HIF-1-Dependent Induction of Jumonji Domain-Containing Protein (JMJD) 3 under Hypoxic Conditions

Ho-Youl Lee1,2, Kang Choi1,2, Hookeun Oh1,2, Young-Kwon Park1, and Hyunsung Park1,*

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INTRODUCTION

In response to hypoxia, cells express several genes to maintain homeostasis. More than 500 genes, including vascular endothelial growth factor (VEGF), erythropoietin (EPO), phosphoglycerate kinase-1 (PGK-1), glucose transporter-1 (glut-1), and BCL-2 adenovirus E1B 19 kDa interacting protein 3 (Bnip3) are induced under hypoxia in MCF7 cells (Elvidge et al., 2006). These diverse target genes are transcribed by a common transactivator, the hypoxia-inducible factor 1-α/β (HIF-1α/β) heterodimer (Semenza, 2012). Under normoxic conditions, HIF-1α is ubiquitinated and rapidly degraded whereas HIF-1β constitutively expressed. HIF-1β is identical to the Aryl hydrocarbon receptor nuclear translocator (Arnt) that is a dimerization partner of HIF-1α (Maxwell et al., 1999). The 803rd asparagine residue of HIF-1α is also hydroxylated by an oxygen-dependent asparaginyl hydroxylase, referred to as Factor-Inhibiting HIF-1 (FIH-1). The hydroxylated asparaginyl residue hinders the recruitment of the CREB-binding protein (CBP)/p300 coactivator. PHD and FIH-1 require molecular oxygen, α-ketoglutarate, vitamin C, and Fe(II) for their catalytic activities. The trans-active form of HIF-1α is stabilized under hypoxia because the activities of these two oxygen-dependent hydroxylases decrease (Hewitson et al., 2002).

FIH-1 has unique structural and catalytic properties compared to PHD (Hewitson et al., 2007). The catalytic region of FIH-1 (145-340 amino acids in human FIH-1) shows structural similarity to the Jumonji (Jmj) domain, which is often found in histone demethylases (Chen et al., 2006; Lee et al., 2003). JmjC domain containing proteins (JMJDs) catalyze the oxidative demethylation of a methylated lysine residue of histones. Although JMJDs and FIH-1 hydroxylate different target proteins, both utilize O2, α-ketoglutarate, vitamin C, and Fe(II) and produce succinate and CO2.

More than 100 JMJDs have been identified, and most of them have demethylase activity with different substrate specificities (Shi and Whetstine, 2007). JMJD1A/JHDM2A/KDM3A demethylate H3K9me2 and me1, JMJD2 isozymes demethylate H3K9me3, Jarid1 isoforms demethylate H3K4me3, and JMJD3/KDM6B demethylate H3K27me3. Histone methylation affects gene expression in distinct localized patterns. The tri-methylation of H3K4 and acetylation of H3 in the promoter region are associated with actively transcribed genes, whereas the methylation of H3K9 and H3K27 in the promoter region are associated with inactive genes (Kooistra and Helin, 2012). The fact that JmjC proteins need molecular oxygen for their catalytic activities suggests that their activities can be inhibited under hypoxic conditions. Recent findings that hypoxia induced several JmjC proteins imply that their induction compensates their reduced catalytic activity under hypoxia (Beyer et al., 2008; Pollard et al., 2008; Wellmann et al., 2008; Xia et al., 2009). Thus, in response to hypoxic stress, histone methylation of individual genes can be dynamically regulated by the net change in the activity and expression of the JmjC proteins.

JMJD2s, JMJD1A, and Jarid1B have been identified as HIF-1α target genes (Beyer et al., 2008; Pollard et al., 2008; Xia et al., 2009). This study first demonstrated that hypoxia also in-
duces an H3K27me3 demethylase, JMJD3, by a HIF-1α-depen-
dent mechanism. Together with the recent findings that
JMJD3 is involved in inflammation, cardiac development, neural
development, and senescence, this study suggests that hypoxia
can influence these diverse biological processes by changing
the expression level of JMJD3.

