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The histone demethylase JMJD1A regulates adrenomedullin-mediated cell proliferation in hepatocellular carcinoma under hypoxia

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ABSTRACT

We studied the roles of JMJD1A and its target gene *ADM* in the growth of hepatocellular carcinomas (HCCs) and breast cancer cells under hypoxic conditions. Hypoxia stimulated HepG2 and Hep3B cell proliferation but had no effect on MDA-MB-231 cell proliferation. Interestingly, the *JMJD1A* and *ADM* expressions were enhanced by hypoxia only in HepG2 and Hep3B cells. Our ChIP results showed that hypoxia-induced HepG2 and Hep3B cell proliferation is mediated by JMJD1A upregulation and subsequent decrease in methylation in the *ADM* promoter region. Furthermore, *JMJD1A* gene silencing abrogated the hypoxia-induced *ADM* expression and inhibited HepG2 and Hep3B cell growth. These data suggest that JMJD1A might function as a proliferation regulator in some cancer cell types.

1. Introduction

Hypoxia plays an important role in the progression of human cancers. When cancer cells are exposed to hypoxic condition, several adaptive mechanisms, including glycolytic metabolism, apoptosis, angiogenesis, invasion, migration, and proliferation, are triggered [1–5]. Hypoxia inducible factor 1 (HIF1) is a key regulator of these processes. In hypoxia, stabilized HIF1 promotes the expression of hundreds of hypoxia-response genes, such as epidermal growth factor (*EGF*), insulin-like growth factor 1 and 2 (*IGF1*, *IGF2*), and platelet-derived growth factor (*PDGF*) [6,7]. Hypoxia also induces chromatin remodeling through epigenetic mechanisms. For example, global histone modifications are altered under hypoxic conditions in various cell lines [8]. In this regulation, methylation of lysine residues on histone H3 plays an important role in target gene expression.

Previous study showed that hypoxic induction of JMJD2B, a member of the Jumonji C (JmjC) domain-containing family of histone demethylases, regulates cyclin A1 (*CCNA1*) expression, thereby enhancing gastric cancer cell growth [9]. This study showed that the JmjC family plays a regulatory role in cancer cell growth induced by hypoxia. JmjC domain-containing proteins are histone lysine demethylases that can remove all three (mono-, di- and tri-) methyl groups from histone lysines [10]. One JmjC domain-containing protein, JMJD1A, specifically antagonizes mono- and di-methyl H3K9 (H3K9me1 and H3K9me2, respectively) in mammalian cells [11]. The demethylase activity of JMJD1A influences diverse functions, including spermatogenesis, obesity, and carcinogenesis [12–15]. Interestingly, much evidence showed that the expression of JMJD1A is upregulated in a HIF1-dependent manner [16–19]. Although little is known about the functional roles of JMJD1A during hypoxia, the evidence suggested that hypoxic induction of JMJD1A stimulates the expression of HIF target genes such as adrenomedullin (*ADM*). Krieg et al. showed that the expression of ADM is regulated by JMJD1A under hypoxia in colon cancer, and that ADM facilitates the growth of colon carcinoma cells [20]. However, the regulatory mechanisms of JMJD1A and its target gene *ADM* during hypoxia have not yet been elucidated in hepatocellular carcinoma (HCC) and breast cancer cells.

In this study, we demonstrate the functional roles of JMJD1A and ADM in HepG2, Hep3B, and MDA-MB-231 cell proliferation under hypoxia. The expression of JMJD1A and ADM were differentially regulated by hypoxia in a cell line-specific manner. Hypoxia-mediated upregulation of JMJD1A and ADM was observed only in HepG2 and Hep3B cells, but not in MDA-MB-231 cells. Moreover, increased JMJD1A expression induced a decrement of H3K9

Abbreviations: JMJD1A, Jumonji domain-containing 1A; ADM, adrenomedullin.

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di-methylation in the ADM promoter region, thereby stimulating HepG2 and Hep3B cell proliferation.

2. Materials and methods

2.1. Cell culture

Human hepatocellular carcinoma cells (HepG2, Hep3B) and human breast cancer cells (MDA-MB-231) were grown in DMEM (WelGENE Inc.) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 5% antibiotic–antimycotic (Gibco). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 21% O_2. For hypoxia treatment, cells were incubated in a chamber (5% CO_2, 1% O_2) for 24 h.

