JMJD2B promotes epithelial-mesenchymal transition by cooperating with β-catenin and enhances gastric cancer metastasis

Li Zhao1#, Wenjuan Li1#, Wen Zang1, Zhifang Liu1,2, Xia Xu1,2, Han Yu1, Qing Yang1,3, and Jihui Jia1*

1Department of Microbiology/Key Laboratory for Experimental Teratology of Chinese Ministry of Education, School of Medicine, Shandong University, Jinan, PR China. 2Department of Biochemistry, School of Medicine, Shandong University, Jinan, PR China. 3Department of Parasitology, School of Medicine, Shandong University, Jinan, PR China.

1#Li Zhao and Wenjuan Li contributed equally to the work.

Running title: Role of JMJD2B in Gastric Cancer Metastasis

Keywords: JMJD2B; EMT; β-catenin; gastric cancer; metastasis

*Corresponding Author: Dr. Jihui Jia, Department of Microbiology/Key Laboratory for Experimental Teratology of Chinese Ministry of Education, School of Medicine, Shandong University, 44 Wenhua West Road, Jinan, PR China. Tel: +86 531, 88382672, fax: +86 531 88382502, e-mail: jiajihui@sdu.edu.cn

Disclosure of Potential Conflicts of Interest:
We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and / or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "JMJD2B promotes epithelial-mesenchymal transition by cooperating with β-catenin and enhances gastric cancer metastasis".

Words account: Text-4,982; Abstract-234.

Figures and tables: 6

Translational relevance
Gastric cancer (GC) is one of the most common malignancies worldwide and ranks second in terms of global cancer-related mortality. Metastasis is a common lethal
outcome of gastric cancer after curative resection. Thus, a better understanding of the molecular mechanisms underlying gastric cancer progression is conducive to the development of targeted therapy. In this study, we reveal a novel role of Jumonji domain containing 2B (JMJD2B) in promoting epithelial–mesenchymal transition by cooperating with β-catenin and enhancing gastric cancer invasiveness and metastasis in vitro and in vivo for the first time. We also find that overexpression of JMJD2B in gastric cancer positively correlates with tumor size, differentiation status, tumor invasion, lymph node metastasis, distant metastasis, TNM stage and vimentin expression in patients with gastric cancer. Taken together, these results implicate JMJD2B as a new effective therapeutic target for reversing EMT and intervention of the progression of gastric cancer.

Abstract

Purpose: This study investigated the role of histone demethylase Jumonji domain-containing protein 2B (JMJD2B) in promoting epithelial-mesenchymal transition (EMT) and underlying molecular mechanisms in the progression of gastric cancer (GC).

Experimental Design: The induction of EMT by JMJD2B in GC cells and its underlying mechanisms were examined by a series of assays. In-vivo and in-vitro assays were performed to clarify invasive potential of JMJD2B in GC cells. The expression dynamics of JMJD2B were detected using immunohistochemistry in 101 cases of primary gastric cancer tissues.

Results: Inhibition of JMJD2B by specific siRNA suppresses EMT of GC cells, while ectopic expression of JMJD2B induces EMT. Importantly, JMJD2B is physically associated with β-catenin and enhances its nuclear localization and transcriptional activity. JMJD2B together with β-catenin binds to the promoter of the β-catenin target gene vimentin to increase its transcription by inducing H3K9 demethylation locally. JMJD2B inhibition attenuates migration and invasion of GC cells in vitro and metastasis in vivo. The expression of JMJD2B was positively correlated with tumor size ($P = 0.017$), differentiation status ($P = 0.002$), tumor invasion ($P = 0.045$), lymph node metastasis ($P = 0.000$), distant metastasis ($P = 0.024$) and TNM stage ($P = 0.002$) in gastric cancer patients.

Conclusion: The data reveals a novel function of JMJD2B in promoting EMT and GC invasion and metastasis, implicating JMJD2B as a potential target for reversing EMT and intervention of the progression of GC.

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide and ranks second in terms of global cancer-related mortality (1). Although the overall incidence of gastric cancer has declined in United States, the incidence remains high in Asian countries (2). Once into the stage of locally advanced or metastatic stage, surgery and combination chemotherapies play a minor role to improve the survival rate (3). Thus,
a better understanding of the progression of gastric cancer is urgent. It is critical to identify the new diagnostic and prognostic markers and find novel targeted therapies.

