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Histone Demethylase JARID1B Is Overexpressed in Osteosarcoma and Upregulates Cyclin D1 Expression via Demethylation of H3K27me3

Wei Wang, Ke Zheng, Yi Pei, and XiaoJing Zhang

Department of Bone and Soft-Tissue Tumor Surgery, Cancer Hospital of China Medical University, Shenyang, Liaoning Province, P.R. China

JARID1B has been proven to be upregulated in many human malignancies and is correlated with tumor progression. However, its expression and clinical significance in osteosarcoma are still unclear. Thus, the aim of this study was to explore the effects of JARID1B in osteosarcoma tumorigenesis and development. In this study, we found that the expression levels of JARID1B in osteosarcoma tissues were significantly higher than those in corresponding noncancerous bone tissues. In addition, JARID1B upregulation occurred more frequently in osteosarcoma specimens from patients with a poor prognosis. After JARID1B transfection in osteosarcoma cells, cell proliferation was significantly promoted in vitro and in vivo. On the contrary, knockdown of JARID1B inhibited cell proliferation in vitro and tumor growth in vivo. JARID1B can also decrease the G_0/G_1 phase cell numbers and increase the S and G_2/M phase cell numbers. We further demonstrated that JARID1B regulates cyclin D1 expression through H3K27me3. These findings indicate that JARID1B may act not only as a novel diagnostic and prognostic marker but also as a potential target for molecular therapy in osteosarcoma.

Key words: JARID1B; Osteosarcoma; Proliferation; Cyclin D1; H3K27me3

INTRODUCTION

Osteosarcoma is the most common primary malignant tumor of the bone affecting adolescents and young adults[1,2]. However, the prognosis of patients with recurrence and metastasis is still poor. Therefore, a novel and more reliable gene marker is needed to help estimate the clinicopathologic characteristics and prognosis of osteosarcoma[3,4].

Histone methylation is a kind of posttranslational modification that affects chromatin formation, translational regulation, injury repair, and other biochemical processes. A previous study has proven that a methylation modification may occur in multiple lysine residues on histones H3, such as H3K4, H3K9, H3K27, H3K36, and H3K79[5–7]. Since LSD1 was found in 2004[8], there have been dozens of histone demethylases found, which include two types: the first type takes flavin adenine dinucleotide (FAD) as a catalytic cofactor, such as LSD1, and the second type contains the JmjC domain. Histone demethylation with JmjC domain enzyme family members includes JmjC domain-containing histone demethylase 1 (JHDM1), JmjC domain-containing histone demethylase 2 (JHDM2), JmjC domain-containing histone demethylase 3/jumonji domain containing 2 (JHDM3/JMJD2), jumonji, AT-rich interactive domain 1 (JARID1), and ubiquitously transcribed tetratricopeptide repeat gene, X chromosome/jumonji domain containing 3 (UTX and JMJD3). The JARID1 protein family has four members: JARID1A, JARID1B, JARID1C, and JARID1D[9].

JARID1B, which belongs to the second type, can specifically remove H3K4 trimethylation, thereby inhibiting the corresponding gene transcription. It plays an important role in the occurrence of breast, prostate, and other cancers and is considered to be an important drug target. The JARID1B subtribe contains five conserved domains, including JmjC, JmjN, ARID, PHD, and ZF (zinc finger-like domain). The JmjC domain is highly conserved and present in more than 100 proteins and is the catalytic domain of histone demethylation. H3K4 methylation is the key signal for RNA polymerase binding to genes and rotates transcription factor binding to the promoter[10,11]. JARID1B is a transcription inhibitor that directly regulates cell cycle, cell differentiation, and gene expression in cell lines[12]. However, other research found that JARID1B also can inhibit the cell cycle gene switch by inhibiting H3K4 methylation, thereby inhibiting cells from going from the G_0 to the G_1 phase[10]. JARID1B expression

Address correspondence to XiaoJing Zhang, Department of Bone and Soft-Tissue Tumor Surgery, Cancer Hospital of China Medical University, No. 44 Xiaoheyan Road, Dadong District, Shenyang 110042, Liaoning Province, P.R. China. E-mail: zyltg2013@163.com

and its clinical significance in osteosarcoma are still unclear. Thus, the aim of this study was to explore the effects of JARID1B in osteosarcoma tumorigenesis and development.

In the current study, we show that JARID1B was overexpressed in osteosarcoma cells and tissues compared with normal bone tissues using quantitative reverse transcription (qRT)-PCR and Western blot. JARID1B expression is correlated with patient survival time. Both gain- and loss-of-function studies showed that JARID1B increased the ability of osteosarcoma cells to proliferate and increased tumor volume and weight in vivo. JARID1B can also decrease the G_0/G_1 phase cell numbers and increase the S and G_2/M phase cell numbers. We further demonstrated that H3K27me3 is a novel target of JARID1B. Therefore, JARID1B is believed to be a potential therapeutic target for use in the treatment of osteosarcoma.

MATERIALS AND METHODS

Patients and Tissue Samples

A total of 52 osteosarcoma tissue samples, along with matched normal tissues, were used in this study. All of the samples were obtained from the Department of Bone and Soft-Tissue Tumor Surgery, Cancer Hospital of China Medical University. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of China Medical University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Cell Culture and Reagents

The osteosarcoma cell lines (143B, HS755, SJSA-1, D17, Saos-2, and MG-63) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The signal silencing siRNA and its control siRNA were purchased from Cell Signaling Technology. All of the remaining reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

Plasmid Construction and Transfection

For overexpression, the cDNA representing the complete open reading frame of JARID1B was cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to generate the JARID1B expression plasmid. The expression plasmid was verified by sequencing both strands and was used to transfect D17 cells to establish the JARID1B overexpression cell line. For JARID1B RNA interference, the control and JARID1B shRNA plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and were used to transfect the MG-63 cells to establish the JARID1B knockdown cell line. The transfection efficiency of JARID1B was confirmed by Western blotting and qRT-PCR analysis.

MTT Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay was used to assess cell proliferation. The cells were seeded, and 20 ml of the MTT solution (5 mg/ml) was then added to each well at the indicated times. The absorbance at 490 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blot Assay

Equal amounts of protein were separated using SDS polyacrylamide gels and were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted overnight at 4°C with primary antibodies, followed by their respective secondary antibodies. β-Actin was used as the loading control.

