Hypoxia Inhibits Myogenic Differentiation through p53 Protein-dependent Induction of Bhlhe40 Protein*

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Background: Hypoxia inhibits myogenic differentiation, but the underlying mechanisms are not well understood.

Results: We used microarray analysis to identify genes and pathways affected by hypoxia. Gain- and loss-of-function studies indicate that Bhlhe40 inhibits myogenic differentiation and that hypoxia up-regulates Bhlhe40 through an HIF1α-independent, p53-dependent mechanism.

Conclusion: Hypoxia inhibits myogenesis through p53-dependent induction of Bhlhe40.

Significance: Inhibition of Bhlhe40 or p53 may facilitate muscle repair after ischemic injuries.

Satellite cells are muscle-resident stem cells capable of self-renewal and differentiation to repair injured muscles. However, muscle injury often leads to an ischemic hypoxia environment that impedes satellite cell differentiation and reduces the efficiency of muscle regeneration. Here we performed microarray analyses and identified the basic helix-loop-helix family transcription factor Bhlhe40 as a candidate mediator of the myogenic inhibitory effect of hypoxia. Bhlhe40 is strongly induced by hypoxia in satellite cell-derived primary myoblasts. Overexpression of Bhlhe40 inhibits Myog expression and mimics the effect of hypoxia on myogenesis. Inhibition of Bhlhe40, conversely, up-regulates Myog expression and promotes myogenic differentiation. Importantly, Bhlhe40 knockdown rescues myogenic differentiation under hypoxia. Mechanistically, Bhlhe40 binds to the proximal E-boxes of the Myog promoter and reduces the binding affinity and transcriptional activity of MyoD on Myog. Interestingly, hypoxia induces Bhlhe40 expression independent of HIF1α but through a novel p53-dependent signaling pathway. Our study establishes a crucial role of Bhlhe40 in mediating the repressive effect of hypoxia on myogenic differentiation and suggests that inhibition of Bhlhe40 or p53 may facilitate muscle regeneration after ischemic injuries.

Satellite cells reside between the sarcolemma and basement membrane of myofibers (1) and are responsible for postnatal muscle growth and regeneration of damaged muscles (2). Postnatal myogenesis involves activation of quiescent satellite cells, proliferation of the activated satellite cells (called myoblasts), and differentiation and fusion of myoblasts into multinuclear myofibers. This process is orchestrated by several basic helix-loop-helix family myogenic regulatory factors (MRFs),2 including Myf5, Myf6, MyoD, and Myog (2). Each MRF alone can transform some types of nonmyogenic cells into myoblasts (3–6). By contrast, knocking out any of these factors impedes postnatal myogenesis (7–9). Because of the crucial role of MRFs in myogenesis, physiological stresses that inhibit their expression also impede myogenesis (10–12).

Low oxygen tension (hypoxia) is one of the physiological stresses repressing myogenesis. A hypoxic microenvironment could result from ischemic insults, cachexia, or acute exposure to high altitude (10, 13). The physiological oxygen (O2) volume is 2–10% in muscle, but it can drop to 1% after an ischemic insult (14, 15). A hypoxic culture condition (1% O2) has been shown to repress myogenesis through various mechanisms, such as activating the Notch signaling pathway, inhibiting the PI3K pathway, or accelerating MyoD degradation (16–19). Although hypoxia has been reported to trigger multiple signaling pathways to repress myogenesis, ectopic expression of MyoD or Myog only partly rescues myogenesis (10). Therefore, additional factors that modulate the activity of MyoD, Myog, and other MRFs may also have been involved in repressing myogenesis under hypoxia.

Bhlhe40 (also called DEC1, STRA13, SHARP2, and Bhlhe2) belongs to the basic helix-loop-helix family and acts as a transcriptional repressor involved in cell proliferation, apoptosis, adipogenesis, fatty acid oxidation, and circadian rhythms (20–26). Although Bhlhe40 is recognized as a transcriptional repressor in multiple biological processes, its role in myogenesis is controversial. It has been reported that Bhlhe40 is required for postnatal myogenesis through promoting myogenic differentiation (27). However, overexpression of Bhlhe40 has been reported to inhibit myogenic differentiation in other studies (10, 28).

