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Original Article

Histone demethylase JMJD1C regulates esophageal cancer proliferation Via YAP1 signaling

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Abstract: Esophageal cancer (EC) is the most lethal cancer, and it is of significant concern worldwide, particularly in China. However, there are no effective treatments to cure it, such as chemotherapy, surgery, or radiotherapy. This is attributed to the lack of understanding of the molecular mechanisms of EC. Recently, the superfamily of Jmj-containing KDMs has been shown to play an important role in tumorigenesis in various cancers, including EC. In this study, we demonstrated that JMJD1C was upregulated in patient EC tissues and different EC cell lines. Furthermore, JMJD1C levels were positively correlated with the TNM stage. Moreover, the colony formation assay, CCK8, and cell number count assay showed that the knockdown of JMJD1C inhibited EC cell proliferation. Western blot analysis and the quantitative real-time polymerase chain reaction assay showed that the knockdown of JMJD1C repressed the protein and mRNA levels of YAP1 via regulating the H3K9me2 activity, but not the H3K9me1 activity. The colony formation assay, CCK8 analysis, and cell number count assay revealed that inhibition of EC cell proliferation by the knockdown of JMJD1C was rescued by overexpression of YAP1. Taken together, our results demonstrated that JMJD1C controls the proliferation of EC via modulation of H3K9me2 activity, targeting the YAP1 gene expression and functions as a tumor suppressor in EC. This novel pathway may serve as a therapeutic target for EC patients.

Keywords: JMJD1C, YAP1, H3K9me2, esophageal cancer, proliferation

Introduction

Esophageal cancer (EC) is the most lethal cancer, and it has a very high incidence in China [1]. Although many clinical techniques have been used to treat it, such as chemotherapy, surgery, and radiotherapy, none represents an effective strategy. Recent reports indicate that the number of patient deaths and the 5-year survival rate of EC are still alarming. The reason the outcomes for patients with EC remain poor is attributed to the lack of understanding of the molecular mechanisms of EC [2].

Previous studies have shown that epigenetic changes play important roles in the progression of tumors [3, 4]. Recently, histone lysine methylation has been considered as an important factor in the process in the tumor development [5, 6]. However, how histone lysine demethylases (KDMs) are involved in the initiation and progression of EC is still unclear. To date, a considerable number of histone demethylases have been identified, the majority of these belonging to the superfamily of Jmj-containing KDMs [7]. Numerous reports have shown that the deregulation of Jmj-containing KDM proteins alters cancer development [8-10]. For instance, Tae-Dong Kim and his colleagues found that overexpression of JMJD2A, also known as KDM4A, can initiate prostate cancer development through binding to transcription factor ETV1 [11]. In other cancers, a variety of Jmj-containing KDM proteins are also deregulated, such as those in breast cancer, and KDM2A and KDM3A are upregulated [12, 13]. Recently, Watanabe et al. reported that JMJD1C has been identified as a DNA-damage response (DDR) component to regulate the survival of cancer cells [14]. Mo Chen et al. showed that JMJD1C is essential for the survival of acute myeloid leukemia (AML) [15]. Also, Ek et al. demonstrated that JMJD1C might be associated with the risk of EC [16]. Considering the above evidence, it seems that JMJD1C may be involved in the development of EC. However, the

molecular mechanism is still far from being understood.

The Hippo pathway is a highly conserved regulator of organ size and cell fate. YAP1, as the core component, is deregulated in a wide range of cancers, such as lung cancer, colon cancer, and liver cancer [17-19]. A limited number of reports in the literature indicate that YAP1 is a tumor suppressor gene in a variety of cancers. For example, Yuan et al. reported that decreased YAP1 expression is detected in clinical luminal breast cancer tissues compared to normal tissues and that knockdown YAP1 strongly increases tumor migration and proliferation in vitro and in vivo [20]. However, the majority of studies support the concept that YAP1 acts as an oncogene in tumor progression. In liver cancer, upregulation of YAP1 protein is required to maintain tumor growth [21]. In addition, Wang et al. clearly showed that overexpression of YAP1 is distinctly correlated with poor survival and metastasis in non-small cell lung cancer (NSCLC) [22]. Other reports also indicate that increased YAP1 proteins promoted tumor growth and malignancy in different types of cancer, such as epithelioid hemangioendothelioa, Kaposi sarcoma, and colorectal cancer [23-25]. Recently, Zhao et al. showed that knockdown YAP1 greatly decreases the growth ability of EC [26]. However, the precise molecular mechanism by which YAP1 is upregulated in EC is not fully known.

