attenuated. This result suggests that hypoxia may be affecting additional modulators of adipogenesis induced by IDM.

We also determined whether overexpression of *PPARγ2* gene is sufficient to drive L1 differentiation under hypoxia. L1 cells received three rounds of *PPARγ2* retroviral gene infection to maximize infection efficiency. The *PPARγ2*-infected cells are induced to differentiate in medium containing 10% fetal bovine serum and 1 μM rosiglitazone. Significant adipocyte differentiation is observed at 20% O_2 (Figure 4C), indicating efficient expression of *PPARγ2* gene. In contrast, vector-infected L1 cells do not differentiate under the same conditions, although these cells can still differentiate upon IDM stimulation (data not shown). Similar to *C/EBPβ*-transduced cells, the majority of the *PPARγ2*-expressing cells differentiate into adipocytes when stimulated by rosiglitazone under the conditions of 2% O_2, $CoCl_2$, or DFO, but they accumulate less fat than normoxic controls (Figure 4C). Our results indicate that overexpression of *PPARγ2* or *C/EBPβ* gene can overcome inhibition of adipocyte differentiation under hypoxia, but the phenotype of mature adipocytes is not fully restored.

DEC1/Stra13 Is an Effector for Hypoxia-Mediated Inhibition of Adipogenesis

Since HIF-1 is a transcription activator, the inhibition of adipogenesis by hypoxia is likely to be mediated by HIF-1-regulated genes. We investigated whether the HIF-1 target gene *DEC1/Stra13*, which contains a bHLH and an Orange domain homologous to those of the HES transcription repressors (Boudjelal et al., 1997; Shen et al., 1997), is involved in inhibition of adipogenesis by hypoxia. HIF-1 is required for hypoxic induction of *DEC1/Stra13*, since the increase of *DEC1/Stra13* mRNA occurs in wild-type MEF cells but not in *HIF-1α*$^{-/-}$ MEF cells (Figure 5A). DEC1/Stra13 protein is also sensitive to O_2 tensions, as it increases rapidly under hypoxia and decreases to its basal level within 6–12 hr upon reoxygenation (Figure 5B). Consistent with the literature, *DEC1/Stra13* mRNA is induced approximately 2-fold within 2 hr of IDM treatment (Figures 5C and 5E) during L1 cell differentiation under normoxia (Inuzuka et al., 1999). In contrast, *DEC1/Stra13* mRNA in differentiating L1 cells exhibits approximately a 4-fold increase on day 2, followed by a steady decline to the basal level under

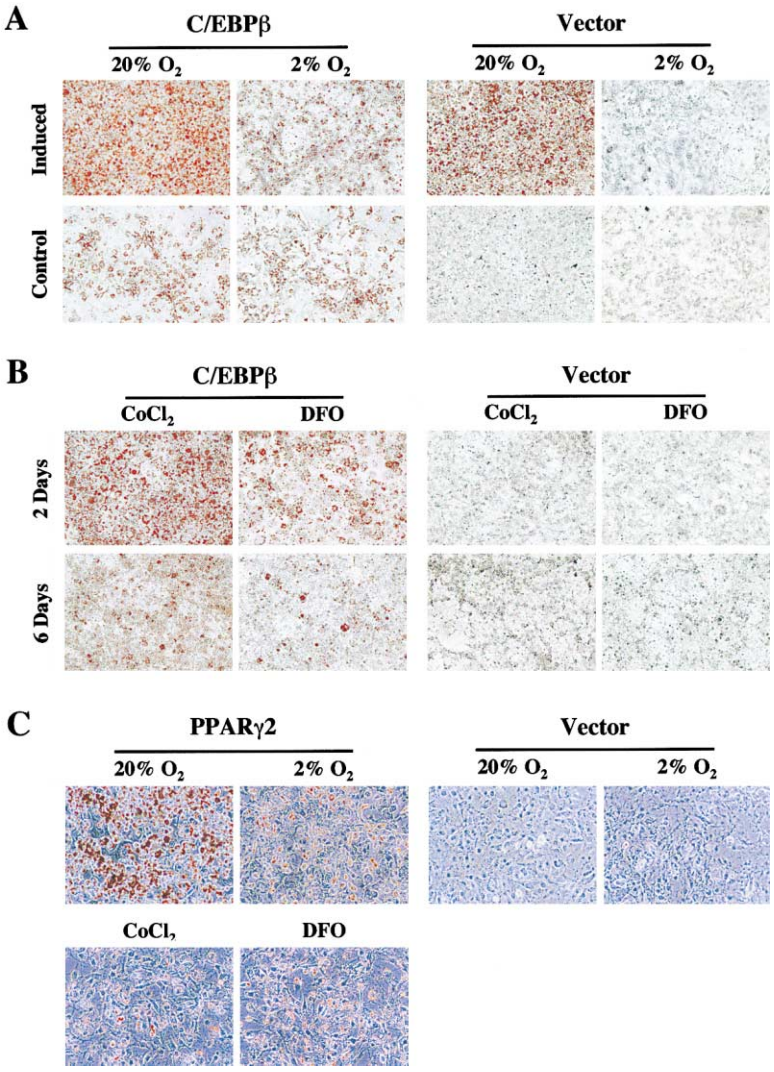

Figure 4. Ectopic Expression of *C/EBP*β or *PPARγ2* Restores the Adipogenic Potentials of 3T3-L1 Cells under Hypoxia

(A) Stable *C/EBP*β or vector cells (L1) were induced to differentiate (Induced) or left uninduced (Control) either at 20% or 2% O_2. Cells were stained on day 6 and photographed (20×).

(B) Stable *C/EBP*β or vector cells (L1) were induced to differentiate at 20% O_2 in the presence of either 50 μM $CoCl_2$ or 100 μM DFO for the first 2 days of induction (2 Days) or the entire 6 days of treatment (6 Days). Cells were stained on day 6 and photographed (20×).

