Repression of hypoxia-inducible factor $\alpha$ signaling by Set7-mediated methylation

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Received January 20, 2015; Revised March 23, 2015; Accepted April 11, 2015

ABSTRACT

Hypoxia-inducible factor (HIF)-1$\alpha$ and HIF-2$\alpha$ are the main regulators of cellular responses to hypoxia. Post-translational modifications of HIF-1$\alpha$ and 2$\alpha$ are necessary to modulate their functions. The methylation of non-histone proteins by Set7, an SET domain-containing lysine methyltransferase, is a novel regulatory mechanism to control cell protein function in response to various cellular stresses. In this study, we show that Set7 methylates HIF-1$\alpha$ at lysine 32 and HIF-2$\alpha$ at lysine K29; this methylation inhibits the expression of HIF-1$\alpha$/2$\alpha$ targets by impairing the occupancy of HIF-1$\alpha$ on hypoxia response element of HIF target gene promoter. Set7-null fibroblasts and the cells with shRNA-knocked down Set7 exhibit upregulated HIF target genes. Set7 inhibitor blocks HIF-1$\alpha$/2$\alpha$ methylation to enhance HIF target gene expression. Set7-null fibroblasts and the cells with shRNA-knocked down Set7 or inhibition of Set7 by the inhibitor subjected to hypoxia display an increased glucose uptake and intracellular adenosine triphosphate levels. These findings define a novel modification of HIF-1$\alpha$/2$\alpha$ and demonstrate that Set7-mediated lysine methylation negatively regulates HIF-1$\alpha$ transcriptional activity and HIF-1$\alpha$-mediated glucose homeostasis.

INTRODUCTION

Lysine methylation in non-histone proteins acts as a novel regulatory mechanism to control protein functions (1–8). Set7, also known as Setd7, Set9 and Set7/9, was originally identified as a monomethylase of histone H3 lysine 4; thus, Set7 is involved in gene activation (9,10). Set7 monomethylates various non-histone proteins, including p53, TAF10, DNMT1, E2F1, AR, ER$\alpha$, RelA, PCAF, Stat3, Yap, FOXO3a, Survivin and pRb (11–28). This methylation produces varied results. For instance, the methylation of p53, TAF10, AR, ER$\alpha$ and pRb enhances their corresponding activities by improving protein stability or via other mechanisms (11,12,15,16,18–20); by contrast, the methylation of DNMT1, Stat3, Yap, Foxo3a and Survivin hinders their corresponding activities (13,24–28). However, contradictory results have been observed in E2F1 and RelA methylation (14,17,21,22,29). Furthermore, the in vivo function of p53 methylation by Set7 remains debatable (16,30,31). In most cases, the significance of non-histone methylation by Set7 should be investigated. Set7 substrates and underlying biological consequences should be detected to elucidate the physiological relevance of Set7 in catalyzing the monomethylation of non-histone proteins.

Oxygen homeostasis is critical for normal functioning and development of aerobic organisms (32–36). Low ambient oxygen stimulates hypoxic responses, an ancient stress response controlled by hypoxia-inducible transcription factors (such as HIF-1 and HIF-2). The $\alpha$ subunit of HIF-1 or HIF-2 is rapidly degraded by the pVHL E3 ubiquitin ligase complex under normoxia; conversely, this subunit is stabilized when O$_2$-dependent prolyl hydroxylase that targets the O$_2$-dependent degradation domain of HIF-$\alpha$ is inhibited under hypoxia (37–43). HIF activation under hypoxia induces numerous genes involved in energy metabolism and promotes angiogenesis to maintain tissue integrity/homeostasis; thus, organisms can adapt to cellular hypoxia (44–47).

HIF-$\alpha$ is mainly regulated post-translationally; post-translational modifications, including ubiquitination, sumoylation, phosphorylation and acetylation, significantly contribute to the biological functions of HIF-$\alpha$ (48,49). However, converse effects may be observed in some cases. HIF-$1\alpha$ sumoylation can stabilize HIF-$1\alpha$ or de-stabilize HIF-$1\alpha$ (50–53). MAPK-induced phosphorylation of Ser-641/643 and CDK1-induced phosphorylation of Ser-668 enhance the transcriptional activity of HIF-$1\alpha$. 

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substrates of Set7. Set7 monomethylates HIF-1α and HIF-2α by enhancing poly-ADP-ribose formation (61). Despite these studies, whether Set7 is involved in hypoxia stress by methylating modifications of HIF-1α (DMEM) (HyClone) with 10% fetal bovine serum (FBS). by Peter J. Ratcliffe. HEK293T, HepG2 and RCC4 cells were obtained from ATCC. RCC4 cells were provided to HEK293T, HepG2, HCT116, H1299, HT29 and 786-O cells. RCC4 cells were cultured in DMEM supplemented with sodium pyruvate (2-NBDG), a glucose analog, was purchased from Invitrogen.

**MATERIALS AND METHODS**

**Cell line and culture conditions**

HEK293T, HepG2, HCT116, H1299, HT29 and 786-O cells were obtained from ATCC. RCC4 cells were provided by Peter J. Ratcliffe. HEK293T, HepG2 and RCC4 cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM) (HyClone) with 10% fetal bovine serum (FBS). 786-O and H1299 cells were cultured in RPMI 1640 (HyClone) with 10% FBS. HCT116 and HT29 cells were cultured in Mc-Coy5A (HyClone) with 10% FBS. Set7 wild-type and Set7-null mouse embryo fibroblasts (MEFs) were maintained in DMEM supplemented with sodium pyruvate (110 mg/l), 10% FBS, 1× nonessential amino acids (Sigma) and 1% penicillin-streptomycin. The cells were grown at 37°C in a humidified incubator containing 5% CO2. The cells were cultured under hypoxic condition (1% O2) by using an incubator with O2 control, filled with 5% CO2, and balanced with N2 (NBS Galaxy 48R).

**Plasmid construction**

The p2.1 reporter was purchased from ATCC. VEGF promoter luciferase reporter, EPO promoter luciferase reporter, BNIP3 promoter luciferase reporter, hypoxia response element (HRE) reporter and PAI-1 promoter luciferase reporter were provided by Amato Giaccia, Eric Huang, Spencer Gibson, Navdeep Chandel and Xin-Hua Feng. Wild-type human Set7 gene (originally obtained from Zengqiang Yuan) and its enzymatically dead mutant from H297A were PCR amplified and subcloned into the pCMV-Myc vector and the lentivirus vector pHAGE-CMV-MCS-IzsGreen. Wild-type human HIF-1α and K32R mutant were subcloned into the pCMV-HA vector; wild-type human HIF-2α and its K29R mutant were subcloned into the pCMV-Tag2C vector (Stratagene). Human HIF-1α domains were subcloned into pGEX-2B, and human Set7 gene was cloned into pET-32α. Set7 short hairpin RNAs (shRNAs) and control shRNA (GFP) were cloned into pSuper-Retro-Puro vector. HIF-1α shRNAs; Set7-shRNAs and control shRNA (luciferase) were cloned into the lentivirus vector lentiLox3.7.