In this study, we showed that JMJD1C expression is upregulated in EC where it is positively associated with YAP1 upregulation. Knockdown JMJD1C strongly inhibited cancer cells growth, and JMJD1C can transcriptionally regulate the YAP1 protein and mRNA levels by dependence on H3K9me2 activity. Moreover, we demonstrated that the impacts of JMJD1C on cancer cells strongly depend on YAP1. Taken together, our results confirmed that JMJD1C functions as an oncogene in EC, the upregulation of which contributes to the activation of the YAP1/TAZ signaling pathway and EC growth.

Materials and methods

Cell lines and cell culture

Primary cultures of normal esophageal epithelial cells (NEECs) were established from fresh specimens of the adjacent normal esophageal tissues >5 cm from the cancerous tissue, according to previously reported studies. The following EC cell lines: Eca109, EC18, KYSE-140, HKESC1, and KYSE-30 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in a humidified incubator under 5% CO_2.

Clinical samples

In this study, the EC samples and adjacent normal tissues were obtained and clinically diagnosed at the Tongji Hospital, Wuhan, China. This study was approved by the Ethics Committee of the Huazhong University of Science and Technology, and all patients provided written informed consent prior to the study. All fresh clinical samples were fast-frozen in liquid nitrogen and then stored at -80°C until required.

Immunohistochemistry

The expression of YAP1 was examined by immunohistochemistry (IHC) analysis in the paraffin-embedded EC samples used in this study. To evaluate YAP1 expression, the scores were determined by the staining intensity and the percentage of positively stained areas. Staining intensity was scored according to the following standard: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). In addition, the extent of staining was scored as follows: 0 (0%), 1 (<10%), 2 (10%-35%), 3 (36%-75%), or 4 (>75%). The final staining index was the sum of the staining intensity and extent scores. Using this method to evaluate the protein expression, the clinical samples were reviewed and scored separately by two experienced pathologists.

Knockdown and overexpression experiment

Two short hairpin RNA (shRNA) oligonucleotides were cloned into a lentiviral vector (Thermo Scientific, Waltham, MA, USA), and lentivirus production and purification were performed according to the manufacturer's instructions. We then generated the stable cell lines expressing JMJD1C shRNA and the negative control cell lines expressing sequence-mismatched shRNA by using a treatment with 1 ug/ml puromycin every 48 h for 2 wk. The total gene of YAP1 was