(C) L1 cells transiently infected with *PPARγ2* or vector control were induced to differentiate under the following conditions: 20% O_2, 2% O_2, 50 μM $CoCl_2$ (6 days), or 100 μM DFO (6 days). Cells were stained on day 6 and photographed (20×).

hypoxia (Figures 5C and 5E). Interestingly, the DEC1/Stra13 protein level remains elevated (approximately 3-fold) from day 2 through day 8 under hypoxia compared to the level under normoxia (Figure 5D and 5E). The increased stability of DEC1/Stra13 protein in IDM-stimulated L1 cells indicates that both *DEC1/Stra13* mRNA and protein are regulated by hypoxia.

To address whether DEC1/Stra13 could repress *PPARγ2* gene induction, we analyzed the effect of DEC1 on the *PPARγ2* promoter activity using the 0.6 kb *PPARγ2* promoter (−603 to +62)-driven luciferase gene as a reporter for *PPARγ2* transcriptional activity (Tong et al., 2000). As shown in Figure 6A, full-length DEC1 represses *PPARγ2* promoter activity by 70% compared to the vector control. Interestingly, two N-terminal fragments (N1 and N2) containing the bHLH domain show similar levels of repression to the full-length DEC1 (Figure 6A). In contrast, the two C-terminal fragments (C1 and C2) do not inhibit *PPARγ2* promoter activity. This result suggests that the bHLH domain of DEC1/Stra13 is functionally sufficient for inhibition of *PPARγ2* gene expression. Using a series of deletion or truncation constructs of the 0.6 kb *PPARγ2* promoter (FL), we find that the BsrGI (−285)-StuI (−116) fragment (BrS) contains the element(s) repressible by DEC1 (data not shown). The BrS fragment shows similar levels of repression by DEC1 to those of the FL promoter, whereas the region with BrS deleted (FLΔBrS) is no longer repressed by DEC1 (Figure 6B). Structural analysis suggests a C/EBPβ site at −229 and a C/EBPα site at −200 followed by a YY-1 box within BrS (Figure 6C). Cotransfection of *C/EBP*β or *C/EBP*α can partially relieve repression by DEC1 (Figure 6B). Further deletion of the C/EBPβ site or both C/EBP sites from BrS results in an 80% and 85% decrease, respectively, in transcription activity alone. Together, these data suggest that DEC1 repression is mediated, at least in part, by the putative C/EBP sites at −229 and −200.

More importantly, overexpression of DEC1 or HA-DEC1 in L1 cells by retroviral infection results in nearly complete inhibition of adipocyte differentiation (Figure 7A). Significant inhibition is also conferred by retroviral transduction of N1 (amino acid [aa] 1–141), as shown in Figure 7A. Inhibition of adipogenesis by DEC1 is consistent with the finding that *PPARγ2* expression is repressed in L1 cells ectopically expressing DEC1, HA-DEC1, or N1 (Figure 7B). As shown in Figure 6A, the bHLH domain of DEC1/Stra13 is functionally equivalent

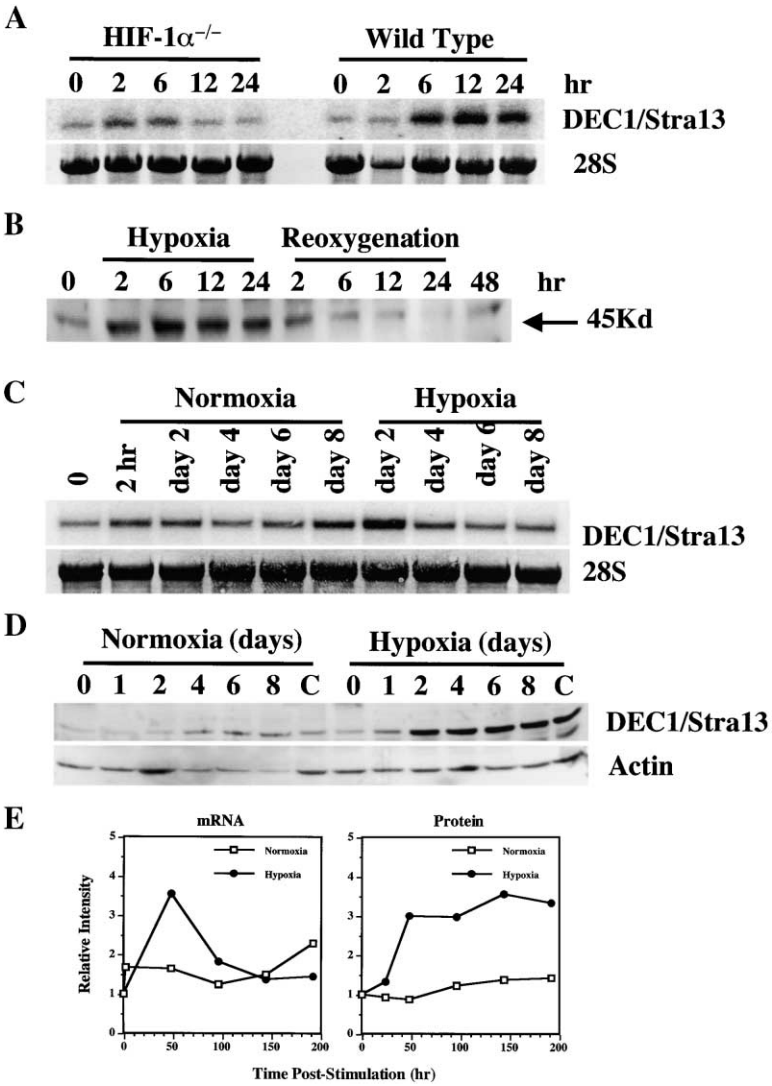

Figure 5. *DEC1/Stra13* Expression Is Regulated by O_2 Tensions via HIF-1

(A) Total RNA was prepared from *HIF-1*$\alpha^{-/-}$ or wild-type MEF cells at indicated time at 0.01% O_2. Equal amounts (10 μg/lane) of total RNA were subjected to Northern blotting analysis with ^{32}P-labeled *Stra13* cDNA as probe.