Quantitative Reverse Transcription-PCR

RNA was extracted using TRIzol reagent according to the manufacturer's recommended protocol (Invitrogen). qRT-PCR was performed using Applied Biosystems (Foster City, CA, USA) StepOne and StepOne Plus Real-Time PCR Systems. GAPDH was used as a loading control. The experiments were repeated a minimum of three times to confirm the results.

In Vivo Tumor Growth Model

Male BALB/c nude mice 4 to 6 weeks old were purchased from the Hunan Slac Jingda Laboratory Animal Co., Ltd. (Changsha, P.R. China). For tumor growth assay, cells stably overexpressing JARID1B or shJARID1B were suspended in PBS, and 1×10^6 cells (200 μl) were subcutaneously injected into the dorsal flank of nude mice as previously reported[13,14]. Tumor size was measured every 3 days, and tumor volumes were calculated with the following formula: volume $=(L\times W^2)/2$, in which L is the longest diameter, and W is the shortest diameter. Twenty-two days later, mice were sacrificed, and tumors were dissected and weighed. Animal handling and research protocols were approved by the Ethics Committee of China Medical University.

mRNA Expression Arrays and Data Preprocessing

Total RNA quality and quantity were determined using an Agilent 2100 Bioanalyzer and NanoDrop ND-1000. Affymetrix HU U133 Plus 2.0 arrays were used according to the manufacturer's protocol. The data were initially

normalized by robust multiarray average (RMA) normalization algorithms in expression console software (Affymetrix, Santa Clara, CA, USA). Significantly altered genes between JARID1B overexpression and its control cells were assessed by scatter plots, and the genes were up- and downregulated ≥5-fold. Clustering analysis was done using the gene list by Gene Cluster v3.0 software, and heat maps were visualized using Java TreeView v1.1.4r3 software. Gene set enrichment analysis was carried out using ConceptGen (http://conceptgen.ncibi.org/core/conceptGen/index.jsp). Gene sets were either obtained from the ConceptGen or from published gene signatures.

Chromatin Immunoprecipitation (ChIP) Assays

The ChIP assay was carried out with antibodies against trimethyl H3K27 (rabbit polyclonal; Cat. No. 07-449; Upstate, Lake Placid, NY, USA). The assay was performed using the EZ-ChIP Kit (Millipore, Boston, MA, USA) according to the manufacturer's instructions. The cells were cross-linked for 10 min by the addition of formaldehyde to a final concentration of 1%. The cross-linking was stopped by adding 1/20 volume of 2.5 M glycine. This was followed by cell lysis and sonication. Antibody incubations were carried out overnight at 4°C. Reversal of cross-linking was carried out at 65°C for 3 h. The purified DNA was analyzed by quantitative PCR.

Statistical Analysis

The results were analyzed using SPSS 18.0 software (Chicago, IL, USA). Each experiment was repeated a minimum of three times. A two-tailed *t*-test was used to determine statistical significance. The results were presented as the means ± SD. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

JARID1B Was Upregulated in Human Osteosarcoma Carcinoma Tissues and Cell Lines

To explore the expression level of JARID1B in human osteosarcoma carcinoma development, 52 paired osteosarcoma carcinoma tissues and adjacent nontumor tissues were used. According to the qRT-PCR analysis, JARID1B was strongly upregulated in tumor tissues compared with the matched nontumor tissues (Fig. 1A and B). JARID1B expression was higher in metastatic tumor tissues compared with nonmetastatic tumor tissues (Fig. 1C). We confirmed the results using Western blot, and we found that JARID1B protein expression was elevated in tumor tissues compared with the matched nontumor tissues (Fig. 1D). We also found that patients with a higher JARID1B expression had a shorter survival time compared to those with normal JARID1B expression (Fig. 2).

Establishment of Stable JARID1B Transfection in Osteosarcoma Cancer Cell Lines

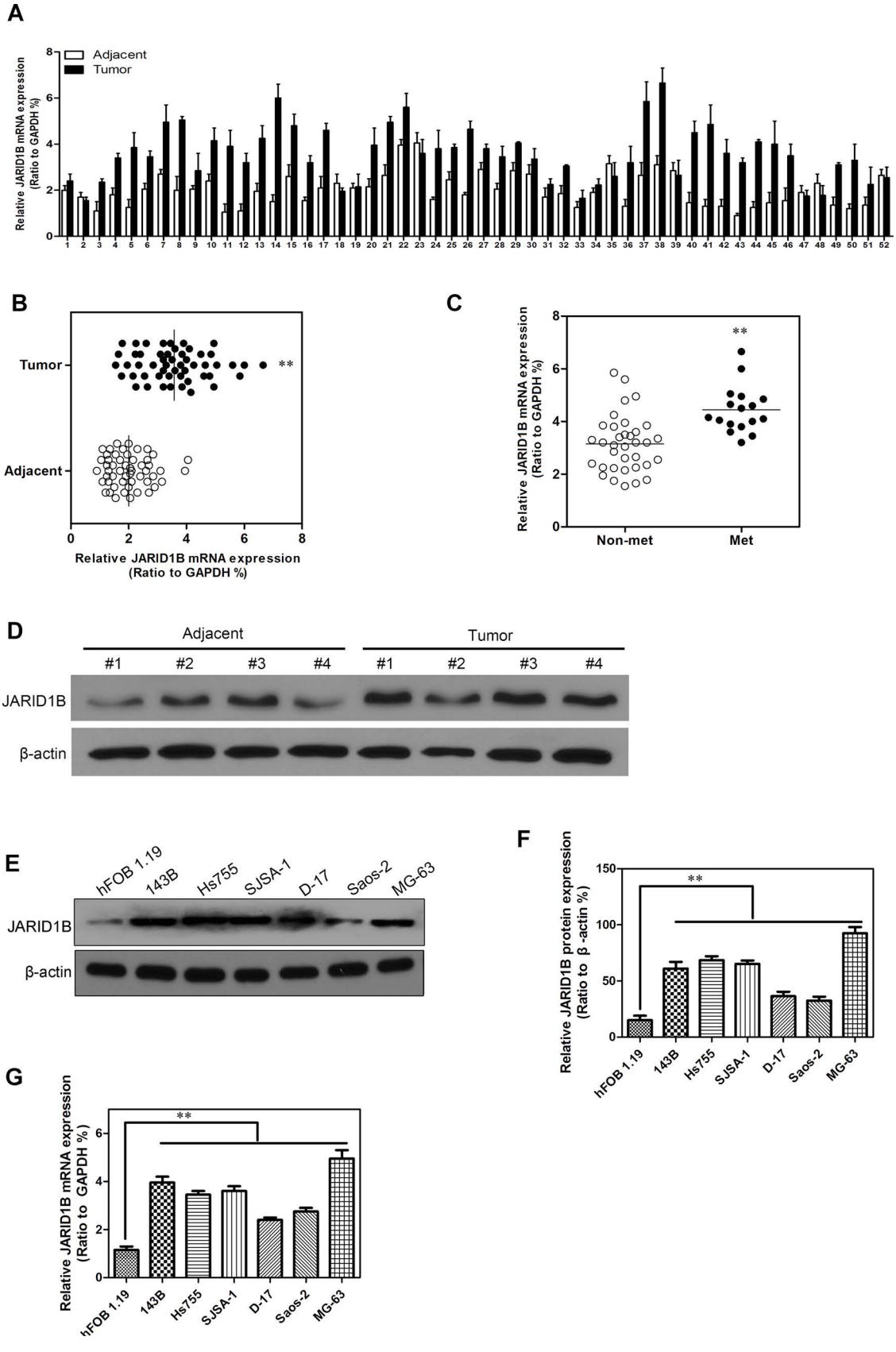
We then examined JARID1B expression levels in a panel of six widely used human osteosarcoma cell lines (143B, HS755, SJSA-1, D17, Saos-2, and MG-63) in comparison to levels in the nonmalignant cell line hFOB 1.19. Correspondingly, JARID1B expression levels were consistently increased in osteosarcoma cell lines, shown by Western blot and qRT-PCR. The MG-63 cell line showed the highest JARID1B expression, and D17 showed the lowest JARID1B expression in osteosarcoma cell lines (Fig. 1E–G). We used an inhibitor to generate a stable JARID1B knockdown in the MG-63 osteosarcoma cancer cell line. We also used D17 cells to establish stable cell lines that constitutively overexpressed JARID1B with the aim of revealing the role that JARID1B expression has in the development or progression of osteosarcoma cancer. The transfection efficiency was confirmed using qRT-PCR analyses. The MG-63 cells that had been transfected with JARID1B inhibitor displayed a significantly decreased JARID1B expression compared with the control cells, shown by Western blot and qRT-PCR (Fig. 3A and B). In addition, the D17 cells that had been transfected with the JARID1B expression plasmid displayed significantly increased JARID1B expression compared with the vector cell lines (Fig. 3C and D).

