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Regulation of histone demethylase KDM6B by hypoxia-inducible factor-2α

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Abstract

Lysine (K)-specific demethylase 6B (KDM6B) is a histone H3K27 demethylase, which specifically catalyzes the demethylation of H3 lysine-27 tri/dimethylation (H3K27me3/2). KDM6B can activate gene transcription by promoting transcriptional elongation which is associated with RNA polymerase II and related elongation factors. So KDM6B is important for the regulation of gene expression. Previous studies have indicated that several histone demethylases such as KDM3A, KDM4B, and KDM4C are regulated by hypoxia-inducible factor (HIF). But, the effect of hypoxia on KDM6B is not fully understood. In this study, we found that the expression levels of KDM6B mRNA and protein are modestly up-regulated under hypoxia (1% O_2) or mimic hypoxia (desferrioxamine mesylate or $CoCl_2$ treatment) ($P < 0.05$). The result of RNAi shows that the up-regulation of KDM6B is dependent on HIF-2α, but not on HIF-1α. The result of chromatin immunoprecipitation assay indicates that there is a hypoxia response element in *KDM6B* promoter (−4041 to −4037). The result of Co-IP assay indicates that KDM6B can form complex with HIF-2α or HIF-1α. The knockdown experiment implies that KDM6B is a potential regulator for HIF-2α target genes. These data demonstrate that *KDM6B* is a new hypoxia response gene regulated by HIF-2α. Our results also show that KDM6B is a potential co-activator of HIF-α, which is important for the activation of hypoxia response genes.

Key words: histone, H3K27 demethylase, KDM6B, hypoxia, hypoxia-inducible factor-2α

Introduction

Histones are subject to a wide variety of posttranslational modifications including acetylation, methylation, phosphorylation, and ubiquitination, which play fundamental roles in most biological processes that are involved in the organization of chromatin structure and expression of DNA [1,2]. Histone methylation is a reversible process catalyzed by histone methyltransferases and demethylases [3]. Both histone methyltransferases and demethylases are important in developmental control and cell-fate decisions, and their abnormality can lead to many diseases such as cancers [4,5]. Histone modifying enzymes also are strictly regulated by many factors, one of which is hypoxia [6,7].

Hypoxia is a pathological condition in which the body or cell is lack of oxygen supply. In response to hypoxia, cells undergo specific

alterations in gene expression patterns which promote cell survival and maintain homeostasis [8]. Hypoxia-inducible factor (HIF) plays a central role in the transcriptional response to changes in hypoxia condition [9]. HIF is a heterodimer consisting of an oxygen-sensitive alpha subunit (HIF-1α, HIF-2α, or HIF-3α) and a constitutively expressed beta subunit (HIF-1β or HIF-2β) [10]. Under hypoxic condition, HIF-α and HIF-β form transcriptionally active complexes and bind a cognate hypoxia response element (HRE) containing the core sequence ACGTG, which drives the expression of hypoxia response genes [11].

It is becoming increasingly apparent that epigenetics plays a crucial role in the cellular response to hypoxia [12]. Several histone demethylases are induced by hypoxia in an HIF-α-dependent manner [13], including lysine (K)-specific demethylase 3A (KDM3A), KDM4B, and KDM4C [14–18]. These progresses highlight the importance of histone methylation in the signaling pathway of hypoxia. But whether other histone demethylases are also regulated by hypoxia remains to be explored.

In this study, we investigated the expression changes of three H3K27 methylation modifying enzymes, KMT6 (lysine *N*-methyltransferase 6), KDM6A and JMJD3 (also name KDM6B) in liver and kidney cancer cell lines after hypoxia or desferrioxamine mesylate (DFO)/$CoCl_2$ treatment for 24 h. Our results show that hypoxia induces transcription of the KDM6B, which leads to the increase of protein expression. We identify an HRE in the KDM6B promoter and provide evidence that HIF-2α, but not HIF-1α, can bind to the HRE. Our results also demonstrate that KDM6B can form complex with HIF-α and regulate the HIF-2α target genes. This implies that KDM6B may be a potential new component for hypoxia signaling pathway.

Materials and Methods

Cell culture and treatment

The human liver hepatocellular cell HepG2 and embryonic kidney cell HEK293 were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China). Both cells were maintained in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, USA), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C. For hypoxia treatment, cells were incubated in a hypoxia chamber maintained at 1% oxygen for 24 h. Cells were also treated with 100 μM DFO (Sigma, St Louis, USA) or 100 μM $CoCl_2$ for 24 h to mimic hypoxia. After removal of culture medium, cells were rinsed with cold phosphate-buffered saline (PBS; pH 7.4) and then collected for the subsequent experiments.

cDNA synthesis and real-time polymerase chain reaction

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The total RNA concentration was determined with Nanodrop 2000 (Thermo Scientific, Wilmington, USA). RNA (2 μg) was reversely transcribed into first-strand cDNA using a reverse transcription system according to the manufacturer's instructions (Invitrogen). The conditions for reverse transcription are 42°C for 60 min and 70°C for 15 min.