(B) NIH-3T3 cell lysates were prepared at indicated time at 0.01% O_2 or during reoxygenation following 24 hr hypoxia. Normoxic controls were prepared at 0 hr and 48 hr, respectively. DEC1/Stra13 protein was analyzed by Western blotting (25 μg protein/lane) as described.

(C) L1 cells were induced to differentiate either under normoxia or hypoxia (0.01% O_2). Equal amounts (10 μg/lane) of total RNA prepared at indicated time after induction were subjected to Northern blotting as in (A). The relative levels of *DEC1/Stra13* mRNA were analyzed by densitometry (E).

(D) L1 cells were induced to differentiate as in (C). Cell lysates were prepared at indicated times after treatment and subjected to Western blotting (20 μg protein/lane) as described. The controls (lane C) were maintained under either normoxia or hypoxia without adipogenic stimulation. The relative levels of DEC1/Stra13 protein were analyzed by densitometry (E).

to the full-length protein in repressing *PPAR*γ*2* expression. To more rigorously investigate the role of this bHLH domain in regulating adipogenesis, we constructed a fusion protein between N1 and an 11 aa protein-transduction domain derived from the TAT protein of human immunodeficiency virus (HIV) (Schwarze and Dowdy, 2000). A dose-dependent inhibition of adipogenesis is found when L1 cells are induced to differentiate in the presence of the TAT-N1 fusion protein (Figure 7C). These results indicate that DEC1/Stra13 functions as an effector of HIF-1 for the inhibition of adipogenesis by hypoxia.

Discussion

There is pathophysiological evidence that suggests a correlation between hypoxia and adipogenesis. For example, children with cyanotic heart disease have less body fat due to apparent adipocyte hypocellularity (Baum and Stern, 1977). High altitude training is well known to cause weight loss that is attributed largely to body fat reduction (Armellini et al., 1997; Westerterp et al., 1994a). Strenuous physical training, on the other hand, is also attributed to significant loss of body fat (Van Etten et al., 1994; Westerterp et al., 1994b). Besides other physiological changes, hypoxia occurs in exercising skeletal muscles, as characterized by an increase in the expression of HIF-1 and VEGF (Gustafsson and Kraus, 2001; Gustafsson et al., 1999). Under hypoxia, fatty acid oxidation is impeded and glycolysis is augmented to maintain energy homeostasis. If the stored fat is not used under hypoxia, there is less need to increase or renew adipose tissue via adipogenesis. Experimentally, rats exposed to hypoxia experience significant fat loss (Mortola and Naso, 1997; Tanaka et al., 1997). Thus, reduction of adipose tissues can be caused by tissue hypoxia.

Our results indicate that hypoxia-mediated adipogenic inhibition involves the repression of *PPAR*γ*2* gene induction and decrease in *C/EBP*β expression, two critical events during adipogenesis. The inhibition of

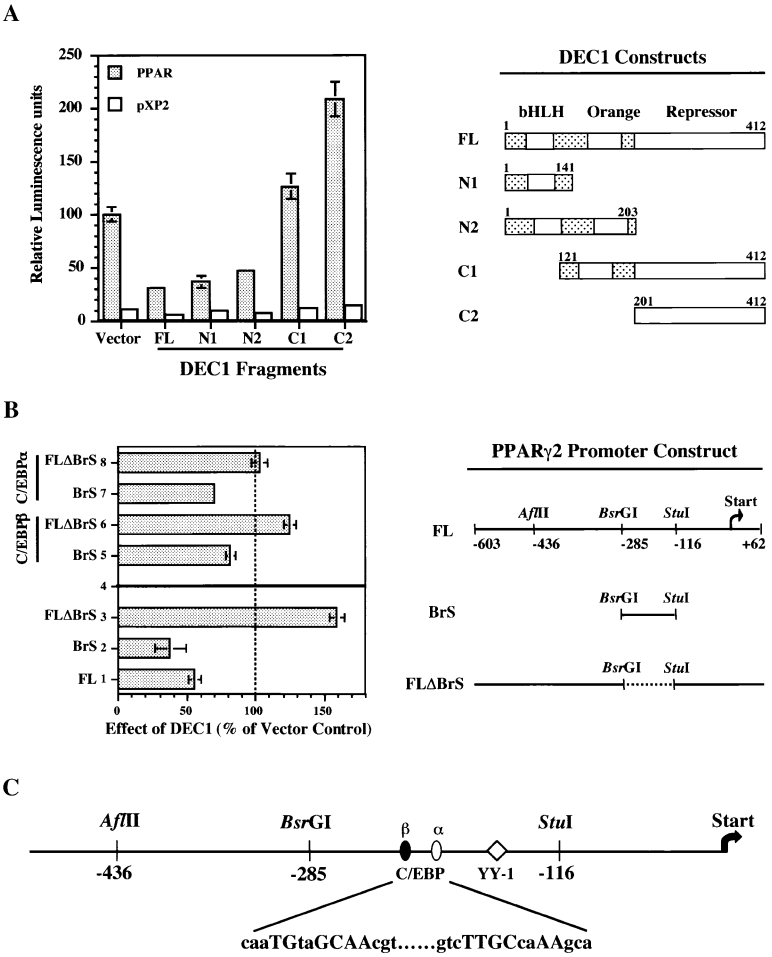

Figure 6. DEC1/Stra13 Represses *PPARγ2* Promoter Activity

(A) NIH-3T3 cells were transiently cotransfected with the 0.6 kb *PPARγ2* promoter-driven luciferase construct (PPAR) or the promoterless vector (pXP2) and DEC1 fragments (0.3 μg DNA each) as indicated. Luciferase activities in cell lysates were measured 40–48 hr after transfection using a luminometer and presented as relative luminescence units ± SD.