Epithelial-mesenchymal transition (EMT), a developmental process in which epithelial cells show reduced intercellular adhesion and acquire migratory fibroblastoid properties, is considered to be critical for invasive and metastatic progression in cancer (4-6). The process of EMT is associated with the down-regulation of epithelial markers, abnormal translocation of \( \beta \)-catenin, and aberrant upregulation of mesenchymal markers (7). Repression of epithelial markers [e.g. E-cadherin (CDH1)], abnormal translocation of \( \beta \)-catenin and aberrant upregulation of mesenchymal markers [e.g. vimentin (VIM) and N-cadherin (CDH2)] are typical gene expression changes observed during EMT (5-7). These processes are initiated by zinc-finger transcriptional repressors such as Snail (also known as Snail-1), which suppresses E-cadherin expression (5). Vimentin, a type III intermediate filament normally expressed in cells of mesenchymal origin, has been reported as a mesenchymal marker. Upregulation of vimentin is associated with a poor clinical prognosis of many tumors including gastric cancer. \( \beta \)-catenin/TCF binds to the vimentin promoter and transactivates its expression, thereby increasing tumor cells invasive potential (8-10). Although great research efforts have been made on EMT program, the underlying molecular mechanism is still not fully understood.

Mounting evidence indicates that histone post-translational modifications (PTMs) play a crucial role in diverse biological processes, and disruption of these processes has been linked to diseases such as cancer (11, 12). Abnormalities in the methylation of histones by histone demethylases have been implicated in various cancers (13). JMJD2B, also known as KDM4B, is a jmjC domain-containing histone demethylase, which belongs to JMJD2 family. JMJD2B primarily targets the tri-methylated lysine 9 of histone H3 (H3-K9) and has been reported to play an important role in many biological processes such as heterochromatin formation, X-chromosome inactivation, homeotic gene silencing, and transcriptional regulation (14-16). Recently, increasing studies have revealed an epigenetic role of JMJD2B in stem cells differentiation (17), inflammation (18) and tumorigenesis (19-26). It is reported that JMJD2B interacts with estrogen receptor \( \alpha \) (ER\( \alpha \)) and is recruited to ER\( \alpha \) target sites, which demethylates H3K9me3 and facilitates transcription of ER responsive genes including MYB, MYC and CCND1 to play an important role in the development and progression of breast cancer (22). It is also reported that JMJD2B/MLL2 complex is copurified with ER\( \alpha \), with H3K9 demethylation and H3K4 methylation in ER\( \alpha \)-activated transcription, which provides a mechanism for the role of JMJD2B in breast carcinogenesis (23). In our previous study, we demonstrated that JMJD2B promoted proliferation and survival of gastric cancer cells (24). However, whether and how JMJD2B is involved in gastric cancer invasion and metastasis arouses our interest.

In the present work, we found that JMJD2B promoted EMT and enhanced metastasis and progression of gastric cancer. We identified that JMJD2B physically interacted with \( \beta \)-catenin and was recruited to the promoter of vimentin and induced H3K9 demethylation on vimentin promoter increasing vimentin transcription.
Moreover, we also found that JMJD2B inhibition attenuated nuclear accumulation of β-catenin and decreased its transcriptional activity. Knockdown of JMJD2B in GC cells inhibits cells migration and invasion both in vitro and in vivo. Finally, we demonstrated that JMJD2B expression positively correlated with the tumor metastasis status in gastric cancer patients. Collectively, we identified potential invasive activities of JMJD2B in gastric cancer cells, and revealed a novel transcriptional mechanism for regulating vimentin expression, suggesting that JMJD2B might be a promising therapeutic target for blocking progression of gastric cancer.

## Materials and Methods

### Cell lines, reagents, small interfering RNA (siRNA) transfection and plasmids

Human gastric cancer cell lines (AGS, BGC823, HGC27, MGC803), and HEK293T cells were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. Cell lines were incubated at 37°C in a 5% CO₂ humidified atmosphere in RPMI1640 (BGC823, HGC27, MGC803), F12 (AGS) or Dulbecco’s Modified Eagle’s Media (HEK293T) containing 10% FBS. TGF-β1 was purchased from Sigma-Aldrich (St Louis, MO, USA). Chemical modified Stealth™ siRNAs were bought from Invitrogen. The sequences for JMJD2B siRNA and control siRNA (containing a scrambled sequence with no specific degradation of any known cellular mRNA) were listed in the Supplementary Table S1 and Supplementary Materials and Methods. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection of siRNA. HA-JMJD2B vector was kindly provided by Professor Kristian Helin (Biotech Research & Innovation Centre and Centre for Epigenetics, 2200 Copenhagen, Denmark); VimPro-Luc vector and VimProMut-Luc vector were kindly provided by Dr. Christine Gilles (University of Liege, 4000 Liege, Belgium).

### RNA extraction, reverse transcription and QRT-PCR

The method of RNA extraction and reverse transcription was used as described (27). The quantitative real-time (QRT-PCR) was performed using the ABI7000 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with a SYBR Green kit (Applied Biosystems) according to the manufacturer's instructions. The primers used in this study were listed in the Supplementary Table S1.