**Antibodies and chemical reagents**

Anti-Myc (9E10), anti-His (H-15), anti-GAPDH (4811) and anti-HIF-1α (H206) antibodies were purchased from Santa Cruz. Anti-hemagglutinin (anti-HA) antibody was purchased from Covance. Anti-flag (F1804) antibody was purchased from Sigma. Anti-α-tubulin (EPR1333) and anti-Set7 (EPR5574) antibodies were purchased from Epitomics. Anti-HIF-1α (NB100-105) was purchased from Novus Biologicals. Anti-SOD2 (EPR2350Y) antibody was purchased from GeneTex. Anti-PAI-1 (612025) antibody was purchased from BD Transduction Laboratories. Anti-Histone H3 [(D1H2)XP] antibody was purchased from Cell Signaling. Anti-HIF-1α (CH3)-K32 polyclonal antibody was obtained at Abmart with the HIF-1α-K32-mel peptide (Shanghai, China). The active Set7 protein (14-469) was purchased from Millipore, and S-adenosylmethionine (SAM) (B9035) was purchased from New England Biolabs (NEB). 2ME2 (S1233) was purchased from Selleck, and (R)-PFI-2 was obtained from Tocris. Glucose assay kit was purchased from BioVision, and adenosine triphosphate (ATP) assay kit from Beyotime. 6-[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose(2-NBDG), a glucose analog, was purchased from Invitrogen.

**Virus production**

Lentiviruses for wild-type Set7, its H297A mutant and control (empty vector) were generated by transfecting HEK293T cells with the transducing vector and packaging vectors, including pMD.2 and pspAX. Lentiviruses used to knock down Set7, HIF-1α and control (targeting luciferase) were generated by transfecting HEK293T cells with a transducing vector and packaging vectors, including VSVG, RSV-REV and pMDL g/p RRE. After transfection was performed for 48 h, virus particles in the medium were harvested, filtered and transduced into target cells. The following shRNA target sequences were used: control shRNA (luciferase shRNA), 5′-GGTTGGCACCAAGCAGCGAC-3′, Set7 shRNA-1, 5′-GGGAGTTTCACTTACGGA-3′, Set7 shRNA-2, 5′-GCCCTGTTAGGAGAATGAA-3′, HIF-1α shRNA-1, 5′-GAGCTTGCTCATCAGTGCC-3′, HIF-1α shRNA-2, 5′-GGGTGGAAACTCAAGCAAC-3′.

** Luciferase reporter assays**

The cells were grown in 24-well plates and transfected with various amounts of plasmids by VigoFect (Vigorous
Biotech, Beijing, China). pTK-\textit{Renilla} was used as an internal control. After the cells were transfected for 18–24 h, luciferase activity was determined using a dual-luciferase reporter assay system (Promega). For luciferase reporter assays in HCT116 cells with knocked down Set7, shRNA targeting green fluorescent protein (GFP; targeting sequence: 5′-GCAAGCTGACCTGAAAGTTCAT-3′) in pSuper was used as control. Transient transfection was conducted for subsequent assays.

Data were normalized to \textit{Renilla} luciferase. Data were reported as mean ± standard error of the means (SEM) from three independent experiments performed in triplicates. Statistical analysis was performed using Graph Pad Prism 5 (unpaired t-test; GraphPad Software Inc., San Diego, CA, USA).

**Immunoprecipitation and western blot**

Immunoprecipitation and western blot analysis were performed as previously described (62). Anti-Myc antibody-conjugated agarose beads were purchased from Sigma. Protein A/G-Sepharose beads were purchased from GE Company. Fuji Film LAS4000 mini-luminescent image analyzer was used to photograph the blots. Multi Gauge V3.0 was used to quantify protein levels based on the band density obtained by western blot analysis.

**In vitro methylation assay**

The samples were incubated at 30°C for 60 min in a reaction buffer containing 50 mM Tris-HCl (pH 8.5), 5 mM MgCl₂, 4 mM DTT, active Set7 protein and 1 μM SAM (NEB). HIF-1α K32 peptide or GST–HIF-1α (amino acids 1–80) fusion proteins (1 μg) were used as substrates. The total volume of the reaction mixture was adjusted to 10 μl. The samples were subjected to dot blot or separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis; western blot analysis was conducted to detect methylation with an anti-HIF-1α(Ch3)-K32 antibody.

**Chromatin immunoprecipitation**

The following primers were used to amplify the LDHA gene promoter region: 5′-TTGGAGGCGACCATCTAGTATAGC-3′ and 5′-GCTCTACTGACATCTCTGTGAAGC-3′. The following primers were used to amplify the RPL13A promoter region were 5′-GAGGCAGGTGTAGAG-3′ and 5′-ACACACAGAGTCTCAATCC-3′. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (63).

**Semi-quantitative real-time PCR**

Total RNA was extracted using TRIZol reagent (Invitrogen). cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas). The following primers were used for human Set7: 5′-TTCTACCTCACAATGCATCCTCAAGCAGA-3′ and 5′-GGGTGCGGATGCTTTT-3′. The following primers were used for internal control 18S rRNA: 5′-TCTCTTTCATGATGTTATGCTGCGGT-3′ and 5′-TCTCTTTCATGATGTTATGCTGCGGT-3′. Other primers were synthesized, as described in previous reports (64,65).

**Glucose uptake assays**

RCC4 cells were transduced with a lentivirus encoding control shRNA or Set7 shRNAs. Intracellular glucose level was determined with a fluorescence-based glucose assay kit (BioVision).

2-NBDG glucose uptake assays were also performed for MEFs and RCC4 cells. In brief, the cells were grown under specified conditions with or without 100 μM 2-NBDG (Invitrogen) in the media for 1 h. Glucose uptake was quantified by flow cytometry (FACS), as previously described (64,66).

**ATP concentration assays**

RCC4 cells were transduced with lentivirus encoding control shRNA or Set7 shRNAs, in the absence or presence of (R)-PFI-2 (1 μM, 24 h), and ATP concentration was measured by ATP assay kit (Beyotime) following manufacturer instruction.