JARID1B Promoted Osteosarcoma Cell Proliferation In Vitro

We first explored the effects of JARID1B expression on cell growth using the MTT assay. Knockdown of JARID1B significantly inhibited the growth of MG-63 cells, whereas JARID1B overexpression significantly enhanced the growth of D17 cells (Fig. 3E and F). Knockdown of JARID1B could increase the G_0/G_1 phase cell numbers and decrease the S and G_2/M phase cell numbers (Fig. 4A and B), whereas overexpressed JARID1B could decrease the G_0/G_1 phase cell numbers and increase the S and G_2/M phase cell numbers (Fig. 4C and D). The findings demonstrated that JARID1B induces a more aggressive phenotype of osteosarcoma carcinoma and indicate that JARID1B is potentially oncogenic.

Increased JARID1B Expression Promotes Xenograft Tumor Formation

To further evaluate the potential effect of JARID1B on osteosarcoma cell proliferation in vivo, MG-63 cells transfected with shJARID1B and NC were subcutaneously inoculated into nude mice. Knockdown of JARID1B could inhibit tumor growth in vivo (Fig. 5A). Weights and sizes of tumors excised from animals in the JARID1B-silenced group were lighter and smaller compared with those in the control group (Fig. 5B and C). On the contrary, D17 cells transfected with JARID1B and NC were subcutaneously


A
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Relative JARID1B mRNA expression (Ratio to GAPDH %)
B
Tumor
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Non-met
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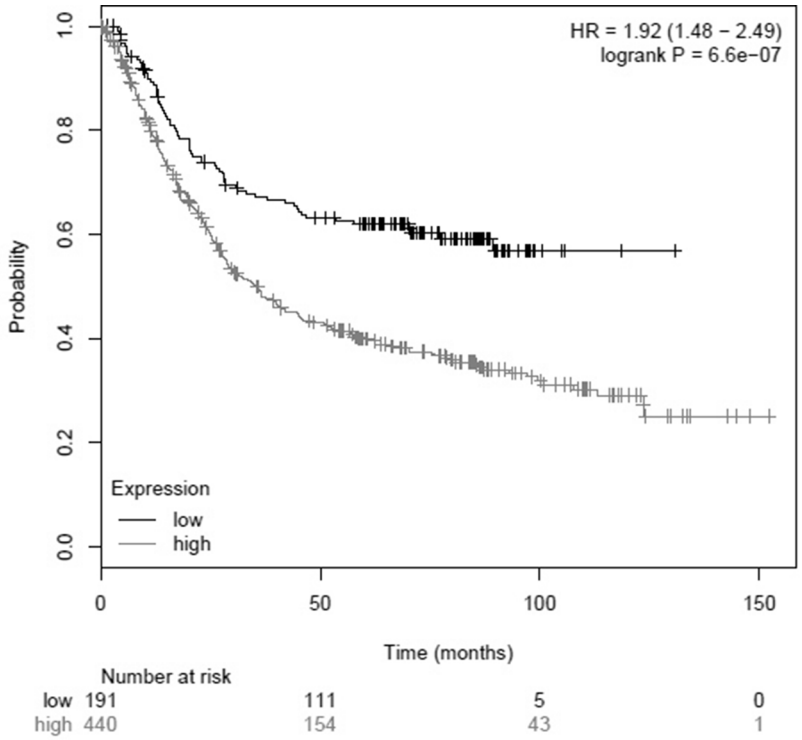

Figure 2. Correlation between JARID1B expression and osteosarcoma patients' survival curve.

inoculated into nude mice. Overexpression of JARID1B could promote tumor growth in vivo (Fig. 5D). Sizes and weights of tumors excised from animals in the JARID1B overexpression group were heavier and larger compared with those in the control group (Fig. 5E and F). Thus, these data indicated that JARID1B may promote xenograft tumor formation in osteosarcoma cells in vivo.

