

Differential Hypoxic Regulation of Hypoxia-Inducible Factors 1 α and 2 α

Qun Lin, Xiangyu Cong, and Zhong Yun

Abstract

The hypoxia-inducible transcription factors (HIF) 1 α and HIF-2 α play a critical role in cellular response to hypoxia. Elevated HIF- α expression correlates with poor patient survival in a large number of cancers. Recent evidence suggests that HIF-2 α appears to be preferentially expressed in neuronal tumor cells that exhibit cancer stem cell characteristics. These observations suggest that expression of HIF-1 α and HIF-2 α is differentially regulated in the hypoxic tumor microenvironment. However, the underlying mechanisms remain to be fully investigated. In this study, we investigated the transcriptional regulation of *HIF-1 α* and *HIF-2 α* under different physiologically relevant hypoxic conditions. We found that transcription of *HIF-2 α* was consistently increased by hypoxia, whereas transcription of *HIF-1 α* showed variable levels of repression. Mechanistically, differential regulation of *HIF- α* transcription involved hypoxia-induced changes in acetylation of core histones H3 and H4 associated with the proximal promoters of the *HIF-1 α* or *HIF-2 α* gene. We also found that, although highly stable under acute hypoxia, HIF-1 α and HIF-2 α proteins become destabilized under chronic hypoxia. Our results have thus provided new mechanistic insights into the differential regulation of *HIF-1 α* and *HIF-2 α* by the hypoxic tumor microenvironment. These findings also suggest an important role of HIF-2 α in the regulation of tumor progression under chronic hypoxia. *Mol Cancer Res*; 9(6); 757–65. ©2011 AACR.

Introduction

The hypoxia-inducible transcription factors (HIF) 1 α and HIF-2 α are the key transcription factors regulating the expression of hypoxia-induced genes critical for a wide range of tumor cell functions ranging from survival to clonal selection to metastasis (1–3). Elevated HIF- α expression correlates with poor patient survival in a large number of cancers. Nonetheless, other evidence indicates a correlation of HIF-1 α expression with favorable prognosis in other cases (4, 5). The stability of the HIF- α proteins is post-translationally regulated by prolyl-4-hydroxylase (PHD)-mediated hydroxylation of 2 proline residues located in the oxygen-dependent degradation domain, which leads to degradation of the hydroxylated HIF- α via interaction with the von Hippel-Lindau (VHL) protein (6, 7). In addition to their nonoverlapping transcription activities (8, 9), HIF-1 α and HIF-2 α are expressed in different tissues with HIF-1 α being more widely expressed (10). Recent evidence suggests

that HIF-2 α appears to be preferentially expressed in neuronal tumor cells with cancer stem cell characteristics (11–13). Furthermore, elevated HIF-2 α expression is colocalized *in vivo* with expression of neural crest progenitor markers, suggesting a preferential association of HIF-2 α expression with the immature stem cell–like neuroblastoma (NB) cells (12). These observations suggest that expression of HIF-1 α and HIF-2 α is differentially regulated in the hypoxic tumor microenvironment.

Oxygenation in solid tumors varies from physiologic levels of approximately 5% to 8% O₂ to near anoxia (14, 15). Tumor hypoxia is also highly heterogeneous with both chronic and acute hypoxia (16). In this study, we investigated the transcriptional regulation of HIF-1 α and HIF-2 α under different hypoxic conditions. In addition to conventional hypoxia treatment, we developed an adaptive chronic hypoxia approach by preconditioning tumor cells at 5% O₂ before reducing partial pressure of oxygen (pO₂) to hypoxia levels ($\leq 2\%$ O₂) to mimic *in vivo* tumor hypoxia. We found that transcription of *HIF-2 α* was consistently increased by hypoxia in a panel of NB cell lines, whereas transcription of *HIF-1 α* showed variable levels of repression. Mechanistically, differential regulation of *HIF- α* transcription involved hypoxia-induced changes in acetylation of core histones H3 and H4 associated with the proximal promoters of the *HIF-1 α* or *HIF-2 α* gene. We also found that, although highly stable under acute hypoxia, HIF-1 α and HIF-2 α proteins become destabilized under chronic hypoxia. Our results have thus provided new mechanistic insights into the differential expression and localization of

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HIF-1 α and HIF-2 α proteins within the hypoxic tumor microenvironment. These findings further underscore the importance of HIF-2 α in the regulation of tumor progression, especially in the regulation of the stem cell-like tumor cell population as observed in neuronal tumors.

Materials and Methods

Cell culture under normoxia or hypoxia

SK-N-BE(2)C, SK-N-ER, and SH-SY5Y cells were maintained in minimum essential medium and F12 (1:1), and IMR-32 cells in minimum essential medium. The media were supplemented with 10% FBS, 1 mmol/L sodium pyruvate, 25 mmol/L HEPES at pH 7.4 to maintain pH stability. The medium was replaced every other day.

For experiments at reduced pO₂ (5% O₂) or hypoxia (\leq 2% O₂), cells were incubated in a hypoxia chamber (Invivo2 400, Ruskinn Technology). Anoxia experiments were carried out in a Bactron Anaerobic Chamber (Sheldon MFG Inc.). Deferoxamine mesylate (DFO; Sigma-Aldrich) was used to mimic hypoxia effects at 21% O₂ (17, 18). Culture media were replaced every other day inside the hypoxia chamber. During long-term hypoxic incubation, cells were trypsinized and culture passages were done inside the chamber to prevent reoxygenation.

