Hypoxia enhances the expression of prostate-specific antigen by modifying the quantity and catalytic activity of Jumonji C domain-containing histone demethylases

Ho-Youl Lee, Eun Gyeong Yang* and Hyunsung Park*

Introduction

Mammalian cells adapt to hypoxia by inducing the expression of genes that enable anaerobic metabolism, oxygen delivery and cell survival. These diverse target genes are induced by a common heterodimeric transcription factor, hypoxia-inducible factor-α (HIF-α) (1). HIF-α is rapidly degraded in normoxic cells, whereas HIF-β, also known as Arnt (aryl hydrocarbon receptor nuclear translocator), is constitutively expressed. Under hypoxic conditions, HIF-α is stabilized and translocated into the nucleus where it forms a heterodimer with nuclear protein, Arnt (2). The stability and activity of the α-subunit is inhibited by posttranslational modification, specifically by hydroxylation. HIF-α hydroxylation is catalyzed by prolyl hydroxylase domain protein 2 (PHD2) and factor-inhibiting HIF-1 (FIH-1), which utilize molecular oxygen and α-ketoglutarate as cosubstrates (3). The hydroxylated proline residues of human HIF-1α are recognized by the E3 ubiquitin ligase function of the von Hippel–Lindau protein (pVHL), which mediates HIF-1α polyubiquitination and degradation by the 26S proteasome (4). The hydroxylation of human HIF-1α-asparagine residue 803 by FIH-1 prevents HIF-α from recruiting the CBP/p300 coactivator. A lack of available oxygen has been shown to reduce the activities of these two oxygen-dependent hydroxylases, resulting in the stabilization of the transactive form of HIF-1α.

Although similarities in their substrate specificities exist, PHD2 and FIH-1 exhibit distinctly different three-dimensional structures. The catalytic domain of PHD2 resembles that of collagen-specific prolyl-4-hydroxylase, whereas FIH-1 has the Jumonji C (JmjC) domain in its catalytic region. X-ray crystallographic analyses revealed that the catalytic domain of Jumonji C domain-containing histone demethylase 2A (JMJD2A/JHDM3A/KDM4a) is closely related to that of FIH-1 (5). JMJD2A demethylates the trimethylated ninth lysine residue of histone-3 (H3K9me3) by two sequential reactions; it first hydroxylates the methyl group using α-ketoglutarate, O2, Fe(II) and vitamin C, and then the resulting intermediate spontaneously releases formaldehyde (6). Amino acid homology analyses revealed that >100 proteins are identified as having the JmjC domain, and most of them exhibit demethylase activity with different substrate specificities (7). JMJD2A and JMJD2C demethylate H3K9me3. JMJD1A/HDM2A/KDM3a demethylates H3K9me2 and me1, and Jumonji/ARID domain-containing protein 1A (JARID1A) and JARID1B demethylate H3K4me3 (Figure 1A). The histone methylation affects gene expression in distinct localized patterns. Methylation of H3K4 in coding and regulatory regions is associated with active transcription, H3K9 methylation in the coding region is associated with active transcription, but in the regulatory region, it is associated with transcriptional inactivation (8).

The fact that JmjC proteins utilize O2 in order to catalyze demethylation suggests that their activities can be inhibited under hypoxic conditions. However, determination of the Km values for O2 associated with the individual JmjC isozymes are necessary in order to estimate their distinct sensitivities for O2. Interestingly, recent findings that several JmjC proteins are induced by HIF in response to hypoxic stress imply that their induction compensates their presumed reduction in catalytic activity under hypoxia (9–12). Thus, in response to hypoxic stress, histone methylation of individual genes can be dynamically regulated by the net change of both the activities and expression of the JmjC proteins involved in the transcriptional regulation of their targets.

JMJD2A, JMJD2B, JMJD2C, JMJD1A and LSD1 are often overexpressed in prostate cancers, and they promote aberrant activation of androgen receptor (AR) target genes such as prostate-specific antigen (PSA) and kallikrein-related peptidase 2 (KLK2) (13–16). PSA is a secreted chymotrypsin-like serine protease A, which cleaves proteins that affect cell proliferation, invasion and metastasis (17). Knockdown of JMJD1A and JMJD2C increased the occurrence of H3K9me2 and H3K9me3 on the PSA gene, respectively, thus, blocking AR-induced transcriptional activity (15,16). These findings indicate that androgen-induced PSA gene is a target regulated by JMJD1A and JMJD2C. Here, we investigated the changes in the histone modification of the PSA gene in response to hypoxia and determined whether or not the hypoxic environment in most malignant prostate cancers affects the activity and expression of JMJDs. This study found that pathological hypoxia (<0.5% O2) caused epigenetic changes of genes such as PSA and KLK2 by altering both the quantity and activity of histone demethylases.