JARID1B Regulates Cyclin D1 Expression Through H3K27me3

To uncover mRNA targets of JARID1B in osteosarcoma, we used mRNA expression arrays and bioinformatics databases to identify potential targets. Cyclin D1 showed the most significant difference between shJARID1B-transfected MG-63 cells and control cells (Fig. 6A and B). To experimentally verify this potential target, 143B and MG-63 cells were transfected with JARID1B and shJARID1B, and the mRNA target and protein levels were assessed by qRT-PCR and Western blot. JARID1B overexpression increased, and JARID1B knockdown reduced the expression of cyclin D1 in osteosarcoma cells (Fig. 6C–F). We then explored how JARID1B regulates cyclin D1 expression at the transcriptional level. To determine whether JARID1B regulates specific histone modifications in osteosarcoma cells, histone modification patterns were measured after modulation of JARID1B expression. Ectopic expression of JARID1B decreased H3K27me3, while silencing of JARID1B increased this modification (Fig. 6E and F).

Because H3K27me3 is associated with active transcription, we tested whether JARID1B expression was correlated with the H3K27me3 modification at the cyclin D1 gene promoter in osteosarcoma cells (Fig. 7A). Quantitative chromatin immunoprecipitation (qChIP) assays were

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Figure 1. Expression of jumonji, AT-rich interactive domain 1B (JARID1B) is elevated in osteosarcoma tissues and cell lines. (A) Relative JARID1B expression in osteosarcoma and para-carcinoma tissues. (B) JARID1B expression was measured by quantitative reverse transcription (qRT)-PCR in osteosarcoma tissues and normal tissues. (C) JARID1B expression levels are significantly elevated in metastatic osteosarcoma tissues compared to nonmetastatic osteosarcoma tissues. (D) JARID1B protein expression was measured in osteosarcoma and para-carcinoma tissues. (E) JARID1B protein levels in osteosarcoma cells were assessed using Western blot analysis. (F) JARID1B mRNA levels in osteosarcoma cells were assessed using qRT-PCR analysis (ratio to β-actin). (G) JARID1B mRNA levels in osteosarcoma cells were assessed using qRT-PCR analysis ratio to GAPDH). All results are from three independent experiments. Error bars, SD. $^{**}p<0.01$, based on the Student's *t*-test.

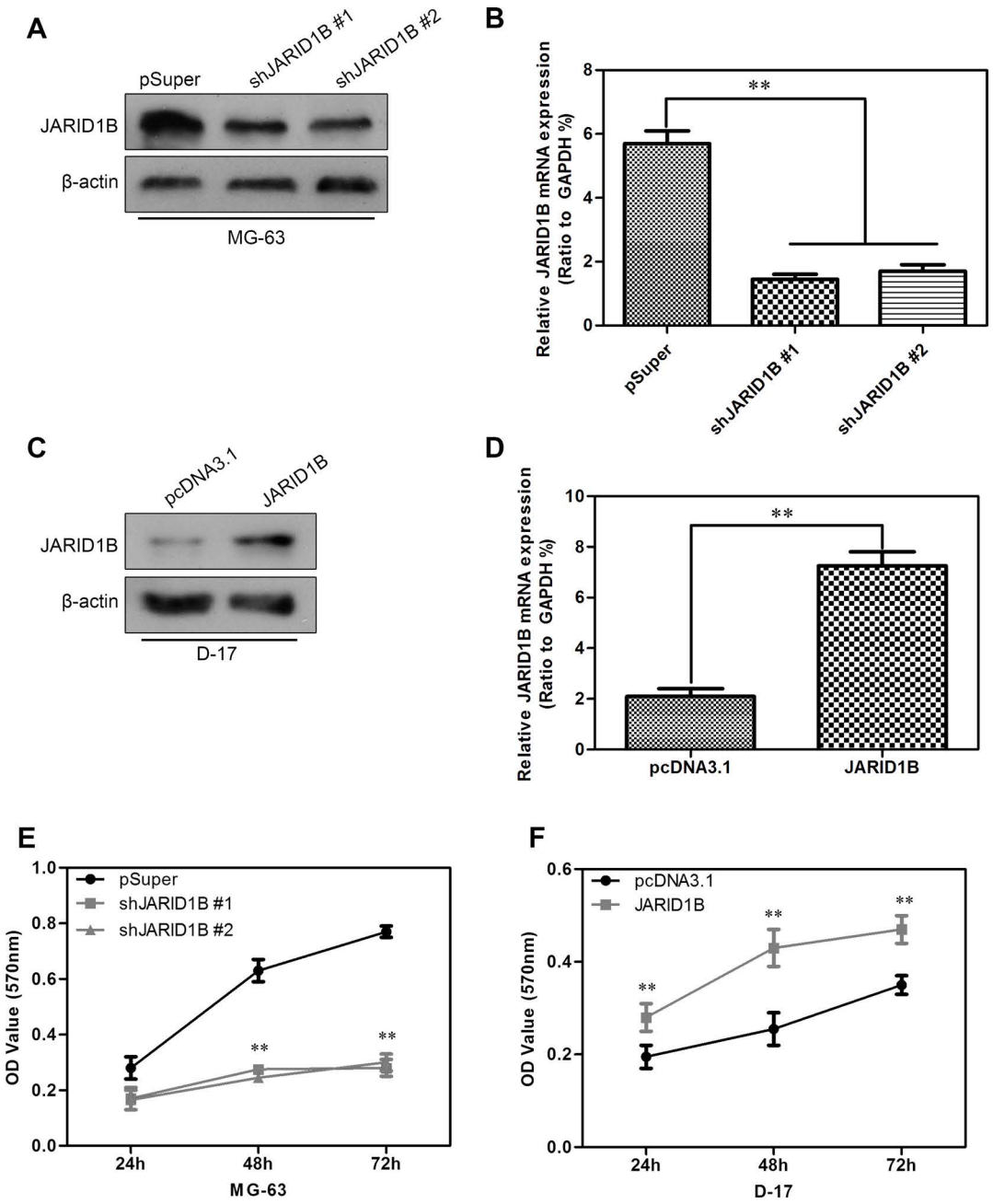

Figure 3. Overexpression of JARID1B enhances tumorigenesis in vitro. (A) Establishment of MG-63-shJARID1B and MG-63 control cells. The results were analyzed by qRT-PCR. (B) Establishment of MG-63-shJARID1B and MG-63 control cells. The results were analyzed by Western blot. (C) Establishment of D17-JARID1B and D17 control cells. The results were analyzed by qRT-PCR. (D) Establishment of D17-JARID1B and D17 control cells. The results were analyzed by Western blot. (E) Proliferation of MG-63-shJARID1B cells was significantly decreased compared to normal MG-63 control cells measured by MTT assay. (F) Proliferation of D17-JARID1B cells was significantly accelerated compared to normal D17 control cells measured by MTT assay. All results are from three independent experiments. Error bars, SD. $^{**}p<0.01$, based on the Student's *t*-test.

