

## RESEARCH ARTICLE

# Expression and Effects of JMJD2A Histone Demethylase in Endometrial Carcinoma

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### Abstract

Previous studies have demonstrated that JMJD2A is a potential oncogene and is overexpressed in human tumors. However, its role in the endometrial carcinoma remains largely unknown. In this study, we discovered that JMJD2A was overexpressed in endometrial carcinoma, using immunohistochemistry, quantitative real-time polymerase chain reaction, and western blotting. Downregulation of JMJD2A led to reduced endometrial carcinoma RL95-2 and ISK cell proliferation, invasion and metastasis as assessed with cell counting kit-8, cell migration and invasive assays. Collectively, our results support that JMJD2A is a promoter of endometrial carcinoma cell proliferation and survival, and is a potential novel drug target.

**Keywords:** JMJD2A - endometrial carcinoma - cell proliferation - tumor migration - tumor invasion

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### Introduction

Endometrial carcinoma is one of the most common gynecological malignancies (Siegel et al., 2013) and its incidence has been growth since the menopausal women populations were increasing, in western countries and China (Thanappaprasr et al., 2013). Although most endometrial carcinoma is diagnosed at an early stage, 30% of all cases are still diagnosed at later stages, which correlate with decreased survival rates (Salvesen et al., 2012). Advanced and recurrent cases carry a poor prognosis, principally because they are often characterized by aggressive local invasion, metastasis and poor response to chemotherapy. Prevention and treatment of endometrial carcinoma require a better understanding of the molecular mechanisms underlying the progression of endometrial carcinoma.

The Jumonji domain-containing (JMJD) proteins have been implicated as histone demethylases (Tsukada et al., 2006). One JMJD subfamily consists of JMJD2A, JMJD2B, JMJD2C and JMJD2D. JMJD2A (also known as JHDM3 or KDM4A) was identified and characterized in 2004 (Katoh et al., 2004). JMJD2A is a transcriptional cofactor and enzyme that catalyzes demethylation of histone H3 lysines 9 and 36 and is overexpressed in human tumors, but its role in oncogenesis remains unclear (Berry et al., 2012). Furthermore, several reports have linked this protein to proliferation, apoptosis,

invasion and metastasis in several cancers. For instance, JMJD2A overexpression enhances estrogen-dependent transcription, and downregulation of JMJD2A reduces breast cancer cell growth by forming a complex with endogenous estrogen receptor (ER) $\alpha$  *in vivo* (Berry et al., 2012). In addition, JMJD2A overexpression enhances cell motility and invasion and metastasis of bladder and colon carcinoma cells (Kauffman et al., 2011; Kim et al., 2012). One study has shown that JMJD2A gene expression is upregulated in human prostate tumors, suggesting that JMJD2A may exert a tumor-promoting function (Cloos et al., 2006). Collectively, these results suggest that endogenous JMJD2A regulates cell proliferation, apoptosis, invasion and metastasis in a variety of cancers.

Although many studies have examined the role of JMJD2A in various cancers, there are few reports on this protein in the context of endometrial carcinoma. Therefore, we put forward a hypothesis that JMJD2A is overexpressed in endometrial carcinoma, regulates endometrial carcinoma cell biological behavior, and that its knockdown inhibits cell biological behavior. In this study, we examined the expression of JMJD2A in 56 specimens of endometrial carcinoma and 20 of normal endometrium. JMJD2A-specific siRNA was chemically synthesized and transfected into human endometrial carcinoma cell lines. We investigated how JMJD2A regulated endometrial carcinoma proliferation, migration and invasion.

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## Materials and Methods

### *Tissue samples*

Seventy-six patients were treated at the Second Affiliated Hospital of Harbin Medical University from 2008 to 2013. Tissue samples from these patients were obtained from the Department of Pathology after obtaining approval from the Ethics Committee. Informed consent was obtained from each patient. No patient had received radiotherapy, chemotherapy, or other treatment prior to surgery. Of the 76 cases, 20 cases were benign and 56 were type II endometrial carcinoma. Staging was according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. Table 1 shows a complete list of 56 patients in this study.