The mRNA expression levels of three H3K27 methylation modifying genes were primarily analyzed by PCR. The primers of human *KMT6*, *KMD6A*, *KDM6B,* and *β-ACTIN* were synthesized by Sangon Biotech (Shanghai, China) as shown in Table 1.

PCR amplification was performed by adding 1 μl aliquot of cDNA sample to 20 μl of reaction mixture (Fermentas, Glen Burnie, USA) containing both the forward and reverse primers. Amplification was carried out in DNA Thermal Cycler (Applied Biosystems, Foster City, USA) under the following conditions: denaturation at 94°C for 10 min, annealing at 58°C for 45 s, and extension at 72°C for 30 s, with final extension at 72°C for 10 min. Each PCR product (5 μl) was subject to electrophoresis on 1.5% agarose gel in Tris acetic acid–EDTA buffer and stained with ethidium bromide.

Quantitative real-time polymerase chain reaction

The mRNA levels of *KMT6* (NM_001203249.1), *KMD6A* (NM_001291415.1), *KMD6B* (NM_001080424.1), *GLUT1* (glucose transporter 1, NM_006516.2), and *EPO* (erythropoietin, NM_000799.2) were then determined by quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was carried out by adding 1 μl aliquot of cDNA sample to 20 μl of qPCR Mix (TaKaRa, Dalian, China) including 1 μl forward and inverse primers (200 nM). Reaction was performed using LightCycler 480 Real-Time PCR System (Roche Diagnostic, Indianapolis, USA) under the following conditions: 95°C for 30 s, 95°C for 5 s, and 68°C for 20 s, 40 cycles. Relative expression levels of *KMT6*, *KMD6A*, *KMD6B*, *GLUT1*, and *EPO* were normalized to the internal reference gene *β-ACTIN* (NM_001101.3) mRNA. The data were analyzed using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

Western blot analysis

Both HepG2 and HEK293 cells were collected and homogenized in the lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, pH 8, and 1% NP-40) containing inhibitors of proteases (1 mM PMSF, 10 mg/ml leupeptin and 10 mg/ml pepstatin A) using supersonic method. Proteins were quantified using the BCA assay kit (Thermo Fisher Scientific, Waltham, USA). A total of 40 μg of protein was mixed with reducing loading buffer, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% non-fat milk in TBST buffer (50 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween-20) for 2 h, incubated with primary antibodies overnight, and then incubated with 1 : 5000-diluted peroxidase-conjugated goat anti-rabbit IgG (secondary antibody; Sigma) for 1 h. After being washed with TBST for four times (5 min/time), the PVDF membrane was detected with enhanced chemiluminescence CL solution (Pierce, Rockford, USA) and then exposed to the X-ray film. The primary antibodies used in this study include anti-KDM6B (ab85392; 1 : 3,000; Abcam, Hong Kong, China), anti-H3K27me3 (A-4039-025; 1 : 2,000; Epigentek, Brooklyn, USA); anti-HIF-1α (sc-10790; 1 : 200; Santa Cruz, Shanghai, China), anti-HIF-2α (sc-28706; 1 : 200; Santa Cruz), and anti-β-ACTIN (ab133626; 1 : 5,000; Abcam).

RNA interference experiments

HEK293 cells were transfected with siRNA (20 nM) or shRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Six hours later, the cells were cultured in normal medium for 18 h, and then treated with DFO to mimic hypoxia for 24 h. These cells were washed and lysed. RNA and protein were extracted. PCR and western blot analysis were carried out to examine the mRNA and protein expression of specific genes, respectively. All the siRNAs and shRNA were synthesized by Genepharma (Shanghai, China) and the sequences were as follows: negative control:

Table 1. Primers for PCR analysis

Gene	Forward primer	Reverse primer	Size (bp)
β-ACTIN	CCACTGGCATCGTGATGGACTCC	GCCGTGGTGGTGAAGCTGTAGC	169
KMT6	AGGAGTTTGCTGCTGCTCTCACC	CCCGTTTCAGTCCCTGCTTCCC	172
KDM6A	AGCGGCGAGAGCGAGGAG	AAGAGGCGGCTGTCCAGTCC	80
KMM6B	ACCGCCTGCGTGCCTTAC	GTGTTGCTGCTGCTGCTACTG	86
HIF-1α	GTTTACTAAAGGACAAGTCACC	TTCTGTTTGTTGAAGGGAG	193
HIF-2α	CGGAGGTGTTCTATGAGCTGG	AGCTTGTGTGTTCGCAGGAA	115
GLUT1	CGGGCCAAGAGTGTGCTAAA	TGACGATACCGGAGCCAATG	283
EPO	CTCCGAACAATCACTGCT	GGTCATCTGTCCCCTGTCCT	116