**Nucleus and cytoplasm separation**

Nucleus and cytoplasm separation was conducted using the Nuc-Cyto-Mem Preparation Kit (P1201, ApplyGen.). Briefly, HEK293T cells transfected with indicated plasmids were washed with phosphate buffered saline and lysed by sonicating for 40 times in 500 μl CER buffer provided by the kit. The homogenate was centrifuged at 800 × g for 5 min and the pellet was saved as the crude nuclei, which was then washed twice with 500 μl NER buffer provided by the kit.

**RESULTS**

**HIF-1α is methylated by a lysine-specific methyltransferase**

Set7 at lysine 32 in vitro and in vivo

To determine the involvement of Set7 in the hypoxia signaling pathway, we analyzed whether the HIF-1α protein sequence harbors a conserved domain. The three ‘RSK’ residues in the bHLH domain (amino acids 20–32) of HIF-1α (Figure 1A1) are identical to the core local recognition sequence of Set7, namely, (K/R)l(K/T)lK. This core sequence is identified in Set7-targeted proteins, such as histone H3, p53, E2F1, estrogen receptor-α and LIN28A (9,11,17,19,20,67,68). These three residues are also evolutionarily conserved among human, macaque, cow, pig, dog, mouse and rat (Figure 1A1). Thus, HIF-1α is a potential substrate of Set7.

To determine the role of Set7 on HIF-1α methylation in vivo, we generated a polyclonal antibody that specifically recognizes monomethylated HIF-1α-K32. We initially screened rabbit antibodies against HIF-1α peptides harboring K32 with different degrees of methylation. An antibody that specifically recognized a monomethylated peptide but failed to detect an unmodified peptide or equivalent peptides that were di- or tri-methylated was selected by dot-blot assays [anti-HIF-1α(Ch3)-K32] (Figure 1A2).

To determine whether HIF-1α is a novel substrate of Set7, we overexpressed wild-type Set7 or enzymatically dead mutant Set7 (H297A) with the wild-type HIF-1α. We
then examined the methylation status of HIF-1α by using anti-HIF-1α (CH3)-K32 antibody. Wild-type Set7, not mutant Set7 (H297A), could methylate HIF-1α (Figure 1B1). After the K32 residue in HIF-1α was mutated to arginine, anti-HIF-1α (CH3)-K32 antibody could not detect the methylation of HIF-1α mutant, HIF-1α(K32R) (Figure 1B2). We concluded that an antibody specifically recognized K32-methylated HIF-1α and HIF-1α may be a novel substrate of Set7.

To further determine whether HIF-1α is methylated by Set7 in vivo, we used RCC4 cell, a pVHL-deficient clear cell renal carcinoma cell line with high HIF-α under normoxic condition (69). We transduced RCC4 cells with lentiviruses overexpress or knock down Set7. The overexpression of the wild-type Set7 enhanced endogenous HIF-1α methylation, as revealed by coimmunoprecipitation assays; by contrast, the overexpression of the mutant Set7 (H297A) did not improve (Figure 1C1). The knockdown of Set7 by two Set7 shRNAs, namely, Set7-shRNA-1 and Set7-shRNA-2, decreased the endogenous methylation level of HIF-1α to lower than the basal level of the control (Figure 1C2). To determine whether Set7 can directly methylate HIF-1α, we performed in vitro methylation assays. The active Set7 protein (14–469) (Millipore) could effectively methylate the bacterial-purified HIF-1α fragment (amino acids 1–80) harboring the core local reorganization sequence of Set7 (Figure 1D1). The active Set7 protein could also effectivemethylate the synthesized unmodified peptide (NH2-RSRRSKESEVF-COOH), as revealed by dot-blot assays (Figure 1D2).

These data suggest that Set7 methylates HIF-1α at K32 in vitro and in vivo.

HIF-1α interacts with Set7 in vitro and in vivo

We examined the interaction between HIF-1α and Set7 to determine the mechanism of Set7-mediated methylation. We transfected Myc-tagged Set7 (Myc-Set7) with HA-tagged HIF-1α (HA-HIF-1α) or HA empty vector (Figure 2A1), as well as HA-HIF-1α with Myc-tagged Set7 (Myc-Set7) or Myc empty vector, into HEK293T cells; we then performed coimmunoprecipitation assays. HA-HIF-1α or Myc-Set7 could efficiently pull down Myc-Set7 (Figure 2A1) or HA-HIF-1α (Figure 2A2), respectively; this result indicated that HIF-1α interacts with Set7. To determine whether these proteins interact endogenously, we performed coimmunoprecipitation assays in RCC4 cells by using a monoclonal antibody against HIF-1α. The results showed that endogenous HIF-1α could interact with endogenous Set7 (Figure 2B).

To determine whether Set7 can directly bind to HIF-1α and to which domains of HIF-1α, we performed GST

Figure 1. Set7 methylates HIF-1α. (A) (A1) Sequence alignment of partial HIF-1α proteins (1–39 amino acids) of human, macaque, cow, pig, dog, mouse and rat. The red box indicates a conserved consensus motif (R/K-S/T-K) methylated by Set7; the blue arrow indicates the conserved lysine (K32) mutated to arginine in methylation assays. The amino acid is numbered based on human HIF-1α amino acid sequence. (A2) Dot-blot assay for HIF-1α peptides containing various degrees of methylation. Equal amounts of unmodified (K32), mono-(K32-mel), di-(K32-mel2) and tri-(K32-mel3) methylated HIF-1α were immunoblotted with monomethyl HIF-1α antibody [anti-HIF-1α(CH3)-K32]. (B) (B1) Western blot analysis of HIF-1α methylated by the wild-type Set7 or mutant Set7 (H297A) in transfected cells with monomethyl HIF-1α antibody [anti-HIF-1α(CH3)-K32]. Anti-HA agarose beads were used for immunoprecipitation. (B2) Western blot analysis of mutant HIF-1α (K32R) methylated by the wild-type or mutant Set7 (H297A) in transfected cells with monomethyl HIF-1α antibody [anti-HIF-1α(CH3)-K32]. (C) (C1) Detection of endogenous methylated HIF-1α from whole-cell extracts of RCC4 stable cell lines overexpressing the wild-type or mutant Set7 (H297A). Methylated HIF-1α was detected by western blot analysis with anti-HIF-1α/antibody as loading controls. Anti-HIF-1α conjugated agarose beads were used for immunoprecipitation. (C2) Detection of endogenous methylated HIF-1α from whole-cell extracts of RCC4 stable cell lines with knockdown of endogenous Set7 by Set7-shRNA-1 and Set7-shRNA-2. (D) (D1) Western blot analysis of recombinant partial HIF-1α peptide (1–80 amino acids) methylated by Set7 (Millipore) in vitro with monomethyl HIF-1α antibody [anti-HIF-1α(CH3)-K32] (top). Western blot signals obtained with anti-HIF-1α antibody as loading controls. Anti-HIF-1α conjugated agarose beads were used for immunoprecipitation. (D2) Western blot analysis of the synthesized HIF-1α peptide (ARSRRSKESEVF) methylated by Set7 (Millipore) in vitro with monomethyl HIF-1α antibody [anti-HIF-1α(CH3)-K32].
Figure 2. Set7 interacts with HIF-1α in vitro and in vivo. (A) Coimmunoprecipitation of Myc-tagged Set7 with HA-tagged HIF-1α and vice versa. Myc-tagged Set7 was transfected with the HA empty vector or HA-tagged HIF-1α into HEK293T cells, and anti-HA antibody conjugated agarose beads were used for immunoprecipitation (A1); HA-tagged HIF-1α was transfected with the Myc empty vector or Myc-tagged Set7 into HEK293T cells, and anti-Myc antibody conjugated agarose beads were used for immunoprecipitation (A2). (B) Endogenous interaction between HIF-1α and Set7. Western blot analysis of Set7 after coimmunoprecipitation with endogenous HIF-1α or mouse IgG control. (C) (C1) Schematic of the HIF-1α domains. The extent of the interaction between Set7 and HIF-1α domains is indicated by the number of plus signs (+). (C2) GST-pull down assay for GST-tagged HIF-1α domains and His-tagged Set7. The association of the GST-HIF-1α domains with His-Set7 was detected by immunoblotting with anti-His antibody. GST and GST fusion proteins were stained with Coomassie blue.