Materials and methods

Cell culture, antibodies and plasmids

Human prostate cancer LNCaP cells, obtained from the American Type Culture Collection (Manassas, VA), and LNCaP-LN3 cells, obtained from Korea Cell Line Bank (Seoul, Korea), were maintained in RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum at 37°C and 5% CO2.
The LNCaP cells were maintained in 5% charcoal-stripped fetal bovine serum containing RPMI 1640 media for 3 days prior to dihydrotestosterone (DHT) treatment (18). The cells were cultured in an anaerobic incubator (Model 1029; Forma Scientific, Waltham, MA) with 5% CO₂, 10% H₂ and 85% N₂ at 37°C in order to produce pathological hypoxic conditions. We measured the residual O₂ concentration in this chamber using a Fyrite Analyzer (Bacharach, New Kensington, PA) with results of 0.1–0.5% residual oxygen. The cells were also treated to produce physiological hypoxia (3 or 5%) in an In Vivo 200 Hypoxia Workstation (Ruskinn Technology, Leeds, UK). Anti-Myc and anti-HIF-1α were purchased from IG Therapy (Chuncheon, Korea) and BD Bioscience (Franklin Lakes, NJ), respectively. Antibodies against JMJD1A, RBP5, JARID1A and JARID1B were purchased from Bethyl Laboratories (Montgomery, TX). Anti-JMJD2C and anti-G9a were purchased from Novus Biologicals (Littleton, CO) and Abcam (Cambridge, UK), respectively. Antibodies against histone H3, H3K9me3, H3K9me2, H3K4me3 and H3K9Ac/K14Ac were acquired from Millipore (Billerica, MA). Anti-histone H3K9me1 was purchased from Abcam.

Quantitative real-time reverse transcription–PCR
Two micrograms of RNA from LNCaP cells were analyzed using quantitative real-time reverse transcription–PCR (qRT–PCR) as described previously (19). The qRT–PCR was performed using the 7000 sequence detection system (Applied Biosystems, Warrington, UK) using Power SYBR Green Master Mix reagent (Applied Biosystems). The genes were amplified using the primers that are described previously: PSA (20); VEGF (21); CA9 (22); JMJD1A,

Fig. 1. Upregulation of PSA expression by hypoxia in LNCaP cells. (A) Specific substrates and cofactors for JMJD2, JMJD1 or JARID1. (B–D) LNCaP cells were pretreated with hypoxia (<0.5 or 3% O₂) for 2 h followed by DHT treatment (10nM) (hypoxia 18 h, DHT 16 h). (B) Western blot analyses were performed using the indicated antibodies. β-Actin was utilized as a loading control. (C) Relative messenger RNA levels of vascular endothelial growth factor, carbonic anhydrase 9, PSA and KLK2 were analyzed by qRT–PCR and normalized using 18S ribosomal RNA. The mean value of qRT–PCR from untreated LNCaP cells was set for 1. Data represent the average and standard deviations of three independent experiments. (D) A schematic diagram of cis-acting elements of the PSA gene. Bars indicate positions of the primer sets used for ChIP analyses. ChIP analyses were performed using the indicated antibodies and primer sets of the PSA gene. DNA was isolated from the LNCaP cells treated as shown on the x-axis. Values on the y-axis are presented as percentage input of the average and standard deviation of triplicate determinations of qPCR from two independent ChIP assays. Input values were obtained from samples treated in the same way as the experimental method, except that no immunoprecipitation steps were performed. The P values were obtained using Student’s t-test and significance between the groups is indicated (*P < 0.05, **P < 0.01, ***P < 0.001).
JMJD2A, JMJD2B and JMJD2D (12); JMJD2C (23); JARID1A, 1B, 1C and 1D (23). The primers for qRT–PCR were as follows: KL2K (5′-TGGAC ACCTGAAAGCTTGAAGACTC-3′; (R)5′-TACACCTGTGTCGGOCTC TTC-3′; Supplementary Table S2, available at Carcinogenesis Online, showed individual threshold cycle (Ct) values of qRT–PCR. Ct values were defined by ABI 7000 Real-Time PCR System.

