Up-Regulation of MBD1 Promotes Pancreatic Cancer Cell Epithelial-Mesenchymal Transition and Invasion by Epigenetic Down-Regulation of E-Cadherin

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Abstract: Methyl-CpG binding domain protein 1 (MBD1) has been implicated in transcriptional regulation, heterochromatin formation, genomic stability, cell-cycle progression and development. It is also predicted that MBD1 might be involved in tumor development and progression. However, whether and how MBD1 is involved in tumorigenesis, especially in pancreatic cancer (PC), is currently unknown. We found that MBD1 was significantly up-regulated in PC tissues compared with the surrounding normal tissues according to RT-PCR data. Tissue microarray (TMA) based immunohistochemical study from 58 surgically resected PC specimens indicated that higher MBD1 expression correlated with lymph node metastasis and poor survival in PC patients. Gain- and loss-of-function studies in vitro validated MBD1 as a potent oncogene promoting PC cell invasion as well as epithelial-mesenchymal transition (EMT). Mechanistically, MBD1 is associated with Twist and NAD-dependent deacetylase sirtuin-1 (SIRT1), thereby forming the Twist-MBD1-SIRT1 complex on the CDH1 promoter, which resulted in reduced E-cadherin transcription activity and increased cell EMT ability. Significantly, targeting MBD1 reversed the EMT phenotype of PC and restored sensitivity to chemotherapy. Taken together, the results of our study revealed a novel function of MBD1 in PC invasion and metastasis by providing a molecular mechanism underlying MBD1-promoted EMT. Thus MBD1 may serve as a potential therapeutic target for PC.

Keywords: Drug resistance, EMT, MBD1, PC, SIRT1, Twist.

INTRODUCTION

Pancreatic cancer (PC) is currently the fourth leading cause of cancer-related mortality in western countries [1]. Late clinical presentation, difficulties in early diagnosis and the aggressive biological nature of PC with its high resistance to traditional chemo- and radiotherapies all contribute to the poor prognosis of PC [2]. Understanding the molecular basis of PC and its metastasis may provide insight into the development of novel therapeutic strategies. Epithelial-mesenchymal transition (EMT) triggered by a wide variety of genetic or epigenetic alterations [3] caused cells to lose their epithelial characteristics and acquire mesenchymal phenotype with increased metastatic and invasive potential [4] in many solid tumors including PC [5, 6]. Loss of E-cadherin expression, which is a major component of cell-cell adhesion junctions in the maintenance of cell polarity and environment [7], is a hallmark of EMT [8]. It has been reported that hypoxia and hypermethylation activated expression of Twist, a master regulator of E-cadherin is crucial for EMT as well as maintaining drug resistance in human PC cell lines [9, 10]. Therapeutic strategies aiming at up-regulating E-cadherin to reverse the EMT phenotype may become a novel treatment for PC.

Methyl-CpG binding domain protein 1 (MBD1), which binds to methylated CpG islands symmetrically and couples DNA methylation to transcriptional repression [11], has been implicated in transcriptional regulation, heterochromatin formation, genomic stability, cell-cycle progression and development [12]. It has been reported that MBD1 plays an important role in silencing tumor suppressor genes that are hypermethylated at their promoter CpG islands in several cancer cells [12]. Our previous gene expression profiling study also identified MBD1 as an important oncogene in PC [13], while significant increase of MBD1 in PC was found to simultaneously down-regulate some tumor suppressor genes such as CDH1 [14]. In addition, a nanoparticle-delivered MBD1-siRNA can significantly inhibit cell growth and induce apoptosis of PC cells in vitro [15]. However, the molecular mechanism how MBD1 is involved in PC tumorigenesis and progression is not clear.