Table 2. *KDM6B* primers for CHIP analysis

Location (promoter)	Forward primer	Reverse primer	Size (bp)
−4266 to −4072	ATAGCGACAGGAAAGGGAGAG	CAGGCGGCTTTACACAGAC	195
−4099 to −3987	CCTCGTGGAGTCTGTGTAAAG	CAGGCTGGGCATTATGGTC	113
−3088 to −2979	GGGTCAAAGGTCTGTGTTAGAG	GAGGTGGGAGGGAATAGAAGG	110

5′-UUCUCCGAACGUGUCACGU-3′; HIF-1α: 5′-CUGAUGACCA GCAACUUGA-3′; HIF-2α: 5′-CAGCAUCUUUGAUAGCAGU-3′; and KDM6B shRNA: 5′-GAGACCTCGTGTGGATTAA-3′.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using EZ-ChIP kit (Upstate, Millipore, USA) according to the manufacturer′s protocol. In brief, HepG2 and HEK 293 cells were incubated with 100 μM CoCl2 for 24 h to increase HIF-α level. DNA-binding proteins were cross-linked to DNA using formaldehyde at a final concentration of 1% (w/v) for 10 min at room temperature, followed by treatment with glycine (125 mM) for a further 5 min. Cells were washed with PBS, lysed in SDS lysis buffer containing protease inhibitor cocktail, and sonicated with 4–5 sets of 10-s pulses on ice. The supernatant was collected by centrifugation (15,000 *g* for 10 min at 4°C) and pre-cleared with protein G agarose for 1 h at 4°C with rotation. Chromatin was then incubated overnight with rabbit polyclonal antibodies against HIF-1α or HIF-2α at 4°C with rotation. And then protein G agarose was added and incubated for a further 1 h. Normal rabbit IgG was used as a negative control. The beads were washed, and protein G agarose–antibody/chromatin complexes were eluted with 1% SDS and 0.1 M $NaHCO_3$ elution buffer. Cross-links of protein/DNA complexes were reversed by overnight incubation at 65°C and followed by protein digestion with proteinase K. DNA was purified using spin columns. For the quantification of HIF-binding sites, ChIP DNA (5 ng) from each of input, rabbit IgG, HIF-1α, or HIF-2α was subject to qPCR using primers designed to amplify putative HRE consensus sequences (Table 2). The fold-enrichment of each HRE in the $CoCl_2$-treated cells was determined using the $2^{-\Delta\Delta CT}$ method. Standard endpoint PCR was further performed and the products were analyzed by 2% agarose gel electrophoresis.

Co-immunoprecipitation assay

HEK293 cells were incubated under normoxia and hypoxia for 24 h, washed with PBS, pelleted by centrifugation and resuspended in lysis buffer supplemented with a protease inhibitor cocktail. Cell lysate was collected by centrifugation, pre-cleared by incubation with protein A-Sepharose Fast Flow (Sigma), pre-equilibrated with lysis buffer on a rotating platform. Centrifuged supernatants were then collected and incubated with the anti-KDM6B primary antibody or IgG overnight. These supernatants were further mixed with protein G-Sepharose Fast Flow beads, which are pre-equilibrated in lysis buffer. Beads collected by centrifugation were washed and resuspended in an equal volume of 5 × SDS loading buffer. Immunoprecipitated proteins were separated by 10% SDS-PAGE. Western blot analysis was performed as described above.

Statistical analysis

All experiments were repeated at least three times. All data were expressed as the mean ± SEM. Statistical significance was determined by Student's *t*-test (two-tailed). *P* value of ≤0.05 was considered statistically different.

Results

Expression level of *KDM6B* mRNA is modestly upregulated under hypoxia

In an attempt to identify novel hypoxia-controlled genes that are involved in histone methylation, PCR analysis of mRNA levels of three H3K27 methylation modifying enzymes were performed in HepG2 and HEK293 cells that were treated with 1% oxygen for 24 h or treated under normal condition. The results indicated that *KDM6B* transcript was modestly increased, while the mRNA levels of *KDM6A* and *KMT6* were not significantly changed (**Fig. 1A**). In order to determine the effect of hypoxia on *KDM6B*, qRT-PCR was performed to analyze gene expression in both cells treated with hypoxia, DFO or $CoCl_2$ for 24 h. The induction of *KDM6B* mRNA expression (ranging from 2.1 to 4.3 folds, $P < 0.05$) by hypoxia was observed in HepG2 (**Fig. 1B**) and HEK293 (**Fig. 1C**). *KMT6* mRNA and *KDM6A* mRNA were not induced by hypoxia (ranging from 0.5 1.5 folds, $P > 0.05$).

