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KDM4B promotes epithelial-mesenchymal transition through up-regulation of ZEB1 in pancreatic cancer

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Abstract

Lysine (K)-specific demethylase 4B (KDM4B) is a histone H3K9 demethylase and is reported to activate gene transcription through regulation of chromatin structures. Previous research has revealed that KDM4B plays special regulatory roles in colorectal, prostate and gastric cancers. However, its physiological role in pancreatic cancer remains largely unknown. In the present study, it is demonstrated KDM4B plays a crucial in epithelial-mesenchymal transition (EMT) in pancreatic cancer. siRNA mediated silencing of KDM4B inhibits cell migration, invasion and EMT. Moreover, KDM4B was demonstrated to epigenetically regulate the expression of ZEB1 in the TGF-β-induced EMT process. In tumor tissues of pancreatic cancer patient, the protein level of KDM4B was positively correlated with ZEB1. In conclusion, our results suggested that KDM4B is a key mediator in EMT process, and may serve as an important prognostic marker and therapeutic target for the metastatic progression of human pancreatic cancer.

Key words: pancreatic cancer, KDM4B, epithelial-mesenchymal transition, ZEB1, TGF-β

Introduction

Pancreatic cancer is the most fatal malignancies in the world, and has the worst prognosis. Although significant progress has been made, its 5-year survival rate is 5% and remains unchanged in the past 40 years [1]. The highly malignant phenotype of pancreatic cancer is strictly correlated with its extensive invasive ability and metastatic potential. At the time of diagnosis, only ~20% pancreatic cancer patients are surgically resectable due to distant metastasis. Even after successful surgery, the 5-year survival rate remains within 10%–25% due to the extensively malignantly biological properties of this tumor, such as local recurrence, peritoneal dissemination, liver and lymph node metastases [2–4]. To improve patient's survival, there is a pressing need to understand and elucidate the molecular mechanisms that control the metastasis of pancreatic cancer.

Epithelial-mesenchymal transition (EMT) is a process during which cells lose their polarized epithelial characteristics and acquire mesenchymal properties such as the decrease of E-cadherin expression and up-regulation of N-cadherin and vimentin, which consequently confers an aggressive phenotype to cancer cells. EMT plays a pivotal role in cancer metastasis, which facilitates malignant tumor progression and metastatic spread by enabling cancer cells to depart from the primary tumor, invade and disseminate to distant organs [5–8]. A significant amount of growth factors such as TGF-β, HGF, EGF, IGF and FGS are known to trigger EMT process through a cascade of down-regulation E-cadherin expression [9]. E-box binding transcription factor such as Snail, Twist and ZEB1, were reported to epigenetically regulate E-cadherin expression through modification of the chromatin structure [10]. However, how these transcription factors are regulated has seldom been discussed.

It is well acknowledged that histone demethylation process is closely correlated with the tumor oncogenesis and metastasis process [11]. As a member of the histone demethylase family, KDM4B was reported to correlate with proliferation and metastasis in prostate cancer, colorectal cancer and gastric cancer [12–14]. However, its role in pancreatic cancer has seldom been reported. Therefore, in this study, the role of KDM4B in pancreatic cancer metastasis was explored. Our data indicated that KDM4B is a positive regulator of EMT process through epigenetic regulation of ZEB1. New pancreatic cancer metastasis related genes identified in this study will shed light on in-depth investigation and understanding of molecular mechanisms of pancreatic cancer metastasis.

Materials and Methods

Pancreatic cancer specimens

Pancreatic cancer specimens were obtained from 49 patients who underwent surgical resection without preoperative chemotherapy in Zhongshan Hospital, Fudan University between 2012 and 2014. Informed consent for tissue use was obtained from all patients. All of the specimens were collected according to the protocols that had been approved by the Ethics Committee of Zhongshan Hospital and confirmed the diagnosis by histopathological examination.

Cell lines

Human pancreatic cancer cell lines PANC-1 and MIA PaCa-2, and HEK-293 T cells were purchased from American Type Culture Collection (ATCC, Manassas, USA) and cultured strictly under ATCC culturing conditions.

Plasmids

Coding sequence of KDM4B was amplified from PANC-1 cDNA with the following primers: F: 5′-AGTGTGCTTCCCGCACAGCTG CAG-3′ and R: 5′- CTGAGCGGCCAGCTGTCCTAGAA-3′, and subsequently cloned into pCMV-F-FLAG vector (Beyotime, Haimen, China). ZEB1 promoter was amplified from PANC-1 genomic DNA with primers F: 5′-CAGAGATCACATCTGTCAGCCGATG-3′ and R: 5′-GTGTCTAAATGCTCGAGTCACCTCC-3′, and cloned into pGL3-Basic vector (Promega, Madison, USA).

Antibody

All antibodies used in this study were listed in **Supplementary Table S1**.

