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Original Article

Ascorbate antagonizes nickel ion to regulate JMJD1A expression in kidney cancer cells

Xiaoqiang Guo[1,2,3], Jingxiao Lu[1], Yuejia Wang[2], Yaoting Gui[1], Xianglin Duan[2*], and Zhiming Cai[1,4*]

[1]Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Institute of Urology, Peking University Shenzhen Hospital, Shenzhen PKU-HKUST Medical Center, Shenzhen 518036, China
[2]Laboratory of Iron Metabolism and Molecular Biology, College of Life Science, Hebei Normal University, Shijiazhuang 050016, China
[3]Department of Biochemistry, Bethune Military Medical College, Shijiazhuang 050081, China
[4]Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University, Shenzhen 518035, China
*Correspondence address. Tel: +86-755-83365228; E-mail: caizhiming2000@yahoo.com.cn (Z.C.)/Tel: +86-311-86267215; E-mail: xlduan0311@163.com (X.D.)

Abnormal expression of histone demethylase Jumonji domain-containing protein 1A (JMJD1A) is associated with many kinds of cancers. JMJD1A is also a hypoxic response gene and its expression is regulated by hypoxia-inducible factor-1α (HIF-1α). In this study, we determined the role of JMJD1A in development and hypoxia pathway. We also measured the expression of JMJD1A and two hypoxia factors glucose transporter 1 (GLUT1) and vascular endothelial growth factor (VEGF) in 786-0 and HEK293 cells treated with different concentrations of $NiCl_2$ (2.5–100 μM) for 24 h, and found that JMJD1A mRNA and protein were up-regulated with increased concentrations of $NiCl_2$. We then observed that ascorbate could retard the up-regulated effect of $NiCl_2$-induced JMJD1A expression in a dose-dependent manner through decreasing the stability of HIF-1α protein. Immunohistochemical analysis further demonstrated ascorbate antagonized Ni^{2+}-induced up-regulation of JMJD1A expression in 786-0, HEK293, and OS-RC-2 cells. These findings suggest that both Ni^{2+} and ascorbate can regulate the expression of histone demethylase JMJD1A, which is important for cancer development or inhibition.

Keywords ascorbate; hypoxia; JMJD1A; kidney cancer; Ni^{2+}

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Introduction

Jumonji domain-containing protein 1A (JMJD1A) is an iron- and 2-oxoglutarate-dependent dioxygenase and specifically demethylates mono- and dimethylated histone 3 lysine 9 residue (H3K9me1/2). JMJD1A can repress transcription of special target genes and is involved in nuclear receptor activation, sperm development, energy metabolism, and so on [1–3]. JMJD1A is also one of the hypoxic response genes and its expression was up-regulated by hypoxia-inducible factor-1α (HIF-1α) [4–8]. It has been proved that hypoxia signal pathway plays an important role in tumor progression [9], which suggests that JMJD1A may participate in cancer development. Several researches have indicated that abnormal expression of JMJD1A exists in many kinds of cancers, including colorectal, prostate, and kidney cancers [10–12]. Our previous experiment also confirmed that high expression of JMJD1A is associated with renal cell carcinoma (RCC) [13].

Much is known about JMJD1A roles in development, hypoxia response, and cancer development, but many important questions remain concerning its expression, regulation, and mechanism. In this study, the regulation of JMJD1A expression by Ni^{2+} and ascorbate in kidney cancer cells was investigated. The results showed that Ni^{2+} increases JMJD1A expression and ascorbate suppresses Ni^{2+}-induced up-regulation of JMJD1A expression, which can be considered as one explanation for the carcinogenic effect of Ni^{2+} and cancer-preventive role of ascorbate.

Materials and Methods

Chemicals and antibodies

Nickelous chloride hexahydrate ($NiCl_2{\cdot}6H_2O$) was purchased from Shanghai Chemical Reagent Co., Ltd (Shanghai, China) and ascorbate from Sangon Biotech Co., Ltd (Shanghai, China). Antibiotics penicillin and streptomycin were purchased from Sigma-Aldrich (St Louis, USA). Primary antibodies used in this study include anti-JMJD1A and anti-H3 (Sigma-Aldrich), anti-HIF-1α (Invitrogen, Carlsbad, USA), anti-glucose transporter 1 (GLUT1) and anti-H3K9me2 (Abcam, Hong Kong, China), and anti-GAPDH (Epitomics, Burlingame, USA) antibodies.