Here we report Bhlhe40 as a hypoxic responsive transcription repressor that mediates the repression of Myog expression through decreasing the transcriptional activity of MyoD, leading to inhibition of myogenic differentiation. Moreover, we
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demonstrate that hypoxia induces Bhlhe40 through the p53 pathway independent of HIF1a. These data together suggest that, other than the classical MRFs, Bhlhe40 also plays a key role in myogenesis, especially under hypoxic conditions.

Experimental Procedures

Animals—Mice were from The Jackson Laboratory (HIF1afl/fl, stock no. 007561; Myf5Cre, stock no. 007893; MyoDCre, stock no. 014140), and PCR genotyping was carried out using protocols described by the supplier. Mice were housed in the animal facility with free access to water and standard rodent chow. All procedures involving the use of animals were performed in accordance with the guidelines presented by the Purdue University Animal Care and Use Committee.

Primary Myoblast Isolation, Culture, and Differentiation—Primary myoblasts were isolated from hind limb skeletal muscles that were minced and digested in type I collagenase and dispase B mixture (Roche Applied Science). The digestion was stopped with F-10 Ham’s medium containing 17% FBS, and the cells were filtered from debris, centrifuged, and cultured in growth medium (F-10 Ham’s medium supplemented with 17% FBS, 4 ng/ml basic fibroblast growth factor, and 1% penicillin-streptomycin) on uncoated dishes for 3 days, and 5 ml of growth medium was added each day. Then the supernatant was collected, centrifuged, and trypsinized with 0.25% trypsin. After washing off the trypsin using growth medium and centrifugation, primary myoblasts were seeded on collagen-coated dishes, and the growth medium was changed every 2 days. Myoblasts were induced to differentiate on Matrigel-coated dishes and cultured in differentiation medium (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin). Unless indicated otherwise, primary myoblasts were cultured in normal humidified tissue culture incubators with 5% CO₂.

For hypoxia treatment (~1% O₂), primary myoblasts were put into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA) that were flushed with a custom gas mixture containing 5% air, 5% CO₂, and 90% N₂, 30 p.s.i./min, for 2.5 min each day. The p53 inhibitor Pifithrin-α was purchased from Sigma and used to treat satellite cell-derived primary myoblasts at 5 μM concentration.

Microarray Analysis of Myoblasts under Hypoxic Conditions—Three replicates of total RNA extracted from myoblasts cultured under hypoxic conditions (1% O₂) or normoxic conditions (21% O₂) for 2 days were shipped to Miltenyi Biotec, and the growth medium was changed every 2 days. Myoblasts were induced to differentiate on Matrigel-coated dishes and cultured in differentiation medium (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin). Unless indicated otherwise, primary myoblasts were cultured in normal humidified tissue culture incubators with 5% CO₂.

Adenovirus Production to Overexpress Bhlhe40—The adenovirus with Bhlhe40 insertion was generated using the AdEasy system (30). Briefly, the Bhlhe40 ORF was cloned using a pair of primers (5'-CACGCCGGCATGAGGATCCCAG and 5'-TTTCGCTGTAAGCTTGGTTTTTCAAGTTT) and inserted into the pAdTrack-CMV plasmid. The formed pAdTrack-CMV-Bhlhe40 (pAdTrack-CMV as the control) plasmid was digested by Pmel, and then we transfected the DH5α– competent cell with pAdEasy-1. The positive recombinant plasmid was detected by PacI digestion. Then 293A cells (60–70% confluent) in 10-cm culture dishes were transfected with 4 μg of PacI-digested recombinant plasmid using Lipofectamine 2000 (Life Technologies) according to the protocol of the manufacturer. After 14 days of incubation, the recombinant adenovirus were released by four freeze-thaw-vortex cycles. Two more rounds of infection were adapted to amplify the recombinant virus, and the titers were determined by the expression of GFP. To analyze the influence of Bhlhe40 on myoblasts, myoblasts were treated with adenovirus for 1 day and cultured in virus-free medium for at least one more day.