Chromatin immunoprecipitation (ChIP)

ChIP was performed by using EZ ChIP kit (Millipore, Billerica, USA) according to the manufacturer's protocol. Briefly, PANC-1 and MIA PaCa-2 cells treated with KDM4B siRNA or control siRNA were cross-linked and lysed in 1% (v/v) formaldehyde containing medium for 10 min at 37°C, followed by sonication to make soluble chromatin with DNA fragments between 200 and 1000 bp. Immunoprecipitation was performed overnight at 4°C with specific antibodies, or irrelevant control antibody. The protein A/G Sepharose beads (Millipore) were added and incubated for 1 h at 4°C with rotation to collect the antibody/histone complex for immunoprecipitation. The protein-DNA complex was eluted, and reverse cross-linked. After treatment with Protease K, DNA was extracted with phenol–chloroform and precipitated with ethanol. The recovered DNA was resuspended in Tris-EDTA buffer, and used for PCR amplification with specific primers listed in **Supplementary Table S2**.

Luciferase reporter assay

Luciferase reporter assay were performed by using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's standard protocol. Briefly, HEK 293 T cells were plated into 96-well plate and incubated overnight at 37°C. On the next day, cells were co-transfected with pGL3-ZEB1 or pGL3-Snail and pCMV-KDM4B plasmids. After 24 h of culture, luciferase activity was determined as above.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cells with Trizol reagent (Invitrogen, Carlsbad, USA). qRT-PCR was performed on a 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, USA) using SYBR Green agent (TaKaRa, Dalian, China). Primers used were listed in **Supplementary Table S3**. All RT-PCR assays were repeated three times.

siRNA transfection

siRNA against KDM4B was synthesized and transfected into pancreatic cancer cells by using Lipofectamine 2000 regent (Invitrogen) according to the manufacturer's recommendations. The sequence of siRNA oligo was 5′-GGCAUAAGAUGACCCUCAUTT-3′.

Scratch wound-healing assay

PANC-1 and MIA PaCa-2 cells were seeded in 6-well plates and transfected with KDM4B siRNA and control siRNA. The confluent cell monolayer was scratched with a 200-μl pipette tip and the plates were washed twice with phosphate buffered saline (PBS) before adding fresh medium. The cell migration was observed and measured after 24 h and 48 h. Each assay was repeated three times.

Migration assay

The migration assay was tested using a Transwell Boyden Chamber (8 mm; Costar, Cambridge, USA). PANC-1 and MIA PaCa-2 cells were collected after transfection with KDM4B siRNA or control siRNA for 48 h and transferred to the chamber inserts in serum-free medium (200 μl containing 5×10^4 cells). The bottom chamber contained medium with 10% FBS as a chemoattractant. The cells were incubated in a humidified incubator at 37°C for 12 h (PANC-1) or 24 h (MIA PaCa-2). The cells that migrated to the underside of the filter were stained with crystal violet and counted by bright-field microscopy.

Invasion assay

PANC-1 and MIA PaCa-2 cells were seeded onto filters of a 24-well Transwell chamber that were coated with Matrigel (1/6 dilution; BD Biosciences, Franklin Lakes, USA). Invasion of the cells through the Matrigel to the underside of the filter was assessed 24 h (PANC-1) or 36 h (MIA PaCa-2) later by staining with crystal violet and counting by bright-field microscopy.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described [14]. The primary antibody, anti-KDM4B (anti-rabbit, 1:100; Bethyl Laboratories, Montgomery, USA) and anti-ZEB1 (anti-rabbit, 1:100; OriGene, Rockville, USA) were used to detect the expression patterns of KDM4B and ZEB1 in pancreatic cancer tissues and matched normal tissues. The staining score (0 = '–', 1 = '+', 2 = '++', 3 = '+++') was