### Western blot and co-immunoprecipitation

Total cellular proteins were extracted and western blot was performed as described (24). Cellular proteins (80 μg) were separated for western blot analysis. The procedure was described in detail in Supplementary Materials and Methods.
HEK293T cells were transfected with different plasmids and then treated cells as well as BGC823 cells were lysed in NP40 lysis buffer (supplemented with protease inhibitors) for 30min on ice. Extracts were clarified by centrifugation at 13,000 g for 10 min at 4°C for co-immunoprecipitation. After centrifugation, the supernatant was collected and precleared by incubating with protein G PLUS-Agarose Immunoprecipitation beads (Santa Cruz) at 4°C for 1hr. And then they were incubated with special antibodies against Flag (Zhongshan Goldenbridge, China), HA (Zhongshan Goldenbridge, China), JMJD2B (A301-478A, Bethyl), β-catenin (ab32572, Abcam, Cambridge, UK) and IgG (Santa Cruz Biotechnology) at 4°C on a rocker platform. The antibody-coated beads were then incubated with the lysates at 4°C overnight. Immunoprecipitates were collected, washed, lysed and boiled. The boiled samples were analyzed by Western blot as described above.

**Luciferase reporter activity assay**

The luciferase reporter activity assay was described in detail in Supplementary Materials and Methods.

**ChIP**

ChIP assay was performed as described previously (28). Briefly, HEK293T cells transfected with different plasmids and BGC823 cells treated with JMJD2B siRNA and control siRNA were cross-linked and lysed in 1% (vol/vol) formaldehyde-containing medium for 10 min at 37°C, followed by sonication to make soluble chromatin with DNA fragments between 200 and 1000 bps. Immunoprecipitation was performed overnight at 4°C with specific antibodies, or irrelevant control antibody. The protein A/G Sepharose beads (Millipore) was added for one hour at 4°C with rotation to collect the antibody/histone complex for immunoprecipitation. The protein-DNA complex was eluted, and reverse cross-linked. Following treatment with Protease K (Sigma-Aldrich), DNA were extracted with phenol-chloroform and precipitated with ethanol. The recovered DNA was re-suspended in TE buffer, and used for PCR amplification with the following specific primers listed in the Supplementary Table S1.

**Immunofluorescence assay**

The immunofluorescence assay was described in detail in Supplementary Materials and Methods.

**Scratch wound-healing assay**
Scratch wound-healing assay was used to assess cell migration and described in detail in Supplementary Materials and Methods.

**Matrigel invasion assay**

Matrigel invasion assay was described in detail in Supplementary Materials and Methods.

**Immunohistochemistry**

Immunohistochemical analysis was performed as previously described (24). The procedure was described in detail in Supplementary Materials and Methods. The pathological scores were assigned by two independent pathologists, who were blinded to the final pathological interpretation. According to the intensity and percentage of JMJD2B staining, JMJD2B expression was scored as 0 (no appreciable staining in tumor cells), 1 (mild, positive staining in the nucleus of 0–29% tumor cells), 2 (moderate, positive staining in the nucleus of 30–69% tumor cells) and 3 (strong, positive staining appreciable in the nucleus of more than 70% tumor cells).

**Patients**

The paraffin-embedded pathological specimens from 101 patients with primary gastric cancer were used in the study, which were approved by the local ethics committee. The patients underwent gastrectomy at Qilu Hospital, Shandong University between 2005 and 2011, none of whom had received chemotherapy or radiotherapy before surgery. We confirmed the diagnosis of the specimens by histopathological examination. These patients included 77 (76.2%) men and 24 (23.8%) women, with mean age of 60.0 years. The pathological TNM status of all GC was assessed according to the criteria of the seventh edition of American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis classification system.

**Mouse model**

BGC823 cells were injected into nude mice through tail vein and metastatic lung tissues were evaluated. The detailed procedure was described in Supplementary Materials and Methods.

**Statistical Analyses**

Differences between experiment groups were analyzed by Mann-Whitney U-test or Student's t-test. The relationships between JMJD2B expression and clinicopathological features of GC patients were analyzed by the \( \chi^2 \) test. All the tests were two-tailed and analysis using the SPSS statistical software package (standard
$P$ values of $< 0.05$ in all cases were considered statistically significant.

**Results**

**JMJD2B inhibition induces the epithelial morphology of gastric cancer cells**

In our previous study, we identified the functional role of JMJD2B in gastric cancer cell proliferation, survival and tumorigenesis (24). Interestingly, we noted that knocking down JMJD2B expression by small interfering RNA (siRNA) in HGC27 and MGC803 gastric cancer cells (spindle-shaped malignant gastric epithelial cell lines with high expression of JMJD2B and loosen cell-cell adhesion) not only inhibited cell proliferation, but also induced tight cell-cell contacts and cobblestone-like appearance which represented epithelial morphology (Fig. 1A). This observation indicated that JMJD2B might play a role in EMT and promoting gastric cancer cell invasion.