Western blot

Nuclear extracts were used for Western blot analysis as described previously (17, 19) with antibodies to the following antigens: HIF-1 α (1:2,000) and HIF-2 α (1:1,000), HIF-1 β (1:500), and DEC1 (1:2,000), all of which were purchased from Novus Biologicals.

Firefly luciferase reporter constructs

All constructs were validated by DNA sequencing. The *HIF-1 α* promoter/enhancer region from +122 to -4,871 relative to the transcription start site (TSS; NT_026437.11) was PCR amplified using the following primers: 5'-TATTC TTGCC TTGGC TGTATC C-3' (forward) and 5'-ACTGT GCACT GAGGA GCTGA G-3' (reverse) and then inserted between *Mlu*I and *Nhe*I sites of the pGL3 basic vector. The 2.6-kbp construct was generated by restriction digestion of the 5-kbp construct using *Mlu*I and *Pst*I, followed by ligation of the remaining construct. The 0.6-kbp construct was generated by restriction digestion of the 2.6-kbp construct using *Kpn*I and *Pml*I, followed by ligation of the remaining construct.

The *HIF-2 α* promoter/enhancer region from +116 to -4,883 relative to the TSS (NT_022184.14) was PCR amplified using the following primers: 5'-AGTCC CATT TAACA CTTTG CTACA-3' (forward) and 5'-AGCTG ACCAT ACAGT CTCAG GAC-3' (reverse) and then inserted between *Mlu*I and *Nhe*I sites of the pGL3 basic vector. The 3.3-kbp construct was generated by deletion of the 5' sequence from the 5-kbp construct using *Mlu*I and *Agl*I. The 1.0-kbp construct was generated by deletion of the 5' sequence from the 3.3-kbp construct using *Kpn*I and *Stu*I. The 0.8-kbp construct was generated by deletion of

the 5' sequence from the 3.3-kbp construct using *Kpn*I and *Pvu*I.

Real-time reverse transcription-PCR

First-strand cDNA was synthesized from total RNA. Real-time PCR was carried out on StepOne Plus (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommended protocol. The primer sequences can be found in Materials and Methods (Supplementary Table S1).

Chromatin immunoprecipitation

SK-N-BE2C cells were incubated at 1% O₂ for 24 hours and were used for chromatin immunoprecipitation (ChIP) analysis according to our previously published protocol (19, 20). The ChIP primer sequences can be found in Materials and Methods (Supplementary Table S2).

Statistical analysis

The statistical difference between 2 groups was analyzed by the 2-tailed, unpaired Student's *t* test using Prism 3.0 (GraphPad Software Inc.). Significant difference between 2 groups was declared if *P* < 0.05.

Results

Differential regulation of transcription of *HIF-1 α* and *HIF-2 α* by hypoxia

We investigated the transcription of *HIF-1 α* and *HIF-2 α* under hypoxia using a selected panel of NB cell lines either with *N-myc* amplification [SK-N-BE(2)C and IMR32] or without *N-myc* amplification (SK-N-ER and SH-SY5Y). Hypoxia induced robust accumulation of HIF-1 α protein in all the 4 cell lines (Fig. 1A). In comparison, accumulation of HIF-2 α protein was readily detected in hypoxia-treated BE(2)C, ER, and SY5Y cells but not in IMR32 cells (Fig. 1A). A lack of detectable HIF-2 α protein in hypoxia-treated IMR32 cells was also observed by others (21). This is likely due to the low *HIF-2 α* mRNA expression in IMR32 cells [$>$ 40-fold less than in BE(2)C cells based on our real-time RT-PCR (qRT-PCR) analysis].

Interestingly, transcription of *HIF-1 α* showed a bias toward downregulation by hypoxia, whereas transcription of *HIF-2 α* was increased by hypoxia, in the above 4 representative NB cell lines (Fig. 1B). In addition to NB cell lines, we found similar differential regulation of *HIF-1 α* and *HIF-2 α* in the glioblastoma cell line U373 (Supplementary Fig. S1), suggesting a common regulatory mechanism of hypoxia-regulated *HIF- α* expression in neuronal tumors.

Because tumor hypoxia is heterogeneous and dynamic (16), we further examined the effect of acute (\leq 24 hours) and chronic ($>$ 24 hours) hypoxia on transcription of *HIF-1 α* and *HIF-2 α* . Using the BE(2)C cell line expressing high levels of both HIF-1 α and HIF-2 α proteins as a model, we found that transcription of *HIF-1 α* was consistently repressed by both acute and chronic hypoxia, whereas transcription of *HIF-2 α* was consistently upregulated under