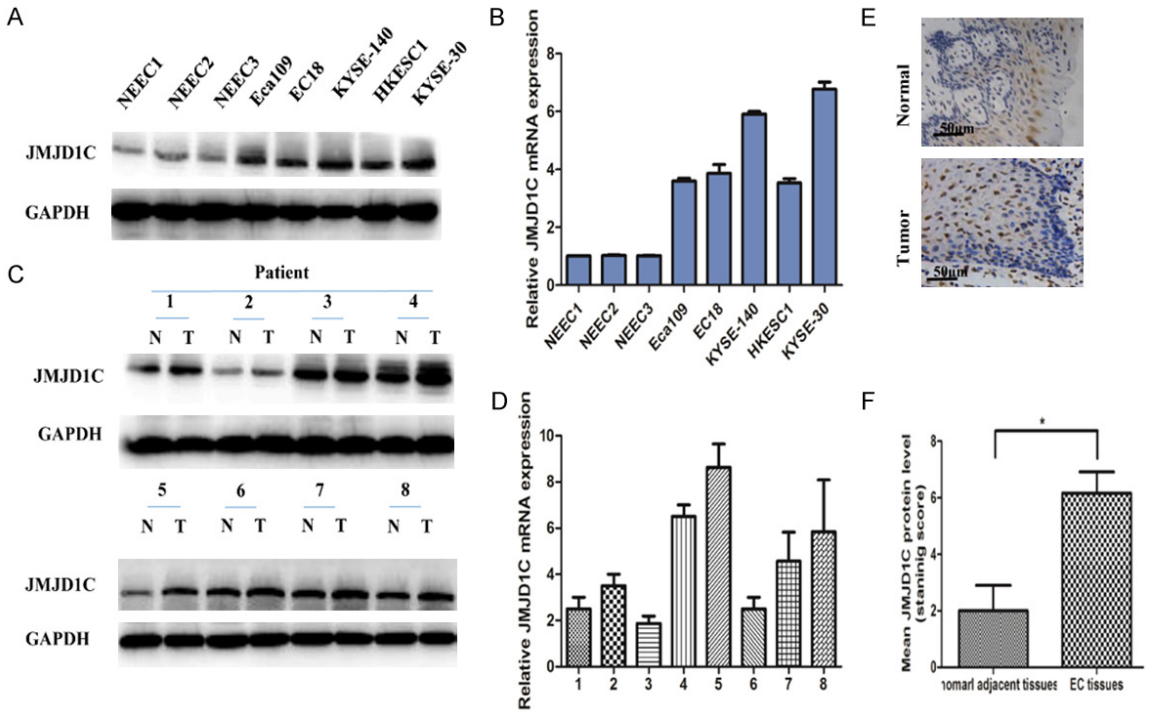

Figure 1. JMJD1C is overexpressed in EC and correlated with TNM stage. JMJD1C protein and mRNA expression were analyzed in 3 NEECs and 5 EC cell lines (A, B). JMJD1C protein and mRNA expression were examined in 8 paired human EC tissues and the adjacent normal ES tissues, GAPDH was used as reference gene in qRT-PCR analysis and as a protein loading control for western blot (C, D). The JMJD1C immunohistochemistry staining of human EC tissues and the adjacent normal ES tissues. Scale bars: 50 μm (E). Analysis of JMJD1C protein expression using the staining score. Student's *t*-test (*P<0.05) (F).

cloned into the pcDNA3.1 vector. For the overexpression experiment, transient transfection of the pcDNA3.1-YAP1 plasmid and the pcDNA3.1-Vector were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Western blotting

Whole cells were harvested 48 h after transfection and lysed directly in NP40 solution, adding 1 mM PMSF prior to use. Then, protein extracts were boiled with 5 × SDS sample buffer at 100°C for 10 min, electrophoresed on SDS-PAGE gel, and transferred to PVDF membranes. In brief, membranes were incubated with primary antibodies against JMJD1C, YAP1, and GAPDH overnight at 4°C. Subsequently, the bands were incubated with a special secondary antibody and visualized with ECL blotting detection reagents.

RNA isolation and real-time PCR (qPCR)

Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA) from EC cells, and then cDNA was synthesized from RNA using Master Mix 5 × (Takara, Tokyo, Japan). The following PCR conditions were used: 37°C for 15 min, 85°C for 5 s, and 4°C forever. For qPCR, the following PCR conditions were used: 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C.

Colony formation assay and cell number count assay

For the colony formation assay, EC cells were seeded in 6-well plates (100 cells/well), then the medium was replaced every 3 d. After 2 wk, the number of colonies were counted in each well. A colony was counted as such when it had more than 50 cells. The capability of colony formation was evaluated by the colony formation number. For the cell number count assay, cells were seeded in 6-well plates (3×10^5 cells/well), and then the number cells were counted daily. All experiments were done in triplicate.

Luciferase reporter assay

For the reporter assay, we first generated a YAP1 promoter reporter plasmid, which con-

Table 1. Correlation between clinicopathological characteristics and expression of JMJD1C in the EC patients (n=100)

Characteristics	n	JMJD1C (staining score >3)	Percent	xe	*P*-value
Sex					
Male	52	38	73.1	0.642	0.547
Female	48	30	62.5		
Age					
<55	38	30	78.9	0.734	0.522
≥55	62	42	67.7		
Differential grade					
Poor	44	30	68.2	0.849	0.654
Middle	34	24	70.6		
Well	22	12	54.5		
LN metastesis					
Positive	48	20	41.7	3.848	0.086
Negative	52	36	69.2		
pTNM stage					
I	22	12	54.5	6.413	0.023
II	33	26	78.8		
III	45	37	82.8		