pull down assays. His-tagged Set7 and GST-tagged HIF-1α domains were bacterially expressed and purified. His-Set7 is strongly associated with GST-HIF-1α-(amino acids 576–785) and relatively weakly associated with GST-HIF-1α-(amino acids 1–80), (amino acids 201–330) and (amino acids 400–575). Nevertheless, the associations of His-Set7 with GST-HIF-1α-(amino acids 81–200), (amino acids 331–399) and (amino acids 786–826) were barely detected (Figure 2C). We concluded that Set7 directly interacts with HIF-1α to catalyze HIF-1α methylation.

Set7 negatively regulates HIF-1α transcriptional activity by catalyzing HIF-1α methylation

To assess the functional importance of HIF-1α methylation by Set7 on HIF-1α transcriptional activity, we examined the effect of Set7 on the activation of five well-defined luciferase reporters, including BNIP3-lucerase (BNIP3-luc), EPO-lucerase (EPO-luc), VEGF-lucerase (VEGF-luc), p2.1-reporter and multiple HRE repeat reporters (70–74). The overexpression of Set7 in HEK293T cells inhibited the activity of these reporters under normoxic and hypoxic conditions (Figure 3A–E). The knockdown of Set7 in HEK293T cells by Set7-shRNA-1 and Set7-shRNA-2 significantly increased the activity of p2.1 and EPO-luc reporters (Figure 4A1 and A2).

We further compared the reporter activity of BNIP3-luc and EPO-luc between the wild-type Set7 MEF cells (Set7+/+) and Set7-null MEF cells (Set7−/−). The activities of BNIP3-luc and EPO-luc were lower in the wild-type Set7 MEFs than those in Set7-null MEFs under hypoxia (Figure 4B1 and B2). The wild-type Set7 was recovered in Set7-null MEF cells (Set7−/−) by overexpression; as a result, BNIP3-luc and p2.1-reporter activities were inhibited. Conversely, the overexpression of the enzymatically dead mutant Set7 (H297A) failed to inhibit the activities of BNIP3-luc and p2.1-reporter (Figure 4C1 and C2). These data suggest that Set7 represses HIF-1 transcriptional activity, and this repressive role may depend on the methyltransferase activity of Set7.

We directly examined the role of Set7 on HIF-1α target gene expression by semi-quantitative real-time PCR assays. The overexpression of Set7 in HepG2 cells significantly decreased LDHA expression under hypoxic condition (Figure 5A). The efficiency of Set7-shRNA-1 and Set7-shRNA-2 mediated knockdown of Set7 in HCT116 cells was confirmed by semi-quantitative real-time PCR assays (Figure 5B1). The knockdown of Set7 by Set7-shRNA-1...
Figure 3. Set7 inhibits HIF-1α transcriptional activity. (A) The overexpression of Set7 inhibits the activity of BNIP3-luc, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O2) or hypoxia (1% O2) conditions. (B) The overexpression of Set7 inhibited the activity of EPO-luc, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O2) or hypoxia (1% O2) conditions. (C) The overexpression of Set7 inhibited the activity of p2.1 reporter, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O2) or hypoxia (1% O2) conditions. (D) The overexpression of Set7 inhibited the activity of VEGF-luc, a well-defined hypoxia-induced reporter, in HEK293T cells under normoxia (20% O2) or hypoxia (1% O2) conditions. (E) The overexpression of Set7 inhibited the activity of HRE-luc but not the mutant HRE in HEK293T cells under normoxic (20% O2) or hypoxic (1% O2) conditions. Luciferase activity was determined at 24 h post transfection. HRE, hypoxia responsible element; wt, wide type; mt, mutant type. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).

and Set7-shRNA-2 in HCT116 cells considerably increased the mRNA levels of HK1 (Figure 5B2). We also compared the expression of HIF-1α target genes in wild-type Set7 MEFs and Set7-null MEFs. The mRNA levels of Slc2a1, Pfk1 and Vegfa were higher in the wild-type Set7 MEFs than in the wild-type Set7 MEFs (Figure 5C). In addition, the knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells significantly induced LDHA and PDK1 expression (Figure 5D).

We also examined whether Set7 can directly suppress HIF-1α transcriptional activity. Under normoxic condition, the overexpression of HIF-1α increased the reporter activity of VEGF-luc and p2.1-reporter, but this increase was inhibited by co-transfection of Set7 (Figure 6A1 and A2). The transactivity of the HIF-1α mutant (K32R) was enhanced compared with that of the wild-type HIF-1α, as indicated by the activities of BNIP3-luc and HRE-reporter in HEK293T cells (Figure 6A3 and A4). This finding indicated that the endogenous Set7 lost its inhibitory role on the mutant; thus, transactivity was enhanced when the Set7-methylated site of HIF-α underwent mutation (K32R).