**JMJD2B repression results in downregulation of mesenchymal markers and upregulation of epithelial markers**

To address whether JMJD2B affected the EMT program, we knocked down JMJD2B expression using JMJD2B specific siRNA in the gastric cell lines AGS, BGC823, HGC27 and MGC803, and determined the expression of the epithelial marker (E-cadherin) and mesenchymal markers (vimentin and snail) by western blot and quantitative real-time (QRT-PCR) (Fig. 1B and C1-C3). JMJD2B inhibition led to decreased Vimentin and Snail protein expression and evaluated E-cadherin protein expression (Fig. 1B). The mRNA profile is consistent with protein expression (Fig. 1C1-C3). Furthermore, ectopic expression of JMJD2B using HA-JMJD2B vector transient transfection upregulated the vimentin and snail expression while reduced E-cadherin expression both in mRNA in BGC823 cells (Fig. 1D) and protein level in AGS and BGC823 cells (Fig. 1E). Collectively, JMJD2B plays an important role in regulating EMT marker expression in gastric cancer cells.

**JMJD2B is physically associated with β- catenin and recruited to the vimentin promoter**

JMJD2B regulated vimentin at mRNA level (Fig. 1C1-C3 and D). To identify whether JMJD2B regulated vimentin expression at the transcription level, we performed a luciferase assay. Our results showed that VimPro-Luc luciferase activity
was decreased in JMJD2B-depleted BGC823 cells (Fig. 2A) while opposite results were obtained in JMJD2B-overexpressed BGC823 cells (Fig. 2B). Previous study shows that β-catenin targets the vimentin gene for its transcription in gastric cancer (10). Given that JMJD2B regulates vimentin expression at the transcriptional level, we hypothesized a potential involvement of JMJD2B and β-catenin in vimentin transcription. We assessed the luciferase activity driven by the VimProMut-Luc luciferase vector where putative binding site of β-catenin/TCF on the vimentin promoter were inactivated by mutagenesis. VimProMut-Luc luciferase activity failed to be repressed in JMJD2B-depleted BGC823 cells (Fig. 2A), while its activity also failed to be enhanced in JMJD2B overexpression BGC823 cells (Fig. 2B), suggesting that the regulation of JMJD2B on vimentin transcription was dependent on β-catenin. To further characterize the interaction between JMJD2B and β-catenin, we performed a co-immunoprecipitation experiment in flag-β-catenin and HA-JMJD2B expression vectors transfected HEK293T cells, and observed the physical interaction of JMJD2B with β-catenin (Fig. 2C). Consistently, their interaction at endogenous levels was showed in BGC823 cells (Fig. 2D). Furthermore, Chromatin Immunoprecipitation (ChIP) assay showed that JMJD2B occupied the vimentin promoter in HEK293T cells transfected with the HA-JMJD2B vector (Fig. 2E). In BGC823 cells, silencing JMJD2B abolished the binding of JMJD2B to the vimentin promoter coupled with the reduced binding of β-catenin. As predicted, a marked reduction of H3K9me2 and sharp accumulation of H3K9me3 at the vimentin promoter was observed following JMJD2B knockdown (Fig. 2F). Taken together, JMJD2B is physically associated with β-catenin and is recruited to the vimentin promoter to demethylate H3K9me3 there, thereby facilitating vimentin induction.

**JMJD2B inhibition attenuates TGF-β1-mediated β-catenin nuclear accumulation**

TGF-β1-induced β-catenin nuclear translocation and its target genes transactivation is one of the key factors activating the EMT program (29-31). Gene ontology analysis revealed that JMJD2B regulates Wnt and transforming growth factor-β (TGF-β) pathway in tumorigenesis (19). We found that JMJD2B inhibition attenuated the nuclear accumulation of β-catenin and its target gene vimentin expression in BGC823 cells (Fig. 3A and B). Furthermore, TGF-β1 mediated β-catenin nuclear translocation and vimentin expression was also reduced when JMJD2B expression was inhibited in BGC823 cells (Fig. 3C and D). Thus, JMJD2B is required for β-catenin nuclear retention and its transcription activity during EMT process.

**JMJD2B inhibition results in diminished invasion and metastasis in vitro and in vivo**

To further study the role of JMJD2B in gastric cancer cell migration and invasion,
we performed wound healing assay and transwell invasion assay in HGC27 and MGC803 cells. When JMJD2B was inhibited, there was a striking decrease of gastric cancer cell migration and invasion (P < 0.01, Fig. 4A and B; Supplementary Fig. S1A, S1B and S1C).