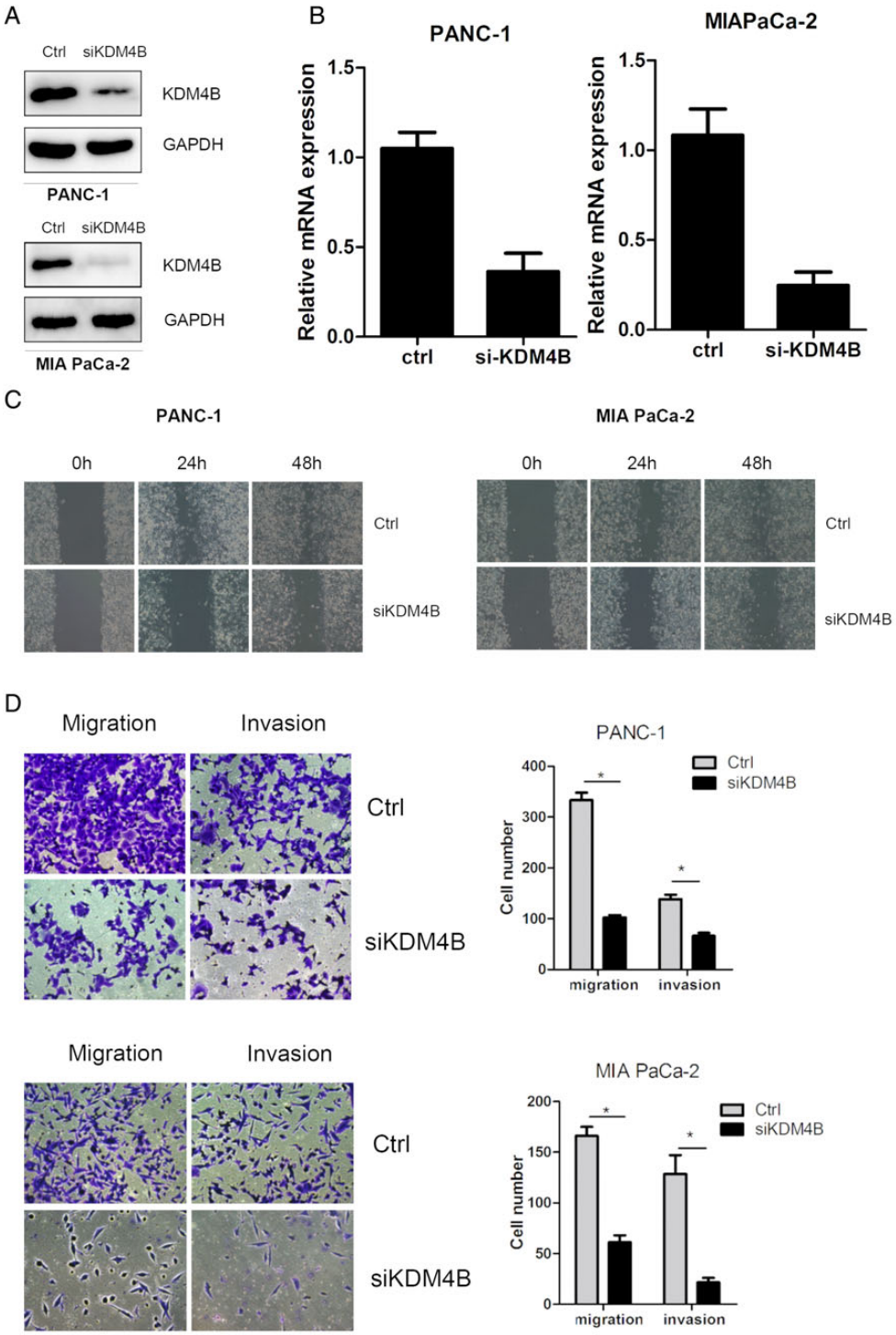


Figure 1. Silencing KDM4B inhibits migration and invasiveness of pancreatic cancer cells (A,B) Efficiency of KDM4B KD in PANC-1 and MIA PaCa-2 cell lines was measured by western blot analysis (A) or real-time RT-PCR (B). Wound-healing assay (C) and Transwell migration and Matrigel invasion assays (D) were performed with cells transfected with control siRNA or KDM4B siRNA. Magnification: ×100 (C) and ×200 (D). All results are from three independent experiments (*$P < 0.05$). Ctrl, control.

measured by two doctors of pathology department who were blinded to clinicopathologic data.

Statistical analysis

All data are presented as the mean ± SD, and experiments were repeated at least three times. Two-tailed unpaired Student's *t*-tests and one-way analysis of variance were used to evaluate the data. The correlation between KDM4B and ZEB1 expression in pancreatic tissues were analyzed by Pearson's chi-squared test. Analyses were performed using the SPSS19.0 statistical analysis software. The statistical significance level was set as *$P < 0.05$.