Immunostaining and Image Acquisition—Myoblasts or myotubes were fixed with 4% paraformaldehyde and then blocked with blocking buffer (5% goat serum, 2% BSA, 0.2% Triton X-100, and 0.1% sodium azide in PBS) for at least 1 h. Then the samples were incubated with primary antibodies (MF20 was from the Developmental Studies Hybridoma Bank) in blocking buffer overnight. After washing with PBS, the samples were incubated with secondary antibodies and DAPI for 45 min at room temperature. Fluorescent images were captured using a Leica DM 6000B fluorescence microscope. As the adenoviral vector contains the green fluorescence protein gene, cells transduced with adenoviral vectors show green fluorescence. We calculated the number of different stainings only in cells with green fluorescence.

Total RNA Extraction, cDNA Synthesis, and Real-time PCR—Total RNA was extracted from cells using TRIzol reagent according to the protocols of the manufacturer. RNA was treated with RNAse-free DNase I to remove contaminating genomic DNA. The purity and concentration of total RNA were measured by Nanodrop 3000 (Thermo Fisher). Random primers and Moloney murine leukemia virus reverse transcriptase were used to convert RNA into cDNA. Real-time PCR was performed using a Roche Lightcycler 480 PCR system with SYBR Green Master Mix and gene-specific primers (18S, 5’-AACC-GTCTAGACAAACAGCTG and 5’-AGTGGTGCTTGGTG-GCTGAC; MyoD, 5’-GGCTAGACACCCGCTACTA and 5’-CGACTCTGGTGCTGATCCT; Myog, 5’-TGCCGAT-GATGCACCTCC and 5’-TTGGGCATGTTGTCCGTTCTGG; P53, 5’-ACTCAGACTGACTGCCTCT and 5’-TCTGAC-TCTGAGCTATAA; and Bhlhe40, 5’-CCACATGTAC-GAGGCAATTG and 5’-CTCTAGCCCTTGCCAGGA). The Ct value of 18S RNA was used as an internal control, and the 2ΔΔCT method was used to analyze the relative mRNA expression of various genes (31).

Protein Extraction and Western Blot Analysis— Cultured myoblasts were washed with PBS and homogenized with radio-immune precipitation assay buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate,
and 0.1% SDS). Protein concentrations were determined using Pierce BCA protein assay reagent. Proteins were separated by 10% SDS-PAGE, electrotransferred onto a PVDF membrane (Millipore Corp., Billerica, MA) and incubated with specific primary antibodies. MyoD (catalog no. M318), Myog (catalog no. F5D), and GAPDH (catalog no. 6C5) antibodies were from Santa Cruz Biotechnology. Bhlhe40/Sharp2 (catalog no. ab23794) was from Abcam, FLAG (catalog no. 2368) was from Cell Signaling Technology, and p53 (catalog no. bs-0033R) was from Bioss. Immunodetection was performed using ECL Western blotting substrate (Pierce) and detected with a Fluochem R imaging system (ProteinSimple).