Animal experiment

BALB/c male mice (2 weeks, 4 weeks, and 6 weeks old) were purchased from Experimental Animal Center of Guangdong Province (Guangzhou, China). All the animal treatments in the experiments were in accord with animal ethical standard. The research animal protocol was reviewed and approved by Ethics Committee of Peking University Shenzhen Hospital. All mice (five mice per age group) were sacrificed after anesthesia with sodium pentobarbital and the kidneys were immediately removed and frozen in liquid nitrogen for subsequent experiments.

Cell culture and treatment

The human embryonic kidney cell line HEK293, the renal carcinoma cell lines 786-0 and OS-RC-2 were purchased from cell resource center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science. The three cell lines were cultured at 37°C in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, USA), 50 U/ml penicillin, 50 μg/ml streptomycin, and 5% CO_2 (oxygen 20%). For hypoxia treatment, 786-0 cells were cultured under 1% O_2 at 6, 12, and 24 h, and untreated cells at 0 h. 786-0 and HEK293 cells were cultured in six-well plates (Corning, Corning, USA) until growing to optimal density, and then different concentrations of $NiCl_2$ (2.5, 10, 25, 50, 100 μM) were added and cultured for another 24 h. After removal of culture medium, these cells were rinsed with cold phosphate-buffered saline (PBS) (pH 7.4) one time and then collected. For ascorbate treatment, 786-0 and HEK293 cells were first incubated with 100 μM of $NiCl_2$ followed by addition of different concentrations of ascorbate (2.5, 10, 25, 50, 100 μM). After 24 h, cells were collected for total RNA and protein extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from mice kidney tissue or cultured cells by Trizol reagent kit (Invitrogen) according to the manufacturer's protocol and quantified with Nanodrop2000 (Thermo Scientific, Wilmington, USA). Total RNA (2 μg) was used to synthesize cDNA with a reverse transcription system (Fermentas, Glen Burnie, USA) according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reactions (qPCR) were performed on ABI PRISM 7700 Sequence Detection System (Applied Biosystem, Carlsbad, USA) using SYBR green (Tiangen, Beijing, China). cDNA levels of target genes were analyzed using comparative C_T methods, where C_T is the cycle threshold number and normalized to *18S* (mouse) or *GAPDH* (human). Primers used in qPCR were listed in **Table 1**.

Western blot analysis

For the measurement of specific protein levels in kidney tissue or cultured cells, appropriate samples were collected in lysis buffer [50 mM Tris-Cl (pH 7.0), 1 mM EDTA, 1% Triton X-100] and sonicated until clear. The crude mixture was centrifuged for 20 min at 12,000 *g* and supernatants were collected. Protein concentration was determined with the Pierce BCA assay (Thermo Fisher Scientific, Waltham, USA), and 50 μg protein from each sample was loaded and then separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrotransferring to polyvinylidene difluoride membrane, the membrane was washed with 25 ml TBS (50 mM Tris-HCl, 150 mM NaCl, pH 8.0) for 5 min at room temperature and then incubated with 5% skim milk in TBST (0.1% Tween-20 in TBS) for 1 h at room temperature. The membrane was incubated with primary antibodies with gentle agitation overnight at 4°C and then washed with TBST three times for 5 min each. The membrane was incubated with 1 : 5000-diluted peroxidase-coupled goat anti-rabbit/mouse IgG (Merck, Darmstadt, Germany) for 2 h at room temperature. After being washed with TBST four times for 5 min each, the membrane was detected with chemical luminescence substrate (Pierce, Rockford, USA), and then exposed to the X-ray film.

Immunofluorescence

786-0, OS-RC-2, and HEK293 cells were seeded in six-well plates with sterile slides at the bottom until cells adhered to slides. Treatments with $NiCl_2$ and ascorbate were carried out as described in the section Cell culture and treatment. After removal of medium, slides were washed with PBS, and then fixed with 4% paraformaldehyde for 30 min at 4°C. The slides were washed twice with PBS and then immersed in PBS-Triton (0.3%) for 10 min at room temperature. After blocking with 3% bovine serum albumin for 30 min at room temperature, slides were incubated with rabbit polyclonal antibody JMJD1A overnight at 4°C, followed by several washes with PBS, and incubation with fluorescently labeled secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, USA) for 30 min. After being washed with PBS three times, 4′,6-diamidino-2-phenylindole (DAPI) (ratio of PBS : DAPI : RNA enzymes = 1 ml : 50 μl : 50 μl) was added to stain nucleus for 15 min. Images were acquired using a confocal microscope (Leica Microsystems, Bensheim, Germany).