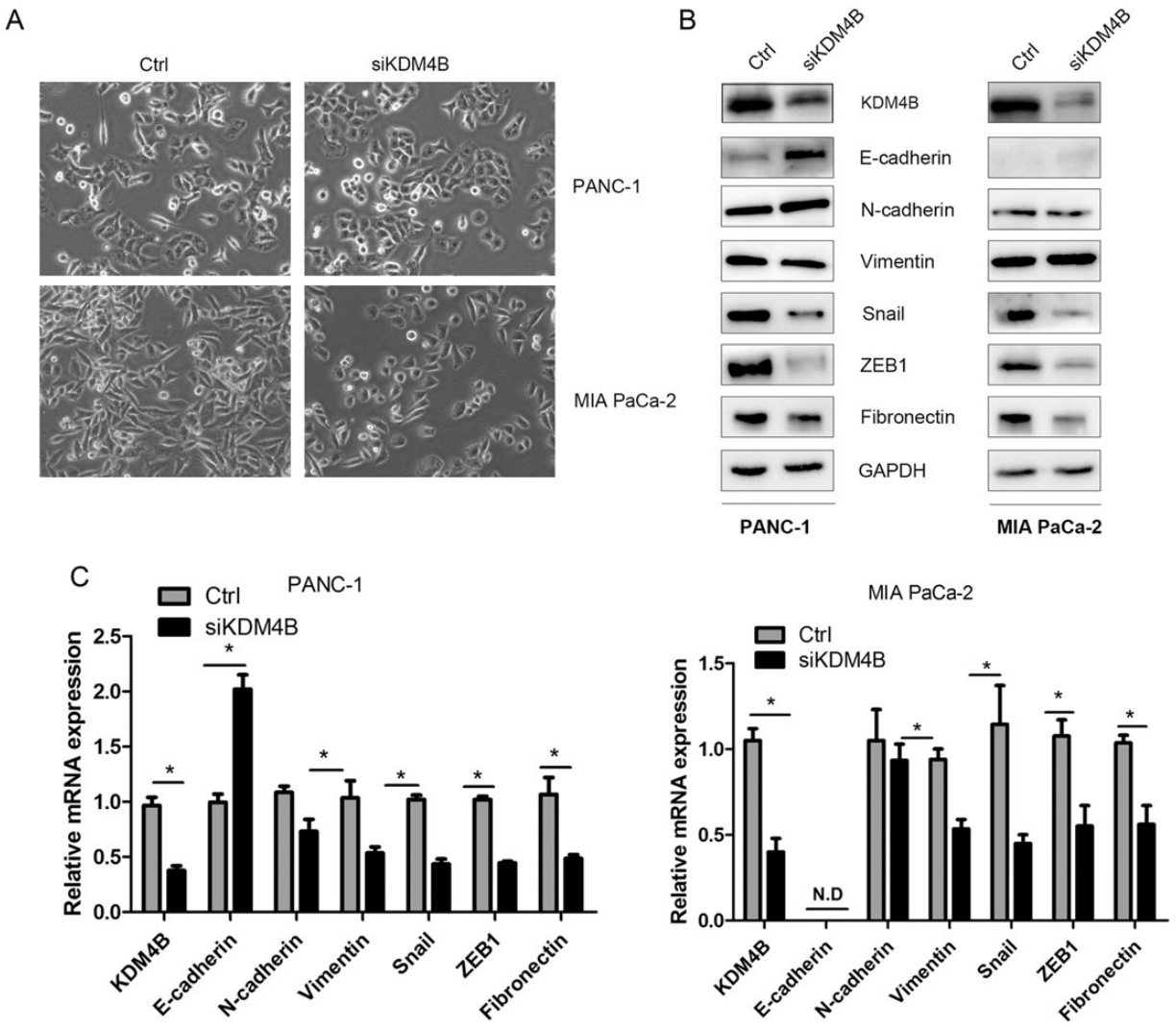


Figure 2. Silencing KDM4B reverses the EMT phenotype of pancreatic cancer cells (A) Knock-down of KDM4B induced morphologic changes in PANC-1 and MIA PaCa-2 cells revealed by phase-contrast microscopy. Magnification: ×400. (B,C) The mRNA and protein expressions of KDM4B, E-cadherin, N-cadherin, vimentin, Snail, ZEB1 and fibronectin were assessed by real-time RT-PCR (B) or western blot analysis (C) in control and KDM4B KD cells. Data are representative of three independent experiments (*$P < 0.05$). Ctrl, control.

Results

Silencing KDM4B inhibits migration and invasiveness of pancreatic cancer cells

In order to examine the role of KDM4B in migration and invasiveness of pancreatic cancer cells, KDM4B was first silenced with siRNA. As shown in **Fig. 1A,B**, the siRNA oligos efficiently decreased KDM4B expression at the protein and mRNA levels in PANC-1 and MIA PaCa-2 cells. Subsequent wound-healing assay demonstrated that silencing KDM4B could significantly decrease cell motility of PANC-1 and MIA PaCa-2 cells (**Fig. 1C**). Moreover, Transwell migration and invasion assay showed that decreased KDM4B expression inhibits migration and invasiveness of PANC-1 and MIA PaCa-2 cells (**Fig. 1D**).

Silencing KDM4B reverses the EMT phenotype of pancreatic cancer cells

EMT process is a pivotal event in the acquisition of invasive and metastatic phenotypes by tumor cells of epithelial origin. As shown in **Fig. 1**, KDM4B regulated pancreatic cancer cell motility, migration and invasion, which are characteristic of EMT process. It was hypothesized that KDM4B regulated EMT process. To address this issue, KDM4B was silenced by using siRNA in PANC-1 and MIA PaCa-2 cells. First, it was observed that decreased KDM4B expression changed the morphology of PANC-1 and MIA PaCa-2 cells. Silencing KDM4B expression induced mesenchymal cells to adopt epithelial cell shapes (**Fig. 2A**). The expressions of epithelial markers (E-cadherin, etc.) and mesenchymal markers (fibronectin, Snail, ZEB1, etc.) were measured by immunoblot and real-time RT-PCR. Results indicated that the decrease of KDM4B expression induces the increase expression levels of E-cadherin protein and mRNA in PANC-1 cells. But in MIA PaCa-2 cells, E-cadherin was found to have no expression. However, the expressions of the mesenchymal markers, such as fibronectin, Snail and ZEB1 were decreased significantly (**Fig. 2B,C**).