### *Immunohistochemistry*

Immunohistochemical stains were performed on 5- $\mu$ m-thick sections of formalin-fixed, paraffin-embedded tissues. Immunohistochemistry was performed on sections of human endometrial cancer tissue using previously reported procedures (Chao et al., 2006). Tissue blocks were incubated with rabbit monoclonal antibody to JMJD2A (Cell Signaling Technology, Danvers, MA, USA). All stained tissue sections were evaluated by two independent pathologists, and separate scores were given to each sample based on: (1) tissue positivity, that is, the percentage of tissue staining positive; and (2) the intensity of cell staining (0, none; 1, weak; 2, moderate; 3, strong)

### *Cell culture and transfection*

We used the human endometrial carcinoma cell lines RL95-2 and Ishikawa. RL95-2 cells (Shanghai Cell Bank, China) were routinely cultivated in complete growth medium comprising Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA), 1% penicillin-streptomycin (Invitrogen), and 0.005 mg/mL insulin (Sigma-Aldrich, St. Louis, MO, USA). Ishikawa (ISK) cells were stored in our laboratory and maintained in RPMI-1640 (HyClone) containing 1% penicillin-streptomycin (Invitrogen) with 10% FBS (Gibco Invitrogen). JMJD2A siRNA and negative control siRNA were purchased from Qiagen (Hilden, Germany) and were transiently transfected into endometrial carcinoma cells using Lipofectamine 2000 reagents (Invitrogen), following the manufacturer's instructions. Cells were harvested after transfection for 48h.

### *RNA extraction and cDNA synthesis*

After transfection with negative control- or JMJD2A-siRNA, total RNA of each group was extracted respectively from cultured cells by Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNAs from three groups were synthesized from total RNA by the one-step Prime Script RT reagent Kit (TaKaRa, Dalian, China) following the manufacturer's protocol.

### *Quantitative real-time polymerase chain reaction (PCR)*

JMJD2A and GAPDH primers were designed

using Primer5 software. The primers were as follows: JMJD2A F: GTGGTCTTCATTACCTGCTTTCGG, R: GGACCAAAGTGGAGACAGTCCTGG, GAPDH F: AACCCCAAGGCCAACCGCGAGAAGATGACC, R: GGTGATGACCTGGCCGTCAGGAGCTCGTA. Primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). Quantitative real-time PCR (qRT-PCR) for miRNAs was performed using the AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Daejeon, Korea) in an Bio-Rad CFX96 Real-Time System. The PCR conditions were: denaturation at 95°C for 10 min, 40 cycles at 95°C for 30 s, 55°C for 30s, and 72°C for 30 s. Data analysis used the  $2^{-\Delta\Delta C_t}$  method. All experiments were performed in triplicate.

### *Western blot analysis*

After transfection for 72h, total protein in the three groups of cells was extracted with lysis buffer (Beyotime, Shanghai, China) containing a 1% dilution of the protease inhibitor phenylmethylsulfonyl fluoride (Beyotime). We determined the protein concentration with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins (40  $\mu$ g) were subject to electrophoresis on 10% SDS-PAGE, and separated proteins were transferred to polyvinylidene difluoride membranes. After blocking the membrane with 5% milk proteins for 2h at room temperature, the membrane was incubated with monoclonal rabbit anti-human JMJD2A antibody (1:1000; Cell Signaling Technology) and  $\beta$ -actin (1:2000; Cell Signaling Technology) at 4°C overnight. Immunolabeling was detected using an enhanced chemiluminescence autoradiography (ECL kit, Amersham), according to the manufacturer's protocol. Western blot data were quantified using ImageJ pixel analysis (NIH Image software).  $\beta$ -Actin was used as an internal control to confirm equal protein loading.

### *Cell proliferation assay*

Human endometrial carcinoma Ishikawa and RL95-2 cells were seeded in 96-well plates ( $2 \times 10^3$  cells/well) in a total volume of 100  $\mu$ l/well. After 48h transfection with either control- or JMJD2A-siRNA, we added an additional 10  $\mu$ l of CCK-8 per well. Cell proliferation assay was evaluated using the WST-8 method (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). The absorbance at 450 nm was recorded using a 96-well plate reader (Infinite M200 PRO; Tecan, Morrisville, NC, USA). All experiments were performed in triplicate.