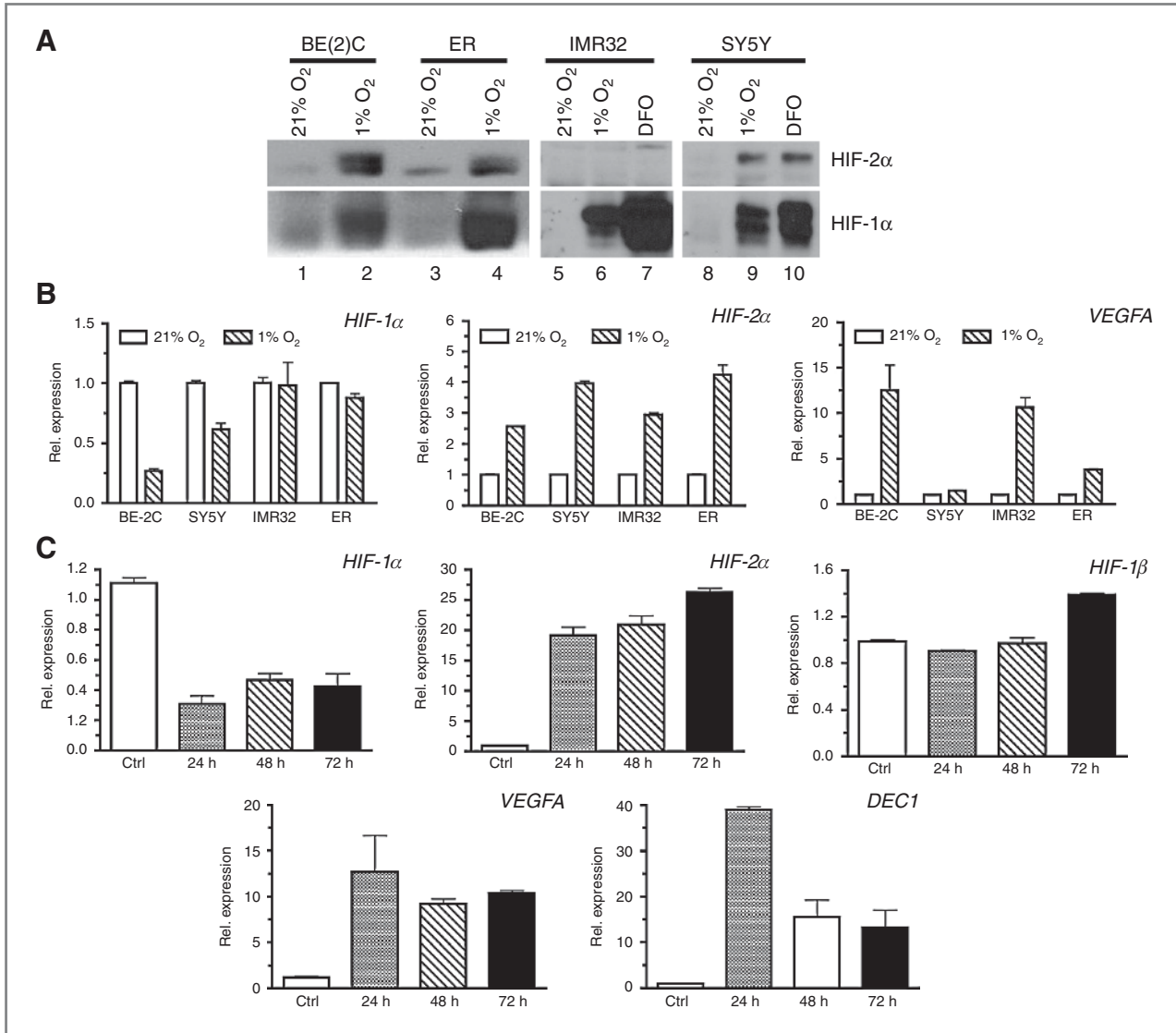


Figure 1. Differential regulation of *HIF-1 α* and *HIF-2 α* transcription by hypoxia. A, hypoxic induction of *HIF-1 α* and *HIF-2 α* protein in NB cell lines: BE(2)C = SK-N-BE(2)C, ER = SK-N-ER, IMR32, SY5Y = SH-SY5Y. Cells were incubated for 20 to 24 hours at 1% O₂ or in the presence of 50 μ mol/L deferoxamine (DFO). *HIF-1 α* and *HIF-2 α* proteins in nuclear extracts were detected by Western blot analysis. B, NB cell cultures were incubated for 20 to 24 hours at 1% O₂. Total RNA was prepared and subjected to qRT-PCR for quantitative analysis of gene expression. Data are shown as mean relative expression \pm SEM ($n = 4$). C, expression of *HIF- α* and related genes in BE(2)C cells incubated at 1% O₂ for 24, 48, or 72 hours. Total RNA was used for analysis of gene expression by qRT-PCR (mean \pm SD). Cells cultured at 21% O₂ was used as the normoxia control.

the same hypoxic condition (Fig. 1C). Transcription of the classical hypoxia-induced genes *VEGFA* and *DEC1/BHLHE40* (*DEC1* is used herein) was increased under both acute and chronic conditions, although more robust induction appeared to occur under acute hypoxia (Figs. 1C and 2). In contrast, transcription of *HIF-1 β* was not significantly affected by either acute or chronic hypoxia (Fig. 1C). These observations indicate that transcription of *HIF-1 α* and *HIF-2 α* is differentially regulated under hypoxia.

The most commonly used approach to *in vitro* hypoxia studies involves transferring cells from the hyperoxic atmosphere (21% O₂) to a hypoxic condition (e.g., $\leq 2\%$ O₂).

However, pO₂ levels in physiologic normal tissues are mostly found to be around 5% to 8% O₂, with intratumoral pO₂ levels often found to be less than 10 mm Hg ($<1.3\%$ O₂; refs. 14, 15). To gain insight into the transcriptional regulation of *HIF-1 α* and *HIF-2 α* by tumor hypoxia *in vivo*, we designed a stepwise adaptive hypoxia model to mimic tumor hypoxia. BE(2)C cells were first allowed to adapt to 5% O₂ (tissue-level normoxia) and then to hypoxia ($\leq 2\%$ O₂) on a chronic (72-hour exposure) basis (Fig. 2A). Decrease of pO₂ from 21% to 5% resulted in subtle accumulation of *HIF-1 α* but did not induce significant stabilization of *HIF-2 α* proteins (Fig. 2B). Only minor changes occurred in the transcription of *HIF-1 α* , *HIF-2 α* ,