Note: EC, esophageal cancer; LN, lymph node; pTNM, pathological tumor, node, metastasis.

tained 2000 bp YAP1 promoter in the pGL6 basic vector. Then, EC cells were grown in 12-well plates to about 40% confluency and cotransfected with 2 ng of YAP1 promoter reporter plasmid, 25 ng pRL-TK Renilla luciferase reporter vector according to the manufacturer's instructions. The cells were harvested after 3 d and luciferase activities were assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Chromatin immunoprecipitation (ChIP) assay

In brief, EC cells were grown in 10 cm culture dishes and treated with 1% formaldehyde to cross-link the proteins to DNA, and then, according to the manufacturer's instructions, the promoter DNA fragments were released from chromatin fragments pulled down with a special primary antibody. The ChIP-DNA was used for the PCR or qPCR.

Statistical analysis

SPSS v. 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data are shown as means ± standard deviation (SD). The chi-square test was used to evaluate the association with JMJD1C expression and clinicopathological characteristics. Differences were considered statistically significant at $P<0.05$.

Results

JMJD1C is overexpressed in EC and correlated with TNM stage

To assess the biological function of JMJD1C in EC, we first determined the protein and mRNA expression of JMJD1C in different EC cell lines, compared to 3 primary cultured NEECs, and in 8 human EC tissues, compared with the matched adjacent normal tissues. As shown in **Figure 1A-D**, these data indicate that JMJD1C is upregulated in EC.

Next, in order to investigate the clinical significance of JMJD1C in EC, we used IHC to detect the relative expression of JMJD1C in 100 samples of paired EC tissues. As shown in **Figure 1E** and **1F**, JMJD1C was strongly upregulated in EC tissues compared to the adjacent normal tissues. We were surprised to find that JMJD1C levels were positively correlated with the TNM stage ($P<0.05$, **Table 1**). Taken together, our results suggest a possible link between JMJD1C overexpression and EC development.

Knockdown of JMJD1C inhibits EC cells proliferation

To further assess the impact of JMJD1C on the growth of EC, we generated Eca109 and EC18 EC cells that stably expressed JMJD1C shRNA. Western blot and qPCR were used to examine the expression of JMJD1C (**Figure 2A** and **2B**). We found that knockdown JMJD1C dramatically reduced cell numbers compared with the control shRNA cells in Eca109 and EC18 cells (**Figure 2C**). Furthermore, CCK8 analysis indicated that depletion of JMJD1C inhibited the viability of EC cells (**Figure 2D**). Consistently, the colony-forming capability of cells with stable depletion of JMJD1C was also significantly weakened compared with the control cells (**Figure 2E**). These results reveal that JMJD1C provokes proliferation in EC cells in vitro.

Silencing JMJD1C downregulates YAP1 in EC cells through regulating H3K9me2

In regard to the molecular mechanism of the effect of JMJD1C on the proliferation of EC cells, previous studies have suggested that the