Chromatin immunoprecipitation analysis and sequential chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously with the following modifications (25): 1 × 10^6 LNCaP cells were cross-linked in 1% formaldehyde at 37°C for 10 min, and then the nuclei were isolated (26). The isolated nuclei were treated with MNase (300 gel units, 15 min; NEB, Ipswich, MA) and sonicated to shear the chromatin (27). Insoluble materials were removed by centrifugation at 12 000 r.p.m. 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 same. For each immunoprecipitation, 50–60 µg of soluble chromatin was 10-fold diluted in ChIP dilution buffer (20 mM Tris–HCl (pH 8.0), 0.01% sodium dodecyl sulfate (SDS), 1% Triton X-100, 2 mM ethylenediaminetetraacetic acid and 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 (1000 µl) were immunoprecipitated with 2–4 µg of the indicated antibodies at 4°C overnight. The antibody–chromatin complexes were recovered by incubation with 20 µl of protein G-agarose (50% slurry) and sequentially washed as described previously (25). The immunocomplexes were eluted with 150 µl of elution buffer (1% SDS and 0.1 M NaHCO₃) and reversed. The precipitated sequential ChIP after the sequential ChIP chromatin immunoprecipitants were eluted twice by incubation with 15 µl of 10 mM dithiothreitol at 37°C for 30 min, diluted 40 times with ChIP dilution buffer followed by a second immunoprecipitation with the indicated antibodies. Three microliters of DNA samples from a total of 70 µl of ChIP preparations were analyzed using qPCR. The primers for the enhancer and promoter regions of the PSA and KLK2 gene were performed as described previously (15,18). The primer sets for upstream region (−3310 to −3183) of the KLK2 gene, which contained putative hypoxia-responsive elements (HREs), were: (F)5′-TGGACACCTGAAAGCTTGAAGACTC-3′ and (R)5′-TACACCTGTGTCGGOCTC TTC-3′. Supplementary Table S2, available at Carcinogenesis Online, showed individual Ct values of qPCR of ChIP. Ct values were defined by ABI 7000 Real-Time PCR System.

Glutathione-S-transferase pull-down assay

Glutathione-S-transferase (GST)–HIF-1α NT1-(1-401) or CT (401–826) were expressed in Escherichia coli (BL21 strain) and purified using glutathione-uniflow resin (GE Healthcare, London, UK). Resin-bound GST–HIF-1α proteins were mixed with whole cell lysate of LNCaP cells for 2 h at 4°C in the presence or absence of DHT (40 nM). Resin-bound protein complex was then washed three times with NETN (100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 20 mM Tris–HCl (pH 8.0), 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride. Proteins were eluted in 1× SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis and detected by western analysis.

Small interfering RNA transfection

LNCaP cells were maintained in RPMI 1640 containing 10% charcoal-stripped fetal bovine serum for 3 days. Cells (5 × 10^4 cells/60 mm dish) were transected with 50 nM of either JMJ1D1A small interfering RNA (siRNA) duplex (sense, 5′-CUG GUA UUU AGA CCG ACG AUC A-3′; antisense, 5′-UAG UCG GUC UAA AUA CCA GCA G-3′) or scramble siRNA duplex (sense, 5′-CCU ACG CCA AUC UCG UCG U-3′; antisense, 5′-AAC AAA UUG CUG GUC UAG G-3′) using PolyMag 200 reagent (Chemicell, Berlin, Germany) according to the manufacturer’s instructions. siRNAs were purchased from Bioneer (Taejon, Republic of Korea).

Results

Upregulation of PSA expression under hypoxia in LNCaP cells

LNCaP cells were exposed to 21% (normoxia), 3% or <0.5% O₂ for 18 h. The cells were also treated with AR ligand, DHT (10 nM, 16 h). Hypoxic treatment increased the expression of HIF-1α protein but not AR protein. DHT treatment also additionally increased the expression of HIF-1α protein (Figure 1B). qRT–PCR analyses showed that HIF-1α target genes, vascular endothelial growth factor and carbonic anhydrase 9 were also induced by severe hypoxia (<0.5% O₂), but not at physiological hypoxia (3% O₂) (Figure 1C). AR agonist, DHT, induced the AR target genes, PSA and KLK2, and that severe hypoxia (<0.5% O₂), but not physiological hypoxia (3% O₂), additionally increased their expression in DHT-treated LNCaP cells (Figure 1C). These results indicated that expression of AR target genes was enhanced by severe hypoxia. The PSA gene contains HREs at its enhancer region in addition to the androgen-responsive elements. ChIP analyses using HIF-1α antibody identified the HIF-1α occupancy on the enhancer region of the PSA gene in response to hypoxia but not on the promoter region. DHT treatment further increased HIF-1α occupancy on the enhancer under hypoxia (Figure 1D). ChIP analyses using AR antibody showed that more AR binds to the enhancer than to the promoter, and that hypoxia augments DHT-induced AR occupancy.