Statistical analysis

Each qPCR experiment was performed three independent times for each tested gene. All values were expressed as mean ± SEM. P values were calculated by independent t-test. $P < 0.05$ was considered significant.

Table 1 Primers used in this study

Gene	Primer (5′–3′)		Product length (bp)
Jmjd1a (mouse)	Forward	TCGGTGTGGGTTTGGAGTATGTG	140
	Reverse	GTGTGGGCATCAGGTTCTCAGG	
Jmjd1b (mouse)	Forward	GTAATGGGAGTGATGGAGGTGAGG	135
	Reverse	AGCGAATGTTGCGGTTAATCTGG	
Jmjd2c (mouse)	Forward	AGATGGATTGACTACGGCAAGGTTG	168
	Reverse	AGATTCTGGAGTGGGCTTTGTATGG	
18S (mouse)	Forward	GTAACCCGTTGAACCCCATT	151
	Reverse	CCATCCAATCGGTAGTAGCG	
JMJD1A (human)	Forward	GTCAACTGTGAGGAGATTCCAGC	63
	Reverse	AACTTCAACATGAATCAGTGACGG	
GLUT1 (human)	Forward	CGGGCCAAGAGTGTGCTAAA	283
	Reverse	TGACGATACCGGAGCCAATG	
VEGF (human)	Forward	CTTGCCTTGCTGCTCTAC	192
	Reverse	TGGCTTGAAGATGTACTCG	
GAPDH (human)	Forward	GCTCTCTGCTCCTCCTGTTC	100
	Reverse	GACTCCGACCTTCACCTTCC	

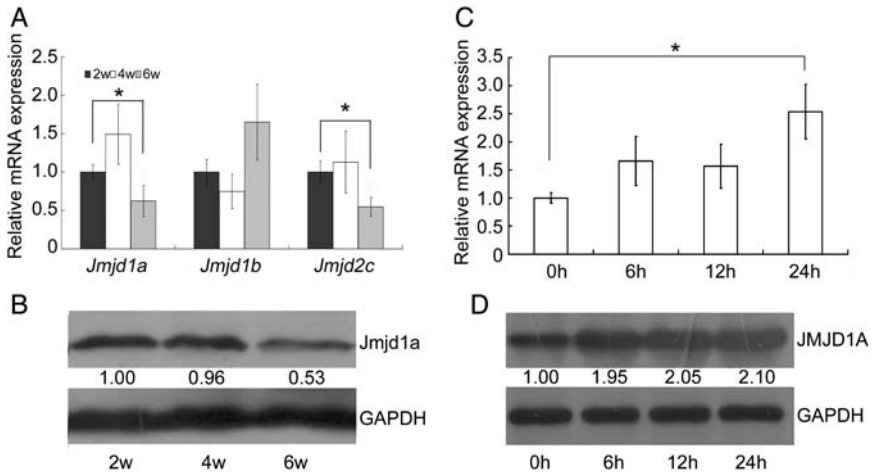

Figure 1 Expression of JMJD1A in kidney tissue from different age groups of mice and in RCC 786-0 cell under hypoxia (A) mRNA expression of three histone demethylase genes *Jmjd1a*, *Jmjd1b*, and *Jmjd2c* in mice (five per group) was measured by qPCR. Data are expressed as mean ± SEM from three independent experiments. $*P < 0.05$. (B) JMJD1A protein content was determined by western blot analysis. A representative image of western blots is provided. (C) mRNA expression of *JMJD1A* under hypoxia for 0, 6, 12, and 24 h in 786-0 cells was determined by qPCR. Data are expressed as mean ± SEM from three independent experiments. $*P < 0.05$. (D) JMJD1A protein content was determined under hypoxia for different time periods in 786-0 cells by western blot analysis. A representative image of western blots is provided.

Results

JMJD1A is a development-related gene in mouse kidney and hypoxia-response gene in embryonic kidney cells

To further determine the biological function of JMJD1A, the expression of mouse Jmjd1a in kidney tissue of different developmental stages (2w, 4w, and 6w) was determined. These results indicated that the expression of Jmjd1a was reduced at mRNA and protein levels at 6w, and the difference has statistical significance ($P < 0.05$) [**Fig. 1(A,B)**]. The expression of other two H3K9me2 demethylase genes *Jmjd1b* and *Jmjd2c* also changed, but only *Jmjd2c* was obvious ($P < 0.05$). As JMJD1A participated in stem cell self-renewal and inhibition of cell differentiation [14,15], decreased expression of mouse Jmjd1a suggests that the potential cell proliferation is declined.