(B) Luciferase constructs (pXP2) with the 0.6 kb *PPARγ2* promoter (FL), BsrGI-StuI fragment alone (BrS), or deletion of BsrGI-StuI fragment (FLΔBrS) were cotransfected into NIH-3T3 cells with DEC1 or its vector control at the ratio of 1:5 (DEC1 or control to pXP2). In other experiments, *C/EBPα* or *C/EBPβ* were also cotransfected at the ratio of 1:1 (C/EBP to pXP2). A *Renilla* luciferase construct was also included to monitor transfection efficiency. Luciferase activities were measured as in (A).

(C) Schematic representation of the *PPARγ2* proximal promoter region. Putative C/EBP sites are displayed with the conserved bases capitalized.

PPARγ2 expression and its activity is a common mechanism for adipogenic inhibition by a variety of stimuli. A recent report has shown that HIF-1 can also repress *PPARα* gene expression (Narravula and Colgan, 2001). However, *PPARα* may only play a minor role in white adipose tissue or preadipocytes, as suggested by its low abundance (Braissant et al., 1996) and by gene knockout studies (Lee et al., 1995). The transcriptional activity of PPARγ2 protein is inhibited when phosphorylated by the mitogen-activated protein kinase (MAPK) (Hu et al., 1996). Expression of *PPARγ2* is repressed by the zinc finger family transcription factors, GATA-2 and GATA-3, that are expressed in preadipocytes but dissipate at the onset of adipogenic stimulation (Tong et al., 2000). Constitutive expression of *GATA-2* or *-3* prevents adipogenesis by inhibiting *PPARγ2* expression. Nevertheless, expression of *PPARγ2* fails to completely rescue the adipogenic inhibition by *GATA-2* or *-3*. Wnt-mediated signaling also inhibits the expression of *PPARγ2* and *C/EBPα* and thereby represses the adipogenesis (Ross et al., 2000). Coexpression of *PPARγ2* or *C/EBPα* with *Wnt*, again, does not fully restore adipogenesis. Similar to these observations, expression of *PPARγ2* can only partially restore the differentiation of L1 preadipocytes under hypoxia. These findings suggest that other *PPARγ2*-independent mechanisms also play important roles during adipocyte differentiation.

Retinoic acid (RA) inhibits the expression of *PPARγ* and *C/EBPα* without affecting *C/EBPβ* expression (Schwarz et al., 1997). Overexpression of *C/EBPα* or *C/EBPβ* does not overcome RA-mediated inhibition. It is interesting to note that *DEC1/Stra13* gene expression can also be induced by RA (Boudjelal et al., 1997) as well as by hypoxia, suggesting that DEC1/Stra13 mediates repression of *PPARγ2* but not *C/EBPβ*. Thus, the similarity between RA- and hypoxia-mediated adipogenic inhibition supports a role for DEC1/Stra13 as a common effector of both RA- and hypoxia-induced inhibition of adipocyte differentiation.

DEC1/Stra13 is well implicated in cell differentiation. *DEC1* was identified in differentiating human embryonic chondrocytes stimulated by a cyclic AMP analog (Shen et al., 1997). Its mouse homologue, *Stra13* (97% identical at protein level), was identified during the RA-induced neuronal differentiation of P19 embryonal carcinoma cells (Boudjelal et al., 1997). In L1 cells, rapid but transient induction of *Stra13* mRNA was found upon IDM stimulation (Inuzuka et al., 1999), which, we speculate, may be important in temporally regulating transcription for adipogenesis. The regulation of the *PPARγ2* promoter is still not understood and seems quite complex. Our study suggests that DEC1/Stra13 represses the activation of PPARγ2 promoter potentially via the putative C/EBP sites at −229 and −200. However, unlike other