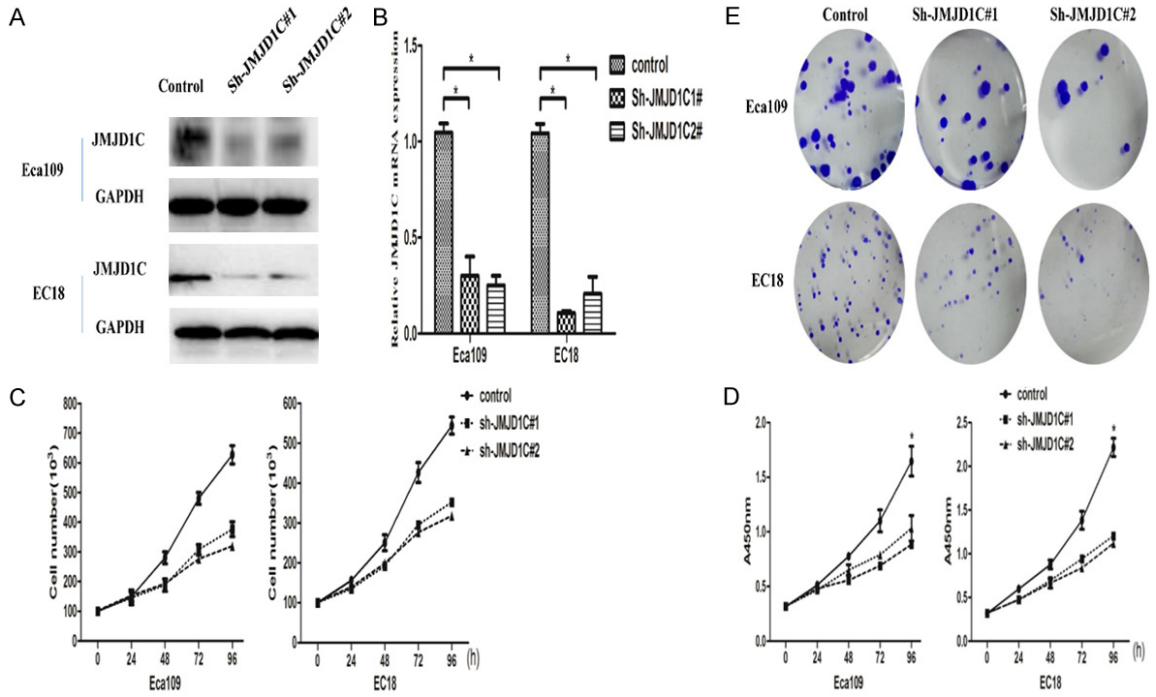

Figure 2. Knockdown of JMJD1C inhibits EC cells proliferation. Western blot analysis of JMJD1C expression in cells stably expressing JMJD1C shRNA and control cells, GAPDH as the loading control (A). Cell growth assays. Cell numbers were counted to assess cellular growth between stably expressing JMJD1C shRNA and control cells (B). Cell viability assays. Cells were analyzed using the CCK8 assay to analyze cell viability between stably expressing JMJD1C shRNA and control cells. Results are shown as averages with SD (C). Colony formation assays. Cells stably expressing JMJD1C shRNA and control cells were subjected to colony formation assays. Student's *t*-test (*P<0.05) (D).

superfamily of Jmj-containing KDM proteins could regulate the expression of YAP1 to modulate the growth of cancer cells [11, 27]. In addition, recent reports also demonstrated that silencing YAP1 strongly inhibited EC cells proliferation. These studies prompted us to consider whether YAP1 was also involved in the JMJD1C-induced growth in EC cells. As shown in **Figure 3A**, Western blot assays showed that silencing JMJD1C greatly downregulated the protein levels of YAP1, compared with the control cells in Eca109 and EC18 cells. Consistent with this, qPCR results obviously indicated that knockdown JMJD1C transcriptionally reduced the mRNA levels of the YAP1 gene in EC cells (**Figure 3B**). Moreover, the luciferase activity caused by the YAP1 promoter was reduced in cells stably expressing the JMJD1C shRNA compared with the cells expressing the control shRNA (**Figure 3C**).

Several studies have demonstrated that JMJD1C has histone H3K9me2 and H3K9me1 demethylase activity. Thus, we hypothesized that the regulation of YAP1 by JMJD1C depends on the function of H3K9me2. In accordance with this expectation, we found that JMJD1C directly binds to the YAP1 promoter (**Figure 3D**). In addition, we examined the H3k9me2 levels by ChIP assays in cells stably expressing JMJD1C shRNA and the control cells. As shown in **Figure 3E** and **3F**, the promoter of YAP1 showed increased H3K9me2 levels, but not H3K9me1, in cells expressing JMJD1C shRNA, compared with the control cells.