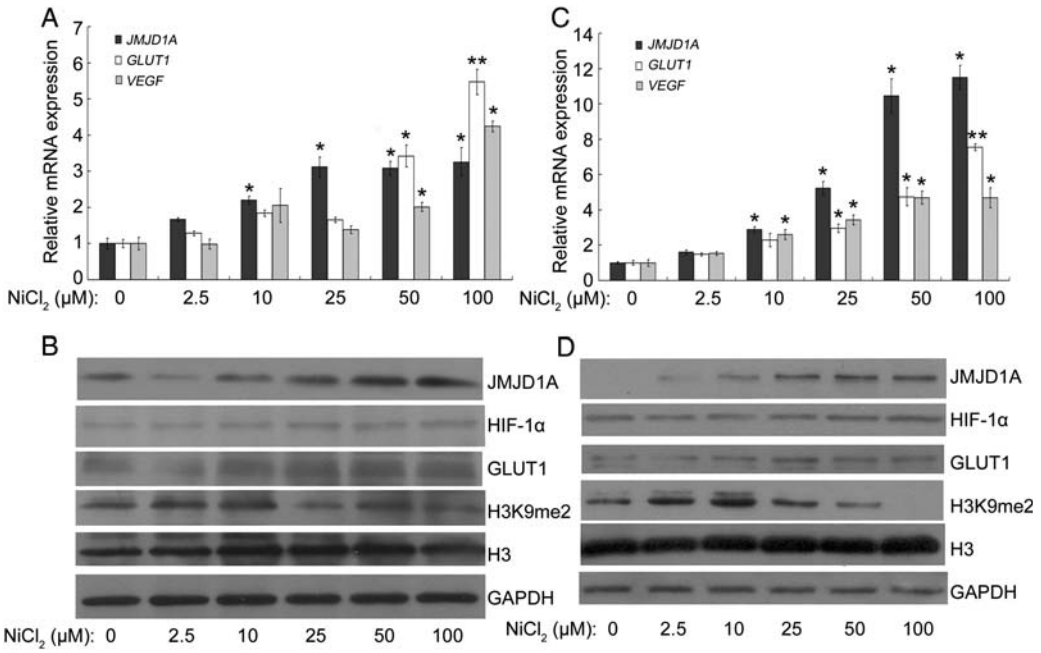

Figure 2 Induction of JMJD1A, GLUT1, and VEGF expression after treatment with different concentrations of $NiCl_2$ Both 786-0 and HEK293 cells were treated with indicated concentrations of $NiCl_2$ for 24 h and then collected for following experiments. mRNA expressions of *JMJD1A*, *GLUT1*, and *VEGF* were measured by qPCR. The protein contents of JMJD1A, GLUT1, and HIF-1α were determined by western blot analysis. (A) *JMJD1A*, *GLUT1*, and *VEGF* mRNA expression levels in 786-0 cells were measured by qPCR. Data are expressed as mean ± SEM from three independent experiments. (B) Representative expression of JMJD1A, HIF-1α, GLUT1, H3K9me2, and H3 proteins in 786-0 cells as detected by western blot analysis. GAPDH was used as an internal control. (C) *JMJD1A*, *GLUT1*, and *VEGF* mRNA expression levels in HEK293 cells were measured by qPCR. Data are expressed as mean ± SEM from three independent experiments. (D) Representative expression of JMJD1A, HIF-1α, GLUT1, H3K9me2 and H3 proteins in HEK293 cells as detected by western blot analysis. GAPDH was used as an internal control. $*P < 0.05$ and $**P < 0.01$ compared with control (0 μM $NiCl_2$).

In the hypoxic condition (1% O_2), *JMJD1A* mRNA expression was obviously increased in 786-0 cell ($P < 0.05$) [**Fig. 1(C)**]. The western blot analysis also proved this result [**Fig. 1(D)**]. These results suggested that JMJD1A is also a hypoxia-response gene in RCC cells, which is consistent with our previous research in mimicking hypoxia [13].