**Knockdown of Bhlhe40 Using Lentivirus**—To deplete endogenous Bhlhe40, the shRNA target 5'-GCGAGGTACAGTTTTATA corresponding to the 3’ UTR of Bhlhe40 was chosen according to the description in Ref. 32. The Plko.1-Bhlhe40 shRNA was purchased from Thermo Scientific (catalog no. TRCN0000081853). The protocol to generate lentivirus was modified on the basis of Ref. 33. Briefly, 293T cells (80–90% confluent) in collagen-coated 10-cm culture dishes were co-transfected with 4 μg of pLKO.1-Bhlhe40shRNA or pLKO.1 (as the control), 4 μg of pHR’-CMV-ΔR8.20vpr, and 2 μg of pHR’-CMV-VSV-G using Lipofectamine 2000 (Life Technologies) according to the protocol of the manufacturer. Viruses were harvested 4 times every 12 h from 24 h post-transfection and filtered through a 0.45-μm pore size filter. Then the viruses were concentrated at 22,000 rpm for 2 h in a Sorvall T 647.5 rotor. The viral pellets were suspended in 100 μl of TNE buffer (50 mM Tris–HCl (pH 7.8), 130 mM NaCl, and 1 mM EDTA) and incubated overnight at 4°C. Along with transduction of Bhlhe40shRNA to myoblast, Polybrene (10 μg/ml) and HEPES (10 mM) were added to increase the transduction efficiency. Puromycin (1 μg/ml) was used to select the infected myoblasts 1 day post-infection.

**Bhlhe40 KD Cell Line Selection**—C2C12 cells were electroporated with pLKO.1-Bhlhe40shRNA or pLKO.1 (1 μg/106 cells). Two days after electroporation, puromycin (1 μg/ml) was added to the culture medium. Seven to ten days after puromycin selection, a single cell clone was detached by trypsin (0.25%) and transferred to 12-well plates. When the cell confluency was over 70%, cells were passaged, and some of them were used to do RNA extraction and Bhlhe40 expression detection.

**Chromatin Immunoprecipitation**—The commercial Bhlhe40 antibody was not suitable for chromatin immunoprecipitation, so we generated the Bhlhe40-FLAG plasmid by cloning the Bhlhe40 gene into the pCMV-FLAG-2b plasmid and electroporated Bhlhe40-FLAG into primary myoblasts. As a control, 1 day after electroporation, we added Bhlhe40 shRNA lentivirus to the electroporated primary myoblasts. The treated primary myoblasts were cross-linked with 1% formaldehyde in Ham’s F-10 medium for 10 min at room temperature, followed by the addition of 125 mM glycine for 5 min at room temperature, after which cells were scraped into SDS lysis buffer. The cells were further sonicated and diluted for immunoprecipitation with the indicated antibodies. The immunoprecipitates were eluted and reverse-cross-linked overnight at 65°C. DNA fragments were purified using the Cycle Pure kit (Omega), and the Myog promoter E1/E2 region (5’-GAATCCACATGTAATCCACTGGA and 5’-ACGCCAACTGCTGGGTGCCA) was quantified by qPCR.

**Statistical Analysis**—The data are presented as mean ± S.E. p values were calculated using unpaired two-tailed Student’s t test. p < 0.05 was considered to be statistically significant.

**Results**

**Microarray Analysis to Identify Hypoxia-induced Genes in Primary Myoblasts**—To identify hypoxia-regulated genes in an unbiased manner, we performed a microarray analysis of primary myoblasts cultured under hypoxia (1% O2) or normoxia. This analysis identified 865 genes that were up- or down-regulated at least 2-fold (-fold change ≥ 2 and p ≤ 0.01) by hypoxia in both replicates (Fig. 1A). Of these, 641 genes (515 protein-coding genes and 126 IncRNAs or non-annotated genes) were up-regulated, and 224 genes (173 protein-coding genes and 51 long non-coding RNAs or non-annotated genes) were down-regulated (Fig. 1A). Gene ontology analysis shows that the top two categories of up-regulated genes are related to the cell cycle and metabolic process and that the top two categories of down-regulated genes are related to the protein catabolic process and muscle organ development (data not shown). Selective cohorts of genes that were up- or down-regulated by hypoxia are listed in the heat map in Fig. 1B. Particularly, many genes related to glycolysis and HIF1α signaling were up-regulated, whereas many genes related to oxidative phosphorylation and muscle organ development were down-regulated (Fig. 1B). These results are consistent with the important role of O2 in oxidative phosphorylation and the known function of hypoxia in activating HIF1α signaling and inhibiting myogenesis. Interestingly, we found that hypoxia also activated the p53 signaling pathway (Fig. 1B), presumably to protect myoblasts from oxidative stress-induced cell death. These results demonstrate the validity of our microarray analysis.