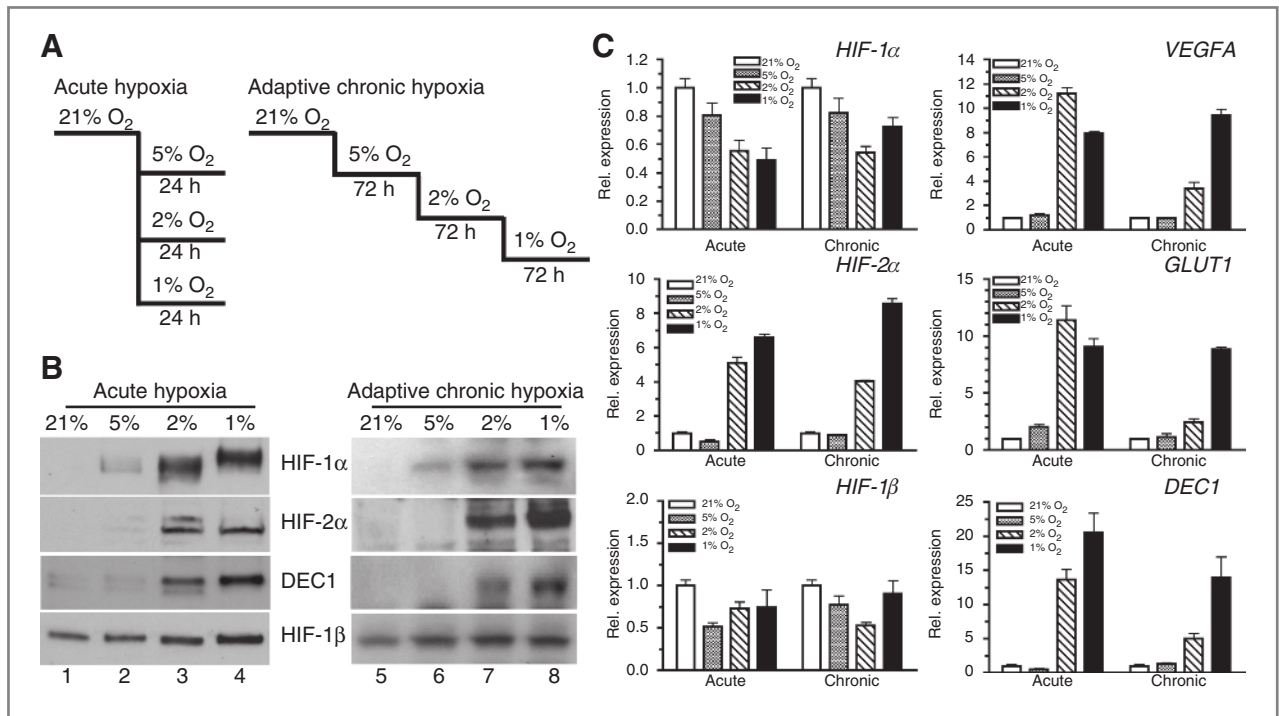


Figure 2. Effect of acute and adaptive chronic hypoxia on the expression of *HIF-α* and related genes. A, schematics of acute hypoxia and adaptive chronic hypoxia *in vitro*. The latter serves as a physiologically relevant model for *in vivo* hypoxia. B, Western blot analysis of HIF- α and related proteins in nuclear extracts of BE(2)C cells treated by acute and adaptive chronic hypoxia. C, quantitative analysis of HIF- α and related genes in BE(2)C cells treated by acute and adaptive chronic hypoxia by qRT-PCR (mean \pm SD).

and the 3 classical hypoxia-induced genes when the environmental pO₂ changed from 21% to 5% (Fig. 2C). In contrast, both acute (24 hours) and adaptive chronic (72 hours) hypoxia of $\leq 2\%$ O₂ reduced the transcription of *HIF-1α* but strongly increased *HIF-2α* transcription (Fig. 2C). Interestingly, *VEGFA*, *GLUT1*, and *DEC1* experienced more robust induction by acute hypoxia ($\leq 2\%$ O₂) than by adaptive chronic hypoxia (Fig. 2C), suggesting a possible involvement of HIF-independent mechanisms of hypoxia-regulated gene expression.

Differential regulation of HIF-1α and HIF-2α protein stabilities by acute and chronic hypoxia

Although it is generally believed that hypoxic induction of HIF-1α and HIF-2α proteins results from increased protein stability or decreased PHD-dependent degradation, it is not clear whether HIF-α protein stabilities are regulated differently under acute or chronic hypoxia. As shown in Figure 3, both HIF-1α and HIF-2α proteins were strongly induced by both acute (24 hours at 1% O₂, lane 4 vs. lane 1) and chronic (72 hours at 1% O₂, lane 9 vs. lane 1) hypoxia. However, the stabilities of both HIF-1α and HIF-2α proteins were much higher under acute hypoxia than under chronic hypoxia. In contrast, HIF-1β protein appears to become even more stable under chronic than under acute hypoxia. These results suggest that stabilities of HIF-1α, HIF-2α, and HIF-1β proteins are differentially regulated under chronic hypoxia.