Hypoxia increases KDM6B protein level and decreases H3K27me3 content

To investigate whether the induction of *KDM6B* mRNA in hypoxic condition resulted in the increase of protein expression, western blot analysis was performed. HepG2 and HEK293 cells were exposed to

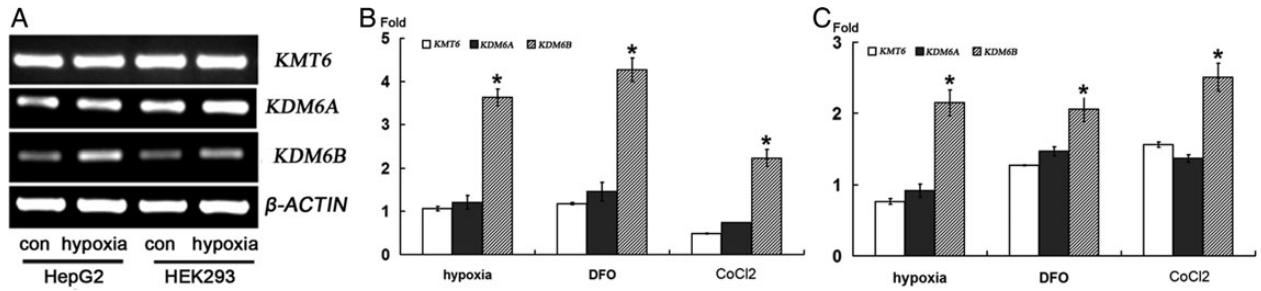

Figure 1. Hypoxia induces the expression of *KDM6B* mRNA (A) HepG2 and HEK293 cells were exposed to 21% O_2 (con) or 1% O_2 (hypoxia) for 24 h. RT-PCR was used for measurement of expression levels of *KMT6, KDM6A,* and *KDM6B.* (B) HepG2 cells were exposed to 1% O_2 (hypoxia), or treated with 100 μM DFO (DFO) or 100 μM $CoCl_2$ ($CoCl_2$) for 24 h. qPCR was used to detect the relative transcript levels of three enzymes and compared with those of control (21% O_2 or untreated). (C) The same experimental procedure as (B) was performed in HEK293 cells. Data are expressed as the mean ± SEM from three independent experiments. The value of each mRNA was normalized to *β-ACTIN*. **P* < 0.05 compared with control.

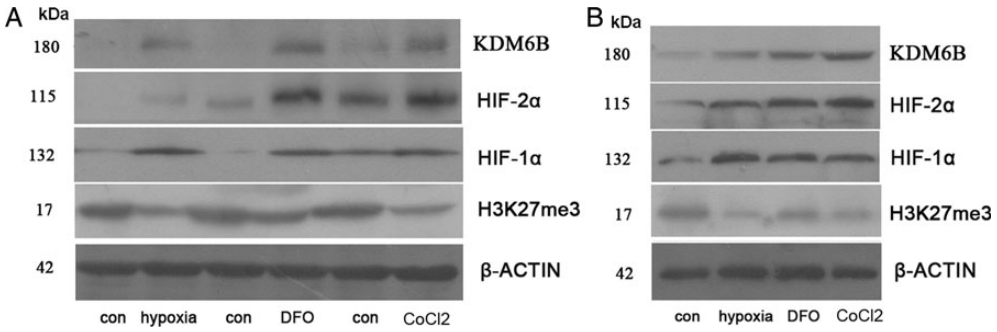

Figure 2. Hypoxia increases the content of KDM6B protein and decreases the level of H3K27me3 (A) HepG2 cells were exposed to 1% O_2 (hypoxia), or treated with 100 μM DFO or 100 μM $CoCl_2$ for 24 h. The anti-KDM6B, anti-HIF-1 α, anti-HIF-2α, and anti-H3K27me3 antibodies were used for immunoblotting. (B) The same experimental procedure as (A) was performed in HEK293 cells.

either 1% O_2 or 100 μM DFO or 100 μM $CoCl_2$ for 24 h, and assayed for the expression levels of HIF-1α, HIF-2α, and KDM6B. As shown in **Fig. 2**, low oxygen tension or DFO/$CoCl_2$ treatment led to a stabilization of HIF-1α and HIF-2α in both cell lines. Hypoxia environment induced the expression of KDM6B protein in both cell lines. On the other hand, hypoxia treatment caused the decrease of H3K27me3. These results further suggest that *KDM6B* is a hypoxia response gene, whose expression is associated with HIF-1α or HIF-2α.