performed in MG-63-shJARID1B and D17-JARID1B and cells. We found that knockdown of JARID1B was associated with increased H3K27me3 levels in MG-63 cells (Fig. 7B and C), and JARID1B expression was associated with decreased H3K27me3 levels in D17 cells (Fig. 7D and E). To determine any clinical correlation of JARID1B and cyclin D1, we analyzed cyclin D1 expression in the same human osteosarcoma tissue microarray. A highly positive correlation between JARID1B and cyclin D1 expression was drawn (Fig. 7F), and it was

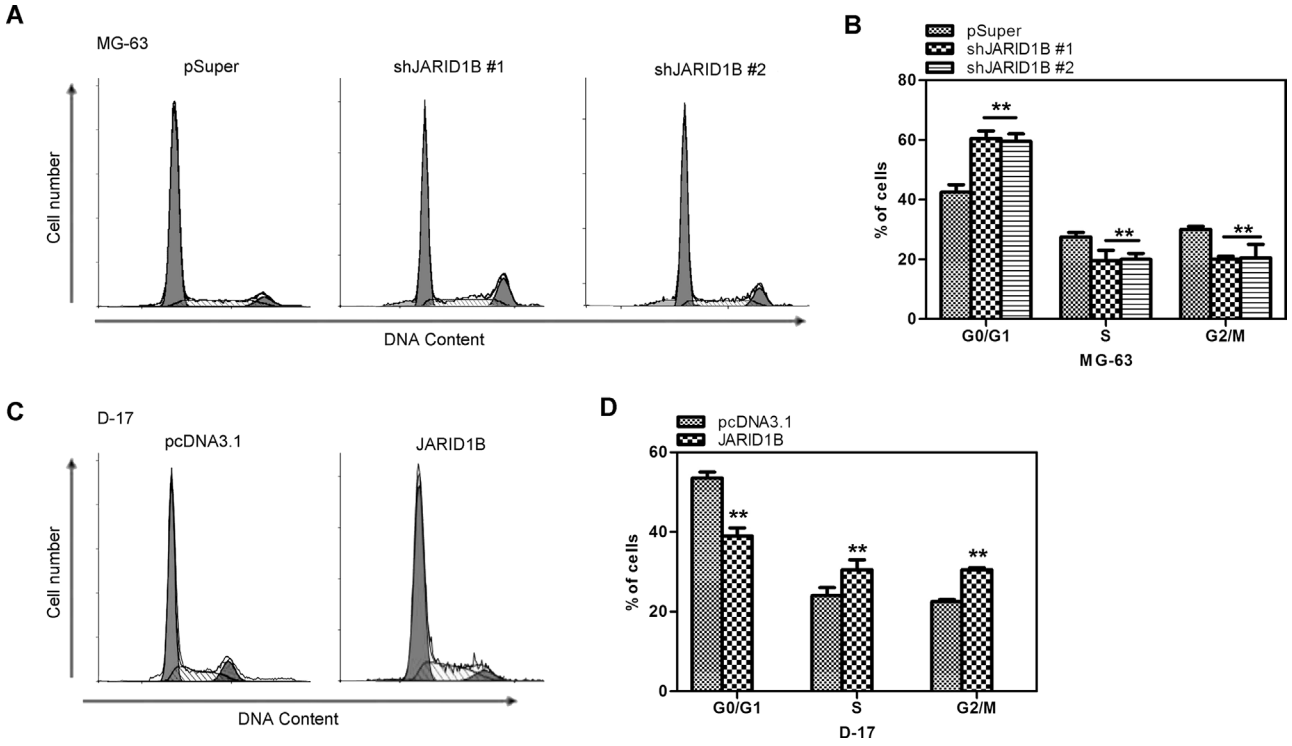

Figure 4. Cell cycle experiment of MG-63-shJARID1B and D17-JARID1B cells. (A) Cell numbers of MG-63-shJARID1B and control cells in different cell phases. (B) Cell numbers of MG-63-shJARID1B and control cells were measured in the G_0/G_1, S, and G_2/M phases. (C) Cell numbers of D17-JARID1B and control cells in different cell phases. (D) Cell numbers of D17-JARID1B and control cells were measured in the G_0/G_1, S, and G_2/M phases. All results are from three independent experiments. Error bars, SD. $^{**}p<0.01$, based on the Student's *t*-test.

found that JARID1B expression was associated with cyclin D1 in osteosarcoma tissues using qRT-PCR. We then tested whether inhibited cyclin D1 expression could suppress the tumor promotion of JARID1B. We used arcyriaflavin A, a specific inhibitor of cyclin D1, to inhibit the expression of cyclin D1. Inhibited expression of cyclin D1 significantly inhibited the growth of D17-JARID1B cells (Fig. 8A). Inhibited cyclin D1 expression could also increase the G_1 phase and decrease the G_2/M phase cell numbers of D17-JARID1B cells (Fig. 8B and C).

DISCUSSION

In this study, the role of JARID1B in osteosarcoma was characterized. Elevated levels of JARID1B mRNA and protein were detected in a majority of the osteosarcoma tissues examined compared to normal brain tissues. Overexpression of JARID1B was found to enhance cell proliferation, as well as to accelerate tumor growth in nude mice in vivo. We also found that knockdown JARID1B in osteosarcoma cells significantly inhibited proliferation, and these properties of JARID1B were partly related to cyclin D1 through H3K27me3 trimethylation. Collectively, these findings suggest for the first time that JARID1B is involved in osteosarcoma genesis.