**Overexpression of Bhlhe40 Inhibits the Differentiation of Myoblasts**—We next focused on transcriptional factors that are activated by hypoxia to mediate its effect on myogenesis. Among the top 50 hypoxia up-regulated genes, we identified Bhlhe40, a member of the basic helix-loop-helix family transcriptional factors, whose expression was up-regulated 7.7-fold (Fig. 1C). Western blotting confirmed that the protein level of Bhlhe40 was also increased by hypoxia (Fig. 1D). Interestingly, another closely related member of this family, Bhlhe41, was also up-regulated 5.3-fold (data not shown). Because basic helix-loop-helix transcriptional factors such as Myf5, MyoD, and Myog are important regulators of myogenesis, we decided to focus the rest of our study on Bhlhe40, whose expression is induced more robustly than Bhlhe41 by hypoxia (Fig. 1C).

We and others have reported previously that hypoxia inhibits the expression of Myog and myogenic differentiation (10, 16). To investigate whether this effect is mediated by Bhlhe40, we first used adenovirus to overexpress Bhlhe40 in primary myoblasts. To examine whether Bhlhe40 affects myogenic differentiation, myoblasts transduced with GFP-expressing (as a control) or Bhlhe40-expressing adenovirus were induced to differentiate by serum withdrawal. Within 1 day after induced differentiation, the control myoblasts had already displayed an obviously elongated morphology, a hallmark of differentiation.
In contrast, the Bhlhe40-overexpressing (BhlheOE) myoblasts were mostly still spherical (Fig. 2B, bottom panels), indicative of a differentiation defect. On day 3 after induced differentiation, more than 70% of the control myoblasts formed uniformly aligned multinucleated myotubes, whereas the BhlheOE myoblasts formed only few poorly aligned myotubes (Fig. 2B). We further quantified the differentiation index, which measures the fraction of myonuclei that are located in myosin heavy chain (MF20/H11001)-expressing cells. Although more than 40% of nuclei in the control group were located in MF20/H11001 cells, only 15% of nuclei in the BhlheOE group were MF20+ (Fig. 2C). At the molecular level, Bhlhe40 mRNA and its protein expression were both elevated in BhlheOE myoblasts compared with control myoblasts (Fig. 2, D and E). Importantly, BhlheOE led to a 30% reduction in Myog mRNA (Fig. 2D) and nearly abolished the expression of Myog at the protein level (Fig. 2E), consistent with the observed defects in myogenic differentiation. Interestingly, the mRNA and protein expression of MyoD was not affected by BhlheOE (Fig. 2, D and E). These results indicate that Bhlhe40 inhibits the MyoD-driven terminal differentiation process without altering the expression of MyoD.