2.2. Cell proliferation assay

Cell proliferation was assessed by MTT assay. Briefly, HepG2, Hep3B, and MDA-MB-231 cells (2×10^5 cells/well) were seeded in 6-well plates and incubated at 37 °C overnight (O/N). Cells were then exposed to normoxia or hypoxia (1% O_2) for 24 h. Subsequently, cells were washed twice with PBS, and 5 mg/mL MTT in PBS was added to each well for 4 h. After removal of the MTT solution, solubilization solution (DMSO/EtOH, 1:1 ratio) was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was measured using a Paradigm™ Detection Platform (Beckman Coulter).

2.3. Western blot analysis

Total cell lysates were loaded onto SDS–PAGE and transferred to PVDF (GE Healthcare). Protein bands were visualized using a Fusion FX5 system (Vilber Lourmat). The following primary antibodies were used: anti-JMJD1A (Bethyl), anti-ADM (Abcam), anti-HIF1α (Novus), and anti-β-actin (Sigma).

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted using an RNeasy Kit (QIAGEN) according to the manufacturer's protocol. The quantity of isolated RNA was measured using NanoDrop (Thermo Scientific), and 1 μg of RNA was reverse-transcribed using an iScript™ cDNA synthesis kit (Bio-Rad). The following qPCR primers were used: sense JMJD1A 5′-GCAAAGGACACGGAGAAGAT-3′ and antisense JMJD1A 5′-CCCAGCCTTGAA CTCCATA-3′; sense ADM 5′-GGAAGAGGGAAC TGCGGATGT-3′ and antisense ADM 5′-GGCATCCGGACTGCTGTCT-3′; sense β-actin 5′-AGCGAGCATCCCCCA AAGTT-3′ and antisense β-actin 5′-GGGCACGAAGGCTCATCATT-3′.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously [21]. The following primers were used: sense ADM 5′-GCTCACGCTC-GACTCTCTTT-3′ and antisense ADM 5′-GCCCGGACTCACTTCTTTC-3′. Anti-JMJD1A (Abcam), anti-H3K9me2 (Abcam), and anti-H3ac (Millipore) antibodies were used to immunoprecipitate chromatin fragments.

2.6. Depletion of JMJD1A

The SHC002 (non-target shRNA) vector and shRNA specific for JMJD1A in the pLKO-puro vector were purchased from Sigma. HepG2 and Hep3B cells was transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. The JMJD1A-shRNA sequence used was: 5′-CCGGCCCA AGATGTATAATG CTTATCTCGAGATAAGCATTATACATCTTGGGTTTTT-3′. The SHC002 vector was used as a control.

2.7. In vivo experiments

Female BALB/c nude mice, 6 weeks old, were obtained from Japan SLC, Inc., Japan. HepG2 cells (5×10^6 cells stably transfected with nonsilencing control and JMJD1A shRNA) were inoculated subcutaneously into BALB/c nude mice. After 24 days, the mice were sacrificed, and tumor volume was estimated by $(\text{short axis})^2 \times (\text{long axis}) \times 0.5$.

3. Results

3.1. Differential expression of JMJD1A and ADM in various cancer cells under hypoxia

Because hypoxia modifies the rate of tumor cell growth, we first checked the proliferation of HCC cells (HepG2 and Hep3B) and breast cancer cells (MDA-MB-231) under normoxic and hypoxic conditions. When cells were exposed to acute hypoxic conditions, proliferation increased in HepG2 and Hep3B cells, but not in MDA-MB-231 cells (Fig. 1A). Next we investigated whether JMJD1A and ADM are responsible for hypoxia-induced HepG2 and Hep3B cell proliferation. As shown in Fig. 1B, the protein levels of JMJD1A and ADM were upregulated under hypoxia in both HepG2 and Hep3B cells, whereas no changes were observed in MDA-MB-231 cells. Similar to the data obtained by **western blot, the mRNA expression of JMJD1A and ADM was significantly induced by hypoxia in HepG2 and Hep3B cells, but not in MDA-MB-231 cells (Fig. 1C and D). These results suggest that JMJD1A and ADM are key regulatory factors for hypoxia-induced cell proliferation in HepG2 and Hep3B cells, but not in MDA-MB-231 cells.