### *In vitro migration and invasion assays*

Cell migration and invasive assays were examined using Transwell chambers (24-well, 8- $\mu$ m pore size, 6.5-mm diameter; Corning Costar, Cambridge, MA, USA). After transfection, three different groups of cells ( $2 \times 10^5$  cells/ml) in 0.2 ml serum-free medium were placed in each upper chamber, either coated with (invasion) or without (migration) Matrigel (BD Biosciences, San Jose, CA, USA). Medium with 20% FBS (600  $\mu$ l) was added to each lower chamber. After 36h (migration) or 72h (invasion) incubation at 37°C, the cells on the top surface of the insert were removed by wiping with a cotton swab. The cells were fixed with 10% carbinol and

stained with crystal violet. The cells were counted using light microscopy (200× magnification) and evaluated based on the mean values from five fields of view. Three independent experiments were performed in triplicate.

#### Statistical analysis

Data were analyzed and expressed as mean±SD for continuous variables. Associations among categorical variables were assessed using Fisher's exact probability test or the (2 test (Table 1). Data for counting variables were analyzed with ANOVA. When the results of the ANOVA were significant, Dunnett's test was used to assess the differences in these expression levels among each group.  $p < 0.05$  was considered statistically significant. All statistical analyses were performed with SPSS 17.0 (Chicago, IL, USA).

## Results

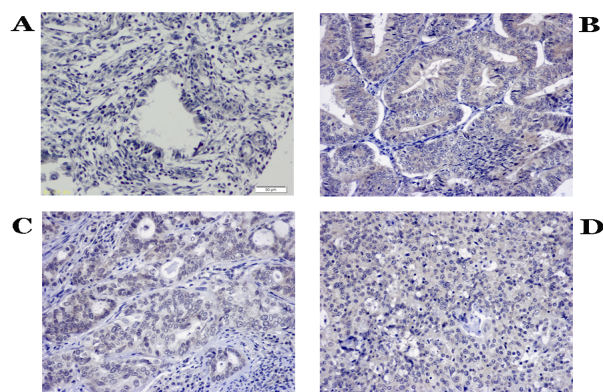
#### Overexpression of JMJD2A in endometrial carcinoma

The JMJD2A expression levels of endometrial carcinoma tissues and benign endometrial tissues from patients were examined by immunohistochemistry.

**Table 1. Relationship between JMJD2A Expression in Endometrial Carcinoma Tissues and Clinicopathological Features**

Variable		Cases	JMJD2A expression	<i>p</i>
Age	<50 years	8	4	0.913
	≥50 years	48	23	
Histological differentiation	Well	29	18	0.938
	Moderate	15	10	
	Poor	12	8	
FIGO stage	I	52	25	0.997
	II	2	1	
	III	2	1	

Statistically significant \* $p < 0.05$



**Figure 1. Overexpression of JMJD2A Protein in Endometrial Tissues.** Matching normal and carcinoma endometrial tissues with well, moderately, and poorly differentiated by immunohistochemistry assay. A: Expression of JMJD2A protein in normal endometrial tissue. B: Expression of JMJD2A protein in well-differentiated endometrial carcinoma tissue. C: Expression of JMJD2A protein in moderately differentiated endometrial carcinoma tissue. D: Expression of JMJD2A protein in poorly differentiated endometrial carcinoma tissue (all images taken at 200× total magnification; bar indicates 50 μm)

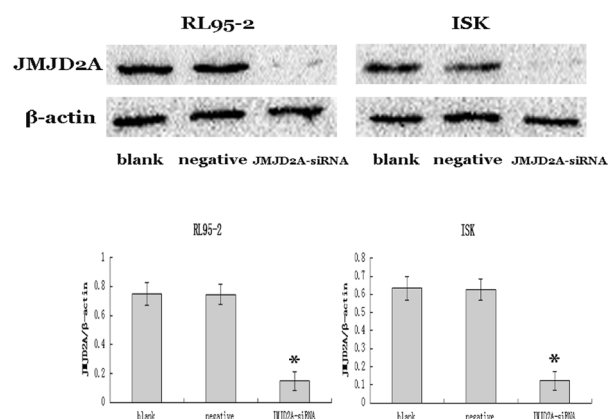
The association of JMJD2A expression with the clinicopathological features in endometrial carcinoma is shown in Table 1. JMJD2A protein was overexpressed in 36/56 endometrial carcinoma samples and in 5/20 benign endometrial samples, and >60% of all human endometrial carcinoma tissues overexpressed the JMJD2A protein. There was a significant difference in JMJD2A expression between endometrial carcinoma and benign endometrial tissues ( $\chi^2=9.155$ ,  $p < 0.05$ ). JMJD2A was expressed in the nucleus as well as the cytoplasm, but there was no significant difference according to tumor age, histological differentiation and stage ( $p > 0.05$ ). Figure 1 shows that the JMJD2A expression in endometrial carcinoma with different histological differentiation, and in benign endometrial tissues.