Knockdown of Bhlhe40 Up-regulates Myog and Leads to Precocious Differentiation—We next performed a Bhlhe40 loss-of-function study using lentiviral shRNA-mediated knockdown. Primary myoblasts were treated with lentivirus for 2 days and selected in puromycin (1 μg/ml) for 2 additional days. The expression of Bhlhe40 in the shRNA knockdown (BhlheKD) group was only 40% of that in the GFP shRNA control group (Fig. 3A). Western blot analysis confirmed that the increased Myog expression was associated with a reduction in Bhlhe40 protein (Fig. 3B). We further selected two clones of single cell-derived C2C12 myoblasts (KD1 and KD2) after transfection with the pLKO.1-Bhlhe40 shRNA plasmid. The mRNA level of Bhlhe40 in the two clones of KD myoblasts was only 20–40% of that in control myoblasts (Fig. 3C). Consistent with the results obtained with a mixture of satellite cell-derived primary myoblasts, Myog expression was increased five to seven times in the Bhlhe40 KD cell clones (Fig. 3D). Myog protein levels were also increased robustly in the two clones of KD myoblasts compared with the control myoblasts (Fig. 3E). The abundance of Myog+ cells under proliferation conditions was 5–21 times higher in the two KD clones than in the control myoblasts (Fig. 3F). MF20+ cells emerged in the two KD clones but were absent in the control myoblasts (Fig. 3F). We further induced the BhlheKD primary myoblasts to differentiate after treatment with lentivirus for 2 days and selection in puromycin (1 μg/ml) for 2 additional days. 24 h (day 1) after induced differentiation, Bhlhe40 KD myoblasts and control myoblasts showed a comparable morphology and differentiation index (Fig. 3, H, top panels, and I). 72 h (day 3) after induced differentiation, although 93% of nuclei in the control group were located in MF20+ cells, only 65% of nuclei in the BhlheKD group were in MF20+ cells (Fig. 3, H, top panels, and I). The fusion index was
also lower in Bhlhe40KD than in control myoblasts (Fig. 3J). Interestingly, the protein levels of Bhlhe40 increased gradually during normal differentiation (Fig. 3K). These data suggest that Bhlhe40 is dynamically regulated during myogenesis and that blockage of its expression leads to precocious myogenic differentiation, resulting in reduced proliferation and overall worsened myogenesis.

Bhlhe40 Regulates Myog Expression through Modulating MyoD Transcriptional Activity—Bhlhe40 belongs to the E-protein family that binds to the E-box (CANNTG) DNA sequence located in the promoter region of target genes to regulate their expression. There are two E-boxes in the proximal promoter of Myog (Fig. 4A). We used ChIP to determine whether Bhlhe40 can bind directly to these E-box DNA sequences. Because of the lack of commercially available ChIP-grade antibodies, we generated the Bhlhe40-FLAG plasmid to overexpress FLAG-tagged Bhlhe40 in primary myoblasts (Fig. 4B). In addition, we used Bhlhe40 shRNA lentivirus, which efficiently blocked Bhlhe40-FLAG expression, as a negative control (Fig. 4B). As expected, the ChIP assay using FLAG antibody showed an 8-fold enrichment of E-box DNA sequences compared with the control group using IgG antibody, and this enrichment was abolished by shRNA knockdown of Bhlhe40 (Fig. 4C). These results demonstrate that Bhlhe40 binds directly to the Myog promoter but do not explain why Bhlhe40 inhibits Myog expression.

We hypothesized that Bhlhe40 disrupts the binding of MyoD to the E-boxes of Myog promoter. Indeed, ChIP assays using MyoD antibody showed that MyoD occupancy at the E-boxes of the proximal promoter of Myog was enhanced significantly...
by Bhlhe40 KD (Fig. 4D). In contrast, MyoD occupancy at the E-boxes of the proximal promoter of Myog was reduced significantly by Bhlhe40 KD (Fig. 4E). We further predicted that the occupancy at the Myog promoter by Bhlhe40 would reduce the transcriptional activity of MyoD, leading to inhibition of Myog expression. To investigate whether Bhlhe40 regulates Myog expression through MyoD, we treated MyoD knockout (MyoD<sup>−/−</sup>) myoblasts with control or Bhlhe40KD lentivirus. Strikingly, Bhlhe40 KD failed to up-regulate Myog expression in the absence of MyoD (MyoD<sup>−/−</sup> myoblasts) (Fig. 4F). These results support our hypothesis that Bhlhe40 inhibits Myog expression through reducing the binding of MyoD to the Myog promoter.