Induction and activity of JMJDs by hypoxia in LNCaP cells

JMJD1A and JMJD2C are known to be involved in the AR-dependent transcriptional activation of PSA gene by demethylating di- and trimethylated H3K9 on its enhancer region (14–16). JARID1B H3K4me3 demethylase, which is overexpressed in prostate cancer, is also associated with AR and increases its transcriptional activity (28). We investigated hypoxic effects on both the expression and activities of these JmJc histone demethylases. qRT–PCR confirmed that severe hypoxia induced JMJD1A >5-fold and induced JMJD2C and JARID1B >2-fold in LNCaP cells (Figure 2A) (9,11,12). Western blot analyses results shown in Figure 2B reveal that hypoxia increased the levels of JMJD1A, JMJD2C and JARID1B proteins. However, physiological hypoxia (3% and 5% O₂) does not induce JMJD1A (Supplementary Figure S1A, available at Carcinogenesis Online). None of these JMJDs were induced by treatment with DHT (10 nM, 16 h).

The finding that JmJc histone demethylases catalyze the hydroxylaton-dependent demethylation reaction using molecular oxygen suggests that their activities could be reduced under hypoxic condition (Figure 1A). In order to test the inhibitory effect of hypoxia on the JmJc histone demethylases, which are known to be involved in the AR-dependent expression of PSA, we transfected JMJD1A, JMJD2C or JARID1B into LNCaP cells and detected the levels of H3K9me3, H3K9me2 or H3K4me3 using immunofluorescence analysis. Overexpression of JMJD1A in LNCaP cells decreased the levels of both H3K9me2 and H3K9me1 under normoxia and 3% oxygen. Interestingly, the activity of JMJD1A was maintained even under severe hypoxia (<0.5% O₂) (Figure 2C and D). In contrast, we found that the activity of JMJD2C is maintained under 3% O₂ but not under severe hypoxia (<0.5%). Overexpressed JARID1B decreased the cellular H3K4me3 level in both normoxia (21% O₂) and physiological hypoxia (3% O₂), whereas their activities were not observed under severe hypoxia (<0.5% O₂). H3K9me3 demethylase JMJD2A and H3K4me3 demethylase JARID1A are also inhibited under hypoxia (Supplementary Figure S1B and C, available at Carcinogenesis Online). These findings suggested that these histone demethylases have different sensitivities to O₂; the Kₘ value of JMJD1A for O₂ is expected to be the least, suggesting that JMJD1A maintains its H3K9me2 demethylation activity even under severe hypoxic conditions.

The finding that pathological hypoxia (<0.5% O₂) inhibited the catalytic activities of JMJD2C and JARID1B even when they were overexpressed in LNCaP cells suggests that their hypoxic inhibition, if any, may not override the hypoxic inhibition of its activities. In contrast, hypoxia increased the expression of JMJD1A without inhibiting its catalytic activity, indicating that hypoxia increased the total activity of JMJD1A. Taken together, these findings implied that hypoxia may decrease H3K9me2 and me1 levels while increasing H3K9me3 and H3K4me3 levels, unless other methyltransferases and demethylases involved in H3K9me2, me1 and H3K4me3 are changed in opposite direction in response to hypoxia.

Hypoxic effect on histone modification of the PSA enhancer

The induction of PSA accompanies the decrease of methylated H3K9 and increase of acetylated H3K9/K14 on its enhancer. The finding that knockdown of either JMJD1A or JMJD2C abolishes the DHT-induced
demethylation of H3K9me on the PSA gene suggests that JMJD1A and JMJD2C are key isoenzymes for the sequential demethylation of the PSA gene from H3K9me3 to H3K9 in response to the AR ligand (15,16). Our finding that the expression and catalytic activities of JMJD2C and JMJD1A are regulated differently by hypoxia suggests that the levels of H3K9me3, me2 and me1 associated with the PSA gene could be modified differently under hypoxia. First, we tested the enhancer occupancy of the histone modifiers. ChIP analyses showed that JMJD1A, JMJD2C and JARID1B were detected in significant quantities on the enhancer of the PSA gene compared with ChIP