KDM4B epigenetically regulates ZEB1 expression

It is well accepted that KDM4B is a chromatin modifier and actively regulates gene transcription. Based on the above observations, whether KDM4B directly regulated Snail or ZEB1 expression was

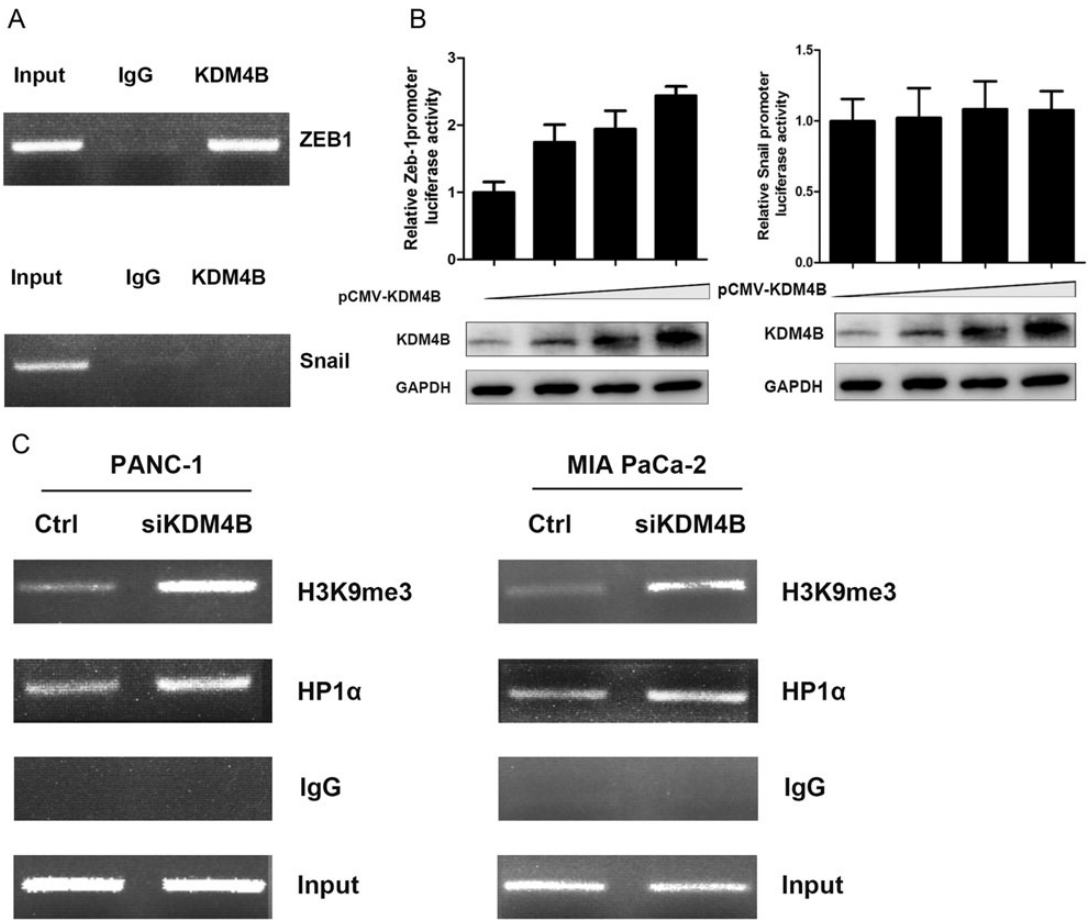


Figure 3. KDM4B epigenetically regulates ZEB1 expression (A) The ChIP assay was performed to test whether KDM4B occupied the promoter region on Snail or ZEB1 in PANC-1 cells. (B) Luciferase reporter assay of ZEB1 or Snail promoter co-transfected with increasing amounts of KDM4B expression plasmid in HEK293 T cells. (C) PANC-1 and MIA PaCa-2 cells were transfected with control siRNA or siKDM4B. Then the ChIP assay was performed to evaluate the enrichment of KDM4B, H3K9me3 and HP1α on ZEB1 promoter. Data are representative of three independent experiments. Ctrl, control.

explored. ChIP experiment was performed to test whether KDM4B occupied the promoter region on Snail or ZEB1. As shown in **Fig. 3A**, KDM4B occupied the promoter region on ZEB1, indicating that it regulated ZEB1 directly. Subsequent reporter assay demonstrated that KDM4B regulated the promoter activity of ZEB1 in a dose-dependent manner (**Fig. 3B**). We measured the occupancy change of H3K9me3 and HP1α, which are heterochromatin markers, on ZEB1 promoter region after KDM4B knock-down (KD). As illustrated in **Fig. 3C**, the decreased KDM4B changed the chromatin structure of ZEB1 promoter into a more condensed form that accounted for its decreased expression.