Ni^{2+} induces JMJD1A expression at mRNA and protein levels in RCC cells

Previous studies also showed that Ni^{2+} or Co^{2+} can effect the histone methylation and JMJD1A expression [13,16,17], but it is unknown as to whether the expression of JMJD1A and related hypoxia-response genes such as GLUT1 and vascular endothelial growth factor (VEGF) is regulated under different Ni^{2+} concentrations. **Figure 2** shows both mRNA and protein levels of JMJD1A, GLUT1, and VEGF were up-regulated with increasing $NiCl_2$ concentration in 786-0 [**Fig. 2(A,B)**] or HEK293 cells [**Fig. 2(C,D)**], and the significance was obvious from 25 μM of $NiCl_2$ ($P < 0.05$). Both GLUT1 and VEGF are hypoxia-response genes whose expression was regulated by HIF1. The increased expression of the two genes means that the protein stability of HIF1-α is also increased [18]. With more HIF-α protein, its target genes including JMJD1A are up-regulated. The change is more significant in HEK293 cells than in 786-0 cells, which can be explained by the fact that von Hippel–Lindau (*VHL*) gene mutation exists in 786-0 cells, so the basic expression of JMJD1A is higher.

Ascorbate down-regulates the Ni^{2+}-induced JMJD1A expression in a dose-dependent manner

After adding 100 μM $NiCl_2$ and different concentrations of ascorbate in both 786-0 and HEK293 cells for 24 h, the expression of JMJD1A, GLUT1, and VEGF was reduced at both mRNA and protein levels. These results indicated that the ascorbate decreased the expression of JMJD1A and related hypoxia-response genes GLUT1 and VEGF in 786-0 [**Fig. 3(A,B)**] or HEK293 cells [**Fig. 3(C,D)**]. Previous researches have indicated that nickel can deplete intracellular ascorbate and stabilize HIF-1α protein which increases the expression of hypoxia-response genes [19,20]. In our experiments, we showed that ascorbate suppressed HIF-1α stability and reversed Ni^{2+}-induced up-regulation of JMJD1A.