MATERIALS AND METHODS

Cell culture, reagents, and antibodies
Human adipose-derived stem cells (hADSC, R7788-115), which
are isolated from human adipose tissue collected during lipo-
suction procedures, were purchased from Life technology (USA).
Cells were plated in a 100-mm plate at a density of 5,000 cells/
cm² in DMEM supplemented with 10% mesenchymal stem cell
(MSC)-qualified fetal bovine serum (FBS). Wild-type mouse embryonic fibroblasts (MEFs) were prepared from 13.5 post-
colum (p.c.) embryos of C57BL/6 background and maintained
in DMEM supplemented with 10% FBS by using the 3T3 proto-
col until reaching passage 7. The other mouse cells were main-
tained as described previously (Choi et al., 2008; Park et al.,
2013). For hypoxic treatments, cells were cultured in an anaer-
obic incubator (< 0.5% O2, Model 1029, Forma Scientific, Inc.)
or in InVivo2 200 hypoxia workstation (3% O2, Ruskin). Defer-
oxamine (DFN), cobalt chloride (CoCl2), and Clioquinol (CQ)
were purchased from Sigma-Aldrich (USA). Antibodies against H3K9me3, H3K27me3, H3K9/ K14Ac,
me3 antibodies were obtained from Cell Signaling Technology
antibodies were purchased from Novus Biologicals (Littleton,
and Abcam (UK), respectively. Anti-mouse and human HIF-1
mouse and human JMJD3 were purchased from Abgent (USA)
were purchased from Sigma-Aldrich (USA). Antibodies against
roxamine (DFN), cobalt chloride (CoCl2), and Clioquinol (CQ)
are isolated from human adipose tissue collected during lipo-
Human adipose-derived stem cells (hADSC, R7788-115), which
Cell culture, reagents, and antibodies

RNA isolation and quantitative real-time PCR
Total RNA was isolated using an RNeasy spin column accord-
ing to the manufacturer’s instructions (Qiagen Inc., USA). cDNA
was reverse transcribed from total RNA (2 μg) using M-MLV
reverse transcriptase (Promega, USA) with dNTPs and random
primers. The qRT-PCR analyses were performed by using the
Power SYBR Green PCR Master Mix and ABI 7000 Real-Time
PCR System (Applied Biosystems) for MEFs experiments and
all hADSC experiments. The Q™ SYBR Green Supermix and
MyQI single color real-time PCR detection system (Bio-Rad, USA)
were used for the analyses of the RNA from the other mouse
cells including 3T3-L1 cells, NIH-3T3 cells, Hepa1c1c7 cells,
and BpRC1 cells. The primer sets for JMJD3 were following:
mJMJD3, (F) 5′-CCC CCA TTT CAG CTG ACT AA-3′; (R) 5′-
CTG GAC CAA GGG GTG TGT T-3′, for Figs. 1A, 1B, 1D
and 2B (De Santa et al., 2007); mJMJD3, (F) 5′-CCT GCA GTC
AAT GAA GCA GGA GTG TGT T-3′; (R) 5′-CTC CAC GTC GTC
TTT G-3′, for Figs. 1C and 1G; hJMJD3, (F) 5′-GAG CCC GCA GAT
AGG GTT -3′; (R) 5′-CAT TTA CAC ACA CCG CTT CCG-3′, (R) 5′-
TGG CTC GGA TCT CCT TCT T-3′. The ChIP primer sets for
the other genes were described previously (Choi et al., 2008; Farrall and Whitelaw, 2009).

Chromatin Immunoprecipitation
ChiP analyses were performed as described previously with the
following modifications (Gao et al., 2011; Park and Park, 2010).
Briefly, hADSC, 3T3-L1, and MEF cells were cross-linked in 1%
formaldehyde at 37°C for 10 min, and then the nuclei were
isolated (Kouskouti et al., 2004). The isolated nuclei of hADSC
and 3T3-L1 cells were treated with MNase (300 gel units) for 15
min, and sonicated to shear the chromatin (Woon Kim et al.,
2011). The insoluble materials were removed by centrifugation
at 10,000 × g for 10 min. We measured the DNA concentration
of the supernatant containing soluble chromatin to ensure that
the amount of chromatin used in each sample was similar. For
each immunoprecipitation, 30-40 μg of soluble chromatin was
10-fold diluted with ChIP dilution buffer [20 mM Tris-HCl (pH
8.0), 0.01% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl].
The diluted lysates of soluble chromatin (100 μl) were analyzed
using qPCR and the resulting quantitative values were used as
input. The diluted lysates (1 ml) were immunoprecipitated with
2-4 μg of the indicated antibodies at 4°C overnight. The antibo-
dy-chromatin complexes were recovered by incubation with 20
μl of protein G-agarose (50% slurry) and sequentially washed
as described previously (Park and Park, 2010). The immuno-
complexes were eluted with 150 μl of elution buffer (1% SDS,
0.1 M NaHCO3) and reversed. Target DNA from 3 μl sample
were analyzed by PCR. The ChIP primer sets for putative HREs
located at upstream and first intron of the JMJD3 gene are
following ; mJMJD3-0.2 kb, (F) 5′-TGG GCC TGA GCC AGA
ATA-3′, (R) 5′-AGC AGC CTG GTT CAG GTA GTA A-3′;
mJMJD3 +4.2 kb, (F) 5′-TGG GCA AGT AGC CAC GTC A-3′;
(R) 5′-TCC TCT ATC CTC TGT GGC CCT TT T-3′; hJMJD3 -4.1
kb, (F) 5′-AGC TGC ACT CCT ACA CGC T-3′; (R) 5′-CAC
AGA CTC CAC GAG GGG AG-3′; hJMJD3 -3.3 kb, (F) 5′-GGG
GAT CCT GCA GAT GGA AG-3′; (R) 5′-ACA TTT CTG AGC
GGT GTC GG-3′; hJMJD3 +1.9 kb, (F) 5′-AAG CAG CGA
GCG TGT AAC CC-3′, (R) 5′-GAG CCC GCA GAT AGG GTT
TT-3′; hJMJD3 +3.3 kb, (F) 5′-CTG ATG AGA AGG ATG CC
CC-3′, (R) 5′-ATG GAA GCG GCT CTT GGC CCC TA-3′; hJMJD3
+4.1 kb, (F) 5′-CAG TTA CAC ACA CCG CTT GCG-3′, (R) 5′-
TGG CTC GCA CCT CCT TCT T-3′. The ChIP primer sets for
the other genes were described previously (Park and Park, 2012).