KDM4B-mediated TGF-β induces EMT process

TGF-β is a key growth factor that induced EMT process. To test whether KDM4B expression changed in the EMT process, PANC-1 cells were treated with 10 ng/ml of TGF-β. Forty-eight hours of treatment with TGF-β could successfully induce morphological change of PANC-1 to mesenchymal state (**Fig. 4A**), and the expressions of KDM4B and EMT markers changed accordingly (**Fig. 4B,C**), indicating that KDM4B may participate in the regulation of TGF-β-induced EMT process. Moreover, an increase of ZEB1 protein level was observed (**Supplementary Fig. S1**). To further address the role of KDM4B in EMT process, KDM4B KD PANC-1 cells was treated with TGF-β. As expected, TGF-β is not sufficient enough to induce EMT in KDM4B KD cancer cells. Moreover, TGF-β induced the enriched occupancy of KDM4B on ZEB1 promoter region. On the contrary, heterochromatic markers H3K9me3 and HP1α were decreased after TGF-β treatment, supporting the hypothesis that KDM4B is an important regulator in EMT process through epigenetic regulation of ZEB1 expression (**Fig. 4D**).

KDM4B is positively correlated with ZEB1 in pancreatic cancer tissues

Since KDM4B could regulate EMT process by epigenetically enhancing the transcription of ZEB1 directly, we raised the question whether KDM4B and ZEB1 have clinical relevance. First, it was observed that ZEB1 protein level is positively correlated with KDM4B level obtained by IHC experiment (**Fig. 5A**). Moreover, a significant positive correlation between KDM4B and ZEB1 was observed by IHC staining in 49 patients tissue samples ($P = 0.0255$; **Fig. 5B**). Thus, it was proposed that KDM4B occupied the promoter region on ZEB1, catalyzed the removal of H3K9me3 and decreased the occupancy of heterochromatin marker HP1α, enhanced the transcription of ZEB1 and ultimately promoted EMT process (**Fig. 5C**).

Taken together, KDM4B is an important regulator of EMT process, depending on its regulatory role in ZEB1 expression at the