We next asked whether JMJD2B knockdown inhibits tumor metastasis in vivo. JMJD2B siRNA and control siRNA-treated BGC823 cells (1× 10^6 cells/mouse) were injected into immunodeficient nude mice through tail vein. The mice were sacrificed after 30 days and then their lungs for metastasis were examined. The lungs from mice injected with control siRNA-treated BGC823 cells were larger and heavier than those from JMJD2B knockdown mice (P < 0.05, Fig. 4C). The representative picture of lungs (Supplementary Fig. S1D) shows grossly cystic lung micrometastasis in the control and JMJD2B-depletion groups. More tumor colonies were observed in the lungs from the control group than that from JMJD2B knockdown group (Fig. 4D).

**JMJD2B expression positively correlates with metastasis in human gastric cancer**

Finally, we determined whether the plot originating from the cell lines and animal experiments has any clinical relevance. Non-metastatic gastric cancer (NMGC) and metastatic gastric cancer (MGC) samples were collected from patients with GC. JMJD2B and vimentin protein expression was determined in 101 cases of paraffin-embedded primary gastric cancer tissues using immunohistochemical staining. As expected, strong nuclear staining of JMJD2B was observed in MGC tissues, especially in metastatic lymph nodes (MLN), while less and weak nuclear staining was found in NMGC (Fig. 4E). Furthermore, cytoplasmic staining of vimentin was consistent with the expression trends of JMJD2B in MGC and NMGC tissues (Fig. 4E). Correlation analysis demonstrated no distinguished relationship between JMJD2B expression and the patients’ age and gender (P > 0.05). However, JMJD2B expression was positively correlated with tumor size (P = 0.017), differentiation status (P = 0.002), invasion (P = 0.045), lymph node status (P = 0.000), distant metastasis (P = 0.024) and TNM stage (P = 0.002) (Table 1) of patients with gastric cancer. Moreover, a significant positive correlation between the expression of JMJD2B and vimentin was analyzed in 101 cases of gastric cancer tissues (P = 0.004, Table 2). Taken together, JMJD2B enhanced the metastasis of gastric cancer.

**Discussion**

Emerging evidence has indicated that aberrant alterations in the methyl status of histones play important roles in tumorigenesis (11, 12, 32). JMJD2B, a member of the JMJD2 family of histone demethylase, has been shown to exhibit oncogenic activities and overexpressed in human cancers (19-26, 33, 34). In our previous study, we found that JMJD2B facilitated gastric cancer cell proliferation, survival and xenografted tumor growth in vivo. However, whether and how JMJD2B is involved in gastric
cancer progression and metastasis is still unknown. In the present study, we provide evidence that JMJD2B is a critical positive regulator of EMT and thereby contributes to the progression and metastasis of gastric cancer. JMJD2B inhibition using specific siRNA induces epithelial morphology of invasive gastric cancer cells with upregulation of epithelial markers and downregulation of mesenchymal markers (Fig.1 and Supplementary Fig.S2A). Moreover, JMJD2B silence results in diminished invasion and metastasis of gastric cancer cells in vitro and in vivo. Furthermore, overexpression of JMJD2B is observed in primary gastric cancer specimens and JMJD2B expression positively correlates with advanced disease stages and metastasis. Collectively, these results indicate that JMJD2B is an important epigenetic factor in the progression of gastric cancer.

EMT represents a critical event in the transition from early to invasive carcinomas (6, 35, 36). The upregulation of the mesenchymal marker vimentin is significantly associated with aggressive phenotype of gastric cancer (7, 10). We found that JMJD2B regulates vimentin expression at the transcription level and JMJD2B expression positively correlates with vimentin expression in primary gastric cancer tissues. It has been demonstrated that JMJD2B regulates gene expression by decreasing trimethylation of histone H3K9 on the target gene promoter (18, 25, 26). Indeed, we found the occupancy of JMJD2B on the vimentin promoter coupled with the accumulation of repressive H3K9me3 on vimentin promoter following JMJD2B depletion.