#### Overexpression of JMJD2A in endometrial carcinoma cell lines and transfection results

Real-time qRT-PCR (data not shown) and western blotting analysis (Figure 2) indicated that RL95-2 and ISK endometrial carcinoma cells expressed high levels of JMJD2A. We transfected each cell line with JMJD2A-siRNA or control-siRNA for comparison, and after 48h transfection, JMJD2A expression levels was significantly reduced in the JMJD2A-siRNA group ( $p < 0.05$ ) compared with the blank and negative control groups in both cell lines. However, there was almost no significant difference between the blank and negative control groups ( $p > 0.05$ ). These data demonstrated that expression of JMJD2A mRNA and protein was significantly reduced after transfection with JMJD2A-siRNA, and JMJD2A silencing achieved >80% knockdown in both cell lines.

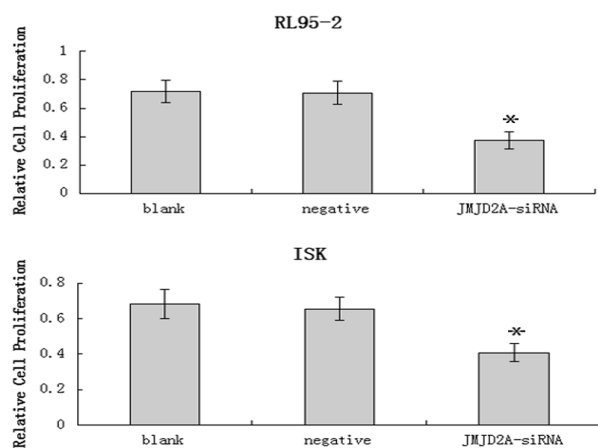
#### JMJD2A silencing suppresses proliferation of human endometrial carcinoma cells

To investigate whether silencing JMJD2A affected human endometrial carcinoma cell proliferation, after transfection with JMJD2A-siRNA and control-siRNA for

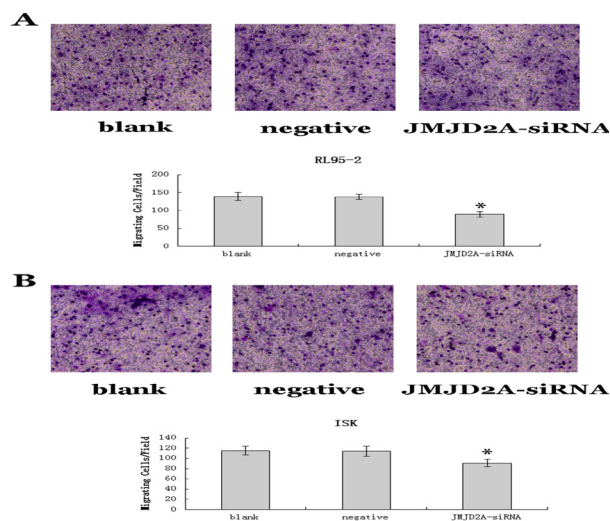


**Figure 2. Overexpression of JMJD2A in Human Endometrial Carcinoma Cell Lines.** Human endometrial carcinoma cell lines RL95-2 and ISK were transfected with JMJD2A-siRNA or negative-siRNA, or were left untreated. After 48 h, expression levels of JMJD2A were detected by western blotting. β-Actin was used as a loading control. Densitometry (mean±SD) revealed >80% knockdown of JMJD2A in the JMJD2A-siRNA group. \* $p < 0.05$  versus blank and negative-siRNA group





**Figure 3. Effects of Knockdown of JMJD2A on RL95-2 and ISK Cell Proliferation.** Cells were separately cultured in 96-well plates. After 48h transfection, the absorbance levels were detected using a microplate reader. Data for each treatment are represented as the mean $\pm$ SD. \* $p$ <0.05 versus blank and negative-siRNA group