The up-regulation of KDM6B is dependent on HIF-2α

To determine which isoform (HIF-1α or HIF-2α) is involved in KDM6B regulation under hypoxia, RNAi experiment was carried out in HEK293 cells. The result indicated that siRNA-HIF-2α substantially reduced expression of KDM6B, whereas siRNA-HIF-1α did not have essential effect (**Fig. 3**). It means that up-regulation of KDM6B by hypoxia is dependent on HIF-2α.

Binding of HIF-2α subunit to the promoter of *KDM6B*

To verify that the induction of KDM6B by hypoxia was mediated directly or indirectly by HIF-α, ChIP assay was performed. The transcriptional activation at HIF target gene loci is directly mediated by binding of the HIF–α/β complex to HREs containing the core motif ACGTG. To identify such sites are within the loci encoding KDM6B, we searched potential sites for HIF-binding within the regions (−6 kb) of the assigned transcriptional starting site (http://genome.ucsc.edu/). Three potential sites within these regions were identified in the *KDM6B* (−4196 to −4192, −4041 to −4037 and −3040 to −3036), suggesting that the *KDM6B* might be a direct target gene. To test whether these loci would bind to HIF-α subunits, we performed ChIP assays using anti-HIF-1α, anti-HIF-2α antibodies or control IgG, and carried out qRT-PCR and common PCR to analyze the precipitated DNA fragments using primers designed to amplify-specific regions containing predicted HREs. Moderate enrichment was observed at the *KDM6B* locus between −4099 and −3987 using anti-HIF-2α but not using anti-HIF-1α (**Fig. 4**). No obvious enrichment was detected in the other two sites with anti-HIF-1α or anti-HIF-2α antibodies. Taken together, the results strongly suggest that KDM6B is a direct target of HIF-2α.

KDM6B may be involved in HIF-2α target gene expression

To understand the potential role of KDM6B in hypoxia signaling pathway, the interactions between KDM6B and HIF-α (including 1α and 2α) were detected by Co-IP assay. Both HIF-1α and HIF-2α were specifically precipitated by anti-KDM6B antibody, but not by control IgG (**Fig. 5A**). It indicates that KDM6B physically interacts with HIF-1α or HIF-2α in HEK293 cells, which suggests that the KDM6B may be a co-activator of HIF-α.

In order to determine the role of KDM6B in regulating target genes of hypoxia, RNA interfere was performed in HepG2. The result indicates that shRNA for KDM6B reduces the expression of KDM6B at both mRNA and protein levels (**Fig. 5B**). After KDM6B knockdown, the induction of HIF-2α target gene *EPO* is decreased under hypoxia,

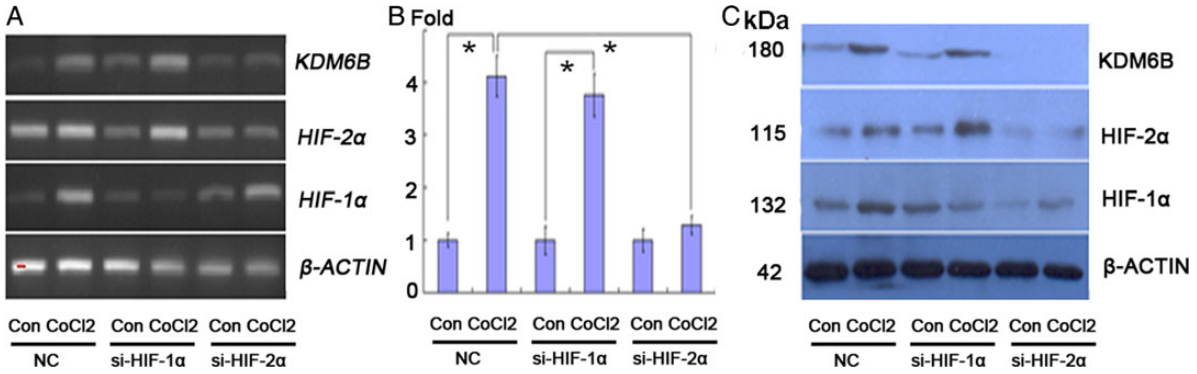

Figure 3. KDM6B is induced by hypoxia in a HIF-2α-dependent manner HEK293 cells were transfected with negative control (NC) or siRNAs specifically targeting HIF-1α (si-HIF-1α) or HIF-2α (si-HIF-2α). After 24 h, these cells were treated with 100 μM $CoCl_2$ for 24 h and then collected for PCR and western blot assays. PCR (A) and qRT-PCR (B) show that hypoxic induction of *KDM6B* mRNA was eliminated by HIF-2α-directed siRNA. Western blot analysis (C) further confirms the result. Data are expressed as the mean ± SEM from three independent experiments. *$P < 0.05$ compared with control (NC).