Histone modifiers often regulate target gene transcription through interaction with their transcription factors (22, 36-39). Gene ontology analysis reveals that JMJD2B regulates Wnt/β-catenin, transforming growth factor-β (TGF-β), Notch and phosphoinositide 3-kinase pathways, all of which play important roles in tumorigenesis or cancer progression (19). β-catenin can directly promotes EMT phenotype besides its involvement in TGF-β1-mediated EMT (6, 8, 10). Our present findings demonstrate that JMJD2B is involved in β-catenin/TGF-β1-mediated EMT. JMJD2B knockdown reduces the nuclear accumulation of β-catenin and the decrease of the transcription of its target gene vimentin. Moreover, TGF-β1-mediated nuclear accumulation of β-catenin is also diminished by JMJD2B inhibition. JMJD2B physically interacts with β-catenin at the vimentin promoter, enhancing its transcription. To further verify the effect of JMJD2B on TCF/β-catenin-dependent transcription, the TOP/FOP reporter system was used. JMJD2B inhibition slightly decreased the TOP/FOP ratio in JMJD2B depleted BGC823 cells (Supplementary Fig. S2E1 and E2). While a significant decrease was found in JMJD2B-depleted BGC823 cells compared with control cells when treated with TGFβ1 or β-catenin plasmid (Supplementary Fig. S2E1 and E2). Ectopic expression of JMJD2B significantly increased the TOP reporter activity when co-transfected with β-catenin plasmid in BGC823 cells (Supplementary Fig. S2E3). The above results indicate that JMJD2B is involved in TGFβ/ Wnt mediated TCF/β-catenin transcription activity and imply its transcriptional activation of vimentin expression through cooperating with β-catenin. Collectively, our result reveals novel mechanisms through which JMJD2B regulates the transcription of β-catenin target genes and EMT program. On one hand, JMJD2B
is recruited to the promoter of β-catenin target gene where it demethylates H3K9me3 and facilitates the transcription of β-catenin target gene. On the other hand, JMJD2B promotes the nuclear translocation and accumulation of β-catenin and thereby enhances its transcription activity. A positive feedback loop is formed to amplify the EMT effect. As is known, β-catenin nuclear accumulation is a hallmark of β-catenin transcriptional activation. While whether JMJD2B has any effect on β-catenin protein expression is worth being considered. Immunoblotting showed that inhibition of JMJD2B expression had no obvious effect on overall expression of β-catenin, and nuclear β-catenin was reduced in JMJD2B-depleted BGC823 cells (Supplementary Fig.S2B1 and B2). However, it is still unclear how JMJD2B stimulates the nuclear translocation of β-catenin and more research work on the detailed mechanism needs to be done.

Cancer-associated EMT process involves multiple factors and pathways (26, 39). In our experiments, we also found the upregulation of E-cadherin and downregulation of Snail in JMJD2B-depleted gastric cancer cells. Therefore, it is likely that other mechanisms are also involved in JMJD2B mediated EMT program in addition to its regulation of vimentin via cooperation with β-catenin. Luciferase activity assay showed no significant effect on the E-cadherin promoter (pEcad−1008/+49) by JMJD2B knockdown or overexpression (Supplementary Fig. S2C). Therefore, JMJD2B might not regulate E-cadherin expression simply dependent on Snail. It was reported that Snail promoted Wnt target gene expression cooperating with β-catenin (40). We assumed that Snail played a role in JMJD2B mediated EMT program by regulating Vimentin via cooperation with β-catenin. To address this hypothesis, we inhibited Snail expression by specific siRNA targeting Snail and western blot analysis showed a subtle reduction of Vimentin expression. The upregulation of Vimentin by JMJD2B overexpression was also partially rescued when Snail was knocked down (Supplementary Fig. S2D). It indicated that Snail played a role in JMJD2B mediated Vimentin regulation. Moreover, a decrease of JMJD2B expression was observed in Snail depleted BGC823 cells (Supplementary Fig. S2D). It suggested that a feedback loop between JMJD2B and Snail might exist. Besides, Snail might also participate in the EMT progress by regulating other EMT related markers through other mechanisms.

JMJD2B was shown to regulate Wnt/β-catenin, transforming growth factor-β (TGF-β), Notch and phosphoinositide 3-kinase pathways by gene ontology analysis (19). Our data also showed the involvement of JMJD2B in Wnt/TGF-β regulated gene expression. It was reported that Snail expression could be regulated through Wnt/β-catenin, transforming growth factor-β (TGF-β) and phosphoinositide 3-kinase pathways (41, 42). Therefore, it is plausible to presume that JMJD2B induces Snail expression through these pathways. However, more work needs to be done to explore the detailed mechanism in future work.

Since the oncogenic potential of JMJD2B has been demonstrated in breast cancer (19, 22, 23), more attention has been drawn on the role of JMJD2B in tumorigenesis. However, little is known about its function and underlying mechanism in tumor metastasis and progression. Our results show that JMJD2B promotes EMT through
Wnt/β-catenin pathway and increases the metastasis and progression of GC. Furthermore, higher JMJD2B expression positively correlates with the aggressive phenotype of GC. Recently, a novel inhibitor targeting the histone demethylase LSD1 has been developed and a suppressive effect on tumor growth in colon cancer cells was demonstrated (43). Therefore, the identification of small-molecule inhibitors targeting JMJD2B will be of interest and importance. JMJD2B may be a novel target for reversing EMT and prevention and treatment of gastric cancer metastasis and recurrence.

Acknowledgement

We thank Professor Kristian Helin (Biotech Research & Innovation Centre and Centre for Epigenetics, 2200 Copenhagen, Denmark) for providing HA-JMJD2B plasmid and Dr. Christine GILLES (University of Liege, 4000 Liege, Belgium) for providing VimPro and VimProMut plasmids. We also thank Dr. Yu-Sun Chang (Chang Gung University, Taiwan) for E-cadherin promoter and Dr. Ajamete Kaykas (University of Washington School of Medicine, Seattle, USA) for TOPflash and FOPflash plasmid.