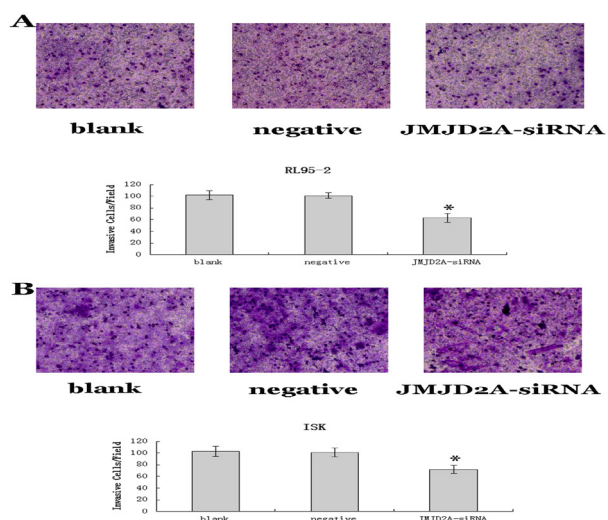


**Figure 4. Effects of JMJD2A Silencing on RL95-2 and ISK Cell Migration.** After transfection with either negative-siRNA or JMJD2A-siRNA for 48 h, or being left untreated, the RL95-2 and ISK cells were tested for migration using a Transwell chamber and were evaluated on the basis of the mean values from five fields of view at 200 $\times$  magnification for each treatment. Bars represent the mean $\pm$ SD. \* $p$ <0.05 versus blank and negative-siRNA

48h, we examined cell proliferation rates using a CCK-8 assay. The results were similar in the two cell lines (Figure 3). In the JMJD2A-siRNA group, cell proliferation rate was markedly reduced compared with that in the blank and negative control groups ( $P$  < 0.05). However, there was no marked difference between the blank and negative control groups ( $p$  > 0.05). These data demonstrated that JMJD2A silencing suppressed proliferation of human endometrial carcinoma cell lines RL95-2 and ISK.

#### *Knockdown of JMJD2A inhibits migration and invasion of human endometrial carcinoma cells*

To extend our study to the function of JMJD2A in endometrial carcinoma cells, we observed cell migration and invasion of cell lines using a Transwell assay after transfection with JMJD2A-siRNA and control-siRNA for



**Figure 5. Effects of JMJD2A Silencing on RL95-2 and ISK Cell Invasion.** After transfection with either negative-siRNA or JMJD2A-siRNA for 48 h, or being left untreated, the RL95-2 and ISK cells were tested for invasion using a Transwell chamber and were evaluated on the basis of the mean values from five fields of view at 200 $\times$  magnification for each treatment. Bars represent the mean $\pm$ SD. \* $p$ <0.05 versus blank and negative-siRNA

48h. After knockdown of JMJD2A, cell migration in the JMJD2A-siRNA group was markedly reduced compared with that in the blank and negative control groups ( $p$  < 0.05) (Figure 4). However, there were no marked differences between the blank and negative control groups ( $p$  > 0.05). Figure 5 shows that cell invasion was significantly inhibited after transfection with JMJD2A-siRNA compared with that in the blank and negative control groups ( $p$  < 0.05). However, in the negative control group, there was no significant difference from the blank control group ( $p$  > 0.05). These data suggested that knockdown of JMJD2A downregulated cell migration and invasion of endometrial carcinoma cell lines RL95-2 and ISK.

## **Discussion**

Previous studies have demonstrated that JMJD2A is widely overexpressed in human tissue and cell lines, including breast cancer (Li et al., 2011; Berry et al., 2012), colon cancer (Kim et al., 2012), bladder cancer (Kauffman et al., 2011), and prostate cancer (Shin et al., 2007), although there is rarely evidence about expression of JMJD2A in endometrial carcinoma. We determined JMJD2A expression level in 56 endometrial carcinoma tissues and 20 normal endometrial tissues by immunohistochemistry. JMJD2A was expressed in approximately 64% of endometrial carcinoma tissues, and overexpressed in endometrial carcinoma tissues compared with benign endometrial tissues, and there was no significant relation with tumor age, histological differentiation and stage.