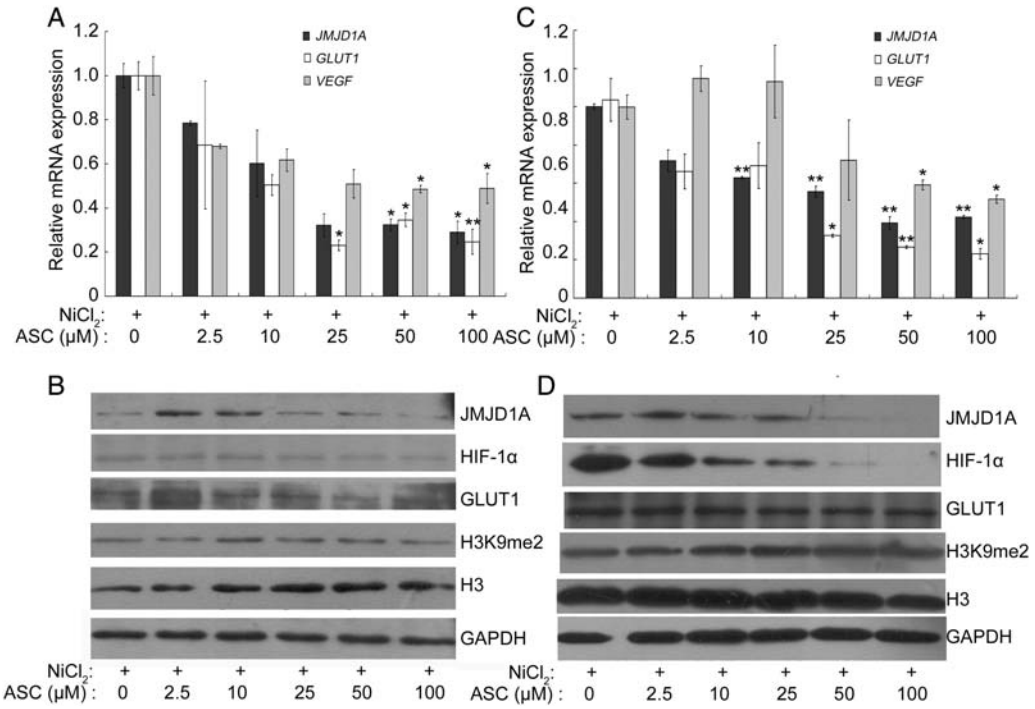

Figure 3 Ascorbate regulates JMJD1A expression after treatment with 100 μM of $NiCl_2$ Both 786-0 and HEK293 cells were treated first with 100 μM of $NiCl_2$ and then with different concentrations (0–100 μM) of ascorbate for 24 h, and then collected for following experiments. mRNA expressions of *JMJD1A*, *GLUT1*, and *VEGF* were measured by qPCR. The protein contents of JMJD1A, GLUT1, and HIF-1α were determined with western blot analysis. (A) *JMJD1A*, *GLUT1*, and *VEGF* mRNA expression levels in 786-0 cell were measured by qPCR. Data are expressed as mean ± SEM from three independent experiments. (B) Representative expression of JMJD1A, HIF-1α, GLUT1, H3K9me2, and H3 proteins in 786-0 cells as detected by western blot analysis. GAPDH was used as an internal control. (C) *JMJD1A*, *GLUT1*, and *VEGF* mRNA expression levels in HEK293 cells were measured by qPCR. Data are expressed as mean ± SEM from three independent experiments. (D) Representative expression of JMJD1A, HIF-1α, GLUT1, H3K9me2, and H3 proteins in HEK293 cells as detected by western blot analysis. GAPDH was used as an internal control. $*P < 0.05$ and $**P < 0.01$ compared with control (100 μM $NiCl_2$ ± 0 μM ascorbate).

Nickel ion increases the expression of JMJD1A and ascorbate antagonizes its effect at cellular level

To further confirm the antagonistic effect of ascorbate to Ni^{2+} on up-regulation of JMJD1A *in vitro*, the immunofluorescent experiment was done to observe the JMJD1A expression change after cells were treated with different concentrations of $NiCl_2$ or ascorbate. The results showed that Ni^{2+} increased JMJD1A expression at a dose-dependent manner in 786-0 cells [**Fig. 4(A)**], HEK293 cells [**Fig. 4(B)**], and OS-RC-2 cells [**Fig. 4(C)**], while ascorbate retarded the effect in all three cell lines [**Fig. 4(D–F)**].

Discussion

Histone methylation can influence expression of specific genes by effecting chromatin folding, which played an important role in many cancers' development [21]. JMJD1A regulated the expression of multiple target genes by catalyzing H3K9m1/2 demethylation. Our results indicated that JMJD1A takes part in kidney development and hypoxia response of renal carcinoma cell lines, which are important for cancer development. Epidemiological data have shown that nickel compounds are important environmental pollutants and carcinogens [22], and the primary target organs of these compounds are the kidney and lung [23], so nickel compounds may be an important cause of kidney cancer development. Our researches provided evidence that Ni^{2+} can increase JMJD1A expression. Considering that dysregulation of JMJD1A is closely associated with kidney cancer [13], Ni^{2+}-induced JMJD1A up-regulation is a factor of renal tumor formation. Although another research has suggested that Ni^{2+} can inhibit JMJD1A enzymatic activity by replacing the ferrous iron in the catalytic centers *in vitro* [24], there are so many Fe^{2+}-dependent dioxygenases including JMJD1A in mammalian that the inhibitory effect of Ni^{2+} on JMJD1A enzymatic activity can be neglected *in vivo*. The increased expression of JMJD1A should be secondary to adding $NiCl_2$. Our results also indicated that the overall H3K9me2 was decreased with the increased JMJD1A expression after treatment with $NiCl_2$ in 786-0 cells and HEK293 cells [**Fig. 2(B,D)**], but it did not change with the JMJD1A reduction when treated with ascorbate [**Fig. 3(B,D)**]. These results suggested that the regulation of JMJD1A expression is complicated.