Grant Support

This work was supported by the National Basic Research Program of China (973 Program, No. 2012CB911202), the National Natural Science Foundation of China (No. 81372680, 81371781, 81000868, 81171536, 81101869 and 30972775), Shandong Provincial Natural Science Foundation (No. ZR2009CZ001, ZR2009CM002 and ZR2010HQ020).

References

7. Ryu HS, Park do J, Kim HH, Kim WH, Lee HS. Combination of epithelial-mesenchymal transition and cancer stem cell–like phenotypes has


Figure legends

Figure 1 Function of JMJD2B in promoting EMT in gastric cancer cells. A, JMJD2B knockdown (siJMJD2B) induces morphological changes in HGC27 and MGC803 gastric cancer cell lines by phase-contrast microscopy (magnification: ×400). E-Cadherin, Snail, and vimentin protein expression was analysed in JMJD2B-depleted (B) and JMJD2B-overexpressed (E) gastric cancer cell lines by immunoblotting. The mRNA of epithelial and mesenchymal markers was detected in JMJD2B silencing gastric cancer cells (C1-C3) and JMJD2B overexpressed BGC823 cells (D) by QRT-PCR analyses. Error bar indicates mean ± SD of three independent experiments. *P-values were calculated using Student’s t-test. *, P < 0.05; **, P < 0.01.

Figure 2 JMJD2B is physically associated with β-catenin to regulate vimentin promoter. JMJD2B-depleted BGC823 cells (A) and JMJD2B-overexpressed BGC823 cells (B) were co-transfected with the human vimentin promoter (VimPro-Luc) or a reporter plasmid containing the vimentin promoter mutated at the putative β-catenin/TCF binding site (VimProMut-Luc). And then luciferase activity was assessed using dual reporter assay kits 48 hours post-transfection. bars, SD. *, P < 0.05, **, P < 0.01, t test. C, JMJD2B is physically associated with β-catenin. Upper panel: HEK293T cells were transfected with HA-JMJD2B or HA-PCMV and flag-β-catenin plasmid. Cell lysates were immunoprecipitated with anti-Flag antibody. Bottom panel: HEK293T cells co-transfected with Flag-β-catenin or Flag-pcDNA3.1 and HA-JMJD2B were co-immunoprecipitated by immunoblotting with anti-HA
antibody. D, BGC823 cell lysates were immunoprecipitated with anti-JMJD2B antibody and the co-immunoprecipitated β-catenin was detected using anti-β-catenin antibody. E, HEK293T cells were co-transfected with HA-JMJD2B plasmid for ChIP to analyse the occupancy of JMJD2B at the vimentin promoter region. F, BGC823 cells were transfected with control or siJMJD2B. The ChIP experiment was performed to evaluate the enrichment of JMJD2B, β-catenin, and histone H3K9me2 and H3K9me3 on the vimentin promoter.

Figure 3 JMJD2B inhibition attenuates TGF-β1-mediated β-catenin nuclear accumulation. A, BGC823 cells treated with JMJD2B siRNA (siJMJD2B) and control siRNA (Control) were immunostained for β-catenin and detection of nuclear accumulation of β-catenin and Vimentin expression by confocal microscopy (magnification: ×400). B, detection of vimentin expression in JMJD2B-depleted BGC823 cells by immunofluorescence assay with confocal microscopy (magnification: ×400). C, diminishing of TGF-β1-induced nuclear accumulation of β-catenin by JMJD2B inhibition. BGC823 cells were treated with JMJD2B or control siRNAs. After 48 hours, cells were incubated with TGF-β1 overnight. β-catenin distribution and vimentin protein expression was examined by immunofluorescence assay with confocal microscopy (magnification: ×400). D, detection of vimentin expression in TGF-β1-induced JMJD2B-depleted BGC823 cells by immunofluorescence assay with confocal microscopy (magnification: ×400).
**Figure 4** JMJD2B silencing inhibits metastasis of gastric cancer cells *in vitro* and *in vivo* and expression of JMJD2B is positively correlated with Vimentin expression in human gastric cancer samples. Suppression of JMJD2B by siRNA reduced capacity of cell migration (A) and invasion (B) in HGC27 and MGC803 cells. Columns, mean of triplicate experiments. bars, SD. **, *P* < 0.01, t test. C, JMJD2B silencing reduces *in vivo* metastasis of BGC-823 cells. The weight of lung harbouring metastases was evaluated between control and JMJD2B depletion group. Columns, mean lung weight (g); bars, SD. *, *P* < 0.05, t test. D, representatives of haematoxylin and eosin staining in lung tissues derived from non-injected, Control and siJMJD2B-BGC823 injected mice. E, correlation of JMJD2B and Vimentin expression in human gastric cancers. Expression of JMJD2B and Vimentin is examined by immunohistochemistry in non-metastatic gastric cancer (NMGC), metastatic gastric cancer (MGC), and metastatic lymph nodes (MLN) samples. Representative images are shown. Original magnification: ×200.
Figure 3