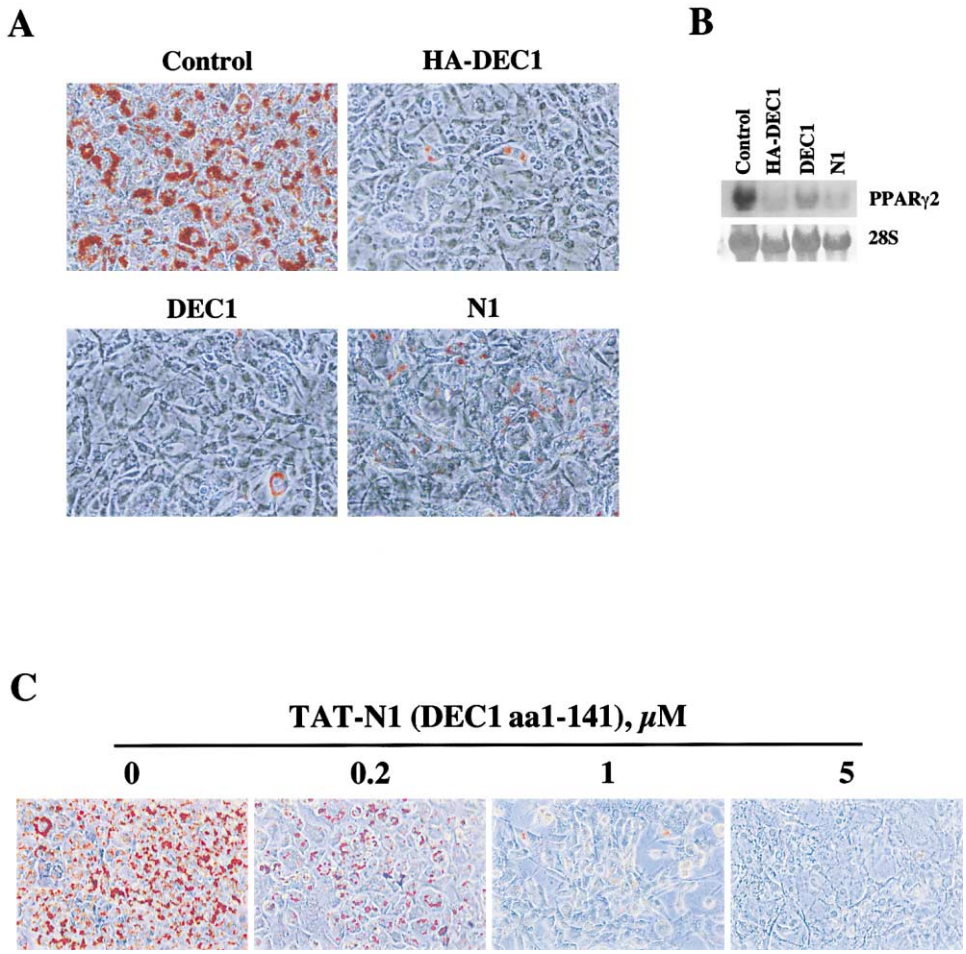

Figure 7. Ectopic Expression of *DEC1/Stra13* Inhibits Differentiation of 3T3-L1 Preadipocytes

(A) L1 cells were retrovirally infected with HA-DEC1, DEC1, N1 or vector control (pLXSN) and then induced to differentiate as described. Oil Red O-stained cells were photographed (20×) on day 5.

(B) L1 cells were retrovirally infected as in (A). Total RNA was isolated on day 2 after IDM stimulation and was analyzed by Northern blotting as in Figure 3A.

(C) L1 cells were induced to differentiate with or without the TAT-N1 (DEC1 aa 1–141) protein at indicated final concentrations. Cells were stained on day 6 and photographed (20×).

members of the HES family, DEC1/Stra13 does not bind to the E box (CANNTG), the N box (CACNAG), or the related C box (CACGCG) (data not shown). It is unlikely that DEC1/Stra13 binds any C/EBP site directly. Nevertheless, it is possible that DEC1/Stra13 may function through interaction with other transcription factors, such as TBP, TFIIB, and USF (Boudjelal et al., 1997; Dhar and Taneja, 2001). The exact nature of DEC1/Stra13-mediated inhibition of *PPARγ2* transcription warrants further investigation.

DEC1/Stra13 protein remains elevated in L1 cells under hypoxia even after its mRNA level decreases to the basal level. Such exceptional protein stability can potentially explain the incomplete recovery of adipogenesis under hypoxia even when *PPARγ* or *C/EBPβ* is overexpressed. The mechanism for increased protein stability of DEC1/Stra13 warrants additional studies, as it belongs to a small of group of proteins, including HIF-1α and p53, that are regulated at the protein level under hypoxia (Graeber et al., 1994; Jewell et al., 2001). Preliminary examination suggests that each of the changes in stabilization of these proteins under hypoxia occurs by a different mechanism (Alarcon et al., 1999; Semenza, 1999; Z. Yun and A.J. Giaccia, unpublished data).

In addition to the adipogenic hormones, the adipocyte microenvironment, such as the extracellular matrix (Selvarajan et al., 2001), can also have significant influence on adipogenesis. Our study strongly argues for hypoxia, a physiological factor of the tissue microenvironment, as an important regulator of adipogenesis. The importance of HIF-1 on adipocyte differentiation extends its role in regulating energy homeostasis. This mechanism of adipogenic repression may be potentially useful for controlling obesity by the regulation of HIF-1, DEC1/Stra13, or pharmacological manipulation of intracellular O_2-sensing mechanisms.

Experimental Procedures

Reagents

Cobalt Chloride ($CoCl_2$), deferoxamine mesylate (DFO), Oil Red O, insulin (INS), dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (MIX) were purchased from Sigma (St. Louis, MO). Stock solution of rosiglitazone was prepared in dimethylsulfoxide (DMSO) from the Avandia tablets (GlaxoSmithKline Pharmaceuticals).

Plasmids

The following constructs were made by PCR amplification of cDNA fragments using pGEM-DEC1 (T. Kawamoto, Hiroshima University, Japan) as template and in-frame cloning at the EcoRI into pcDNA3.1His or pLXSN: pLXSN-DEC1, pLXSN-DEC1 aa 1–141 (N1), pcDNA3.1-DEC1, pcDNA3.1-DEC1 aa 1–141 (N1), pcDNA3.1-DEC1 aa 1–203 (N2), pcDNA3.1-DEC1 aa 121–412 (C1), and pcDNA3.1-DEC1 aa 201–412 (C2). The TAT-N1 was constructed by PCR amplification of DEC1 aa 1–141 and cloned in frame at the EcoRI into pTAT-HA (S.F. Dowdy, Washington University, St. Louis, MO). The pLXSN/HA-DEC1 was made as follows: full-length DEC1 was PCR amplified with 5′ NheI and 3′ XbaI and was cloned into pAS1 at the NheI in frame to the HA tag. The pAS1-DEC1 was cut with NdeI, filled in by Klenow, and then cut with EcoRI to release the HA-tagged DEC1. Finally, the HA-tagged DEC1 was ligated into the pLXSN prepared by XhoI digest, Klenow fill in, and EcoRI digest. The following PPARγ2 promoter constructs were made from pXP2-PPARγ2 (-603 to $+62$) (G. S. Hotamisligil, Harvard University, Boston, MA) by restriction digest, Klenow fill in, and ligation: BsrGI (-285)-StuI (-116) fragment (BrS) and deletion of BsrGI-StuI fragment (FLΔBrS). All constructs were verified by sequencing.