3.2. Increased localization of JMJD1A to the ADM promoter in HCC cells under hypoxia

To assess the effect of JMJD1A on *ADM* gene transcription, we determined whether JMJD1A interacts with the *ADM* promoter in HepG2, Hep3B, and MDA-MB-231 cells using chromatin immunoprecipitation (ChIP) assays. In hypoxic conditions, the binding of JMJD1A to the *ADM* promoter increased significantly in HepG2 and Hep3B cells, compared to that under normoxic conditions (Fig. 2A and 2B, left panel). However, increased occupancy of JMJD1A on the *ADM* promoter was not detected in MDA-MB-231 cells under hypoxia (Fig. 2C, left panel). Because JMJD1A demethylates H3K9, we next examined the level of H3K9me2 at the JMJD1A binding site on the *ADM* promoter using ChIP analysis. The level of H3K9me2 was remarkably reduced under hypoxic conditions in HepG2 and Hep3B cells, but not in MDA-MB-231 cells, which is consistent with the binding pattern of JMJD1A to the *ADM* promoter (Fig. 2A, 2B, and 2C, middle panel). By contrast, the level of H3 acetylation, a known activation marker for gene transcription, was increased under hypoxia on the *ADM* promoter in HepG2 and Hep3B cells, but not in MDA-MB-231 cells (Fig. 2A, 2B, and 2C, right panel). Taken together, these results demonstrate that the increased binding of JMJD1A to the *ADM* promoter during hypoxia lowers the level of histone H3K9 methylation, thereby activating the transcription of *ADM* in HepG2 and Hep3B cells.

3.3. Enhanced HCC cell proliferation mediated by JMJD1A and ADM in vitro

Because the increases in HepG2 and Hep3B cell proliferation induced by hypoxia could be regulated by cellular factors other than JMJD1A and ADM, we used lentiviral shRNA vectors to generate