Histone acetylation is known to modify N-terminal lysine residues of histones, transforming condensed chromatin into a more relaxed structure which is
associated with increased gene transcription [16]. NAD-dependent deacetylase sirtuin-1 (SIRT1), a conserved NAD-dependent deacetylase that regulates life span in accordance with nutritional provision [17]. Recently, Zhao et al. [18] found that SIRT1 may promote cell proliferation and tumor formation in PC. Recent data also suggests that SIRT1 regulates apoptotic thresholds by deacetylating molecular targets including E-cadherin [19]. SIRT1 interacts and deacetylates Suv39h1, which is the major enzyme responsible for the accumulation of histone H3, and these activities contribute to elevated Suv39h1 activity [20]. Furthermore, MBD1 interacts with histone deacetylases through Suv39h1, resulting in methylation and deacetylation of histones for gene inactivation [21]. Therefore we hypothesize that, as a methylated binding protein, MBD1 might down-regulate the expression of E-cadherin through a SIRT1-CDH1 pathway.

In this study, we examined the clinical significance of MBD1 in PC patients. We then investigated the mechanisms involved in the effects of MBD1 on PC EMT and metastasis through epigenetic down-regulation of E-cadherin. Finally we validated the therapeutic potential of MBD1 in PC treatment.

MATERIALS AND METHODS

Tissues, Cells and Reagents

Human PC tissues and the surrounding benign tissues were obtained from 58 patients at the Department of Pancreas and Hepatobiliary Surgery, Shanghai Cancer Center, China between 2009 and 2011. The patient cohort is composed of 33 male and 25 female patients, between 39 and 77 years old, with an average age of 61.04±12.05 years. None of the patients received chemotherapy or radiotherapy before surgical resection. The diagnosis of PC was confirmed by pathologists. Patients were followed up every 2 months during the first postoperative year and at least every 3 to 4 months afterward. The last follow-up was done on March 15, 2012. The median follow-up was 12 months (range, 3-22 months). All tissue samples were collected and archived under protocols approved by the institutional review board with written informed consent for collection of blood, tissue, and clinical follow-up information. Immediately after surgical removal, tissue samples were fixed in 10% formalin solution and paraffin-embedded for histological analysis. The human PC cell lines, PANC-1, AsPC-1, BxPC-3, and SW1990 were purchased from Shanghai Institutes for Biological Science (China). Cells were cultured in RPMI-1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) in a 37°C incubator with 5% CO2. RPMI media, fetal bovine serum, horse serum, L-glutamine (2 mM), penicillin (50 IU/ml) and streptomycin (50 μg/ml) were from Life Technologies, Inc.

Tissue Microarray (TMA) and Immunohistochemistry

TMA was constructed as described previously [22]. Immunohistochemical staining was carried out using the two-step protocol on paraffin-embedded tissue as previously described [23]. A four-point staining intensity scoring system was used for determining the relative expression of MBD1 in tissue specimens; the staining intensity score ranged from 0 (no expression) to 3 (high expression). The results were classified into two groups according to the intensity and percentage of staining. All of the immunostaining results were reviewed and scored independently by two pathologists.

Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from PC tissues, normal tissues and PC cell lines using Trizol reagent (Gibco). First-strand cDNA was synthesized from 1 μg total RNA using RevertAid™ M-MULV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions. Primer sequences for E-cadherin were 5′ TCCATTCTTGTGCTACGCC 3′ for upstream, 5′ CACCTTCAGCCATCCTGTTT 3′ for downstream and annealing temperature was 60°C. Primer sequences for Vimentin were 5′ TGGAGAGAAACTTTGCCTG 3′ and 5′ AAGGTGACGAGCCATTCTC 3′ and annealing temperature was 60°C. Primer sequences for FasL were 5′ TCTGTGTGCCAAAGTCCAAA 3′ and 5′ ACATCCATCTTCCCTCCGA 3′ and annealing temperature was 61°C. GAPDH was used as an endogenous control.