Knockdown of Bhlhe40 Alleviates Hypoxic Repression of Myoblast Differentiation—To examine whether the up-regulation of Bhlhe40 under hypoxia mediates the inhibitory effect of hypoxia on myogenic differentiation, we carried out rescue experiments. Primary myoblasts were infected with control or Bhlhe40KD lentivirus and induced to differentiate under hypoxic conditions. Bhlhe40KD robustly improved the differentiation and fusion index of treated myoblasts shown in F, J, fusion index of treated myoblasts shown in H, bottom panels. The fusion index measures the percentage of myonuclei present in multinucleated myofibers. D, day. K, protein levels of Bhlhe40 during differentiation. n = 3 different batches of myoblasts with two replicates using each batch of myoblasts. Error bars represent mean ± S.D. *, p < 0.05.
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FIGURE 4. Bhlhe40 regulates Myog expression through modulating the DNA binding and transcriptional activity of MyoD. A, schematic of the Myog promoter. E-boxes (E1 and E2) are depicted relative to the +1 transcriptional start site. The solid bar represents the region amplified by the indicated primer sets as described under “Experimental Procedures” and used in the PCR. B, relative levels of Bhlhe40 protein in myoblasts transfected with empty vector, Bhlhe40-FLAG plasmid, and Bhlhe40-FLAG plasmid plus Bhlhe40 shRNA lentivirus infection. C—E, -fold enrichment of the Myog E-box promoter region (shown in A) by Bhlhe40-FLAG (C) and MyoD (D and E), as determined by chromatin immunoprecipitation using antibodies against FLAG and MyoD, respectively. Bhlhe40 enhanced the binding activity of MyoD on the E-box region of the Myog promoter (D), and Bhlhe40 KD reduced the binding activity of MyoD on the E-box region of the Myog promoter (E). F, relative expression of Bhlhe40 and Myog in MyoD KO myoblasts infected with control or Bhlhe40 KD lentivirus. n = 3 different batches of primary myoblasts with two replicates using each batch of myoblasts. Error bars represent mean ± S.D. *p < 0.05.

Discussion

In this study, we identified Bhlhe40 as a hypoxia-regulated factor that inhibits myogenic differentiation through modulating the DNA binding and transcriptional activity of MyoD. Previously, Bhlhe40 has been recognized as a factor regulated by hypoxia in tumor cells and adipocytes (25, 35, 36). Here we report that Bhlhe40 is induced by hypoxia in myoblasts. We found Bhlhe40 from the microarray analysis of myoblasts treated by hypoxia. Bhlhe40 has been identified as a transcriptional repressor in multiple biological processes (20–26). Because Bhlhe40 is induced by hypoxia, and hypoxia inhibits myogenic differentiation, we hypothesize that Bhlhe40 may mediate the inhibitory effect of hypoxia on myogenic differentiation. To test our hypothesis, we used gain- and loss-of-function approaches to demonstrate a direct role of Bhlhe40 in regulating the expression of Myog, a master factor controlling...
myogenic differentiation. Importantly, Bhlhe40 knockdown rescued the myogenic differentiation defects under hypoxic conditions.

Bhlhe40 has been reported to repress MRFs and the differentiation of human myoblasts (28). However, contradictory roles of Bhlhe40 in myogenesis have been reported in two other studies (10, 27). We found that Bhlhe40OE inhibited Myog expression and myogenic differentiation. In contrast, Bhlhe KD up-regulated Myog expression and promoted precocious differentiation at the expense of proliferation. The differentiation defects shown in Bhlhe KD myoblasts indicates that a proper level of Bhlhe40 is necessary for not only preventing the precocious differentiation of myoblasts but also for inhibiting apoptosis and inactivating the Notch signaling pathway (27).