Cell Culture

NIH-3T3 (ATCC, Rockville, MD), *HIF-1*$\alpha^{-/-}$ mouse embryonic fibroblasts (MEFs) (Ryan et al., 1998), wild-type MEFs, and MEFs with *HIF-1*α alleles flanked by *lox*P sites (Ryan et al., 2000) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. 3T3-L1 preadipocytes (ATCC) were maintained in growth medium (GM) containing 10% bovine calf serum (ATCC) and 1 mM sodium pyruvate in DMEM.

Adipocyte Differentiation and Oil Red O Staining

Based on the previously described procedure (Wu et al., 1996), L1 cells were maintained in GM for 2 days after confluence. The confluent cells were treated (day 0) with differentiation medium (DM) containing 10% FBS, 10 μg/ml INS, 1 μM DEX, and 0.5 mM MIX in DMEM for 2 days. Cells were then maintained in DMEM containing 10% FBS and 1 μg/ml INS, and the medium was replaced every other day.

MEFs were induced to differentiate 2 days after confluence (day 0) with DM supplemented with 5 μM rosiglitazone (Alexander et al., 1998; Lehmann et al., 1995). The medium was replaced on day 3 with DMEM plus 10% FBS, 1 μg/ml INS, and 5 μM rosiglitazone and was then changed every other day. To remove the *HIF-1*α gene, subconfluent culture of MEFs with *HIF-1*α alleles flanked by *lox*P sites were treated with adenovirus containing cre recombinase or control adenovirus at the multiplicity of infection (MOI) of 50 as previously described (Seagroves et al., 2001). The treated MEF cells were grown to confluence for 2 days before they were induced to differentiate as above.

To evaluate the effects of hypoxia on adipogenesis, cells were maintained at 20% O_2 in standard incubator, 2% O_2 in standard incubator, or 0.01% O_2 in an anaerobic chamber immediately following treatment with DM. Alternatively, $CoCl_2$ or DFO was added to DM at indicated concentrations either during the initial stage of stimulation or for the entire course of differentiation.

For visualization of differentiated adipocytes, cells were washed with phosphate-buffered saline (PBS) and stained in 60% of the Oil Red O stock solution (0.5 g Oil Red O in 100 ml of isopropanol) for 30 min at 37°C. Cells were briefly washed in 60% isopropanol and then rinsed in distilled water for microscopic observation and photography.

Retroviral Infection of 3T3-L1 Cells

Retroviruses were produced using the phoenix cell system (Baker et al., 1992; Pear et al., 1993). L1 cells at 30%–50% confluence were retrovirally infected two to three times with 8 μg/ml Polybrene by centrifugation at 1800–2000 rpm for 1 hr followed by overnight incubation at 32 °C. For generation of stable *C/EBP*β-expressing cells, L1 cells were infected with pBABEpuro or pBABEpuro-C/EBPβ (S.R. Farmer, Boston University, Boston, MA) followed by selection with 1 μg/ml of puromycin (Sigma). The stable cells were grown to confluence and induced to differentiate as described above. For transient infection with pBABEpuro, pBABEpuor-PPARγ2 (B.M. Spiegelman, Harvard University, Boston, MA), pLXSN/HA-DEC1, pLXSN-DEC1, and pLXSN-N1, L1 cells were spin infected and induced to differentiate as described above.

Northern and Western Blotting Analysis

Total cellular RNA was isolated with Trizol reagent (Life Technologies). The following plasmids were used for cDNA template preparations by restriction digest: MSV-C/EBPβ and MSV-C/EBPδ (S.L. McKnight, University of Texas Southwestern Medical Center, Dallas, TX), pSVsport-PPARγ2 (B. M. Spiegelman), and pBS-Stra13 (P. Chambon, INSERM, Strasbourg, France). Hybridization was carried out at 65°C for 6–12 hr. The radioactive blot was visualized on Storm 860 PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

For Western blotting analysis, cell lysates were prepared on ice using 25 mM HEPES buffer (pH7.4), containing 1% NP-40, 150 mM NaCl, 2 mM EDTA, and a protease inhibitor cocktail (Complete, Boehringer Mannheim). Equal amounts (25 μg/lane) of total cellular proteins were subjected to Western blotting with polyclonal rabbit anti-Stra13 (P. Chambon) at 1:2000 followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG. Protein bands were visualized using ECF substrates (Amersham) on Storm 860 PhosphoImager.

Preparation of TAT Fusion Protein

The TAT-N1 fusion protein was prepared from bacteria as described (Vocero-Akbani et al., 2001). The TAT-N1 protein was purified under native conditions using Ni-NTA metal affinity chromatography (Qiagen). After imidazole elution, the TAT-N1 protein was desalted and kept at –80 °C in phosphate-buffered saline containing 15% glycerol until use.

PPARγ2 Promoter Assay

The luciferase reporter construct (pXP2) under the control of the -603 to $+62$ *PPARγ2* proximal promoter fragment (PPAR) or other deletional subclones were used as described (Tong et al., 2000). NIH-3T3 cells were transiently cotransfected with 0.3 μg each of pXP2 or PPAR and pcDNA3.1His construct expressing a DEC1 fragment using LipofectAmine Plus reagents (Life Technologies). Luciferase activities in cell lysates were measured in triplicates after 40–48 hr of incubation using Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). The luciferase activity is expressed in relation to protein concentrations that vary little from well to well. Alternatively, a *Renilla* luciferase reporter was cotransfected as a control for transfection efficiency.

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References

Alarcon, R., Koumenis, C., Geyer, R.K., Maki, C.G., and Giaccia, A.J. (1999). Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation. Cancer Res. *59*, 6046–6051.

Alexander, D.L., Ganem, L.G., Fernandez-Salguero, P., Gonzalez, F., and Jefcoate, C.R. (1998). Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. J. Cell Sci. *111*, 3311–3322.

Armellini, F., Zamboni, M., Robbi, R., Todesco, T., Bissoli, L., Mino, A., Angelini, G., Micciolo, R., and Bosello, O. (1997). The effects of high altitude trekking on body composition and resting metabolic rate. Horm. Metab. Res. *29*, 458–461.