A

β-catenin-RBITC

DAPI

MERGE

Control

siJMJD2B

B

Vimentin-RBITC

DAPI

MERGE

Control

siJMJD2B

C

Control siRNA

Control siRNA+TGFβ1

JMJD2B siRNA+TGFβ1

β-catenin

DAPI

MERGE

D

Control siRNA

Control siRNA+TGFβ1

JMJD2B siRNA+TGFβ1

Vimentin

DAPI

MERGE
Table 1. Correlation between JMJD2B expression and clinicopathological features in
gastric cancer patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>JMJD2B expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-1</td>
<td>2-3</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>77</td>
<td>19(24.7%)</td>
<td>58(75.3%)</td>
</tr>
<tr>
<td>Women</td>
<td>24</td>
<td>5(20.8%)</td>
<td>19(79.2%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>44</td>
<td>8(18.2%)</td>
<td>36(81.8%)</td>
</tr>
<tr>
<td>≥60 †</td>
<td>57</td>
<td>16(28.1%)</td>
<td>41(71.9%)</td>
</tr>
<tr>
<td>Tumour size (diameter in cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>26</td>
<td>11(42.3%)</td>
<td>15(57.7%)</td>
</tr>
<tr>
<td>≥3,≤5</td>
<td>39</td>
<td>9(23.1%)</td>
<td>30(76.9%)</td>
</tr>
<tr>
<td>&gt;5</td>
<td>36</td>
<td>4(11.1%)</td>
<td>32(88.9%)</td>
</tr>
<tr>
<td>Tumour differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>23</td>
<td>11(47.8%)</td>
<td>12(52.2%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>25</td>
<td>7(28.0%)</td>
<td>18(72.0%)</td>
</tr>
<tr>
<td>Poor</td>
<td>53</td>
<td>6(11.3%)</td>
<td>47(88.7%)</td>
</tr>
<tr>
<td>pT status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>7(50.0%)</td>
<td>7(50.0%)</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>2(22.2%)</td>
<td>7(77.8%)</td>
</tr>
<tr>
<td>3-4</td>
<td>78</td>
<td>15(19.2%)</td>
<td>63(80.8%)</td>
</tr>
<tr>
<td>pN status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>39</td>
<td>18(46.2%)</td>
<td>21(53.8%)</td>
</tr>
<tr>
<td>1</td>
<td>19</td>
<td>3(15.8%)</td>
<td>16(84.2%)</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>1(6.25%)</td>
<td>15(93.75%)</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>2(7.40%)</td>
<td>25(92.6%)</td>
</tr>
<tr>
<td>pM status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM0</td>
<td>87</td>
<td>24(27.6%)</td>
<td>63(72.4%)</td>
</tr>
<tr>
<td>pM1</td>
<td>14</td>
<td>0(0.00%)</td>
<td>14(100%)</td>
</tr>
<tr>
<td>pTNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>20</td>
<td>9(45.0%)</td>
<td>11(55.0%)</td>
</tr>
<tr>
<td>II</td>
<td>13</td>
<td>6(46.2%)</td>
<td>7(53.8%)</td>
</tr>
<tr>
<td>III</td>
<td>54</td>
<td>9(16.7%)</td>
<td>45(83.3%)</td>
</tr>
<tr>
<td>IV</td>
<td>14</td>
<td>0(0.00%)</td>
<td>14(100%)</td>
</tr>
</tbody>
</table>

*χ² test
† Mean age.
JMJD2B, Jumonji domain-containing protein 2B.
0 – 1: low-JMJD2B expression; 2 – 3: high-JMJD2B expression.
Table 2. Association between expression of JMJD2B and Vimentin in gastric cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total</th>
<th>JMJD2B expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-1</td>
<td>2-3</td>
</tr>
<tr>
<td>Vimentin staining</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>38</td>
<td>15(39.5%)</td>
<td>23(60.5%)</td>
</tr>
<tr>
<td>2-3</td>
<td>63</td>
<td>9(14.3%)</td>
<td>54(85.7%)</td>
</tr>
</tbody>
</table>

*χ^2^ test

JMJD2B, Jumonji domain-containing protein 2B.
Clinical Cancer Research

JMJD2B promotes epithelial-mesenchymal transition by cooperating with β-catenin and enhances gastric cancer metastasis

Li Zhao, Wenjuan Li, Wen Zang, et al.

Clin Cancer Res  Published OnlineFirst September 27, 2013.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-0254

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/09/24/1078-0432.CCR-13-0254.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.