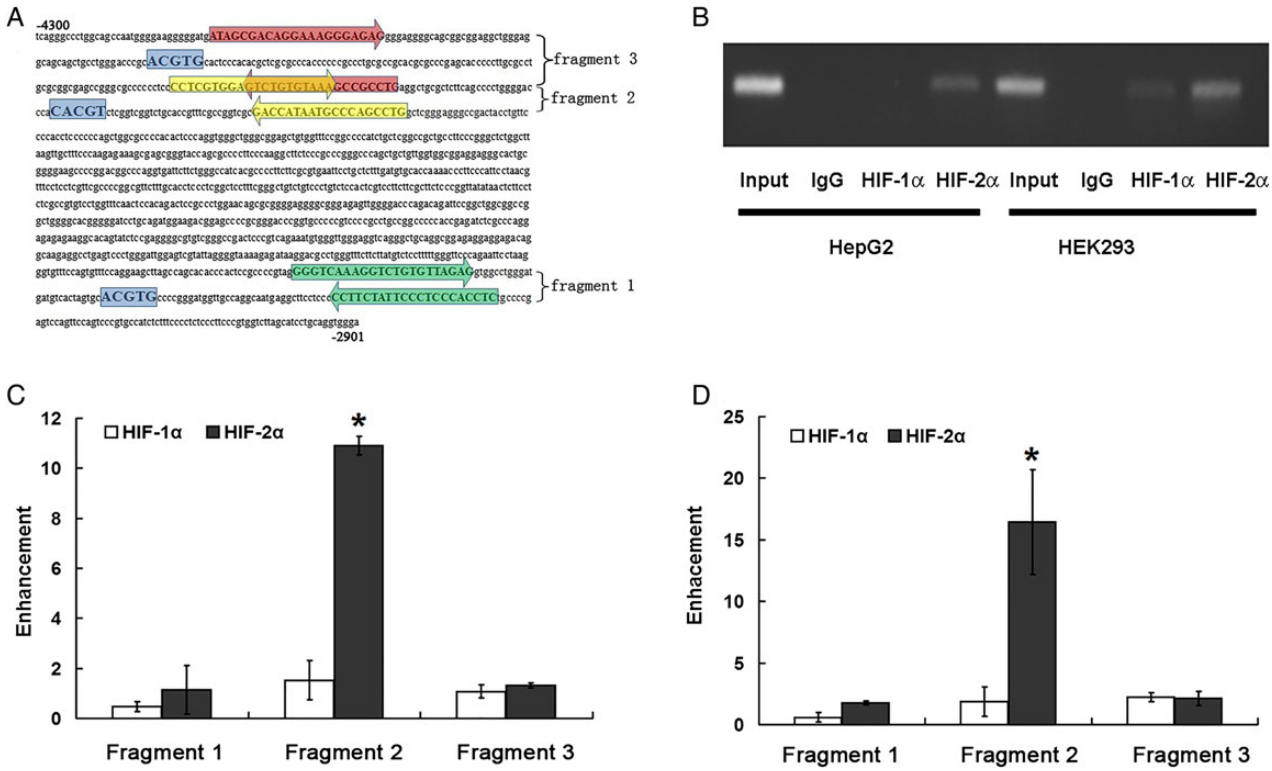

Figure 4. HRE is located in *KDM6B* promoter Both HepG2 and HEK293 cells were treated with 100 μM $CoCl_2$ for 24 h, and then ChIP assay for specific sequences in *KDM6B* promoter was performed. (A) Potential HERs' location in *KDM6B* and corresponding primes for CHIP. (B) Electrophoresis results of PCR product of fragment 2 of *KDM6B* promoter in two cells were shown. qPR-PCR results of *KDM6B* promoter in HepG2 cell (C) or in HEK293 cell (D) were given. Data are expressed as the mean ± SEM from three independent experiments. For each enhancement the value was normalized to IgG. *$P < 0.05$ compared with control.

but HIF-1α target gene *GLUT1* is not affected (**Fig. 5**C). All these results demonstrate that KDM6B may be an important regulator of HIF-2α-mediated signaling pathway.