Baker, B.W., Boettiger, D., Spooncer, E., and Norton, J.D. (1992).

Efficient retroviral-mediated gene transfer into human B lymphoblastoid cells expressing mouse ecotropic viral receptor. Nucleic Acids Res. *20*, 5234.

Baum, D., and Stern, M.P. (1977). Adipose hypocellularity in cyanotic congenital heart disease. Circulation *55*, 916–920.

Boudjelal, M., Taneja, R., Matsubara, S., Bouillet, P., Dolle, P., and Chambon, P. (1997). Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells. Genes Dev. *11*, 2052–2065.

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat. Endocrinology *137*, 354–366.

Bunn, H.F., and Poyton, R.O. (1996). Oxygen sensing and molecular adaptation to hypoxia. Physiol. Rev. *76*, 839–885.

Caniggia, I., Mostachfi, H., Winter, J., Gassmann, M., Lye, S.J., Kuliszewski, M., and Post, M. (2000). Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFβ3. J. Clin. Invest. *105*, 577–587.

Christy, R.J., Kaestner, K.H., Geiman, D.E., and Lane, M.D. (1991). CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proc. Natl. Acad. Sci. USA *88*, 2593–2597.

Dhar, M., and Taneja, R. (2001). Cross-regulatory interaction between Stra13 and USF results in functional antagonism. Oncogene *20*, 4750–4756.

Fambrough, D., McClure, K., Kazlauskas, A., and Lander, E.S. (1999). Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. Cell *97*, 727–741.

Genbacev, O., Zhou, Y., Ludlow, J.W., and Fisher, S.J. (1997). Regulation of human placental development by oxygen tension. Science *277*, 1669–1672.

Graeber, T.G., Peterson, J.F., Tsai, M., Monica, K., Fornace, A.J., Jr., and Giaccia, A.J. (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol. Cell. Biol. *14*, 6264–6277.

Gustafsson, T., and Kraus, W.E. (2001). Exercise-induced angiogenesis-related growth and transcription factors in skeletal muscle, and their modification in muscle pathology. Front. Biosci. *6*, D75–89.

Gustafsson, T., Puntschart, A., Kaijser, L., Jansson, E., and Sundberg, C.J. (1999). Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am. J. Physiol. *276*, H679–H685.

Hu, E., Kim, J.B., Sarraf, P., and Spiegelman, B.M. (1996). Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. Science *274*, 2100–2103.

Hu, E., Tontonoz, P., and Spiegelman, B.M. (1995). Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. Proc. Natl. Acad. Sci. USA *92*, 9856–9860.

Imagawa, M., Tsuchiya, T., and Nishihara, T. (1999). Identification of inducible genes at the early stage of adipocyte differentiation of 3T3-L1 cells. Biochem. Biophys. Res. Commun. *254*, 299–305.

Inuzuka, H., Nanbu-Wakao, R., Masuho, Y., Muramatsu, M., Tojo, H., and Wakao, H. (1999). Differential regulation of immediate early gene expression in preadipocyte cells through multiple signaling pathways. Biochem. Biophys. Res. Commun. *265*, 664–668.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science *292*, 464–468.

Ivanova, A.V., Ivanov, S.V., Danilkovitch-Miagkova, A., and Lerman, M.I. (2001). Regulation of STRA13 by the von Hippel-Lindau tumor suppressor protein, hypoxia, and the UBC9/ubiquitin proteasome degradation pathway. J. Biol. Chem. *276*, 15306–15315.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., and Semenza, G.L. (1998). Cellular and developmental control of O_2 homeostasis by hypoxia- inducible factor 1α. Genes Dev. *12*, 149–162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., et al. (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science *292*, 468–472.

Jewell, U.R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R.H., and Gassmann, M. (2001). Induction of HIF-1α in response to hypoxia is instantaneous. FASEB J. *15*, 1312–1314.

Kageyama, R., and Ohtsuka, T. (1999). The Notch-Hes pathway in mammalian neural development. Cell Res. *9*, 179–188.

Kallio, P.J., Pongratz, I., Gradin, K., McGuire, J., and Poellinger, L. (1997). Activation of hypoxia-inducible factor 1α: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proc. Natl. Acad. Sci. USA *94*, 5667–5672.

Kozak, K.R., Abbott, B., and Hankinson, O. (1997). ARNT-deficient mice and placental differentiation. Dev Biol *191*, 297–305.

Lee, S.S., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H., and Gonzalez, F.J. (1995). Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Mol. Cell. Biol. *15*, 3012–3022.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M., and Kliewer, S.A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J. Biol. Chem. *270*, 12953–12956.

Lennon, D.P., Edmison, J.M., and Caplan, A.I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. J. Cell. Physiol. *187*, 345–355.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403–407.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271–275.

Mitchell, J.A., and Yochim, J.M. (1968). Measurement of intrauterine oxygen tension in the rat and its regulation by ovarian steroid hormones. Endocrinology *83*, 691–700.

Mortola, J.P., and Naso, L. (1997). Brown adipose tissue and its uncoupling protein in chronically hypoxic rats. Clin Sci (Colch) *93*, 349–354.

Mostafa, S.M., Papoutsakis, E.T., and Miller, W.M. (2000). Oxygen tension has significant effects on human megakaryocyte maturation. Exp. Hematol. *28*, 1498.

Narravula, S., and Colgan, S.P. (2001). Hypoxia-inducible factor 1-mediated inhibition of peroxisome proliferator-activated receptor α expression during hypoxia. J. Immunol. *166*, 7543–7548.

Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V., and Kaelin, W.G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel-Lindau protein. Nat. Cell Biol. *2*, 423–427.

Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA *90*, 8392–8396.

Phillips, M., Enan, E., Liu, P.C., and Matsumura, F. (1995). Inhibition of 3T3-L1 adipose differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Cell Sci. *108*, 395–402.