Fig. 2. Induction and activity of JMJDs by hypoxia in LNCaP cells. (A and B) LNCaP cells were pretreated by hypoxia (<0.5% O₂) for 2 h, followed by 10 nM DHT treatment for 16 h (hypoxia 18 h, DHT 16 h). (A) Relative messenger RNA levels of the indicated histone demethylases were analyzed by qRT–PCR and normalized using 18S ribosomal RNA. The *P values were obtained using Student’s t-test and significance between the groups is indicated (*P < 0.05, **P < 0.01). (B) Western blot analyses were performed using the indicated antibodies. β-Actin was measured for use as a loading control. (C) LNCaP cells were seeded on a glass coverslip at a density of 3 × 10⁴ cells/coverslip. After 20 h, the cells were transfected with 1 µg of Flag-tagged JMJD1A, Flag-tagged JMJD2C or Myc-tagged JARID1B. The transfected cells were treated by hypoxia (3 or <0.5% O₂) for 16–18 h, then immunostained using the corresponding tag antibodies and indicated histone antibodies. The nuclei were stained with 4’,6-diamidino-2-phenylindole. The confocal microscopic images of the stained LNCaP cells were produced using a Zeiss LSM510 microscope. The scale bar corresponds to 10 µm. Using a Pseudo-Color 3D confocal fluorescence microscope, the immunofluorescence intensities of H3K9me3, me2, me1 or H3K4me3 were estimated as shown in the right columns. Arrows indicate typical cells from 3 to 22 observed cells transfected with the indicated histone demethylases (as shown in the Supplementary Table S1, available at Carcinogenesis Online). (D) Based on methylated histone values for the non-transfected cells of 100%, the relative intensities of the methylated histone from the cells transfected with the indicated histone demethylase were estimated. Data represent the average and standard deviations of the estimated activities of histone demethylases from 3 to 22 transfected cells.
with IgG (Figure 3A–C). We also found that RbBP5, a subunit of the H3K4 methyltransferase MLL4 complex and G9a, a H3K9 methyltransferase, were significantly bound to the enhancer (Supplementary Figure S2, available at Carcinogenesis Online) (29). In contrast to the others, the occupancy of JMJD1A on the enhancer was augmented, not only by the AR ligand, but also by hypoxia. Similarly, DHT and hypoxia synergistically increased the occupancy of histone acetyltransferase p300 on the PSA enhancer (Figure 3D).

We also evaluated the changes of H3K9me1, H3K9me2, H3K9me3 and H3K4me3 on the enhancer region of the PSA gene in response to hypoxia and/or DHT. In either the absence or presence of DHT, hypoxic treatment (<0.5% O2) further diminished the levels of both H3K9me2 and H3K9me1 but increased the appearance of H3K9me3 on the enhancer region of PSA (Figure 3E and F). These results were consistent with the finding that severe hypoxia exposure induced the expression of JMJD1A without inhibiting its catalytic activity but inhibited the catalytic activities of JMJD2C (Figure 2).

ChIP analyses using an antibody specific for acetylated K9 and acetylated K14 residues of histone-3 (H3K9Ac and H3K14Ac) showed that hypoxia augmented the ligand-induced acetylation of H3K9/K14 residues on the PSA enhancer region (Figure 3G). In contrast to methylation, acetylation of the same K9 residue in the enhancer region increased transcription. Under severe hypoxia, despite the increased quantity of H3K9me3, reduction of both H3K9me2 and me1 eventually produced unmethylated K9 residues of H3, which were able to be acetylated. Similarly, hypoxia dramatically increased the quantity of H3K4me3 on the PSA enhancer region in both the DHT-treated and untreated LNCaP cells (Figure 3H). These results were consistent with the finding that the catalytic activity of JARID1B was inhibited by severe hypoxia (Figure 2C). The fact that H3K4me3 and H3K9Ac/H3K14Ac were often found in the enhancer region of the active genes was consistent with the finding that hypoxia increased the expression of PSA. ChIP analyses using anti-H3 antibody revealed that upon response to DHT, the nucleosome in the enhancer region was dismissed in part (Figure 3I).