Discussion

The HIFs are transcriptional activators that function as major regulators of oxygen homeostasis in all metazoan species [19]. The abnormality of HIF can contribute to pathologic conditions, such as tumors and vascular disease. So, for the understanding and treatment of specific diseases, it is important to investigate the hypoxia signaling pathway. The HIFs are the major regulators of hypoxia condition. They regulate the transcription of several hundred genes to adjust cellular metabolism and signaling, and to cope with oxygen limitation. The discovery of histone demethylases as a new class of HIF-responsive genes provides a new mechanism for regulating the response to hypoxia [20]. This study indicates that KDM6B is up-regulated in a range of different cell lines by hypoxia (1% O_2) or mimic hypoxia (DFO/$CoCl_2$ treatment). It means that H3K27me3 demethylase KDM6B is a new hypoxia response factor.

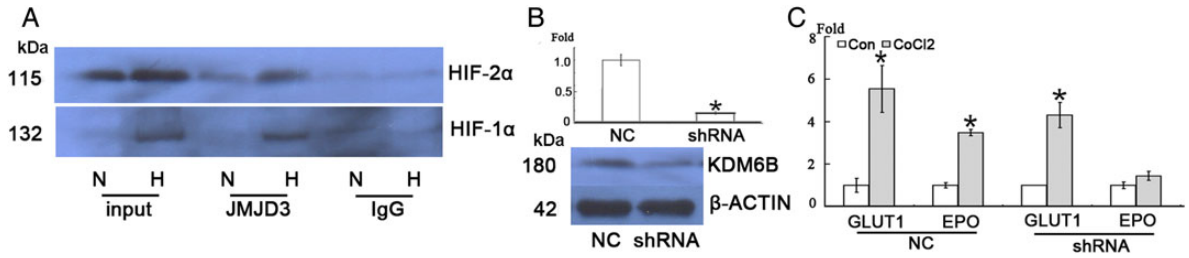

Figure 5. KDM6B is important for HIF-2α-mediated signaling pathway (A) HEK293 cells were treated under normoxia (N) or hypoxia (1% O_2) for 24 h and nuclear lysates were subject to co-IP assays. The result indicates that there is interaction between KDM6B and HIF-1α or HIF-2α. (B) HepG2 cells were transfected with negative control (NC) or shRNA for KDM6B. After 48 h, cells were collected for qPCR (upper) and western blot assay (lower) were done to confirm the effect of KDM6B knockdown. (C) HepG2 cells transfected with negative control (NC) or shRNA for KDM6B were treated with 100 μM CoCl2 for 24 h, and then collected for qPCR to measure the expressions of GLUT1 and EPO. Data are expressed as the mean ± SEM from three independent experiments. *$P < 0.05$ compared with control (Con).