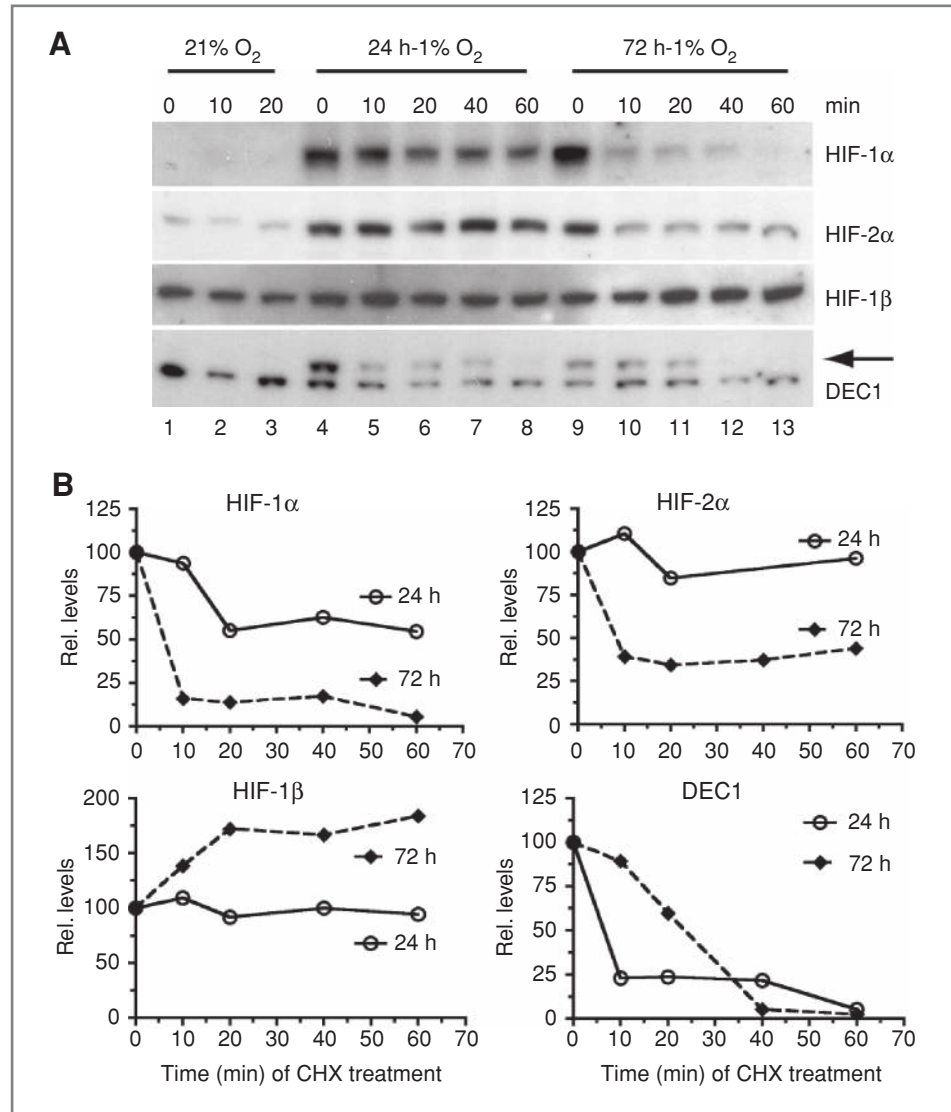
Hypoxic regulation of HIF-1α and HIF-2α mRNA stabilities

We determined the mRNA stabilities of *HIF-1α*, *HIF-2α*, and *HIF-1β* as well as 3 HIF-regulated genes (*VEGFA*, *GLUT1*, and *DEC1*) using the actinomycin D approach. We found that the stabilities of *HIF-2α* and *HIF-1β* mRNA did not change significantly under either acute (24 hours) or chronic (72 hours) hypoxia at 1% O₂, as compared with that at 21% O₂ (Fig. 4). In contrast, *HIF-1α* mRNA appeared to be less stable under acute hypoxia than under chronic hypoxia or at 21% O₂. Among the 3 HIF target genes, *GLUT1* mRNA experienced the most dramatic change in mRNA stability among the 3 experimental conditions with the lowest mRNA stability found under acute hypoxia, whereas stabilities of *VEGFA* and *DEC1* mRNA were similar under both acute and chronic hypoxia (Fig. 4). These observations show that the stability of *HIF-1α* mRNA is more sensitive to regulation by the duration of hypoxia, as compared with that of *HIF-2α* mRNA.

Differential regulation of HIF-1α and HIF-2α promoters by acute and chronic hypoxia

As shown by our results, *HIF-2α* expression is transcriptionally increased by acute and chronic hypoxia, whereas *HIF-1α* mRNA levels were either decreased or little changed under the same conditions. On the basis of sequence comparison, the proximal region of the *HIF-1α* gene

Figure 3. Impact of acute and chronic hypoxia on the stabilities of HIF-1 α and HIF-2 α proteins. A, BE(2)C cells were either maintained at 21% O₂ (normoxia control) or preconditioned at 1% O₂ for 24 and 72 hours, respectively. Nuclear extracts were harvested at the indicated time point after the treatment with cycloheximide (CHX; 25 μ g/mL) to inhibit protein synthesis. Levels of HIF-1 α , HIF-2 α , HIF-1 β , and DEC1 (arrow) proteins were detected by Western blot analysis. B, band intensities were analyzed using NIH Image J and were plotted against time (minutes) after addition of CHX.



(NT_026437.11) is relatively GC rich and lacks a TATA box, whereas the proximal region of the *HIF-2 α* gene (NT_022184.14) contains a putative TATA box (TTTAAA) located around -25 bp from the TSS. To understand how *HIF-1 α* and *HIF-2 α* are differentially regulated by hypoxia at the transcriptional level, we investigated the transcriptional activities of their respective gene promoters under hypoxia. As shown in Figure 5A, we cloned an approximately 5-kbp upstream promoter/enhancer fragment from either the *HIF-1 α* or the *HIF-2 α* genomic sequence. Shorter promoter/enhancer fragments were further generated by restriction digest. As indicated by the luciferase reporter activities (Fig. 5B), transcriptional activities of the 3 *HIF-1 α* promoter/enhancer fragments showed a slight decrease under hypoxia. In contrast, all 4 *HIF-2 α* promoter/enhancer fragments exhibited higher transcriptional activities under hypoxia than under normoxia.