The impact of JMJD1C on the growth of EC cells partly depends on YAP1

Next, to further assess the effect of YAP1 on JMJD1C-induced growth in EC cells, we carried out the following "rescue experiments". We constructed pcDNA3.1 plasmid containing the cDNA of the YAP1 gene, and we transiently transfected pcDNA-YAP1 and pcDNA3.1-WT in cells stably expressing JMJD1C shRNA. Then, Western blot was used to detect the corresponding protein levels (**Figure 4A**). Interestingly, we found that overexpression YAP1 can promote EC cells growth, which is consistent

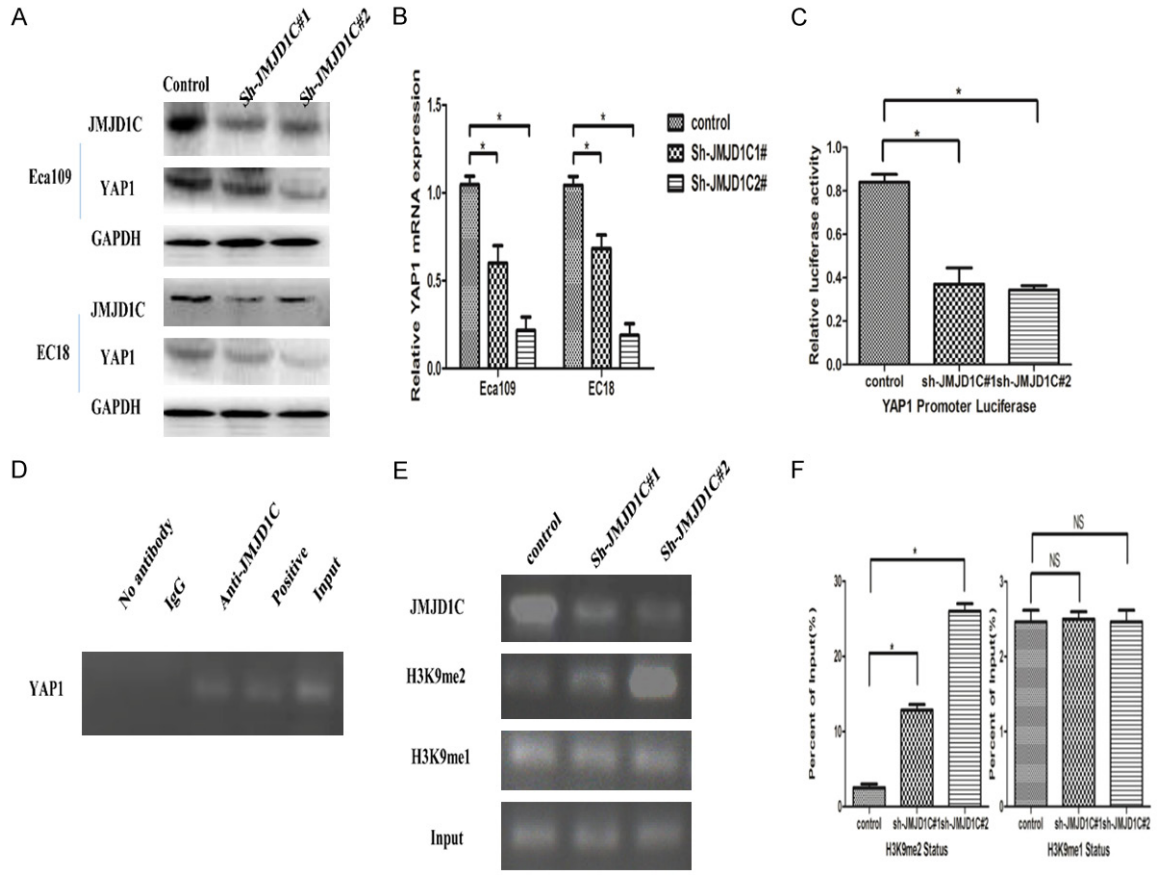

Figure 3. Silencing JMJD1C downregulates YAP1 in EC cells through regulating H3K9me2. Western blot analysis of JMJD1C and YAP1 expression in cells stably expressing JMJD1C shRNA and control cells, GAPDH as the protein loading control (A). qRT-PCR analysis of the mRNA levels of JMJD1C and YAP1 gene in cells stably expressing JMJD1C shRNA and control cells, GAPDH as the reference gene control (B). YAP1 promoter luciferase activity analysis between EC18 cells stably expressing JMJD1C shRNA and control EC18 cells (C). ChIP assay with JMJD1C, H3K9me2, and H3K9me1 Abs at YAP1 gene promoter in EC18 cells. Student's *t*-test (*P<0.05); NS, not significant (D-F).

with the recent literature; furthermore, we found that overexpression of YAP1 markedly rescued cell growth (**Figure 4B** and **4C**) and clonogenic capacity (**Figure 4D**) inhibited by silencing JMJD1C. Taken together, these results strongly indicate that silencing JMJD1C downregulated YAP1 and then decreased the proliferation of EC cells.