shRNA mediated knock-down
HIF-1α was knocked down using a retrovirus infection system,
pSIREN-RetroQ (BD Biosciences), as described previously (Park
and Park, 2010). The short hairpin RNA (shRNA) sequence
against mouse HIF-1α was 5′-TGG GAC TCA TCT TGA TT-3′.

RESULTS

Hypoxia induced JMJD3 in HIF-1α-dependent manner
We found that hypoxia increased the expression of JMJD3 in
several types of cells including mouse fibroblast NIH3T3 cells,
mouse preadipocytes 3T3-L1 cells, and MEFs (Figs. 1A-1C). In
order to test whether hypoxia increased the expression of
JMJD3 in HIF-1α-dependent manner, we used mouse 3T3-L1
cells infected with a retrovirus encoding shRNA against mouse
HIF-1α. The qRT-PCR analyses confirmed that the mRNA
levels of either HIF-1α or its target Bnip3 were reduced in the
HIF-1α knockdown 3T3-L1 (shHIF-1α 3T3-L1) cells (Fig. 1D).
Hypoxia failed to induce JMJD3 mRNA in shHIF-1α 3T3-L1
cells. Western analyses also confirmed that hypoxia stabilized
HIF-1α protein in shControl 3T3-L1 cells but not in shHIF-1α
3T3-L1 cells, and that hypoxia failed to increase the levels of
JMJD3 protein in shHIF-1α 3T3-L1 cells (Fig. 1E). HIF-1α pro-
tein was stabilized by nonhypoxic mimickers such as deferox-
amine (DFN) and CoCl2 that inhibit the prolyl hydroxylation-
dependent ubiquitination of HIF-1α (Fig. 1F). We also showed
that not only hypoxia but also nonhypoxic HIF-1α activators
induce JMJD3 (Fig. 1G).
HIF-1α-dependent hypoxic induction of the mouse JMJD3 gene. (A-C) NIH-3T3 cells, and MEF were incubated under hypoxia (<0.5% O2) for 48 h. 3T3-L1 cells were incubated under hypoxia (<0.5% O2) for 36 h. The relative mRNA levels of BNIP3 and JMJD3 were analyzed by qRT-PCR and normalized by 18S ribosomal RNA. The mean value of qRT-PCR from normoxic cells was set to 1. The data represent the average and standard deviations of triplicate measurements from qPCR. (D, E) HIF-1α knockdown 3T3-L1 cells and control 3T3-L1 cells were treated with hypoxia for 16 h; (D) qRT-PCR was performed using the primer sets for the indicated mouse genes; (E) Western blot analyses were performed by using the indicated antibodies and lysates from hypoxia treated mouse 3T3-L1 cells. HSP70 and β-Actin protein were used as the loading control. The results of two independent experiments were shown. The numbers indicated the relative intensity of each band which was measured using LAS-3000 luminescent image analyzer and Multi Gauge ver.3.0 imaging software. (F, G) 3T3-L1 cells were treated with DFN (100 μM), CoCl2 (200 μM), or hypoxia for 48 h; (F) Western blot analyses were performed using the indicated antibodies; (G) The RT-PCR analyses of JMJD3 and BNIP3 are shown. The 18S ribosomal RNA was used as the loading control. N, normoxia; H, hypoxia (<0.5% O2). The p-values were obtained using the Student’s t-test and the significance between the groups is indicated (**p < 0.05, *p < 0.1).
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Student’s t-test and the significance between the groups is indicated (**p < 0.05, *p < 0.1).