Recent studies have implicated a role of JMJD2A in tumorigenesis, although the underlying mechanisms are unknown. A recent study has found a correlation between JMJD2A and endogenous ER $\alpha$  expression in breast cancer (Berry et al., 2012). In the present study, JMJD2A

formed complexes with ER $\alpha$  *in vivo* and thus potentially functions as a transcriptional cofactor of ER $\alpha$ . Catalytic activity of JMJD2A was required for its ability to stimulate maximally estrogen-dependent gene transcription. It is well known that endometrial carcinoma is frequent estrogen-sensitive malignancy (Wang et al., 2011). ER $\alpha$  plays a pivotal role in the genesis of the majority of endometrial carcinomas (Arafa et al., 2010). We selected two ER-positive endometrial carcinoma cell lines, RL95-2 and ISK, to verify our hypothesis. JMJD2A mRNA and protein were overexpressed in endometrial carcinoma cell lines and their silencing inhibited cell proliferation, invasion and metastasis. JMJD2A exhibits the features of an oncoprotein, whose action may be particularly relevant in ER $\alpha$ -positive endometrial carcinoma. Next, we discuss the probable mechanism of action of JMJD2A in endometrial carcinoma.

Endometrial carcinoma is an estrogen-dependent disease. Epidemiological studies have found that estrogens play an important role in endometrial carcinogenesis. Pervious studies have shown that concentration of estrogen in endometrial carcinoma tissue was significantly higher than that in benign endometrium (Berstein et al., 2003). The majority of endometrial cancers are thought to arise due to excess estrogen stimulation, unopposed by progesterone, promoting mitogenesis, atypical hyperplasia, and the transition to malignant adenocarcinoma (Amant et al., 2005; Ryan et al., 2005; Di et al., 2007). Estrogen plays a major role in the development and growth of breast cancer. Estrogen promotes cell proliferation that increases the chance for random genetic errors to be permanently introduced in the genome of somatic cells (Shah et al., 2004). In endometrial carcinoma, as well as breast carcinoma, estrogen contributes greatly to the growth and development of estrogen-dependent tumors (Thomas et al., 1984; Hecht et al., 2006). Several studies have demonstrated that estrogen can stimulate the *in vitro* proliferation of cells, including ER $\alpha$ -positive carcinoma cells. However, because estrogen is not directly mitogenic, it is proposed that growth factors, in particular insulin-like growth factor-I, mediate estrogen-induced carcinoma cell proliferation (Soto et al., 1987; Dickson et al., 1995; Sirbasku et al., 2000). The evidences shows that estrogen metabolism and synthesis are important in the etiology and progression of endometrial carcinoma. One recent study showed that the JMJD2A histone demethylase as a novel coactivator of ER $\alpha$ , formed a complex with ER $\alpha$ , and overexpression of JMJD2A enhanced estrogen-dependent transcription in breast carcinoma. According to these results, JMJD2A may exhibit features of an oncoprotein via an estrogen-dependent signaling pathway.

JMJD2A coactivates ER $\alpha$  and the p53 receptor. Kim et al. have found that JMJD2A forms complexes with p53 *in vivo* and binds to it *in vitro* in colon cancer (Kim et al., 2012). Knockdown of JMJD2A increases p53 levels and further stimulates colon cancer cell proliferation. The tumor suppressor p53 induces target genes for cell cycle arrest, apoptosis, and DNA repair (Vousden et al., 2009). The expression of ER is correlated with the status of p53, and knockdown of p53 decreases, whereas overexpression of p53 increases, ER expression

in carcinoma cells (Angeloni et al., 2004; Shirley et al., 2009). It has been reported that DNA damage increases expression of ER in a p53-dependent manner (Shirley et al., 2009). It has been shown that upon treatment with estrogen, p53 expression is enhanced, suggesting that ER may regulate p53 expression (Qin et al., 2002). It has been clearly demonstrated that knockdown of ER inhibits, whereas overexpression of ER enhances DNA-damage-induced growth suppression in a p53-dependent manner, suggesting that p53 is a direct transcriptional target of ER in ER-positive carcinoma cells (Berger et al., 2012). This may explain how JMJD2A can regulate proliferation, invasion and metastasis of endometrial carcinoma cells via the estrogen-p53 signaling pathway in ER-positive endometrial carcinoma.

Consequently, the ability of JMJD2A to stimulate endometrial carcinoma cell proliferation, invasion and metastasis strongly indicates that JMJD2A plays an important role during tumorigenesis via the estrogen-p53 signaling pathway. This deepens our understanding of the mechanism of endometrial carcinoma development and progression. Taken together, our data suggest that JMJD2A is a novel potential drug target.

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