Because all the shorter promoter/enhancer fragments within the 5-kbp region of either gene showed similar transcription activities under hypoxia, specific promoter/enhancer elements were not likely to be significantly involved in the transcriptional regulation by hypoxia. Consistent with this notion, we did not find any plausible conserved transcription sites using the transcription factor-binding site prediction algorithms (22). These observations suggest that hypoxia-mediated transcriptional regulation of *HIF-1 α* and *HIF-2 α* expression likely involves chromatin modifications in their promoter/enhancer regions.

Because changes in transcriptional activities are often accompanied by changes in acetylation of core histones near the TSS (23, 24), we therefore examined the effects of hypoxia on histone acetylation in the *HIF-1 α* and *HIF-2 α* promoter/enhancer regions using ChIP. We found (Fig. 5C) that hypoxia decreased acetylation of histones

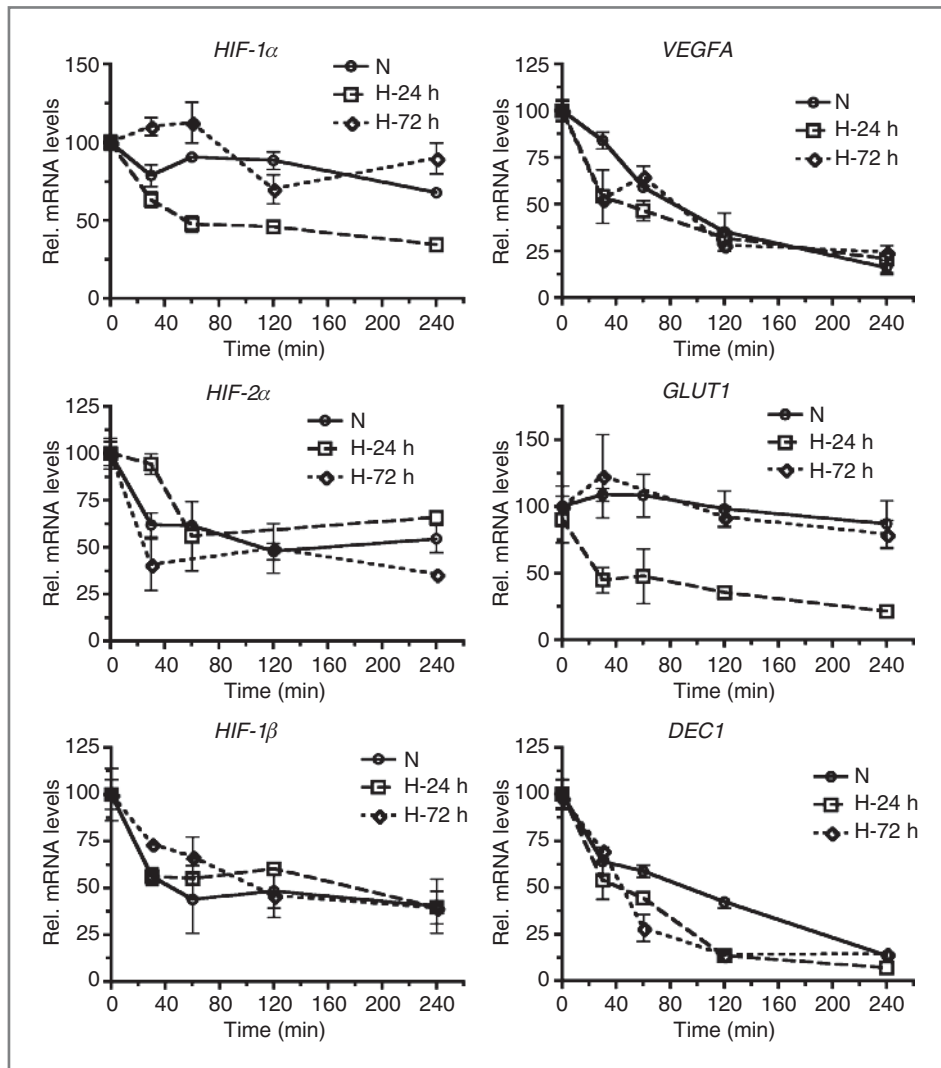


Figure 4. Impact of acute and chronic hypoxia on the stabilities of *HIF-1α* and *HIF-2α* mRNA. BE (2)C cells were either maintained at 21% O₂ (normoxia control; N) or preconditioned at 1% O₂ for 24 and 72 hours, respectively. Cellular RNA was harvested at the indicated time point after the treatment with 5 μg/mL actinomycin D to inhibit RNA synthesis. Levels of *HIF-1α*, *HIF-2α*, *HIF-1β*, and hypoxia-induced genes were determined by qRT-PCR (mean ± SD).