To determine whether the effect of Set7 on the hypoxia signaling is mediated by HIF-1α, we used the potent and specific inhibitor of HIF-α, namely, 2ME2 (75). As shown in Figure 6B, treatment with 2ME2 eliminated the effect of Set7 knockdown on the expression of HIF-1α target genes, including SLC2A1, PDK1, EPO and PGK1 (Figure 6B1 to B4). Hence, the effect of Set7 on hypoxia signaling depends on the activity of endogenous HIF-α.

To gain insights into the mechanisms of Set7 on affecting the expression of HIF-1α downstream targets, we conducted ChIP assays. The knockdown of Set7 in RCC4 cells significantly enhanced HIF-1α occupancy at the HRE region of the LDHA promoter (Figure 6C). By contrast, the knockdown of Set7 did not affect the HIF-1α occupancy in the RPL13A promoter (Figure 6C2), which does not contain the HRE region. Therefore, the methylation of HIF-1α by Set7 may diminish HIF-1α occupancy on the HRE region of HIF-1 targets, resulting in gene downregulation.

Next, we examined whether the suppression of Set7 on HIF-1α transcriptional activity was due to the effect of Set7 on HIF-1α mRNA and protein levels. Overexpression of
Figure 4. Depletion of Set7 enhances HIF-1α transactivity. (A) (A1) The knockdown of Set7 by Set7-shRNA-1 or Set7-shRNA-2 in HEK293T cells significantly enhanced the activity of p2.1 reporter under hypoxic condition ($P = 0.0053$ or $P = 0.0011$, respectively). (A2) The knockdown of Set7 by Set7-shRNA-1 or Set7-shRNA-2 in HEK293T cells significantly enhanced the activity of EPO promoter luciferase reporter under hypoxic conditions ($P = 0.0057$ or $P = 0.0057$, respectively). (B) (B1) The activity of BNIP3 promoter luciferase reporter was enhanced in Set7-null MEF cells (Set7$^{-/-}$) compared with that in the wild-type Set7 MEF cells (Set7$^+/+$) under hypoxic conditions. (B2) The activity of EPO promoter luciferase reporter was enhanced in Set7-null MEF cells (Set7$^{-/-}$) compared with that in the wild-type Set7 MEF cells (Set7$^+/+$) under hypoxic conditions. (C) (C1) The overexpression of the wild-type Set7 but not the mutant Set7 (H297A) in Set7-null MEF cells (Set7$^{-/-}$) inhibited the activity of BNIP3 promoter luciferase reporter. (C2) The overexpression of the wild-type Set7 but not the mutant Set7 (H297A) in Set7-null MEF cells (Set7$^{-/-}$) inhibited the activity of p2.1 reporter. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).
Figure 5. Set7 suppresses HIF-1a target gene expression. (A) The overexpression of Set7 reduced LDHA expression in HepG2 cells under hypoxia (1% O2), as revealed by semi-quantitative real-time PCR assays. (B) The efficiency of Set7-shRNA-1 and Set7-shRNA-2-mediated knockdown of Set7 in HCT116 cells was confirmed by semi-quantitative real-time PCR assays. (B2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in HCT116 cells increased HK1 expression under normoxic and hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (C1) Slc2a expression in Set7-null MEF cells (Set7−/−) was higher than that in the wild-type MEF cells (Set7+/+) under hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (C2) Pgk1 expression in Set7-null MEF cells (Set7−/−) was higher than that in the wild-type MEF cells (Set7+/+) under hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (C3) Vegfa expression in Set7-null MEF cells (Set7−/−) was higher than that in the wild-type MEF cells (Set7+/+) under hypoxic conditions, as revealed by semi-quantitative real-time PCR assays. (D1) The efficiency of Set7-shRNA-1 and Set7-shRNA-2-mediated knockdown of Set7 in RCC4 cells was confirmed by semi-quantitative real-time PCR assays. (D2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells increased LDHA expression, as revealed by semi-quantitative real-time PCR assays. (D3) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells increased PDK1 expression, as revealed by semi-quantitative real-time PCR assays. Data were presented as mean ± SEM of three independent experiments performed in triplicate; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).
Figure 6. Inhibitory role of Set7 in the hypoxia signaling pathway is mediated by HIF-1α. (A) (A1) The overexpression of Set7 inhibited the activity of VEGF promoter luciferase reporter activated by the ectopic expression of HIF-1α in HEK293T cells. (A2) The overexpression of Set7 inhibited the activity of p2.1 reporter activated by the ectopic expression of HIF-1α in HEK293T. (A3) The overexpression of the mutant HIF-1α with Set7-methylated site mutated (K32R) significantly enhanced the transactivity of BNIP3 promoter luciferase reporter compared with that of the wild-type HIF-1α in HEK293T cells (P = 0.0015). (A4) The overexpression of the mutant HIF-1α with Set7-methylated site mutated (K32R) significantly enhanced the transactivity of the HRE reporter compared with that of the wild-type HIF-1α in HEK293T cells (P = 0.0014). (B) The knockdown of Set7 by Set7-shRNA-2 in RCC4 cells increased the expression of SLC2A1 (B1), PDK1 (B2), EPO (B3) or PGK1 (B4); but inhibition of HIF-1α activity by adding HIF-1α inhibitor, 2ME2 (50 ng/μl, 18 h), abolished this kind of effect under normoxia conditions, as revealed by semi-quantitative real-time PCR assays. (C) (C1) ChIP assays show that the knockdown of Set7 in RCC4 cells considerably enhanced the binding ability of HIF-1α to the LDHA promoter. Mouse IgG served as a control for ChIP assays. (C2) The knockdown of Set7 in RCC4 cells does not affect the binding ability of HIF-1α to the RPL13A promoter. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).

Set7 in HEK293T cells did not reduce HIF-1α mRNA level under hypoxic condition (Figure 7A). Consistently, knockdown of Set7 in RCC4 cells also did not enhance HIF-1α mRNA level (Figure 7B). In addition, co-expression of Set7 together with HIF-1α did not alter HIF-1α protein level (Figure 7C). Furthermore, in the presence of cycloheximide (CHX, 50 μg/ml), overexpression of Set7 had no obvious effect on HIF-1α protein stability (Figure 7D). To determine whether the suppression of Set7 on HIF-1α transcriptional activity was due to the effect of Set7 on HIF-1α nuclear translocation (59), we performed subcellular localization assay. After overexpression of HIF-1α together with Set7 or empty vector control, we separated nucleus and cytoplasm fraction and conducted western blot analysis. To ensure the efficiency of fraction separation, we used anti-α-tubulin antibody to monitor cytoplasmic proteins and used anti-Histone H3 antibody to monitor nuclear proteins.