Fig. 2. Amt-dependent hypoxic induction of the mouse JMJD3 gene. (A, B) Hepa1c1c7 cells and BpRc1 cells were incubated under hypoxia (< 0.5% O2) for 16 h; (A) Western blot analyses were performed by using the indicated antibodies. The 14-3-3y protein was used as the loading control; (B) The relative mRNA levels of mouse JMJD3 were analyzed by qRT-PCR. (C) The putative HREs in the mouse JMJD3 gene are identified by using the MatInspector program (www.genomatix.de) (Cartharius, Frech et al., 2005). The bars indicate the positions of the primer sets used for the ChiP analyses. The nucleotide sequences of putative HRE sites (-0.2 Kb, +3.9 Kb, +4.2 Kb) and flanking regions were shown (lower panel). (D) 3T3-L1 cells were incubated under hypoxia (< 0.5% O2) for 16 h. ChiP analyses were performed with the indicated antibodies and primer sets of mouse JMJD3 and mouse Stra13 genes. Input values were obtained from samples treated in the same way as the experimental method, except that no immunoprecipitation steps were performed. The values on the y-axis are presented as the percentage input of the average and standard deviation of triplicate determinations of qPCR. (E) MEF were incubated under < 0.5% O2, 3% O2, and 21% O2 (normoxia) for 48 h. ChiP analyses (upper panel) and Western blot analyses (lower panel) were performed using the indicated antibodies. N, normoxia; H, hypoxia (< 0.5% O2). The p-values were obtained using the

JMJD3 in hypoxic MEFs. Additionally, we showed that 3% oxygen slightly increases HIF-1α occupancy on both JMJD3 and Stra13 (Fig. 2E).

Hypoxic induction of human JMJD3 in hADSC
We tested whether hypoxia induces JMJD3 expression in human cells. qRT-PCR analyses revealed that hypoxia increased the mRNA levels of JMJD3 in hADSC. Clioquinol, an HIF-1α activator, also induced JMJD3 in these cells (Fig. 3A). Previous findings showed that Clioquinol inhibits the asparaginyl hydroxylase activity of FIH-1 and also the prolyl hydroxylation-dependent ubiquitination of HIF-1α, thus Clioquinol stabilized the transactive form of HIF-1α protein even under normoxia (Choi et al., 2006). We confirmed that Clioquinol also increased both the mRNA and protein levels of human JMJD3 in hADSC (Fig. 3B). We also found several HREs located in both upstream and the first intron of the human JMJD3 gene (Fig. 3C) (Cartharius et al., 2005). Differently from mouse JMJD3, in hADSC, ChiP analyses revealed that both HIF-1α and Amt bind HREs in the upstream region rather than on the first intron of human JMJD3 (Figs. 4A-4D). In order to detect any change of histone mark in the upstream region of human JMJD3, we performed ChiP analyses using antibodies detecting the methylation and acetylation of histone 3. We found that repressive histone marks such as H3K9me3 and H3K27me3 were maintained at undetectable low level in the upstream of the human JMJD3 gene, while hypoxia and Clioquinol increased the active histone marks H3K4me3 and H3K9/K14Ac (Figs. 4E-4F).
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**Fig. 3.** Hypoxic induction of human JMJD3 in hADSC. (A, B) hADSC were treated with hypoxia (<0.5% O₂, 48 h) or Clioquinol (50 μM, 16 h); (A) The relative mRNA levels of BNIP3 and JMJD3 were analyzed by qRT-PCR. The data represent the average and standard deviations of two independent experiments; (B) Western blot analyses were performed using the indicated antibodies. The results of two independent experiments were shown. The relative intensity of each band was measured as described in Fig. 1E. (C) The putative HREs in the human JMJD3 gene are identified as described in Fig. 2C. The bars indicate the positions of the primer sets used for the ChIP analyses. The nucleotide sequences of putative HRE sites (-4.1 kb, -4.0 kb, -3.3 kb) and flanking regions were shown (lower panel). The \( p \)-values were obtained using the Student’s \( t \)-test and the significance between the groups is indicated (** \( p < 0.05 \)).