The JARID1B gene was highly expressed in spermatogonia and cell meiosis of normal adult testes and had a lower expression in the ovary, placenta, lymph node, and thymus. The JARID1B gene is highly expressed in 90% of breast cancer cells and also highly expressed in prostate cancer, especially in metastatic prostate cancer. However, it is not detected in benign prostatic hyperplasia or normal prostate tissue. The JARID1B gene is present in benign melanocytic tumors, but it was not found in advanced or metastatic melanoma cell lines[15]. It is believed that one reason JARID1B inhibits tumorigenesis is that it binds to phosphorylated retinoblastoma (pRb) and maintains pRb-mediated cell cycle regulation[16]. ChIP-on-chip experiments found that JARID1B promotes many gene expressions, including TNF-α, NOTCH, TGF-β, MAPK, and WNT signaling pathways, thereby affecting apoptosis and cell cycle regulation[17]. The previous results showed that JARID1B may play a role as an oncogene or cancer-suppressor gene in different tumors.

Histone has six lysine residues that can be methylated. Methylation of histone 3 lysine 4 (H3K4), H3K36, and H3K79 is associated with the initiation of gene transcription; methylation of H3K9, H3K27, and H4K20 is associated with inhibition of gene transcription[18]. A previous

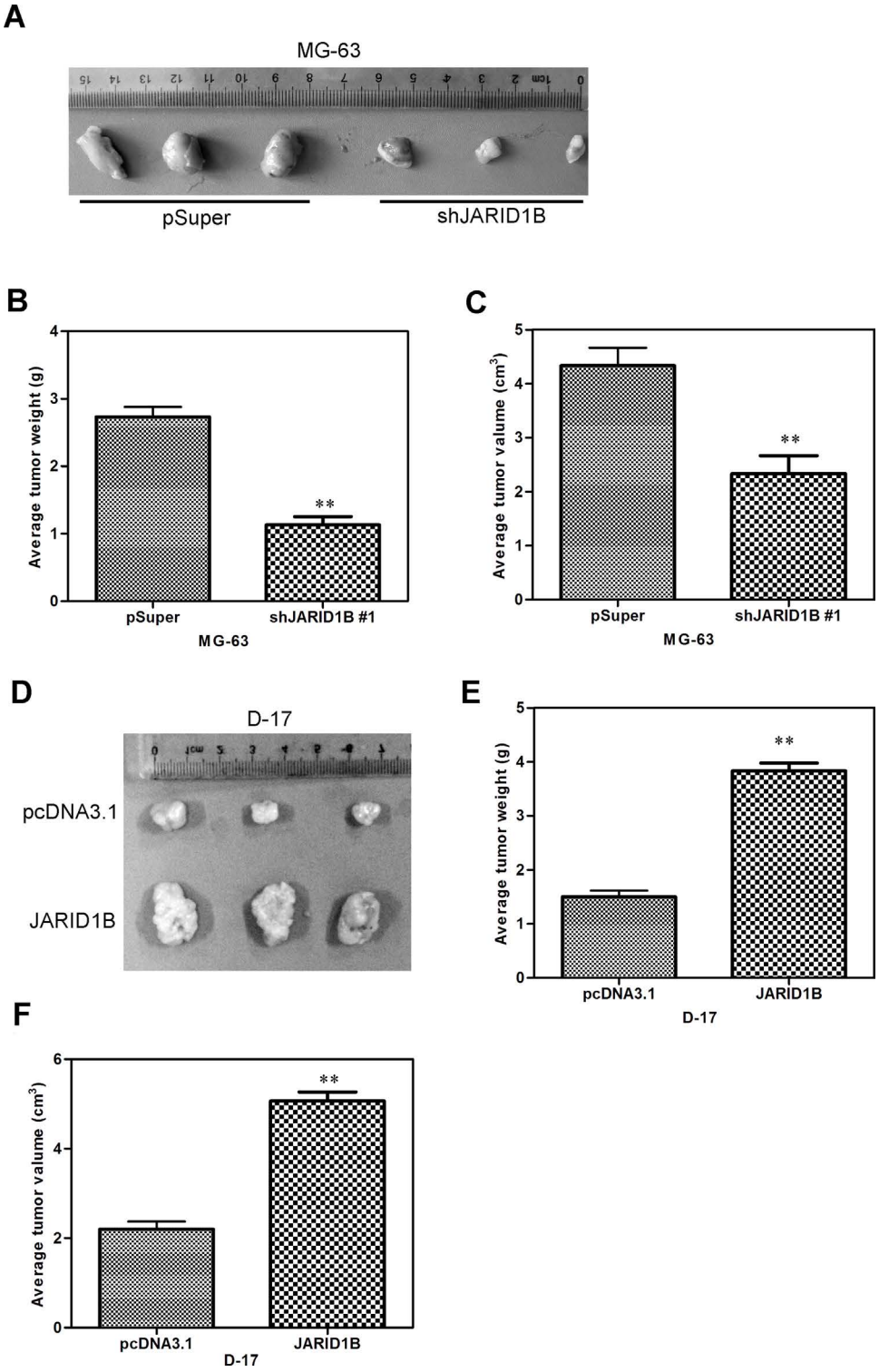

Figure 5. JARID1B promotes tumor growth in vivo. (A) Subcutaneous tumors in the MG-63-shJARID1B ($n=3$) and NC MG-63 mice ($n=3$) are displayed. (B) The weights of tumors are shown. (C) The volume of tumors is shown. (D) Subcutaneous tumors in D17-JARID1B ($n=3$) and NC D17 ($n=3$) mice are displayed. (E) The weights of tumors are shown. (F) The volume of tumors is shown. All results are from three independent experiments. Error bars, SD. $^{**}p<0.01$, based on the Student's *t*-test.