As shown in Figure 7E, overexpression of Set7 did not alter HIF-1α nuclear localization. Taken together, these data suggest that the suppression of Set7 on HIF-1α transcriptional activity was neither due to the effect of Set7 on HIF-1α mRNA and protein levels, nor due to its effect on HIF-1α nuclear localization.

Effect of Set7 on HIF-1α transactivity depends on the activity of Set7 that functions as a methyltransferase

To determine whether the effect of Set7 on HIF-1α activity is dependent on the methyltransferase activity of Set7, we used (R)-PFI-2, a potent and specific inhibitor of Set7 (76). Treatment with (R)-PFI-2 inhibited methylation of HIF-1α by Set7 in HEK293T cells (Figure 8A). The addition of (R)-PFI-2 in RCC4 cells significantly enhanced the expression of HIF-1α target genes, including SLC2A1, LDHA,
Set7 has no effect on mRNA level, protein level and nuclear translocation of HIF-1α. (A) Under hypoxia condition, overexpression of Set7 had no effect on HIF-1α mRNA level in HEK293T cells. (B) In RCC4 cells, knockdown of Set7 by Set7-shRNA-1 has no effect on HIF-1α mRNA level. (C) Overexpression of Set7 had no obvious effect on HIF-1α protein level in HEK293T cells. (D) The protein level of HIF-1α was not affected by overexpression of Set7 in HEK293T cells in the presence of CHX (50 μg/ml). Short, shorter exposure; long, longer exposure. (E) Overexpression of Set7 did not affect HIF-1α nuclear translocation. α-tubulin detection was used to monitor cytoplasmic protein and Histone H3 detection was used to monitor nuclear protein.

These data suggest that methyltransferase activity is essential for Set7 to suppress HIF-1α activity.

Set7 functions as an HIF-1α methyltransferase to regulate glucose homeostasis

The role of HIF-1α in cellular metabolism has been widely investigated (77–81). Under hypoxic conditions, HIF-1α can induce the expression of some genes to promote glucose uptake (44–47). Some genes can also regulate HIF-1α activity to modulate cellular glucose homeostasis (48,62,64,82,83). Set7 affects the expression of glycolysis-associated HIF-1α downstream target genes, such as LDHA, HK1, PDK1, Slc2a1 and Pgk1 (Figure 5), thus, we postulate that Set7 may affect cellular glucose homeostasis by inhibiting HIF-1α activity. To verify this hypothesis, we examined glucose uptake and ATP levels in cells after Set7 was knocked down or Set7 activity was inhibited. The knockdown of Set7 in RCC4 cells increased glucose uptake and intracellular ATP levels (Figure 9A and B). The results of 2-NBDG glucose uptake assays showed that Set7+/− MEFs exhibited higher levels of cellular glucose than Set7+/+ MEFs under hypoxic conditions (Figure 9C). Furthermore, (R)-PFI-2 treatment upregulated cellular ATP levels and glucose uptake in RCC4 cells (Figure 9D and E). These data suggest that the methyltransferase Set7 regulates glucose homeostasis via HIF-1α.

Set7 is a hypoxia-suppressed gene

To delineate the role of Set7 in the hypoxia signaling pathway, we examined whether hypoxia can regulate Set7 expression. Under hypoxic conditions, Set7 expression was significantly suppressed in the three examined cell lines, as revealed by semi-quantitative RT-PCR assays (Figure 10A1–A3). In HEK293T cells, hypoxia exposure also reduced the protein level of Set7 (Figure 10B). By contrast, the knockdown of HIF-1α by HIF-1α-shRNA-1 and HIF-1α-shRNA-2 increased Set7 protein level (Figure 10C). The inhibition of HIF-α activity by 2ME2 in RCC4 cells induced Set7 expression (Figure 10D). To ensure the reliability of hypoxic conditions in these experiments, we established positive controls by using several well-defined hypoxia-induced genes, including SLC2A1, LDHA, PGK1, HK1, EPO and VEGF (Figure 10A and D). Our findings suggest that Set7 is a hypoxia-suppressive gene.
Figure 8. Inhibitory role of Set7 on HIF-1α transactivity is dependent on its methyltransferase activity. (A) (R)-PFI-2 (1 μM), a potent and selective inhibitor of Set7, inhibited HIF-1α methylation at K32 catalyzed by Set7, as revealed by immunoprecipitation assays. (B) After (R)-PFI-2 (1 μM) treatment was administered for 18 h, the expressions of SLCL2A1, LDHA, PKD1, PGK1, EPO and VEGF were increased in RCC4 cells, as revealed by semi-quantitative real-time PCR assay. (C) The enhanced expressions of SLC2A1, LCH2A (C1), PKD1 (C2) and LDHA (C3) by addition of (R)-PFI-2 (1 μM, 18 h) in RCC4 cells were counteracted by the knockdown of Set7. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).

To determine when HIF-1α is methylated, we performed a time course assay using HEK293T cells. As shown in Figure 10E, HIF-1α was methylated to a higher level after the cells were exposed to hypoxic condition from 1 h to 2 h, then the methylation of HIF-1α decreased after the cells was exposed to hypoxic condition from 4 h even though the total protein level of HIF-1α increased constantly. After the cells were exposed to hypoxic condition for 24 h, the protein level of Set7 decreased dramatically, the methylation of HIF-1α also dropped to low level (Figure 10E). These results suggest that the regulation between Set7 expression and HIF-1α methylation is dynamic. To further figure out its intrinsic relationship will be very interesting.

HIF-2α, as well as HIF-1α, is methylated by Set7

The synthesized peptide based on the HIF-1α protein sequence used to generate antibody is evolutionarily conserved between HIF-1α and HIF-2α (Figure 11A1). The core local recognition sequence of Set7, namely, (K/R)(S/T)K, is also present in HIF-2α, which is evolutionarily conserved among human, macaque, cow, pig, mouse and rat (Figure 11A1 and A2). These findings suggested that HIF-2α may be a substrate of Set7, resembling HIF-1α. To test this hypothesis, we examined the methylation ability of Set7 on HIF-2α by using anti-HIF-1α(CH3)-K32 antibody after ectopic expression. In HEK293T cells, the ectopic expression of the wild-type Set7 could methylate the wild-type HIF-2α but not the mutant HIF-2α(K29R); in the latter, the potential methylation site lysine 29 was mutated to arginine (column 2 versus column 5 in the first line of Figure 11B). By contrast, the ectopic expression of the enzymatically dead mutant of Set7, namely, Set7(H297A), did not affect wild-type HIF-2α and mutant HIF-2α(K29R) (column 3 versus column 6 in the first line of Figure 11B). These data suggested that the K29 of HIF-2α is methylated by Set7, and anti-HIF-1α(CH3)-K32 antibody can detect HIF-2α methylation at lysine 29.