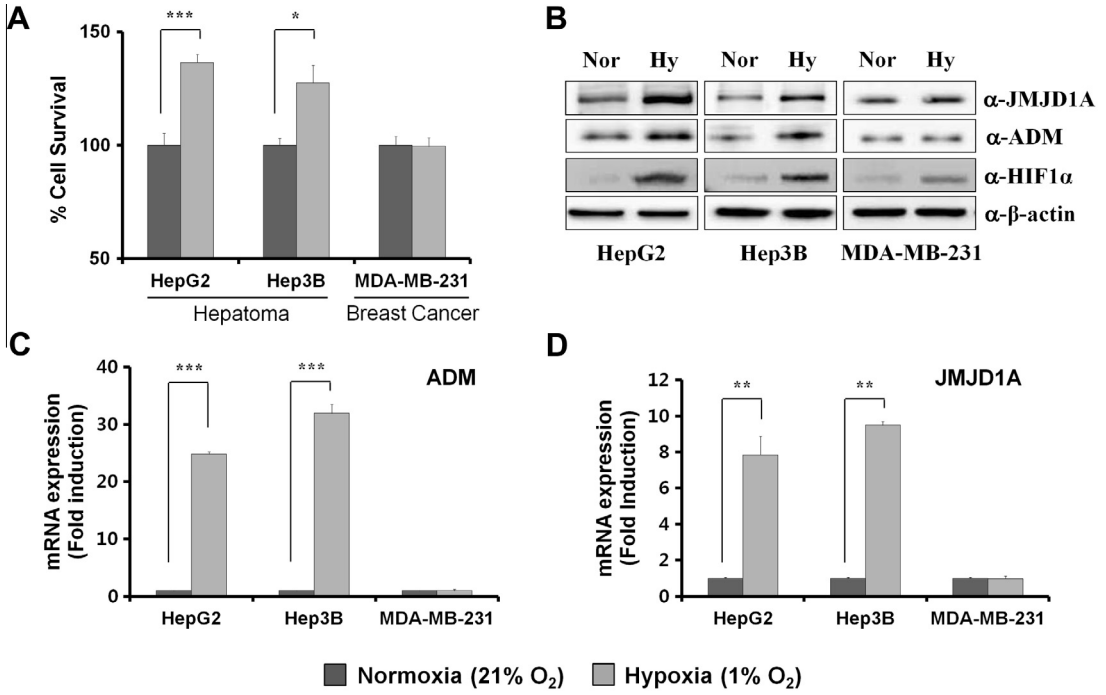

Fig. 1. Expression of JMJD1A and ADM in various cancer cells under hypoxia. (A) The results of the MTT assay were assessed in hypoxic (1% O_2) or normoxic (21% O_2) conditions. Graph represents the data (mean ± standard deviation) from triplicate wells. The average absorbance values were plotted on the *y*-axis with each culture condition on the *x*-axis. (B) JMJD1A and ADM protein expression patterns in hepatoma cells (HepG2, Hep3B) and breast cancer cells (MDA-MB-231) under hypoxic or normoxic conditions. Samples were western blotted and analyzed with anti-JMJD1A, anti-ADM, anti-HIF1α, and anti-β-actin antibodies. (C and D) *JMJD1A* and *ADM* mRNA expression patterns in hepatoma and breast cancer cells under hypoxic or normoxic conditions were assessed by quantitative RT-PCR. *P*-values were calculated using Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

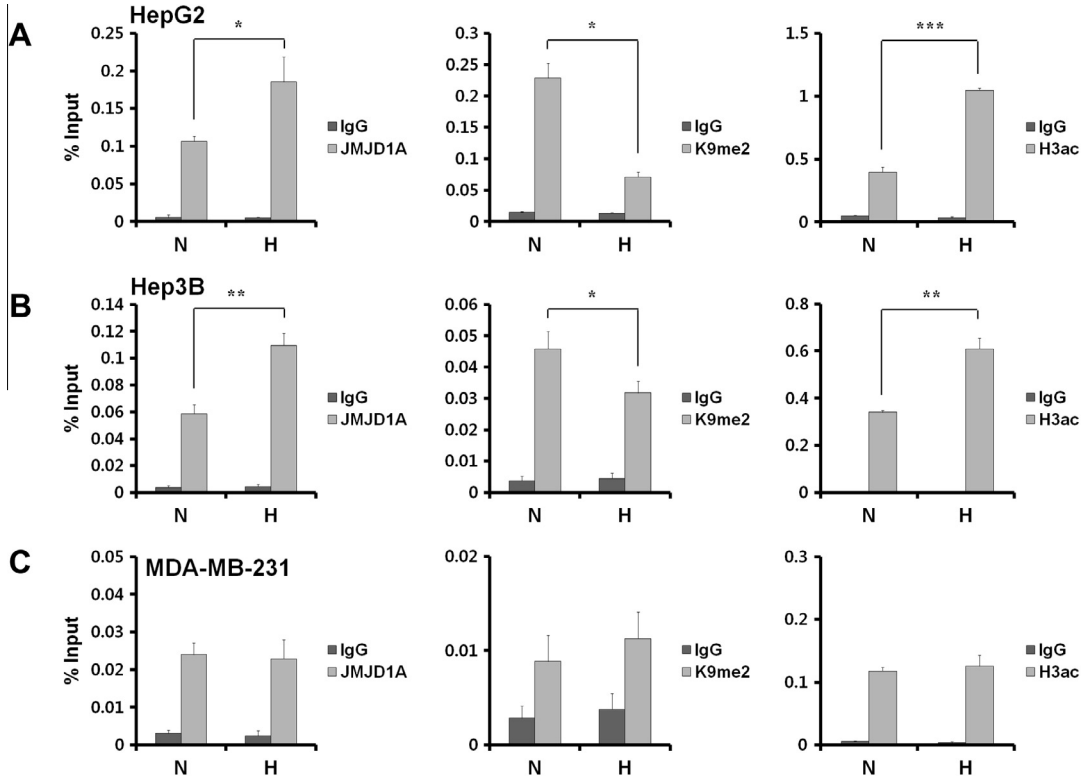

Fig. 2. JMJD1A binds to the *ADM* promoter in HCC cells under hypoxia. (A) Chromatin immunoprecipitation (ChIP) assays were performed to evaluate the occupancy of JMJD1A, H3K9me2 and H3ac at the *ADM* promoter region in HepG2, (A), Hep3B (B) and MDA-MB-231 (C) cells under normoxic (N) or hypoxic (H) conditions. Cross-linked and sheared chromatin was immunoprecipitated with anti-JMJD1A, anti-H3K9me2 or anti-H3ac antibodies, and the DNA was analyzed by PCR using ADM-specific primers. The result is shown as a percentage of the input chromatin. *P*-values were calculated using Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