H3 and H4 in the *HIF-1α* promoter/enhancer region with stronger decreases near TSS (*HIF1A-P*: -30 to -176 bp) than around the -8.5-kbp distal region (*HIF1A-D*), which correlates well with the downregulation of *HIF-1α* expression by hypoxia (Figs. 1 and 2). On the other hand, acetylation of histones H3 and H4 in the *HIF-2α* proximal promoter/enhancer region (*HIF2A-P*: +84 to -59 bp) was significantly increased by hypoxia, whereas there was little change in H3 and H4 acetylation in the distal region around -8.0 kbp (*HIF2A-D*). As expected, acetylation of histones H3 and H4 in the promoter/enhancer region of the hypoxia-induced gene *VEGFA* was increased, whereas histone acetylation was decreased in the promoter of *RAD51*, a hypoxia-repressed gene (25). Our results suggest that differential regulation of *HIF-1α* and *HIF-2α* expression is likely due to hypoxia-induced changes in acetylation of histones associated with their respective promoter/enhancers. Specifically, increased acetylation of histones H3 and H4 around the TSS of *HIF-2α* promoter can potentially facilitate the recruit-

ment of transcription coactivators and formation of the RNA polymerase complex for efficient transcription under hypoxia. In contrast, decreased acetylation of histones H3 and H4 is likely to render the promoter of *HIF-1α* less accessible to transcription cofactors and to result in suppressed *HIF-1α* expression under hypoxia.

Discussion

Posttranslational modifications are thought to be the key mechanisms of regulation for both *HIF-1α* and *HIF-2α* proteins in response to changes of environmental pO₂. As shown by our current study and other reports (26, 27), the transcription of *HIF-1α* and *HIF-2α* genes can be differentially regulated by hypoxia. We reasoned that the structural basis for such differential transcriptional regulation would lie in the different DNA sequences of the upstream promoter/enhancer regions between the *HIF-1α* and *HIF-2α* genes. By examining the transcription activities of the approximately 5-kbp promoter/enhancer regions of *HIF-1α* and *HIF-2α*,