Similar to the inhibitory role of Set7 on HIF-1α, the overexpression of Set7 in HEK293T cells inhibited the reporter activity of the PAI-1 promoter, a well-defined HIF-2α target gene reporter (Figure 11C) (62,65). In RCC4 cells, the inhibition of Set7 activity by adding (R)-PFI-2 increased the expression of HIF-2α target genes, including PAI-1, CITED2, GST and POU5F1, as revealed by semi-quantitative RT-PCR assays (Figure 11D) (62,65). By contrast, the knockdown of Set7 in RCC4 cells by Set7-shRNA-1 and Set7-shRNA-2 promotes the expression of HIF-2α target genes, including POU5F1 (Figure 11E1), PAI-1 (Figure 11E2) and CITED2 (Figure 11E3) (62,65). However, the addition of 2ME2 in RCC4 cells inhibited the activity of HIF-2α; as a result, the upregulation effect of Set7-shRNA-2 on GST expression was completely abrogated (Figure 11F) (62,65).
Figure 9. Increased glucose uptake and ATP levels in Set7-deficient cells and cells with Set7 inhibition. (A) The knockdown of Set7 by Set7-shRNA-2 via lentivirus infection in RCC4 cells significantly increased cellular glucose levels ($P = 0.0057$), as determined using a glucose uptake assay kit. (B) The knockdown of Set7 by Set7-shRNA-2 via lentivirus infection in RCC4 cells significantly increased ATP levels ($P = 0.0160$), as measured using an ATP assay kit. (C) The wild-type Set7 (Set7$^{+/+}$) or Set7-null (Set7$^{-/-}$) MEF cells were grown in the presence or the absence of the fluorescent glucose analog NBDG(100 μM) for 1 h; glucose uptake was quantified by flow cytometry (FACS). In the presence of NBDG, Set7$^{-/-}$ MEFs exhibited higher intracellular glucose levels than Set7$^{+/+}$ MEFs (green curve versus orange curve; $P < 0.0001$). Blue and red curves are controls without the fluorescent NBDG glucose analog. (D) The inhibited Set7 activity by the addition of (R)-PFI-2 (1 μM) for 24 h in RCC4 cells significantly increased ATP levels ($P = 0.0002$), as determined using an ATP assay kit. (E) RCC4 cells were treated with or without (R)-PFI-2 (1 μM) for 24 h, and the cells were grown in the presence or the absence of the fluorescent glucose analog NBDG for 1 h; glucose uptake was then quantified by flow cytometry (FACS). In the presence of NBDG, RCC4 cells with Set7 inhibition (treatment with (R)-PFI-2) exhibited higher intracellular glucose levels than RCC4 cells without Set7 inhibition (treatment with DMSO control) (orange curve versus green curve, $P = 0.0004$). Blue and red curves are controls without the fluorescent NBDG glucose analog. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).

Previous study reported that 786-O cells only harbor wild-type HIF-2α, but not HIF-1α (84). To elucidate the effect of Set7 on HIF-2α, we knocked down Set7 in 786-O cells by Set7-shRNA-1 and Set7-shRNA-2 (Figure 11G1) and examined the expressions of HIF-2α target genes. The knockdown of Set7 in 786-O cells enhanced the expressions of POU5F1 (Figure 11G2) and CITED2 (Figure 11G3). Consistently, the overexpression of the wild-type Set7 by lentivirus infection in 786-O cells decreased the protein levels of PAI-1 and SOD-2, which are two well-defined HIF-2α targets. However, the overexpression of the mutant Set7(H297A) elicited no evident effect (Figure 11H). These data suggest that HIF-2α and HIF-1α are novel substrates of Set7, and Set7 inhibits HIF-2α transactivity by monomethylation of HIF-2α at lysine 29.

DISCUSSION

Set7 methylates HIF-1α/2α and negatively regulates HIF-1α/2α transcriptional activity

In this study, we investigate the regulation of the main regulators of the hypoxia signaling pathway by lysine methylation and demonstrate that Set7 methylates HIF-1α at K32 and HIF-2α at K29. HIF-1α and HIF-2α are possible substrates of Set7, as confirmed by the following findings: Set7 methylated HIF-α in vivo and in vitro; enzymatically dead mutant could not methylate HIF-1α and the specific inhibitor of Set7 blocked HIF-1α methylation.