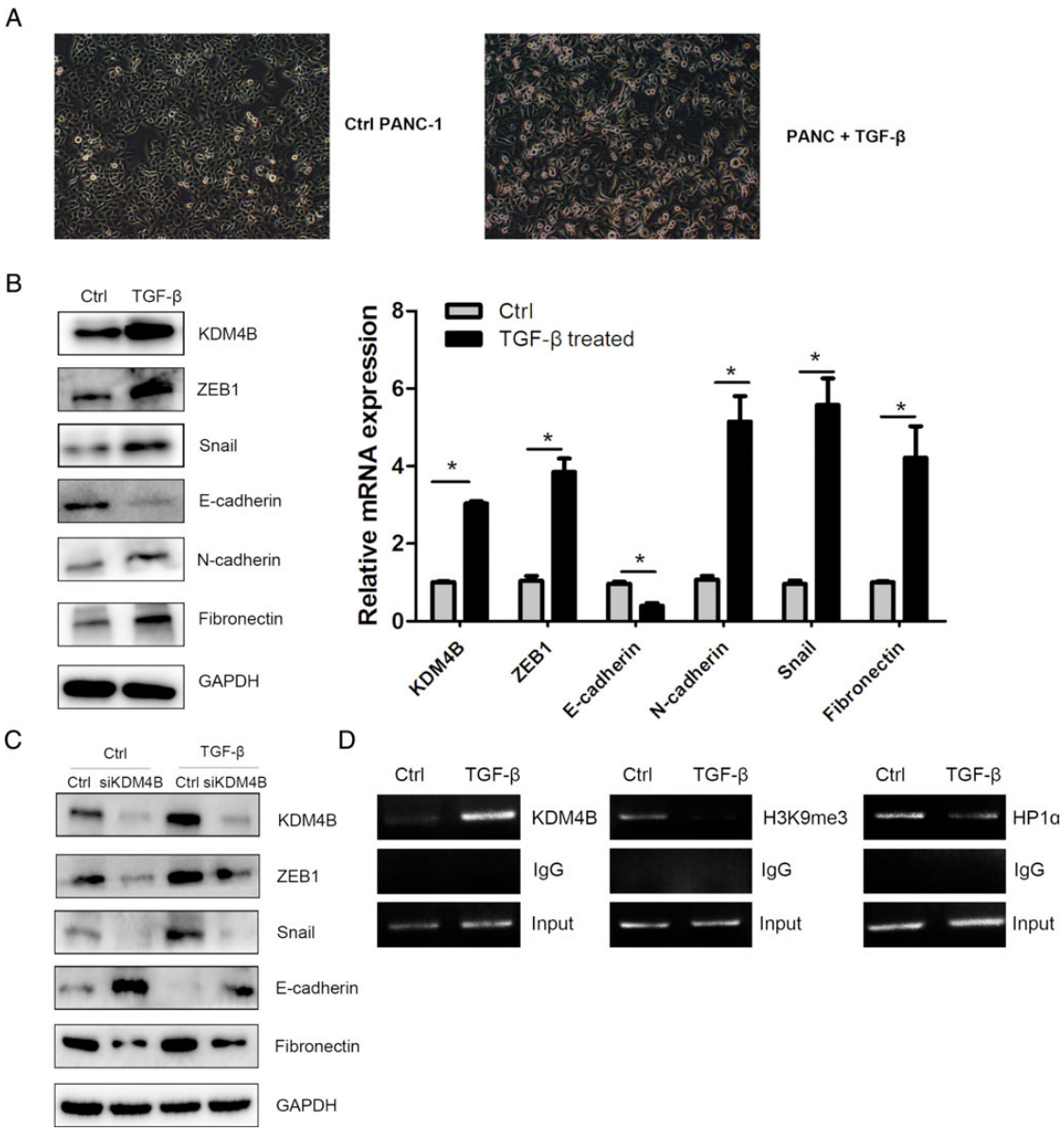


Figure 4. KDM4B-mediated TGF-β induces EMT process (A) Morphological changes in PANC-1 cells after being treated with TGF-β (10 ng/ml) for 48 h. Magnification: ×400. (B) The mRNA and protein expressions of KDM4B, ZEB1, Snail, E-cadherin, N-cadherin and Fibronectin were assessed by western blot analysis or real-time PCR in control and TGF-β-treated PANC-1 cells. (C) Control or siKDM4B cells were cultured for 48 h with or without TGF-β at a concentration of 10 ng/ml, and then KDM4B and EMT markers expressions were detected by immunoblotting. (D) The ChIP assay was performed to evaluate the KDM4B, H3K9me3 and HP1α occupancy on ZEB1 promoter in control and TGF-β-treated PANC-1 cells. Data are representative of three independent experiments (*$P < 0.05$). Ctrl, control.

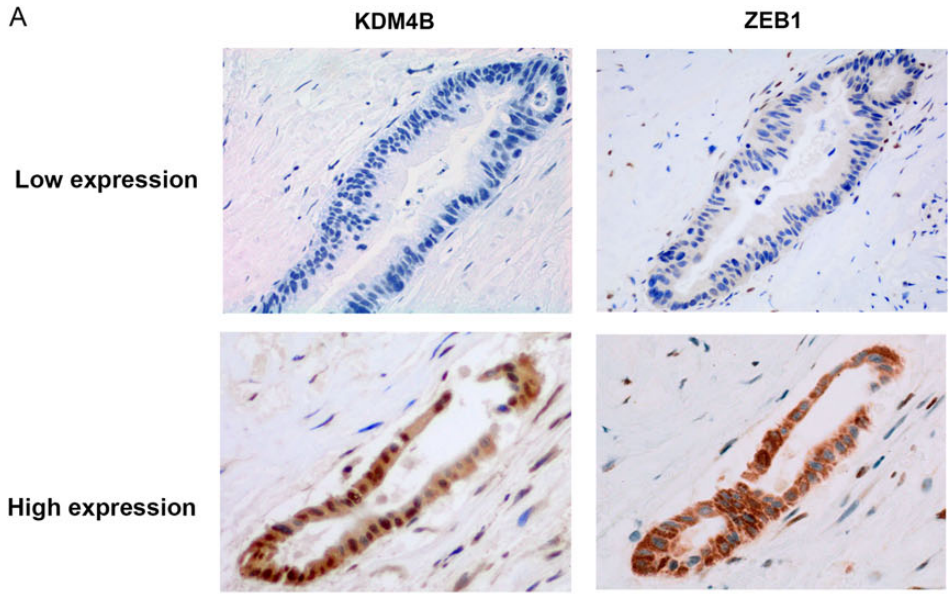
level of transcription, which contributes to the metastasis of pancreatic cancer cells.