**DISCUSSION**

In this study, we showed that hypoxia induced the expression of JMJD3 in an HIF-1α-dependent manner, and that the presence of Clioquinol, an HIF-1α activator, increased JMJD3 expression even under normoxia. ChIP analyses showed that both HIF-1α and HIF-1α/Arnt bound to the first intron region of the mouse JMJD3 gene, but to the upstream region of the human JMJD3, indicating that human and mouse JMJD3 have hypoxia regulatory regions in different locations. HIF-1α binding sites are often found far from the transcription start sites. In ARRD3 and ERRFI1 genes, HREs are located around -100 kb from the transcription start sites and activate the transcription (Schodel et al., 2011). HIF-1α binds HREs in open chromatin region of which H3K4 residues are tri-methylated (Xia and Kung, 2009). Consistently, our results indicated that HIF-1α bound to chromatin region of human JMJD3 gene which has a high level of H3K4me3.

The fact that JMJD3 uses molecular oxygen as a substrate suggests that the catalytic activity of JMJD3 can be inhibited under hypoxia. Interestingly, many hydroxylases, including collagen-prolyl hydroxylase and the H3K9me2 demethylase JMJD1A, maintain their catalytic activities even under severe hypoxia, because their \( K_m \) values for O₂ are very low. The \( K_m \) value for O₂ associated with JMJD3 remains to be estimated. The facts that HIF-1 can be activated by nonhypoxic signals such as mTOR pathways, oxidative stresses, and inflammatory signals, and that HIF-1 increases JMJD3 expression suggest that some of the nonhypoxic HIF-1-activating stimuli can increase the amounts of JMJD3 without reducing its catalytic activity. The biological importance of JMJD3 primarily relies on the function of its target genes. Since histone modifiers such as histone methyltransferase and demethylases do not have the ability to recognize specific DNA sequences, the targets for histone modifiers depend on their interacting transcription factors.

The tri-methylation of H3K27 (H3K27me3) of a certain gene is determined by the balance between the Polycomb Repressive Complex 2 (PRC2) and histone demethylases JMJD3 and UTX (Agger et al., 2007). H3K27me3 and H3K9me3 on the promoter and enhancer region are generally associated with the transcriptional inactivation, whereas H3K4me3 and the acetylation of H3K27 are associated with the transcriptional activa-
A subset of promoters marked with both H3K4me3 and H3K27me3 is also known as bivalent promoters (Bernstein et al., 2006). The bivalent promoters are inactive but able to drive the gene expression with the loss of H3K27me3 and gain of

**Fig. 4.** ChIP analyses of the human JMJD3 gene. hADSC were treated with hypoxia (< 0.5% O2, 48 h) or Clioquinol (50 μM, 16 h). (A-F) ChIP analyses were performed using the indicated antibodies and primer sets for the human JMJD3 gene; (A, C, and E) ChIP-eluted DNA was analyzed by PCR and visualized on 2% agarose gel electrophoresis. (B, D, and F) ChIP-eluted DNA was analyzed by qPCR. The values on the y-axis are presented as the percentage input of the average and standard deviation of triplicate determinations of qPCR. The primer set for the human BNIP3 promoter was used as the positive control. The p-values were obtained using the Student’s t-test and the significance between the groups is indicated (**p < 0.05, *p < 0.1).
H3K27Ac.

De Santa et al. (2007) showed that in macrophages JMJD3 can be induced by inflammatory stimuli such as lipopolysaccharide (LPS) and interferon (IFN)γ. JMJD3 is highly expressed in differentiating bone marrow cells, while it is repressed in terminally differentiated macrophages. Upon treatment with LPS or IFNγ, activated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) occupies the promoter region of JMJD3 and increases its transcription. LPS-induced JMJD3 activates a subset of genes by reducing the levels of the repressive histone mark H3K27me3. In macrophages, Bone morphogenic protein 2 (Bmp-2) is one of the targets of JMJD3. The promoter of the Bmp-2 gene contains a bivalent chromatin domain with high levels of both H3K4me3 and H3K27me3. LPS-induced JMJD3 demethylates H3K27me3 of the bivalent Bmp-2 promoter to increase the transcription of Bmp-2.

The locus of inhibitor of cyclin-dependent Kinase 4a (INK4a) or P16INK4a, P15INK4b inhibit CDK4/6 kinases leading to p21WAF1/CIP1 or p15(Ink4b) respectively. Histone mark H3K27me3. In macrophages, Bone morphogenic protein 2 (Bmp-2) is one of the targets of JMJD3.

Together with these findings, our study suggests that the expression of JMJD3 is regulated by many different transcription activators, including AP-1, NF-κB, and HIF-1α that are activated by senescence, inflammatory signals, hypoxia, and non-hypoxic HIF-1α activators. Therefore, hypoxia-induced JMJD3 can influence the demethylation of JMJD3 target genes such as Bmp-2, INK4a, ARF, and late HoxA4 genes such as HoxA7 and HoxA11.

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