We normalized the occupancy of the modified histones, H3K9me3, me2 and me1, H3K4me3 and H3K9Ac/H3K14Ac by the H3 quantity appearing on the enhancer region (Supplementary Figure S3, available at Carcinogenesis Online). Normalization by the H3 amount did not change the finding that hypoxia decreased the H3K9me2 and me1 but instead increased H3K9me3, H3K4me3 and H3K9/K14Ac in the PSA enhancer region. We also found similar epigenetic changes of the PSA gene in response to hypoxia in other prostatic cancer cell lines, LNCap-LN3, which are AR positive and castration resistant (Supplementary Figure S4, available at Carcinogenesis Online) (30).

Cooperation of JMJD1A, AR and HIF-1α on the PSA enhancer

Our finding that hypoxia increased the occupancy of HIF-1α and JMJD1A on the PSA enhancer suggests the possibility that

Fig. 3. Effect of hypoxia on histone modification of the PSA enhancer. LNCaP cells were pretreated by hypoxia (<0.5% O2) for 2 h, followed by DHT treatment (10nM) for 16 h (hypoxia 18 h, DHT 16 h). (A–I) ChIP analyses for the PSA enhancer region were performed using the indicated antibodies (black bar) or IgG (white bar). Data were analyzed as described in Figure 1D. The P values were obtained using Student’s t-test and significance between the groups is indicated (*P < 0.05, **P < 0.01, ***P < 0.001).
the enhancer-bound HIF-1α recruits JMJD1A together with AR (16,31,32). We tested whether or not HIF-1α interacts with JMJD1A using GST-fused HIF-1α protein expressed in bacteria. Resin-bound human HIF-1α protein was incubated with the lysate from hypoxic LNCaP cells in the presence or absence of DHT, and then the captured proteins were analyzed using anti-JMJD1A antibody. Western blot results (Figure 4A) indicated that JMJD1A interacts with the N-terminal region of HIF-1α (1–401 amino acids). In order to confirm the interaction between endogenous JMJD1A and HIF-1α, we treated the LNCaP cells with DHT under hypoxia (<0.5% O2). We immunoprecipitated the lysate with anti-JMJD1A antibody and then detected the coprecipitated HIF-1α. We confirmed that HIF-1α interacted with the endogenous JMJD1A (Figure 4B).

In addition to the previous findings that AR interacts with HIF-1α and that AR binds to JMJD1A, our finding that HIF-1α also interacts with JMJD1A suggests that the enhancer-bound AR encouraged HIF-1α to occupy the HRE of the PSA enhancer, and that both AR and HIF-1α additively recruited JMJD1A to the enhancer region of the PSA gene. In order to test this hypothesis, we performed sequential ChIP. First, we precipitated the isolated chromatin from LNCaP cells with anti-JMJD1A antibody and then, incubated the precipitant with anti-AR antibody, anti-HIF-1α antibody or IgG. The presence of the PSA enhancer in the sequential precipitant was determined by qPCR. Hypoxic treatment was found to increase the co-occupancies of both JMJD1A/AR and JMJD1A/HIF-1α on the enhancer. Furthermore, treatment with DHT increased both co-occupancies on the enhancer (Figure 4C). Therefore, these sequential ChIP assays suggested that DHT and hypoxia additively increased the recruitment of the JMJD1A on the enhancer of PSA, presumably by interaction between the PSA enhancer, AR, HIF-1α and JMJD1A.