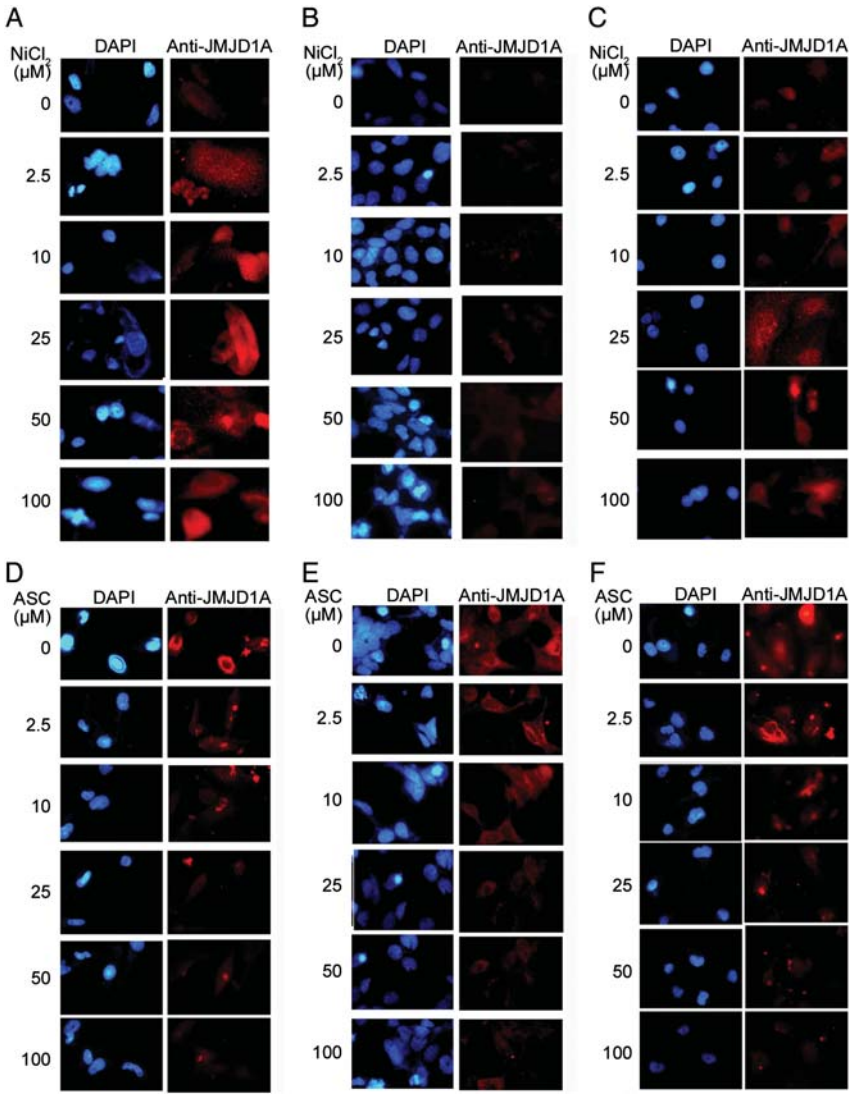

Figure 4 The expression of JMJD1A detected by immunofluorescent staining 786-0, HEK293, and OS-RC-2 cells were treated with indicated concentrations of $NiCl_2$ and ascorbate for 24 h, and then fixed for following immunofluorescent experiment. These cells were incubated with rabbit anti-JMJD1A antibody overnight at 4°C, and the protein–antibody complex then was labeled by CY3-sheep anti-rabbit antibody (red); nuclei were labeled with DAPI (blue) for counterstaining. (A, B, C) JMJD1A immunofluorescence in 786-0 (A), HEK293 (B), and OS-RC-2 (C) cells treated with indicated concentrations of $NiCl_2$. (D, E, F) Representative of JMJD1A immunofluorescence in 786-0 (D), HEK293 (E), and OS-RC-2 (F) cells treated first with 100 μM $NiCl_2$ and then with indicated concentrations of ascorbate.

Ascorbate, also known as vitamin C, is an important antioxidant and has a wide range of biological effects including inhibition of cancer [25]. Our results demonstrated that ascorbate could inhibit the Ni^{2+}-induced JMJD1A up-regulation in two kidney cancer cell lines and HEK293 cells, which may be one reason for the cancer-preventive role of ascorbate. Epidemiological data also showed that ascorbate had an important preventive effect for renal cell carcinoma, but this effect was only significant for males [26]. As JMJD1A is an androgen receptor co-activator [1], it can be presumed that JMJD1A activity is more important in the male and ascorbate is more significant for RCC prevention in the male.

The regulatory function of JMJD1A by Ni^{2+} and ascorbate may be achieved by hypoxia signal pathway. HIF-1α is an important transcriptional factor and regulates expression of many target genes including GLUT1 and VEGF. HIF-1α protein stability is regulated through ubiquitin modification. Prolyl hydroxylase domain (PHD) protein belongs to dioxygenases and catalyzes the hydroxylation of specific proline residues of HIF-α [27,28], which can be recognized by ubiquitin ligase VHL and then modified by ubiquitin for protein degradation [29]. PHD-catalyzed hydroxylation of HIF-α requires many factors, such as oxygen, iron, α-ketoglutarate, and ascorbate [30]. In addition, PHD activity is also regulated by a number of