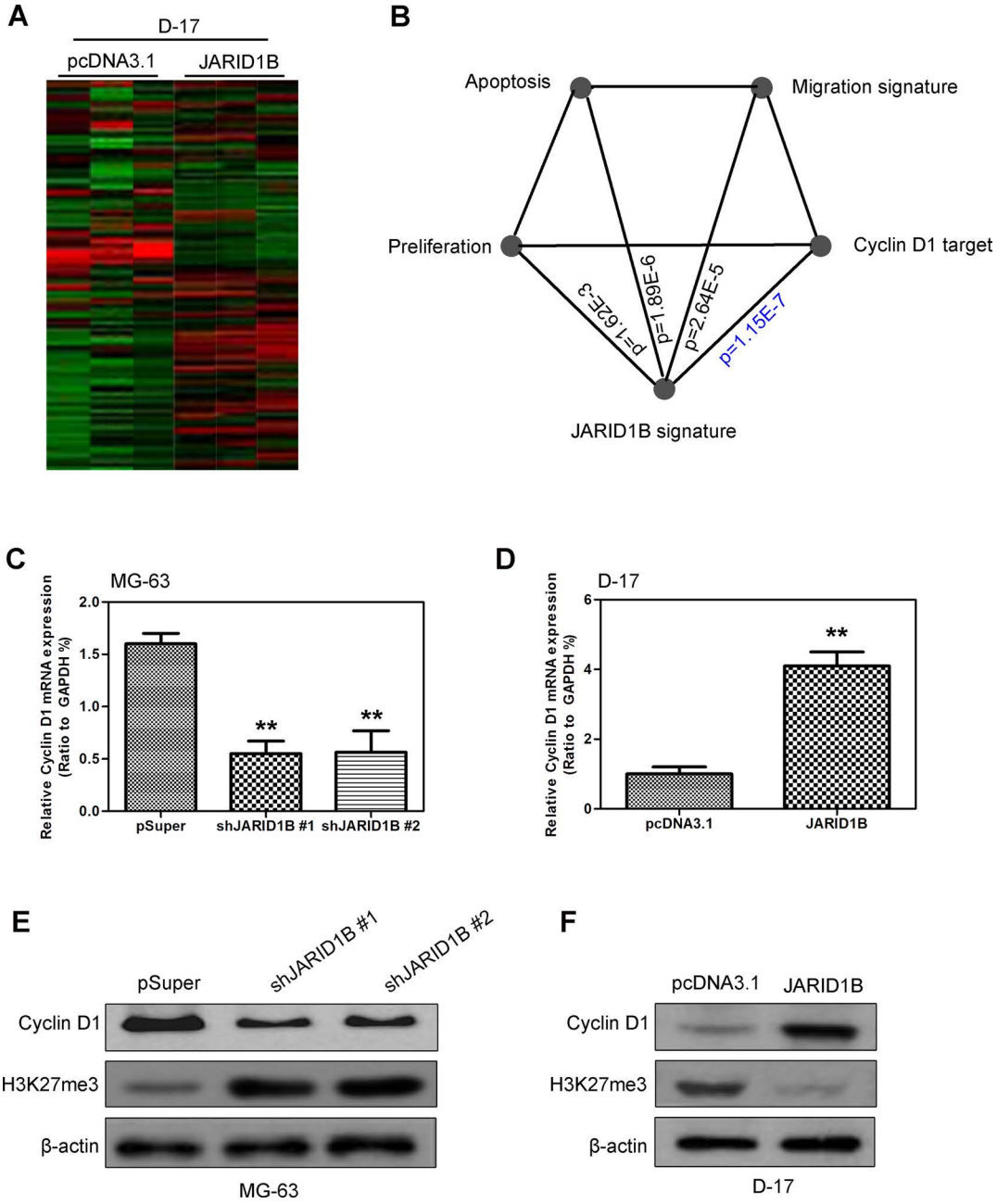

Figure 6. JARID1B reduces the expression of cyclin D1 through H3K27me3. (A) mRNA expression arrays show the different expression levels between D17-JARID1B and NC D17 cells. (B) Data preprocessing shows cyclin D1 expression diversity between D17-JARID1B and NC D17 cells. (C) Cyclin D1 expression levels are significantly declined in MG-63-JARID1B cells using qRT-PRC. (D) Cyclin D1 expression levels are significantly elevated in D17-JARID1B cells using qRT-PRC. (D) Cyclin D1 expression levels are significantly declined in lenti-JARID1B MG-63 cells and elevated in MG-63-shJARID1B cells. (E) Western blot analysis was performed to detect the expression of cyclin D1 and H3K27me3 in MG-63-shJARID1B and NC MG-63 cells. (F) Western blot analysis was performed to detect the expression of cyclin D1 and internal control β-actin in D17-JARID1B and NC D17 cells. All results are from three independent experiments. Error bars, SD. $^{**}p<0.01$, based on the Student's *t*-test.