Bhlhe40 has been shown to inhibit the transcriptional activity of MyoD through disrupting the interaction between P300/CBP-associated factor (P/CAF) and MyoD (34). Because MyoD is an important transcription activator of Myog, it is speculated that Bhlhe40 regulates Myog through modulating the transcriptional activity of MyoD. To examine this possibility, we verified, by ChIP assay, that Bhlhe40 binds directly to the Myog promoter. This result is consistent with a previous report (34). However, we found that this binding does not alter Myog expression in the absence of MyoD, assuring that Bhlhe40 acts through MyoD to regulate Myog expression. Indeed, MyoD binding to the Myog promoter was enhanced when Bhlhe40 was knocked down and disrupted when Bhlhe40 was overexpressed, suggesting that Bhlhe40 interferes with MyoD on Myog promoter binding. Previous work has indicated that formation of the MyoD-P/CAF complex stabilizes the binding of MyoD to the promoter of Myog (37) and that Bhlhe40 disrupts the formation of the MyoD-P/CAF complex (34). Our results suggest that Bhlhe40 destabilizes the binding of MyoD to the promoter of Myog through disrupting the formation of the MyoD-P/CAF complex. Because Bhlhe40 and MyoD both belong to E-proteins that are known to form heterodimers (34), the Bhlhe40/MyoD heterodimer would prevent the formation of the transcriptionally active MyoD-P/CAF complex. These results together demonstrate that Bhlhe40 regulates Myog expression through modulating the transcriptional activity of MyoD.

It has been reported that ectopic expression of Myog only partly rescues the myogenic differentiation under hypoxic conditions (10). Because Bhlhe40 is up-regulated by hypoxia and negatively regulates Myog expression, we examined whether knockdown of Bhlhe40 rescues the myogenic differentiation defects under hypoxia conditions. We found that Bhlhe40 knockdown only partly rescues myogenic differentiation. This may be due to the relatively low efficiency of knockdown, which is about 40% in our study. Another possibility is that multiple factors are involved in mediating the effect of hypoxia. Hypoxia triggers global changes in gene expression, as indicated by our microarray results. The Notch signaling pathways, PI3K pathways, and stability of MyoD mRNA have all been reported to regulate myogenesis under hypoxia conditions (16–19). Although both MyoD and Myog are key factors in myogenic differentiation, their overexpression could only partly rescue myogenesis under hypoxia (10), again suggesting that multiple factors are involved in mediating the effect of hypoxia. Nevertheless, our study provides strong evidence that Bhlhe40 is the key factor mediating the effect of hypoxia on myogenesis.

Defining the intrinsic signaling pathways regulating the expression of Bhlhe40 in myoblasts is key to understanding how hypoxia regulates myogenesis. It has been widely accepted that hypoxia regulates gene expression through stabilizing the transcriptional factor HIF1α. HIF1α has been reported as an inducer of Bhlhe40 in adipocytes (25, 35, 36). Interestingly, we found that hypoxia induces Bhlhe40 in the absence of HIF1α, therefore excluding HIF1α as a regulator of Bhlhe40. Because our microarray data indicate that hypoxia activates the p53 signaling pathway, we sought to determine whether p53 is involved in regulating Bhlhe40. p53 has been shown to target Bhlhe40 in the breast cancer cell line MCF7 and the colon carcinoma cell line RKO (38). We found that pharmacological
inhibition of p53 blocks the induction of Bhlhe40 by hypoxia, demonstrating that p53 is an inducer of Bhlhe40 in hypoxia-treated myoblasts. Importantly, inhibition of p53 partially rescued the differentiation defects of hypoxia-treated myoblasts, indicating that hypoxia regulates Bhlhe40 at least partially through p53. In a recent study, p53 has been reported to inhibit the transcription of Myog directly by binding to the promoter of Myog under genotoxic stress (39). Our study sheds light on an alternative avenue through which p53 regulates Myog. Our results further raise the possibility of promoting muscle regeneration under ischemic hypoxia conditions through temporal blockage of p53.

**Author Contributions**—C. W. and S. K. conceived the study and wrote the paper. C. W., W. L., and Z. L. performed the experiments. L. C. and X. L. provided technical assistance. All authors reviewed the results and approved the final version of the manuscript.

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