Thus far, the roles of Set7-mediated methylation on non-histone proteins and corresponding mechanisms have not been elucidated. The ability of Set7 to positively or negatively regulate substrates varies in different substrates (6,8). Several mechanisms have been proposed to explain the role
of lysine methylation by Set7 on non-histone proteins. The enhancement of protein stability and nuclear retention positively regulates the activity of p53 and LIN28A by Set7-mediated monomethylation (11,61); by contrast, the promotion of protein degradation negatively regulates the activity of Sox2 and DNMT1 by Set7-mediated monomethylation (13,85–87). Cytoplasmic retention by Set7-mediated methylation also enhances the Yap function (25). However, all of these mechanisms could not fully explain the effect of Set7 on other substrates, such as AR and FOXO3a (17,18,26,27). Some controversy observations have been reported for the effect of Set7 on FOXO3a (26,27). Methylation of FOXO3a by Set7 decreases FOXO3a protein stability, while moderately increasing FOXO3a transcriptional activity (26). However, it is also reported that methylation of FOXO3a leads to the inhibition of FOXO3a DNA-binding activity and transactivation without affecting FOXO3a protein stability obviously (17). In this study, we provide evidence to show that lysine methylation of HIF-1α diminished its occupancy on target gene promoters, resulting in the inhibition of gene expression. The conformational change of the HIF-α protein after methylation may reduce the HIF-α protein on the target gene promoter. Of note, the methylation sites (RSK) of HIF-1α/2 by Set7 locate in bHLH domain of HIF-α, which may also result in decreasing DNA-binding activity of HIF-α. Alternatively, HIF-1α/2 may mediate the recruitment of Set7 to the HREs of the HIF target genes, resulting in monomethylation of histone H3 at lysine 4; this monomethylation impairs HIF-α binding to HREs and thereby inhibits the transcription of HIF target genes. Notably, a similar mechanism has
Figure 11. Set7 methylates HIF-2α and inhibits HIF-2α transactivity. (A) (A1) Sequence alignment of partial human HIF-1α (amino acids 1–43) and HIF-2α protein (amino acids 1–40). The blue box indicates the peptide sequence (12 amino acids) used for antibody generation [K32 was methylated (Me1) when antibody was generated]; the red box indicates a conserved consensus motif (R/K-S/T-K) methylated by Set7; the blue arrow indicates the conserved lysine methylated to arginine in the methylation assays. (A2) Sequence alignment of the HIF-2α protein (amino acids 1–40) of human, macaque, cow, pig, mouse and rat. The red box indicates a conserved consensus motif (R/K-S/T-K) methylated by Set7; the blue arrow indicates the conserved lysine (K29) mutated to arginine in the methylation assays. (B) The wild-type HIF-2α is methylated by wild-type Set7, not by Set7 mutant (H297A); HIF-2α mutant (K29R) could not be methylated by Set7, as detected by anti-HIF-1α (CH3)-K32 antibody after immunoprecipitation assays. (C) The overexpression of Set7 suppressed the activity of PAI-1 promoter luciferase reporter in HEK293T cells. (D) The inhibition of Set7 activity in RCC4 cells by addition of (R)-PF1-2 (1 μM for 24 h) induced the expression of typical HIF-2α downstream targets, such as PAI-1, CITED2, GST and POU5F1, as revealed by semi-quantitative real-time PCR assays. (E) (E1) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells considerably increased POU5F1 expression, as revealed by semi-quantitative real-time PCR assays. (E2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells considerably increases PAI-1 expression, as revealed by semi-quantitative real-time PCR assays. (E3) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in RCC4 cells considerably increased CITED2 expression, as revealed by semi-quantitative real-time PCR assays. (F) The knockdown of Set7 by Set7-shRNA-2 in 786-O cells confirmed the semi-quantitative real-time PCR assays. (G) (G1) The efficiency of Set7-shRNA-1 and Set7-shRNA-2 mediated Set7 knockdown in 786-O cells was confirmed by semi-quantitative real-time PCR assays. (G2) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in 786-O cells considerably increased CITED2 expression, as revealed by semi-quantitative real-time PCR assays. (G3) The knockdown of Set7 by Set7-shRNA-1 and Set7-shRNA-2 in 786-O cells considerably increased GST expression, as revealed by semi-quantitative real-time PCR assays. (H) The overexpression of Set7 by lentivirus infection in 786-O cells decreased the protein levels of PAI-1 and SOD2, but the overexpression of Set7 mutant (H297A) did not decrease, as revealed by western blot analysis. Data were presented as mean ± SEM of three independent experiments performed in triplicates; statistical analysis was performed using GraphPad Prism 5.0 (unpaired t-test).
been proposed to explain the coactivator role of the histone demethylase JMJD2C on HIF-1α (88).

In comparing the target sequences of Set7 bound to histone H3, TAF10 and p53, a structurally conserved binding motif of Set7, namely, K/R-S/T-K (in which K is the target lysine residue) has been recognized (11,12). Several putative substrates of Set7 have been identified on the basis of the consensus target site (68,89). In this study, HIF-1α may be a substrate of Set7 based on the ‘RSK’ sequence localized in HIF-1α. Subsequent assessments confirmed this hypothesis. The proteins in the protein pool of mammalian cells harboring this consensus motif may be potential substrates of Set7. In the present study, we noticed that the transcriptional activities of the HIF-1α (K32R)/HIF-2α (K29R) mutants were still suppressed by the overexpression of Set7 (data not shown), although the transcriptional activity of HIF-1α (K32R) mutant was higher than that of the wild-type HIF-1α. It appears that Set7 can also methylate other sites, in addition to the K32 in HIF-1α and K29 in HIF-2α. In fact, Set7 binds to amino acids 201–330, amino acids 400–575 and amino acids 576–785 of HIF-1α, in addition to the N-terminus of HIF-1α (amino acids 1–80) that harbors K32. Therefore, other sites in HIF-1α and HIF-2α may be the possible target sites of Set7. However, sites other than K32 or K29 could not be detected in the assays because the antibody used in this study is specific to K32 methylation in HIF-1α and K29 methylation in HIF-2α. But, it is evident that Set7-mediated methylation suppresses HIF-1α/2α transcriptional activity, regardless of the methylated site because (R)-PFI-2, a specific Set7 methyltransferase activity inhibitor, releases the suppressive role of Set7 on HIF-1α/2α activity. Future studies should identify unknown sites methylated by Set7 to fully elucidate the role of Set7 on HIF-α methylation.

**Set7 may significantly function in response to hypoxia stress by regulation of HIF-1α/2α**

Post-translational modifications of proteins regulate cells in response to different stresses because of rapid response and reversible capability (14,27). As the main regulators of the hypoxia signaling pathway in cells, post-translational modifications of HIF-1α/2α in response to hypoxia have been extensively investigated (48,59,65,66,75,90). Although hypoxia is involved in epigenetic gene regulation by inducing histone demethylase or by activating histone methyltransferase (91–95), whether HIF-1α/2α can directly be methylated or de-methylated and the underlying biological consequences remain poorly described in mechanistic terms; involved factors remain unknown. In this study, evidence indicated that HIF-1α and HIF-2α are methylated by Set7, resulting in the downregulation of HIF target genes; on the other hand, set7 expression is also suppressed by hypoxia; thus, we reveal a novel role of protein methylation in response to hypoxia stress. These data also suggest that Set7 may restrain cells in response to hypoxia to avoid exaggerating reactions. Such reactions cause harmful effects on cells by causing cell apoptosis and autophagy, among others (96–100). Moreover, Set7-HIF-α-Set7 may form a regulatory loop that strictly controls cell responses under hypoxia stress. The destruction of this regulatory loop under some pathogenic conditions may result in the loss of control of HIF-α activity. As a result, cells may encounter abnormal processes that may lead to pathogenic progression. For this reason, animal models should be established to further explore the role of Set7-mediated HIF-α methylation and to elucidate the physiological relevance of Set7 methyltransferase in the hypoxia signaling pathway.

**ACKNOWLEDGEMENTS**

We are grateful to Drs Peter J. Ratcliffe, Amato Giaccia, Eric Hung, Spencer Gibson, Navdeep Chandel, Xin-Hua Feng, Weiguo Zhu, Zengqiang Yuan for the generous gifts of reagents.

**FUNDING**

CAS Major Scientific and Technological Project [XDA08010208 to WXJ]; NSFC [3146163003, 31071212, 91019008 to WXJ]. Funding for open access charge: CAS Major Scientific and Technological Project [XDA08010208 to WXJ].

**Conflict of interest statement.** None declared.

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