Probst, M.R., Reisz-Porszasz, S., Agbunag, R.V., Ong, M.S., and Hankinson, O. (1993). Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. Mol. Pharmacol. *44*, 511–518.

Rangwala, S.M., and Lazar, M.A. (2000). Transcriptional control of adipogenesis. Annu. Rev. Nutr. *20*, 535–559.

Rodesch, F., Simon, P., Donner, C., and Jauniaux, E. (1992). Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet. Gynecol. *80*, 283–285.

Rosen, E.D., and Spiegelman, B.M. (2000). Molecular regulation of adipogenesis. Annu. Rev. Cell Dev. Biol. *16*, 145–171.

Ross, S.E., Hemati, N., Longo, K.A., Bennett, C.N., Lucas, P.C., Erickson, R.L., and MacDougald, O.A. (2000). Inhibition of adipogenesis by Wnt signaling. Science *289*, 950–953.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J. *17*, 3005–3015.

Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M., and Johnson, R.S. (2000). Hypoxia-inducible factor-1α is a positive factor in solid tumor growth. Cancer Res. *60*, 4010–4015.

Schwarz, E.J., Reginato, M.J., Shao, D., Krakow, S.L., and Lazar, M.A. (1997). Retinoic acid blocks adipogenesis by inhibiting C/EBPβ-mediated transcription. Mol. Cell. Biol. *17*, 1552–1561.

Schwarze, S.R., and Dowdy, S.F. (2000). In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. Trends Pharmacol. Sci. *21*, 45–48.

Seagroves, T.N., Ryan, H.E., Lu, H., Wouters, B.G., Knapp, M., Thibault, P., Laderoute, K., and Johnson, R.S. (2001). Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol. Cell. Biol. *21*, 3436–3444.

Selvarajan, S., Lund, L.R., Takeuchi, T., Craik, C.S., and Werb, Z. (2001). A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation. Nat. Cell Biol. *3*, 267–275.

Semenza, G.L. (1999). Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu. Rev. Cell Dev. Biol. *15*, 551–578.

Semenza, G.L., and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. *12*, 5447–5454.

Shen, M., Kawamoto, T., Yan, W., Nakamasu, K., Tamagami, M., Koyano, Y., Noshiro, M., and Kato, Y. (1997). Molecular characterization of the novel basic helix-loop-helix protein DEC1 expressed in differentiated human embryo chondrocytes. Biochem. Biophys. Res. Commun. *236*, 294–298.

Staal, F.J., Weerkamp, F., Langerak, A.W., Hendriks, R.W., and Clevers, H.C. (2001). Transcriptional control of T lymphocyte differentiation. Stem Cells *19*, 165–179.

Studer, L., Csete, M., Lee, S.H., Kabbani, N., Walikonis, J., Wold, B., and McKay, R. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J. Neurosci. *20*, 7377–7383.

Tanaka, M., Mizuta, K., Koba, F., Ohira, Y., Kobayashi, T., and Honda, Y. (1997). Effects of exposure to hypobaric-hypoxia on body weight, muscular and hematological characteristics, and work performance in rats. Jpn. J. Physiol. *47*, 51–57.

Tong, Q., Dalgin, G., Xu, H., Ting, C.N., Leiden, J.M., and Hotamisligil, G.S. (2000). Function of GATA transcription factors in preadipocyte-adipocyte transition. Science *290*, 134–138.

Tontonoz, P., Hu, E., Devine, J., Beale, E.G., and Spiegelman, B.M. (1995). PPARγ2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. *15*, 351–357.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. (1994). mPPARγ2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. *8*, 1224–1234.

Van Etten, L.M., Verstappen, F.T., and Westerterp, K.R. (1994). Effect of body build on weight-training-induced adaptations in body composition and muscular strength. Med. Sci. Sports Exerc. *26*, 515–521.

Vocero-Akbani, A., Chellaiah, M.A., Hruska, K.A., and Dowdy, S.F. (2001). Protein transduction: delivery of Tat-GTPase fusion proteins into mammalian cells. Methods Enzymol. *332*, 36–49.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995a). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. USA *92*, 5510–5514.

Wang, N.D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S.V., Wilde, M.D., Taylor, L.R., Wilson, D.R., and Darlington, G.J. (1995b). Impaired energy homeostasis in C/EBPα knockout mice. Science *269*, 1108–1112.

Westerterp, K.R., Kayser, B., Wouters, L., Le Trong, J.L., and Richalet, J.P. (1994a). Energy balance at high altitude of 6,542 m. J. Appl. Physiol. *77*, 862–866.

Westerterp, K.R., Meijer, G.A., Schoffelen, P., and Janssen, E.M. (1994b). Body mass, body composition and sleeping metabolic rate before, during and after endurance training. Eur. J. Appl. Physiol. Occup. Physiol. *69*, 203–208.

Wu, Z., Bucher, N.L., and Farmer, S.R. (1996). Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPβ, C/EBPδ, and glucocorticoids. Mol. Cell. Biol. *16*, 4128–4136.

Wu, Z., Xie, Y., Bucher, N.L., and Farmer, S.R. (1995). Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis. Genes Dev. *9*, 2350–2363.

Wu, Z., Xie, Y., Morrison, R.F., Bucher, N.L., and Farmer, S.R. (1998). PPARγ induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPα during the conversion of 3T3 fibroblasts into adipocytes. J. Clin. Invest. *101*, 22–32.

Yeh, W.C., Cao, Z., Classon, M., and McKnight, S.L. (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. *9*, 168–181.

Yu, A.Y., Shimoda, L.A., Iyer, N.V., Huso, D.L., Sun, X., McWilliams, R., Beaty, T., Sham, J.S., Wiener, C.M., Sylvester, J.T., and Semenza, G.L. (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1α. J. Clin. Invest. *103*, 691–696.