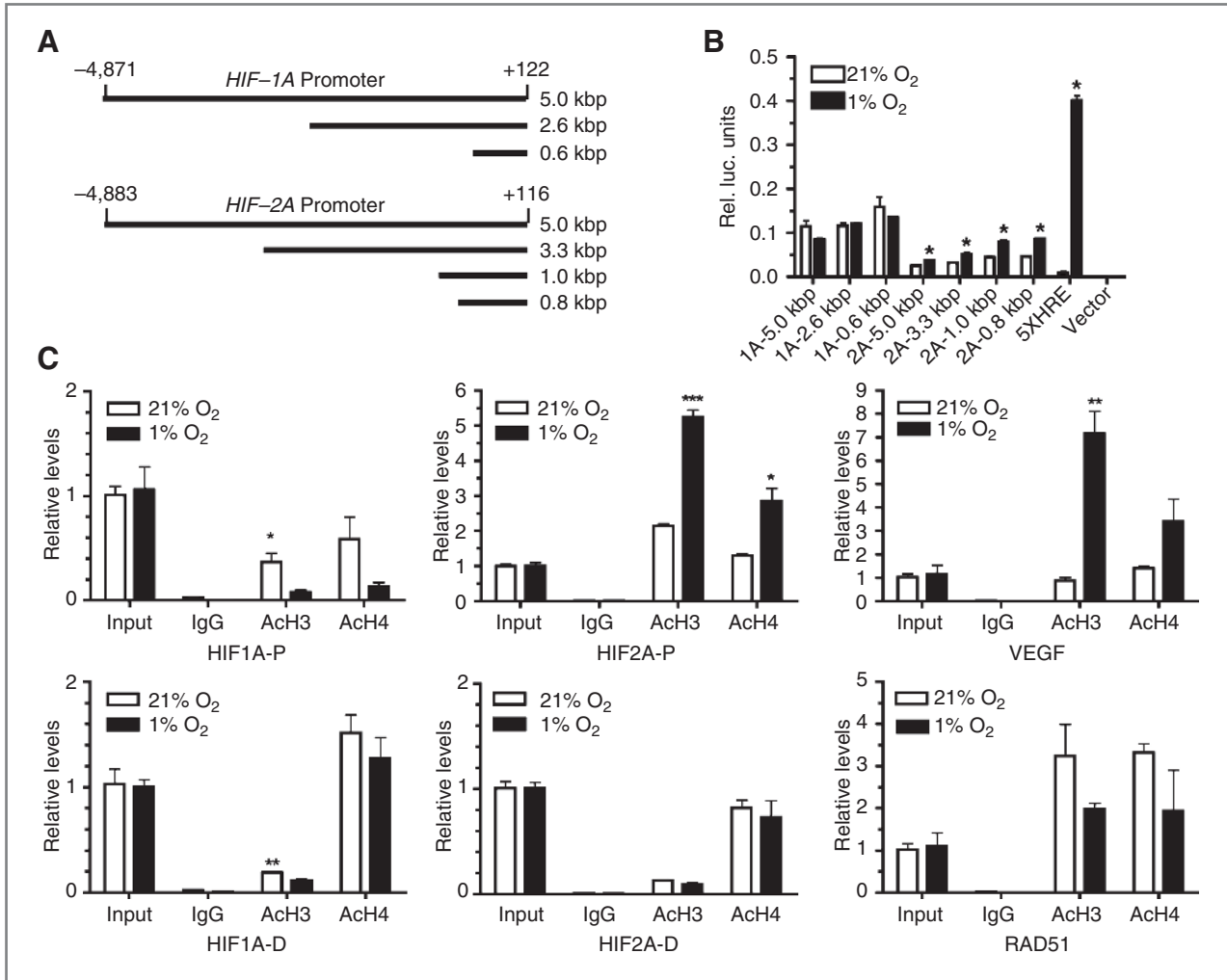


Figure 5. Differential regulation of *HIF-1 α* and *HIF-2 α* promoter/enhancer by hypoxia. **A**, schematics of firefly luciferase reporter constructs driven by different regions of the *HIF-1 α* and *HIF-2 α* promoter/enhancer located upstream to the TSS. **B**, each indicated firefly luciferase construct was cotransfected with a *Renilla* luciferase reporter construct into ER cells. After 24 hours of incubation, transfected cells were either maintained at 21% O₂ or in a hypoxia chamber at 1% O₂ for another 24 hours. Firefly luciferase activity was normalized to that of *Renilla* luciferase. Data shown were average of 3 independent experiments ($P < 0.02$). 1A, *HIF-1 α* ; 2A, *HIF-2 α* ; 5XHRE, a firefly luciferase reporter containing 5 tandem repeats of an HRE; Vector, pGL3 basic. **C**, ChIP was carried out in normoxic (21% O₂) or hypoxic (1% O₂, 24 hours) BE(2)C cells using specific antibodies against acetylated histone H3 (AcH3) or acetylated histone H4 (AcH4) with a naive antibody (IgG) as control. The immunoprecipitated promoter fragments were quantitatively analyzed by qPCR. For the *HIF-1 α* promoter/enhancer, HIF1A-P, proximal region (-176 to -30) and HIF1A-D, distal region (approximately -8.5 kbp); for the *HIF-2 α* promoter/enhancer, HIF2A-P, proximal region (-22 to +76) and HIF2A-D, distal region (approximately -8.0 kbp). Data shown are averages of 3 independent experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

respectively, we have found that specific hypoxia-responsive motifs are unlikely to be responsible for the differential transcription of *HIF-1 α* and *HIF-2 α* genes under hypoxia. In contrast, our data have shown that hypoxia specifically increases acetylation of the core histones H3 and H4 within the proximal (around TSS) but not the distal (approximately -8.0 kbp) promoter region of *HIF-2 α* , coinciding with increased *HIF-2 α* transcription. In contrast, hypoxia decreases histone acetylation of the *HIF-1 α* proximal (near TSS) but not the distal (approximately -8.5 kbp) promoter/enhancer region. These findings indicate that chromatin remodeling is likely a key mechanism for transcriptional regulation of *HIF-1 α* and *HIF-2 α* expression under hypoxia.

However, the exact mechanisms of transcriptional regulation are likely to be quite complex. Consistent with our findings that *HIF-2 α* transcription likely involves chromatin-level regulation, Johnson and colleagues have found that hypoxia induces a wide range of histone modifications associated with both transcriptional activation and repression (28). Our earlier work (20) showed that differential histone modifications were involved in adaptive gene expression under chronic hypoxia. It has been reported that hypoxia can regulate expression and activities of several histone-modifying enzymes. Kim and colleagues have shown that hypoxia can enhance HDAC function to promote angiogenesis (29). Recent studies have shown that members

of Jumonji C domain-containing histone demethylases are also involved in epigenetic regulation of hypoxia-dependent gene transcription (30–33). It is likely that chromatin-level regulation may underline one of the fundamental mechanisms about hypoxia-modulated global gene expression, especially the expression of HIF-independent genes.

It is worth noting that several other mechanisms may also be involved in the transcriptional regulation of *HIF-1 α* and *HIF-2 α* genes. In A549 human lung adenocarcinoma cells, *HIF-2 α* transcription can be increased via an unknown autofeedback mechanism (34). Other evidence suggests possible trans-regulation between *HIF-1 α* and *HIF-2 α* in human renal cell carcinoma cells (35). Because our promoter analysis does not reveal a likelihood of a functional hypoxia-responsive element (HRE) in either *HIF- α* gene, chromatin modifications could potentially play a role in either autofeedback or trans-regulation of *HIF-1 α* and/or *HIF-2 α* genes, albeit the exact molecular mechanisms remain to be delineated.

Another interesting observation of the current study is that stabilities of *HIF-1 α* and *HIF-2 α* proteins are also differentially regulated by acute and chronic hypoxia. As widely reported, acute hypoxia (≤ 24 hours) results in stabilization of *HIF- α* proteins mainly due to inhibition of PHD-mediated proline hydroxylation (36). In this study, we have found that both *HIF-1 α* and *HIF-2 α* proteins become destabilized under chronic hypoxia (72 hours). It is likely that hypoxia-induced expression of PHDs (37) could restore the negative regulation of *HIF- α* protein under chronic hypoxia at 1% O_2 . It is also likely that effective O_2 affinity of PHDs might be increased under chronic hypoxia due to potential changes in other cofactors including Fe^{2+} , 2-oxoglutarate, and/or ascorbate, or due to yet uncharacterized posttranslational modifications of PHDs. Furthermore, other regulators of HIF protein stabilities (reviewed in ref. 38) may also play a role under chronic hypoxia. The increased rates of degradation of *HIF- α*

proteins by chronic hypoxia may constitute a mechanism to fine-tune hypoxic responses.

Taken together, our data suggest the *HIF-1* and *HIF-2* have the potential to play different roles under acute and chronic hypoxia. *HIF-1* is likely to be more involved in response to acute hypoxia via hypoxia-induced stabilization of *HIF-1 α* protein. In contrast, *HIF-2* appears to play a more important role in cellular adaptation to chronic hypoxia via increased *HIF-2 α* transcription, which may offer growth and/or survival advantages under chronic hypoxia. Our results have thus provided new mechanistic insights into the differential expression of *HIF-1 α* and *HIF-2 α* proteins within the hypoxic tumor microenvironment. In light of recent findings that *HIF-2 α* appears to be preferentially expressed in stem cell-like tumor cells *in vivo* (11–13) and has the potential to facilitate cell growth by enhancing c-myc transcriptional activities (39), our findings further underscore the importance of *HIF-2 α* in the regulation of malignant tumor progression, especially in the regulation of stem cell-like tumor cells.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

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