stable HepG2 and Hep3B cell lines depleted of JMJD1A. Nonsilencing (NS) control vector was also transfected to control for any off-target effects of shRNA. The depletion of JMJD1A protein and mRNA was measured by western blot (Fig. 3A) and real-time PCR (Fig. 3B), respectively. We then determined the effect of JMJD1A depletion on *ADM* transcription in HepG2 and Hep3B cells. Under normoxic conditions, minimally reduced expression of *ADM* mRNA was observed in both cell lines. However, ADM expression was decreased by 30–40% in JMJD1A-depleted cells (shJMJD1A) compared with expression in control (NS) cells, under hypoxic conditions (Fig. 3C). To further evaluate the effects of JMJD1A depletion on HepG2 and Hep3B cell proliferation, we measured the proliferation of control and JMJD1A-depleted cells. Consistent with the pattern of ADM expression, the proliferation of HepG2 and Hep3B cells decreased to almost nomoxic levels upon depletion of JMJD1A, whereas the proliferation of mock shRNA-transfected cells remained elevated (Fig. 3D). Collectively, these results strongly indicate that the enhanced proliferation of HepG2 and Hep3B cells under hypoxia is mediated by the upregulation of JMJD1A and ADM.

3.4. JMJD1A depletion suppresses HepG2 cell tumorigenicity in vivo

We next examined whether depletion of JMJD1A can affect the tumorigenicity of HepG2 cells *in vivo*. HepG2 cells stably expressing shJMJD1A or NS control vector were injected subcutaneously into nude mice ($n = 4$), and tumor development was observed for 24 days. Consistent with results observed in vitro, tumor growth in mice injected with JMJD1A-depleted HepG2 cells was significantly lower than that in mice injected with NS control cells (Fig. 4A). Twenty-four days after injection, mice were sacrificed, and tumor mass was visualized (Fig. 4B). The results indicate that knock-down of JMJD1A suppresses the growth of HepG2 tumor xenografts in nude mice.

4. Discussion

JmjC domain-containing proteins are histone demethylases and putative therapeutic targets for cancer [22]. Among them, JMJD1A is a specific demethylase of H3K9me1/me2. It plays an important role in spermatogenesis, obesity, and carcinogenesis by regulating the expression of diverse genes, such as *Tnp1*, *Prm1*, *PPARα*, *Ucp1*, *ADM*, and *GDF15*, through its demethylase activity [12,13,20]. Moreover, JMJD1A is induced by hypoxia in various cancer cells [17–20]. However, the biological role of JMJD1A during hypoxia remains largely unknown. In this study, we demonstrated the regulatory role of JMJD1A during hypoxia in liver and breast cancer cells. We first evaluated the effect of hypoxia on the proliferation of hepatocellular carcinomas (HepG2 and Hep3B) and breast cancer cells (MDA-MB-231). The results showed that the growth of cancer cells was differentially regulated by hypoxia in a cell line dependent manner (Fig. 1A). Interestingly, we observed that the expression of JMJD1A was significantly increased under hypoxia in HepG2 and Hep3B cells, but not in MDA-MB-231 cells (Fig. 1B and C). We also showed that the expression patterns of ADM were consistent with that of JMJD1A in three cell lines (Fig. 1D). Therefore, we speculated that JMJD1A may play an important role in hypoxia-induced HepG2 and Hep3B cell proliferation through ADM. ADM is highly expressed in various cancer cells, including lung carcinoma, colorectal carcinoma, and glioblastoma, and is upregulated by hypoxia in a HIF1 dependent manner [23–25]. Many studies have suggested