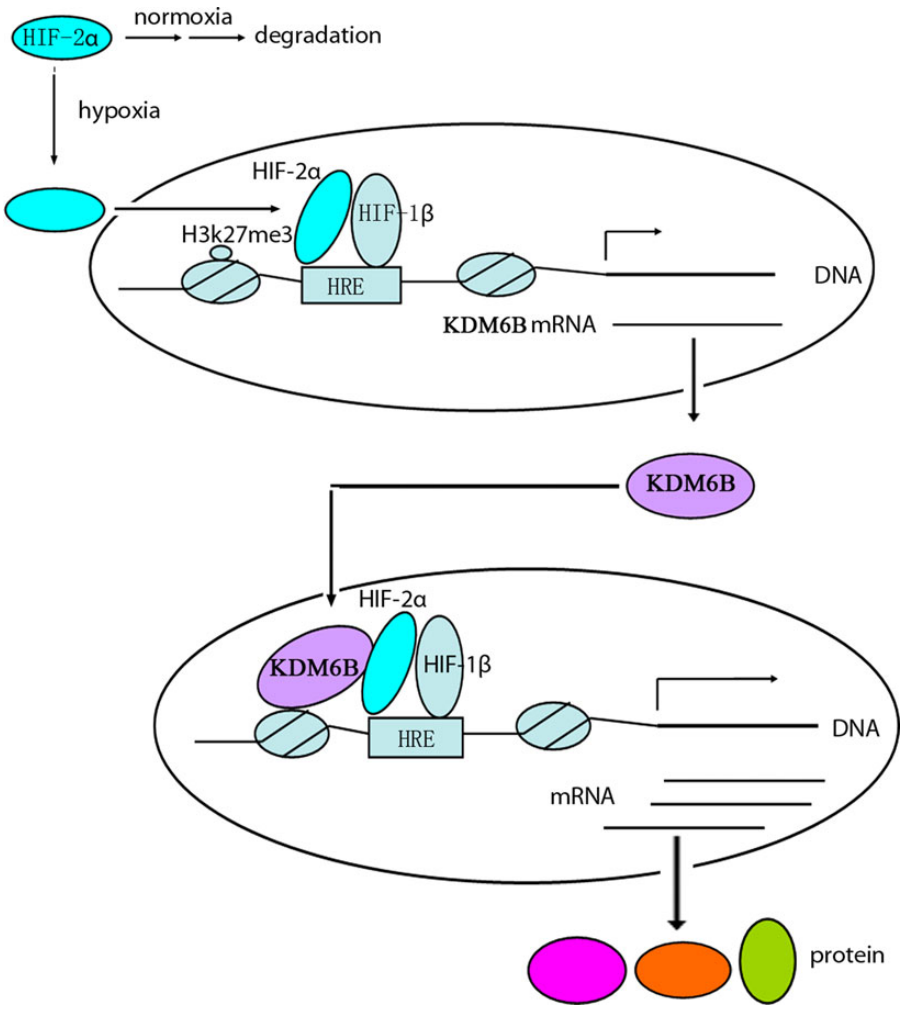

Figure 6. The proposed mechanism by which KDM6B is involved in hypoxia signaling pathway Under hypoxia, HIF-2α is stable and transported into nucleus, where it forms hypoxia activating complex. After binding to HRE, the *KDM6B* is transactivated. Then, KDM6B is also transported into nucleus and forms a complex with HIF-α/HIF-1β, which in turn regulates the expression levels of hypoxia response genes.

It has been demonstrated that hypoxia can influence histone methylation marks, and H3K27me3 is one of them [21]. Dynamic change of histone methylation status is proposed to regulate gene expression. H3K27me3 is a transcriptionally repressive mark and its target genes are expressed at lower levels [22]. KDM6B is an H3K27me3 demethylase and a transcriptional co-activator, which removes H3K27me3 at target genes [23]. A recent paper described that hypoxia increases the expression of KDM6B and reduces the repressive H3K27me3, which is important for the up-regulation of hypoxia response gene endothelial nitric oxide synthase (eNOS) [24].

Previous results also suggested that KDM6B activates gene transcription by promoting transcriptional elongation, which is associated with RNA polymerase II and related elongation factors [25,26]. These results indicated that up-regulation of KDM6B should be essential for gene expression of specific hypoxia response gene under hypoxia. Several hypoxia regulated histone demethylases have been identified as co-activators of HIF, including JMJD1A and JMJD2C [27,28]. All these results demonstrated that KDM6B may be also a co-activator of HIF-α. Accordingly, we proposed a possible mechanism by which KDM6B is involved in hypoxia signaling pathway. Hypoxia increased the stability of HIF-α protein, which activates the expression of hypoxia response genes including KDM6B. Then KDM6B enters nucleus and forms complexes with HIFs to trans-activate-specific target genes through demethylating H3K27me3 at the HREs (**Fig. 6**).

Our previous study has indicated that KDM6B expression is significantly higher in renal cell carcinoma (RCC) [29]. RCC has the most frequent loss of the VHL tumor suppressor, which leads to constitutive activation of HIF-α [30]. Several studies indicated that HIF-2α appears to be more oncogenic than HIF-1α in RCC and other cancers [31,32]. Another study indicated that HIF-2α could induce epithelial–mesenchymal transition (EMT) through transcription of related regulators [33]. EMT is a critical event that occurs in carcinoma invasion and metastasis [34]. This study confirms that KDM6B is regulated by HIF-2α, which further supports the idea that KDM6B plays important roles in carcinogenesis especially in EMT [35]. It is also consistent with the fact that epigenetic reprogramming is important in EMT [36].

While we were preparing this manuscript, another group reported similar results [37]. In that study, it was demonstrated that KDM6B is a HIF, which supports our finding. They discovered that KDM6B up-regulation is HIF-1α dependent, but our results indicated that HIF-2α plays important role in regulating KDM6B expression under hypoxia. The possible reason for the different results from the two groups may be that the cell lines used were different. Lee *et al.* [37] used NIH-3T3 cells and mouse embryonic fibroblasts, while we used HepG2 and HEK293. Despite the interaction between KDM6B and HIF-1α *in vitro*, there is not obvious effect on the expression of HIF-1α target gene *GLUT1* after KDM6B knockdown. The possible explanation is that there is no interaction between KDM6B and HIF-1α in HepG2 cell *in vivo*.

In summary, our studies suggest that histone H3K27 demethylase KDM6B is a new hypoxia response factor and may be one of HIF co-activators which demethylates H3K27me3. But the hypoxia response genes regulated by KDM6B remain to be explored in future study. These findings establish an important association between histone demethylase KDM6B and HIF, which is essential for the understanding of hypoxia signaling pathway and epigenetic modification.

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