**Hypoxic effect on histone modification of the KLK2 enhancer**

Hypoxia also enhanced DHT-induced expression of other AR target genes such as KLK2 (Figure 1C). We searched the minimal HRE consensus sequences (ACGTG) between −1 and −8000 bp from the transcription start site. We found two putative HRE consensus sequences at −2778 and −3230 bp in KLK2 gene (Figure 5A). ChIP analysis using the HIF-1α antibody did not detect HIF-1α on either of the putative HRE sites or on the KLK2 promoter (Figure 5B). Instead, ChIP analyses using anti-AR and anti-JMJD1A antibodies detected DHT-induced occupancy of AR and JMJD1A, respectively, on the enhancer region. Furthermore, under hypoxia, more JMJD1A bound to the KLK2 enhancer in the presence of DHT (Figure 5C). Sequential ChIP analyses showed that hypoxic treatment increased the co-occupancy of JMJD1A/AR, but not JMJD1A/HIF-1α, on the enhancer of the KLK2 gene (Figure 5D). Hypoxia further decreased the existence of H3K9me2 and H3K9me1 and increased the amount of H3K9me3 and H3K4me3 (Figure 5E). Nonetheless, hypoxia did not enhance the DHT-induced acetylation of H3K9/
K14 residues or the recruitment of p300 on the enhancer region of \textit{KLK2} (Figure 5E). These findings reflected the fact that the \textit{KLK2} gene did not strongly recruit HIF-1\(\alpha\) that would additively recruit p300 on the \textit{KLK2} gene. The findings that hypoxia increased the recruitment of JMJD1A on the enhancer regions of both PSA and \textit{KLK2}, indicate that JMJD1A contributes hypoxic induction of both AR target genes. We knocked down the expression of JMJD1A in LNCaP cells by using siRNA against it (Figure 6A). We found that hypoxia enhances the DHT-induced expression of both PSA and \textit{KLK2} in the LNCaP cells treated with control siRNA but not in the cells treated with siRNA against JMJD1A (Figure 6A). These findings that knockdown of JMJD1A canceled hypoxic induction of these genes indicate that hypoxic signal is conveyed to the AR target genes by increasing the expression of JMJD1A, which maintains catalytic activity even under hypoxia. This study provided insight into how \(O_2\)-dependent histone demethylase activity affects epigenetic changes and expression of androgen-induced PSA genes (Figure 6B).

### Discussion

Using an \(O_2\) microelectrode, the oxygen concentration of prostate cancer tissues from 59 patients was measured. The partial \(O_2\) pressure in the prostate cancer tissues was significantly lower than that in normal tissue. The average median \(pO_2\) in prostate cancer tissue was 2.4 mmHg (0.3% \(O_2\)), whereas the average median of periprostatic muscle tissue was 30 mmHg (3.9% \(O_2\)) (33). This study showed that pathological hypoxia (<0.5% \(O_2\)) regulated gene expression by changing the amount and activity of several histone demethylases in human prostate cancer LNCaP cells. We investigated the hypoxic influence on both amount and activity of JMJD1A, JMJD2A, JMJD2C and JARID1B, which are the major known histone modifiers for induction of AR target genes such as PSA and \textit{KLK2} (14–16, 28). This study showed that under pathological hypoxia, the catalytic activities of JMJD2A, JMJD2C and JARID1B were blocked due to lack of their cosubstrate, i.e. oxygen. Consistent to this result, ChIP analyses of the PSA enhancer showed that hypoxia increased the appearance of

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**Fig. 5.** Hypoxic effects on histone modifications of the \textit{KLK2} gene. (A) A schematic diagram of the \textit{cis}-acting elements of the \textit{KLK2} gene. Bars indicate positions of the primer sets for the putative HRE site, enhancer and promoter used for ChIP analyses. (B–E) LNCaP cells were pretreated by hypoxia (<0.5% \(O_2\)) for 2 h, followed by DHT treatment (10 nM) for 16 h (hypoxia 18 h, DHT 16 h). (B) ChIP analyses for the putative HRE site and promoter region of the \textit{KLK2} were performed using HIF-1\(\alpha\) antibody. (C and E) ChIP analyses for the \textit{KLK2} enhancer region were performed using the indicated antibodies (black bar) or IgG (white bar) as described in Figure 3. (D) Sequential ChIP analyses were performed as described in Figure 3C, except for the use of the primer set for the \textit{KLK2} enhancer region. The \(P\) values were obtained using Student’s \(t\)-test and significance between the groups is indicated (*\(P<0.05\), **\(P<0.01\), ***\(P<0.001\)).
Hypoxic reprogramming of histone modifications

The expression of JMJD1A was induced by hypoxia, leading to the demethylation of H3K9me2 and H3K9me1. This demethylation allowed for the recruitment of histone acetyltransferase, CBP/p300, which resulted in the activation of the PSA gene. Hypoxia increased the expression of JMJD1A, which in turn contributed to the epigenetic conversion of the PSA gene from a repressive status to a naive status, i.e., H3K9me2 to H3K9.