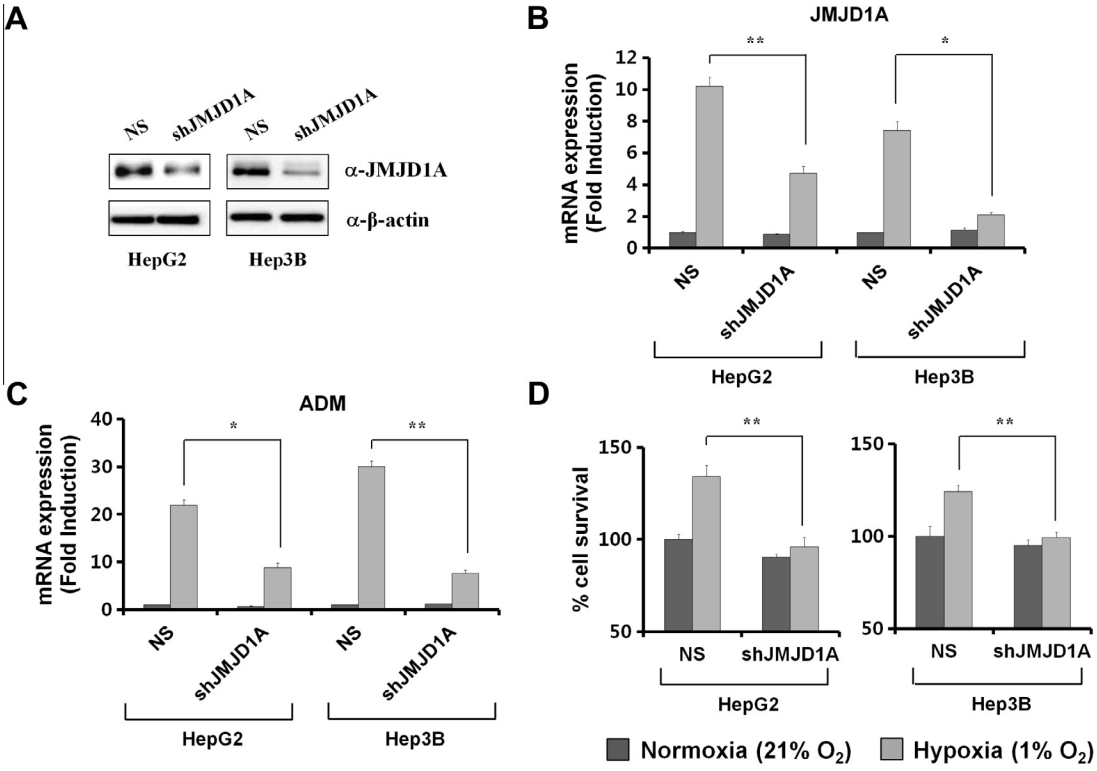

Fig. 3. Effects of JMJD1A depletion on hypoxia-induced HCC cell proliferation. (A and B) The expression of JMJD1A in HepG2 and Hep3B cells transfected with shJMJD1A or non-silencing control (NS) was evaluated by western blot and real-time PCR. Actin was used as an internal control. (C) The effect of JMJD1A knockdown on *ADM* mRNA expression was assessed by quantitative RT-PCR. Results represent mRNA levels normalized to the levels of β-actin mRNA. (D) The effect of JMJD1A knockdown on hypoxia-induced hepatoma cell proliferation was determined with the MTT assay. Graph represents the data (mean ± standard deviation) from triplicate wells. The average absorbance values were plotted on the *y*-axis with each culture condition on the *x*-axis. *P*-values were calculated using Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