Discussion

Pancreatic cancer is one of the most malignant human cancers, with a 5-year survival rate of ~5%. Patients who were diagnosed with pancreatic cancer develop metastases due to the biological features of pancreatic cancer, such as rapidly uncontrolled progression, extensive invasiveness and intrinsically profound resistance to traditional treatments, such as chemotherapy and radiotherapy. Thus, there is a pressing need to identify new biomarkers for early diagnosis and prognosis to reduce the death rate of pancreatic cancer. Much evidence has shown that the increased motility, migration and invasiveness of pancreatic cancer cells are associated with EMT, which has been attributed to metastasis to distant organs, such as liver, lungs and peritoneum [15]. Down-regulation of E-cadherin expression and acquisition of mesenchymal markers, such as N-cadherin, vimentin and fibronectin, which leads to loss of cell adhesion to facilitate cell motility, are considered to be important characteristic of EMT. Over the past decades, the transcriptional and posttranscriptional regulation of E-cadherin has been extensively studied. Transcriptional factors, such as TWIST, Snail and ZEB1 have been extensively studied in E-cadherin regulation [16–18]. However, how the transcriptional factors are regulated has seldom been discussed.

KDM4B is a lysine specific demethylase and reported to be involved in the regulation of malignant behaviors of many cancer

A



B

Variable	KDM4B expression Score 0-1	Score 2-3	*P* value
ZEB1 expression			
Score 0-1	7 (38.9%)	11 (61.1%)	
Score 2-3	3 (9.7%)	28 (90.3%)	0.0255

*$P < 0.05$ is considered significant

Fisher's exact test

C

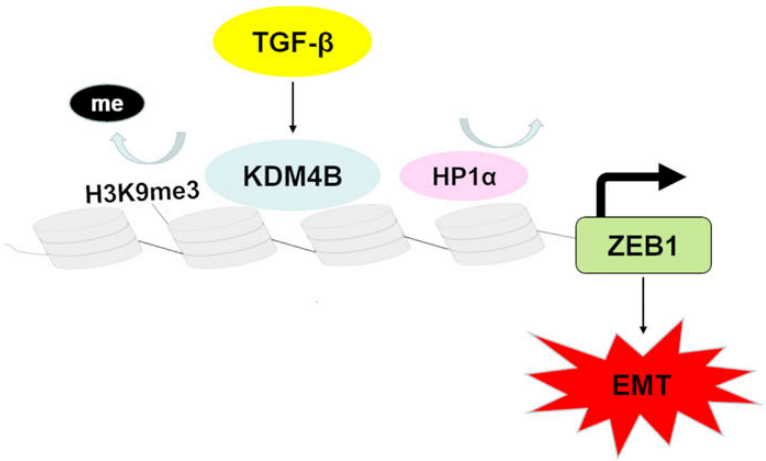


Figure 5. KDM4B is positively correlates with ZEB1 in pancreatic cancer tissues (A) Immunohistochemistry was performed on serial sections using antibodies against KDM4B and ZEB1. Magnification, ×200. (B) The correlation between KDM4B and ZEB1 expression in pancreatic cancer tissues was determined by Fisher's exact test. (C) A proposed model to illustrate that KDM4B epigenetically regulates ZEB1 expression to promote EMT process.

types, and is proposed to be a marker for prognosis and diagnosis [19]. However, its role in pancreatic cancer remains largely unknown. In the present study, we reported that KDM4B is a positive regulator of EMT process because decreased KDM4B by siRNA inhibited the metastasis of pancreatic cancer cells *in vitro*. Zhou *et al.* [14] reported that in gastric cancer, KDM4B is a co-factor of β-catenin, and enhances EMT and metastasis. In this study, the role of KDM4B in EMT was reported from a different point of view. In this study, KDM4B was found

to be a positive regulator of ZEB1, an important EMT modifier. KDM4B epigenetically regulated ZEB1 expression in EMT process. The role of ZEB1 in EMT process has been well studied. It negatively regulated E-cadherin expression, and was a poor prognosis marker in cancers [20–22]. In the present study, we first reported that ZEB1 is under the control of KDM4B, which makes KDM4B be a possible target for pancreatic cancer. However, there are still some questions. For example, KDM4B is a global chromatin modifier, and its regulatory role in gene transcription still relies on transcription factors to specifically recruit it to target genes. Thus, it is necessary to screen KDM4B cofactors to elucidate the role of KDM4B in EMT control.

Our results also showed that KDM4B was significantly correlated with ZEB1 in pancreatic cancer patients. However, its role in prognosis need to be further investigated. We plan to detect the expressions of KDM4B and ZEB1 in more pancreatic cancer patients, and determine the value of KDM4B in prognosis as well as its predictive value in conjugation with ZEB1. Answers to these questions will make KDM4B to be a valuable marker in pancreatic cancer. Furthermore, *in vivo* experiments are also needed to elucidate the role of KDM4B.

In conclusion, KDM4B was demonstrated to positively regulate the EMT process by activating ZEB1 at the transcription level. Furthermore, KDM4B was also found to be significantly correlated with ZEB1 in pancreatic cancer patient sample. All these findings suggest that KDM4B may be a valuable marker in pancreatic cancer.

Supplementary Data

Supplementary data is available at *ABBS* online.

Funding

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