Recent ChIP-sequencing analyses found that 70% of AR-binding sites were co-occupied with forkhead box protein A1 (FoxA1) in LNCaP cells. Knockdown of FoxA1 in LNCaP cells revealed three categories of AR-binding sites: (i) FoxA1-enhanced; (ii) FoxA1-dispensable and (iii) FoxA1-suppressed AR-binding sites. Both PSA and KLK2 enhancers belong to FoxA1-dispensable AR-binding sites on which FoxA1 knockdown does not change AR occupancies. The findings that active histone marks, H3K4me2 and me1, and a repressive histone mark, H3K9me2, determine FoxA1 binding to chromatin (37), and that hypoxia can change these histone marks imply that hypoxia can redistribute AR binding to chromatin by changing the levels of H3K9me2, H3K4me2 and H3K4me1 in FoxA1-enhanced or FoxA1-suppressed AR-binding sites. We selected two AR bindings sites within each category: Ras association (RalGDS/AF-6) domain family member 3 (RASSF3) and breast cancer metastasis-suppressor 1-like (BRMS1L) within FoxA1-enhanced AR enhancer category; sex-determining region Y-box 9 (Sox9) and Dynamin 2 (DNM2) within FoxA1-suppressed AR enhancer category. DHT treatments decreased both repressive and active histone marks, H3K9me2 and H3K4me1, and increased FoxA1 occupancy on both RASSF3 and BRMS1L enhancers. However, hypoxia did not change either the occupancies of FoxA1 and AR or the histone marks on these enhancers. Neither DHT nor hypoxia changed the levels of H3K4me2 and H3K9me2 on Sox9 and DNM2 enhancers, which belong to FoxA1-suppressed AR-binding sites (Supplementary Figure S5, available at Carcinogenesis Online). These results indicated that hypoxia did not change FoxA1 occupancy on any of these enhancers. Several studies using non-prostatic cell lines showed that hypoxia increases whole amounts of H3K9me2, H3K4me2 and H3K4me1 (38,39). Nonetheless, histone methylation marks of an individual enhancer may be differently regulated by hypoxia, depending on unique combination of histone methyltransferases and demethylases, which are recruited on its enhancer.

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Protein kinase C-related kinase 1 (PRK1) is recruited to the PSA enhancer in response to AR ligand, and there it phosphorylates the H3T11 residues on the PSA enhancer region. Phosphorylated H3T11 residues enhance demethylation of H3K9me3 by JMJD2C (40). The finding that knockdown of PRK1 attenuated DHT-induced demethylation of H3K9me3, me2 and me1 suggests that phosphorylation of H3T11 by PRK1 is prerequisite for AR-dependent demethylation of H3K9me3 and me2. Recent findings revealed that hypoxia increases PRK1 activity in PC12 cells implies that hypoxia enhances the H3K9me3 level in the PSA enhancer in a manner other than by reducing PRK1 activity (41). Further studies will be needed in order to determine whether or not hypoxia also enhances the ability of PRK1 to phosphorylate the H3T11 residues on the PSA enhancer region.
to contribute H3T11 phosphorylation-dependent H3K9me2 demethylation by JMJD1A in LNCaP cells. Protein kinase Cβ1 is known to phosphorylate H3T6 during AR-dependent gene activation. The phosphorylated H3T6 residue prevents JARID1B from demethylating the neighboring H3K9me1 residue (42). It also remains to be determined whether or not hypoxia increases the phosphorylation of H3T6 on the PSA enhancer region through the activation of protein kinase Cβ1.

Androgen exposure induces the PSA gene by histone modification and altering the nucleosome positioning at its enhancer region. Jia et al. (18) showed that DHT treatment reduces H3 density at both the PSA enhancer and promoter. By using a new quantitative model for analyzing the nucleosome region, He et al. (43) showed that nucleosomes at AR-binding sites are dismissed in response to androgen treatment. They found that nucleosomes at the AR-binding site are more probably to contain the H2A.Z variant, which is easily dissociated upon AR binding. Our ChIP analyses using H3 antibody (Figure 3I) confirmed the reduction of H3 proteins at the enhancer of PSA upon DHT treatment. In order to account for the changes in histone methylation, but not for the changes in total amount of H3 in the PSA enhancer region, we normalized methylated K residues by the H3 amount detected on the PSA enhancer region. Normalization did not significantly change the epigenetic effects of either hypoxia or DHT, except that under hypoxia, DHT exposure increased the quantity of H3K4me3 residues at a given amount of the occupied H3 protein (Supplementary Figure S3, available at Carcinogenesis Online).

Previous studies demonstrated that the hypoxic microenvironment contained in prostate cancer tissues promotes aberrant AR activity in the absence of androgens, and thus, the onset of androgen independence. Under pathological hypoxia, AR is often found to be bound to the androgen-responsive element and induces the PSA gene in the absence of ligand (13). Khandrika et al. (2009) Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. Proc. Natl Acad. Sci. USA, 106, 4260–4265.


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