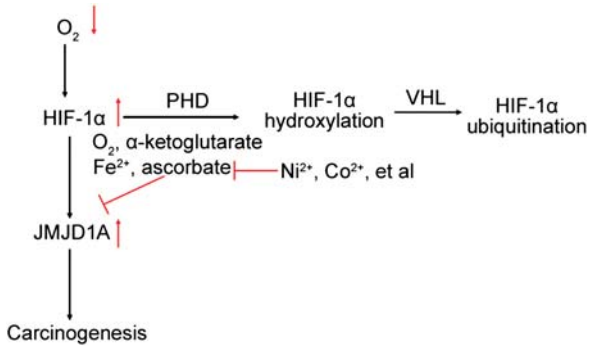

Figure 5 The possible model for regulation of JMJD1A expression by nickel ion and ascorbate Under normoxia, HIF-1α is hydroxylated by oxygen-dependent prolyl hydroxylase (PHD), which leads to its ubiquitination by the VHL protein and subsequently proteasomal degradation. But under hypoxia (oxygen content reduction) or in the presence of PHD inhibitors such as Ni^{2+} or Co^{2+}, the protein stability of HIF-1α is increased and the expressions of hypoxia-response genes including JMJD1A are promoted, which is favorable for carcinogenesis. Ascorbate can reduce the stability of HIF-1α through inhibiting the activity of Ni^{2+} or Co^{2+} and lower the content of JMJD1A, which is good for cancer prevention.

metabolic acids such as fumaric acid and malic acid [31], so kidney cancer is often seen as a metabolic disease. Some mutant genes such as *VHL*, fumarate hydratase and succinate dehydrogenase can change the cell metabolism and affect PDH activity or HIF-1α stability [32]. These facts suggested that PHD and HIF-1α play a very important role in the kidney cancer development.

Both Co^{2+} and Ni^{2+} contribute to ascorbate oxidation and depletion [33,34], thereby reducing the intracellular ascorbic acid content and thus undermining the PHD enzymatic activity [35,36]. Ni^{2+} can promote tumor angiogenesis by increasing HIF-1α stability [37], and also increase drug resistance of cancer cells [38]. Another similar metal ion Co^{2+} promotes the expression of GLUT1 and increases the adaptability of breast cancer cells [18], and contributes to primary and metastatic breast cancer cell invasiveness [39]. Another reason for the regulatory effect of Ni^{2+} and ascorbate on JMJD1A may be that nickel is an inducer of reactive oxygen species (ROS) [40] and ascorbate is a radical scavenger [41], and ROS can inhibit the PHD enzymatic activity [42]. Our previous research has shown that $CoCl_2$ and $NiCl_2$ have a similar effect on the JMJD1A expression induction [13]. This current experiment further suggests that Ni^{2+} up-regulates the expression of JMJD1A though increasing the stability of HIF-1α [**Fig. 2(B,D)**].

Many compounds such as histidine can reverse the Ni^{2+}- or Co^{2+}-induced hypoxic effect [43], but ascorbate is the most widely used protective agent [22,44,45]. A previous study has also indicated that low ascorbate (<6.7 mg/100 g cancer tissue) can increase HIF-1α activity and endometrial cancer aggression [46]. These results further demonstrated that ascorbate is involved in the down-regulation of the HIF-1α-mediated target gene expression, which is important in reducing the cancer development. Our experiments showed that ascorbate can reduce the expression of Ni^{2+}-induced JMJD1A though decreasing the stability of HIF-1α [**Fig. 3(B,D)**]. Combining these facts, the regulated model of JMJD1A can be interpreted as **Fig. 5**. Under hypoxia condition, many metal ions including Ni^{2+} and Co^{2+} can increase the stability of HIF-1α protein and gene expression of JMJD1A which are beneficial to carcinogenesis. The supplementation of ascorbate can reduce the hypoxia effect and JMJD1A expression through weakening metal ions toxicology, which plays important roles in cancer prevention and even in cancer treatment.

In summary, our studies indicate that both carcinogenic $NiCl_2$ and anticancer ascorbate can influence the expression of histone H3K9 demethylase JMJD1A. The ascorbate can suppress the Ni^{2+}-induced JMJD1A up-regulation though hypoxia pathway, i.e. decreasing HIF-1α protein stability. As hypoxia pathway plays an important role in renal cancer development, many hypoxia-response genes can be used in future cancer diagnosis and treatment and JMJD1A may be one of them.

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