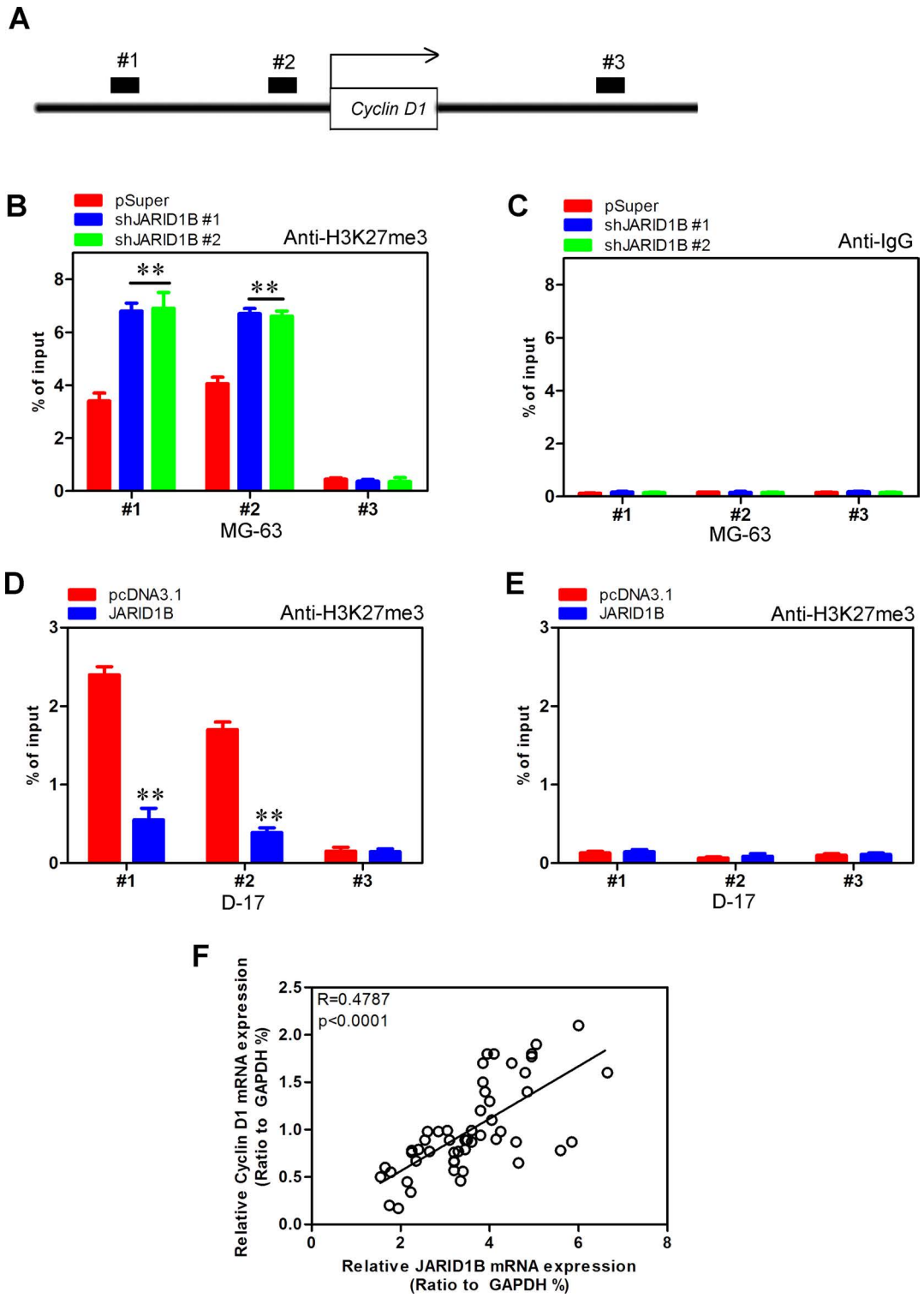

Figure 7. Cyclin D1 is the potential target of JARID1B. (A) H3K27me3 modification of the cyclin D1 gene promoter in osteosarcoma cells. (B) Quantitative chromatin immunoprecipitation (qChIP) assays were performed in MG-63-shJARID1B and NC MG-63 cells. (C) Knockdown of JARID1B was associated with increased H3K27me3 levels in MG-63 cells. (D) qChIP assays were performed in D17-JARID1B and NC D17 cells. (E) JARID1B expression was associated with decreased H3K27me3 levels in D17 cells. (F) A highly positive correlation between JARID1B and cyclin D1 expression was drawn, and JARID1B expression is associated with cyclin D1 in osteosarcoma tissues using qRT-PCR. All results are from three independent experiments. Error bars, SD. $^{**}p<0.01$, based on the Student's *t*-test.

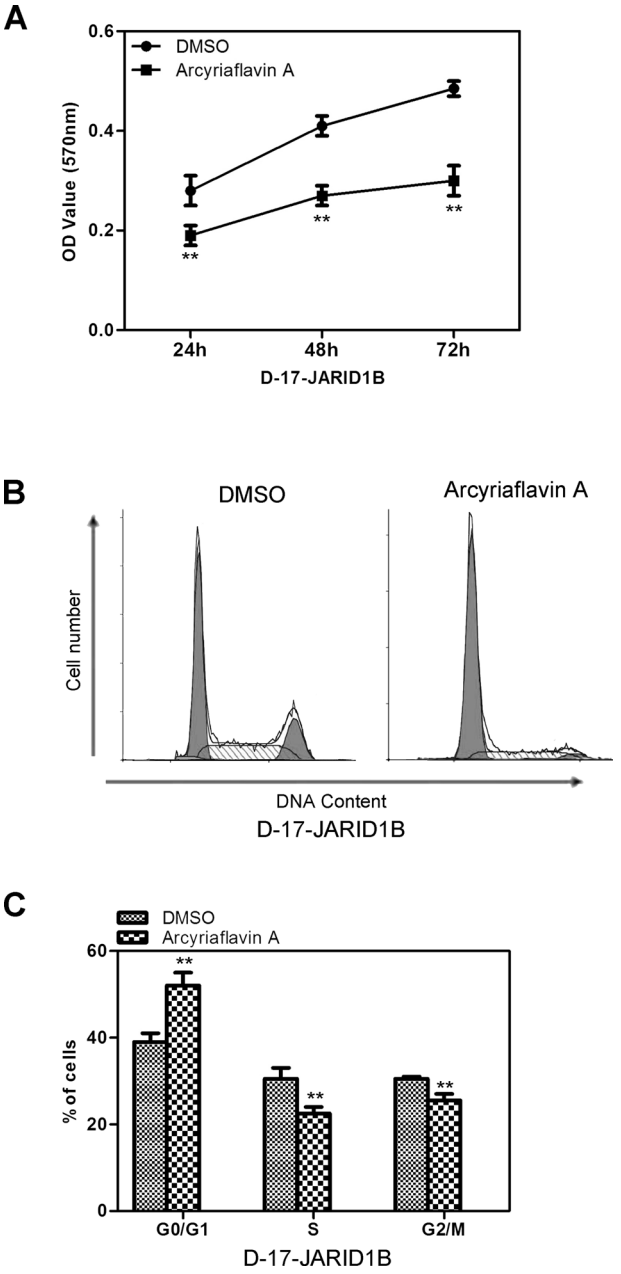

Figure 8. Inhibiting cyclin D1 expression can reverse the JARID1B tumor-promoting effect. (A) Arcyriaflavin A was used to inhibit the expression of cyclin D1, and inhibiting cyclin D1 significantly inhibited the growth of D17-JARID1B cells. (B) Cell numbers in different cell phases were measured in D17-JARID1B and arcyriaflavin A D17-JARID1B cells. (C) Arcyriaflavin A could also increase the G_1 phase and decrease the G_2/M phase cell numbers of D17-JARID1B cells. All results are from three independent experiments. Error bars, SD. $^{**}p<0.01$, based on the Student's *t*-test.

study showed that JARID1B enables H3K4me1/2/3 demethylation[11]. In this study, we found that JARID1B can promote H3K27me3 trimethylation and then elevate the expression of cyclin D1. Arcyriaflavin A is the specific inhibitor of cyclin D1[19]. We used it on D17-JARID1B cells and found that arcyriaflavin A can significantly reverse the cell proliferation and cell phase changes induced by JARID1B overexpression. Thus, these findings are novel and show the mechanism of JARID1B in stimulating osteosarcoma growth.

In conclusion, we have demonstrated for the first time that JARID1B is overexpressed in osteosarcoma tissues, and the overexpression of JARID1B increases osteosarcoma cell growth in vitro and promotes osteosarcoma tumorigenesis in vitro and in vivo. Therefore, our data suggest that JARID1B is potentially an important molecular target for the design of novel antiosteosarcoma therapy.

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