Discussion

In this study, we found that overexpression of JMJD1C occurs in human EC tissues, compared with the matched adjacent normal tissues, and is positively associated with the TNM stage. Meanwhile, we uncovered a clear molecular mechanism by which the histone demethylase JMJD1C promotes EC cell growth via regulation of YAP1. Therefore, our data showed a novel signaling pathway for proliferation of EC.

Previously, numerous studies have demonstrated that epigenetic changes are markedly associated with tumor initiation and progression [28]. Many efforts have been made to uncover how histone lysine methyltransferases modulate the precise molecular mechanism involved in cell growth, differentiation and apoptosis, especially in tumor cells [29-31]. Strikingly, the histone lysine demethylase superfamily plays an important role in the development of tumors. A recent study indicated that overexpressed JMJD2A can initiate prostate cancer development through interacting with transcription factor ETV1 [11]. In gastric cancer, Huang et al. showed that KDM2A can also promote tumor cell growth and migration [32]. The other members of the histone lysine demethylase superfamily are also deregulated in various cancers. For instance, KDM3A is upregulated in prostate and liver cancers [33, 34], and KDM4C is

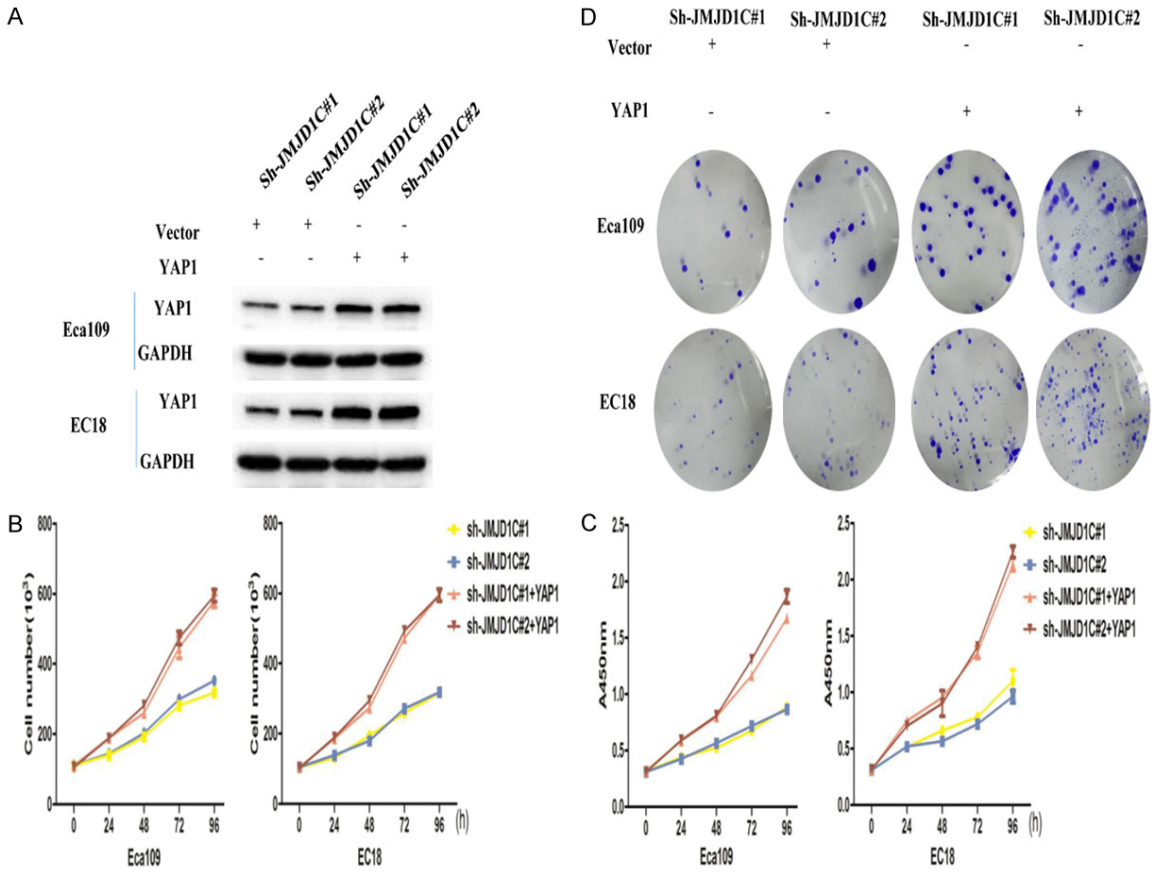

Figure 4. The impact of JMJD1C on growth of EC cells partly depends on YAP1. Western blot analysis of JMJD1C and YAP1 expression in cells stably expressing JMJD1C shRNA with or without transiently overexpression YAP1 (A). Cell growth assays. Cell numbers were counted to assess cellular growth between stably expressing JMJD1C shRNA with or without transiently overexpression YAP1 (B). Cell viability assays. Cells were analyzed using the CCK8 assay to analyze cell viability between stably expressing JMJD1C shRNA with or without transient overexpression YAP1. Results are shown as averages with SD (C). Colony formation assays. Cells stably expressing JMJD1C shRNA with or without transient overexpression YAP1 were subjected to colony formation assays. Student's *t*-test (*P<0.05) (D).

essential to EC [35]. By contrast, KDM6A is downregulated in a great diversity of tumors, such as liver and esophageal tumors, and in leukemia [36]. JMJD1C, which has been rarely studied, was only recently found to be required for the survival of acute myeloid leukemia [15]. Moreover, Ek et al. showed that JMJD1C might be associated with the risk of EC [16]. Consistent with these studies, we found that JMJD1C was upregulated in EC tissues, and that it was significantly correlated with the TNM stage in EC. Furthermore, we determined that knockdown JMJD1C decreased the growth of EC cells.