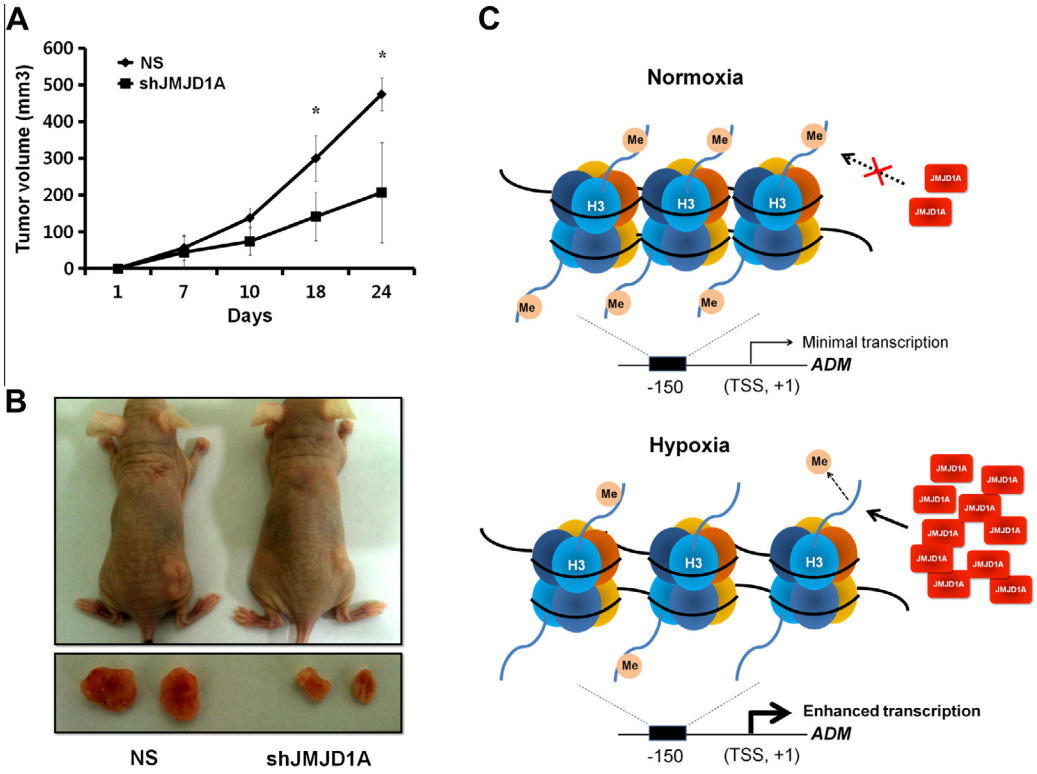

Fig. 4. The effect of JMJD1A knockdown on tumor growth in nude mice. (A) 5×10^6 HepG2 cells expressing shJMJD1A or NS were injected subcutaneously into nude mice ($n = 4$), and the average tumor size was measured at different times. (B) Twenty-four days after injection, tumor mass was visualized. (C) Model depicting the effect of JMJD1A expression on cell proliferation through the regulation of *ADM* expression in HCC cells during hypoxia. *P*-values were calculated using Student's *t*-test. $^*P < 0.05$.

that ADM play an essential role in the tumor cell growth and survival [26–30]. For example, ADM treatment inhibits hypoxic cell death in endometrial adenocarcinoma (Ishikawa cells) and induces the upregulation of Bcl2, which might be responsible for the repression [30]. Moreover, recent study has shown that JMJD1A-dependent upregulation of ADM under hypoxia stimulates colon cancer growth [20]. Based on these results, we selected *ADM* as a putative target for hypoxia-induced HCC cell proliferation.

Our ChIP analysis revealed that hypoxia leads to an increased JMJD1A occupancy on the *ADM* promoter region in HepG2 and Hep3B cells. However, although JMJD1A bound to the *ADM* promoter in all three cell lines under normoxic conditions, hypoxia did not significantly change JMJD1A occupancy in MDA-MB-231 cells (Fig. 2). Furthermore, JMJD1A demethylase activity reduced K9me2 marks on the *ADM* promoter under hypoxia in HCC cells. Because methylation of lysine residue 9 is repressive [31,32], the hypoxia-induced HCC proliferation that we observed may be linked to JMJD1A-mediated ADM expression. To provide additional evidence of a functional role for JMJD1A in hypoxia-induced HCC proliferation, the effect of JMJD1A knockdown on *ADM* transcription and cell survival was determined Our results revealed that JMJD1A is essential for the hypoxia-induced growth of HCCs and, this growth induction depends on the upregulated expression of ADM (Fig. 3C and D). Furthermore, our *in vivo* studies showed that depletion of JMJD1A significantly reduced the tumorigenicity of HCC cells (Fig. 4A and B). These results strongly support that JMJD1A is a key player in hypoxia-induced HCC cell growth.

In conclusion, experimental evidences suggest that hypoxia differentially regulates the growth of specific cancer cell lines, and *JMJD1A* and its target gene *ADM* mainly mediate this regulation (Fig. 4C). Thus, regulation of specific histone demethylases in many different types of cancers might be an appropriate strategy to inhibit cancer cell growth.

Acknowledgments

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