Recently, a considerable number of reports have shown that the deregulation of Hippo pathway signaling plays crucial roles in cell survival, proliferation, and metastasis of various carcinomas. As the core component, YAP1 is highly expressed in several types of cancer, such as lung cancer, breast cancer, pancreatic cancer, and others. Moreover, current reports indicated that Hippo pathway activity frequently is regulated via interaction with other oncogenic singling pathways [17]. For instance, Konsavage et al. found that Wnt/β-catenin signaling strongly regulates the expression of the YAP1 gene in colon cancer cells [37]. Also, it has been reported that GPCRs as the upstream regulators of the Hippo pathway positively enhance its activity [38]. To this extent, YAP1 can be considered as an oncogene. Actually, YAP1 is involved in cellular biology as a means to maintain cellular proliferation and to increase cellular invasion and metastasis. However, pre-

vious research also suggests that YAP1 might function as a tumor suppressor involved in various carcinomas. For example, decreased YAP1 expression was examined in clinical luminal breast cancer tissues, compared to normal tissues, and, interestingly, it was found that knockdown YAP1 strongly enhances tumor migration and proliferation in vitro and in vivo [20]. In addition, Yu et al. also demonstrated that overexpression of YAP1 markedly reverses anoikis resistance and metastasis induced by miRNA-200a in human breast cancer [39]. Although studies of EC are limited, it seems likely that YAP1 also can function as an oncogene in EC. For example, Zhao et al. showed that knockdown YAP1 strongly decreases the growth capacity of EC [36]. However, the precise molecular mechanism by which YAP1 is upregulated in EC remains poorly understood. In this study, we demonstrated that YAP1 is directly regulated by JMJD1C at the transcriptional level. Furthermore, we confirmed the hypothesis that the impact of JMJD1C on the growth of EC cells partly depends on YAP1.

In combination with previous studies, our data contribute strong evidence that JMJD1C plays key roles in EC pathological processes. Of course, additional work is needed to determine the reason JMJD1C is upregulated in human EC, which might offer a novel anticancer therapeutic strategy.

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Disclosure of conflict of interest

None.

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