Western Blot Analysis

Total cell lysate was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel using electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h at room temperature in 10% FBS, then incubated overnight at 4°C with different primary antibodies (anti-MBD1, Santa Cruz Biotechnology; anti-E-cadherin, BD Pharmingen; anti-Vimentin, Santa Cruz Biotechnology; anti-c-myc, US Biological; anti-SIRT1, Santa Cruz Biotechnology; anti-Twist, BD Pharmingen; anti-Snail, BD Pharmingen; anti-N-cadherin, Novocasta; anti-p-β-catenin, Santa Cruz Biotechnology; p-Erk, BD Pharmingen; Erk, BD Pharmingen; bcl-2, Santa Cruz Biotechnology; bax, Santa Cruz Biotechnology; Cleaved Caspase-3, 9, US Biological). GAPDH was used as an endogenous control. After washing for three times, the membrane was incubated with secondary antibody for 1 h at room temperature. The signal was using the ECL detection system (Chemicon).

Lentiviral Production and Infection of PC Cells

Lentiviral vector pLKO.1 TRC (Addgene Plasmid 10878) was used according to a protocol online (http://www.addgene.org/tools/protocols/plko/). In brief, shRNA oligos targeting human MBD1 or SIRT1 were designed and cloned into pLKO.1-TRC cloning vector digested with EcoRI and AgeI. The recombinant construct together with two packaging vectors psPAX2 and pMD2.G were transiently transfected into 293T cells. pLKO.1-scramble (SCR) shRNA (Addgene Plasmid 1864) were used as a negative control.
Lentiviral particles were harvested, filtered and infected into target PC cells. To overexpress MBD1, FLAG-tagged MBD1 was cloned into lentiviral vector pWPI.1. Lentiviral particles were produced by cotransfection of pWPI.1-MBD1-FLAG with psPAX2 and pMD.G into 293T cells.

**In Vitro Invasion Assay**

The invasion assay was performed as previously described [24] using transwell cell culture chambers (8 mM pore size polycarbonate membrane, Costar). The cells that had passed the membrane were counted in 10 randomly selected microscopic fields. Each assay was performed in triplicate.

**Determination of Cell Proliferation and In Vitro Wounding Assay**

10⁴ cells/well were seeded into a 96-well plate and allowed to adhere overnight. After treatment with sodium butyrate, TSA or MS-275 for 24-48 h, 10 µl thiazolyl blue tetrazolium bromide (Sigma) were added and cells were incubated at 37°C for 2 h. Colorimetric measurement was done at 450 nm in a microplate reader (Spectra Max 190, Molecular Devices). In vitro wounding assay was performed by creating a scratch with a 100 µl tip on the surface of a confluent dish of PANC-1, PANC-1-MBD1-sh, PANC-1-MBD1-res respectively. Images were captured and compared at the beginning and regular intervals during cell migration to close the wound.

**Immunofluorescence Microscopy**

Cells were grown on coverslips and fixed in 4% paraformaldehyde, permeabilized and stained with primary antibodies followed by secondary FITC or Alexa 555-conjugated mouse or rabbit antibodies. F-actin was detected with FITC-phalloidin (Invitrogen). Slides were examined and photographed using a fluorescence microscope (Olympus BX 40). Nuclei were counterstained with 40, 6-diamino-2-phenylindole (DAPI).

**Co-Immunoprecipitation Assays**

PANC-1 cells and 293T cells were lysed by brief sonication in co-immunoprecipitation buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 supplemented with protease inhibitors). Lysates were centrifuged for 20 min at 10,000 g and the resulting supernatant was precleared by incubation with immobilized Protein A/G gel (Pierce) for 1 h at 4°C. The precleared supernatant was subjected to overnight immunoprecipitation using the indicated antibodies or control IgG antibodies at 4°C. The next day, protein complexes were collected by incubation with 25 µl of immobilized Protein A/G gel for 1 h at 4°C. The collected protein complexes were washed for four times with co-immunoprecipitation buffer and eluted by boiling in protein sample buffer under reducing conditions, after which proteins were resolved on SDS-PAGE and analyzed by western blot.

Chromatin Immunoprecipitation (ChIP) and Re-ChIP

ChIP assay was performed according to the manufacturer’s suggestions (Upstate Biotechnology) with some modifications. Cells were treated with TSA (10 mM) for 24 h or remained untreated and were crosslinked by incubation with 1% formaldehyde for 10 min. Cells were lysed in SDS lysis buffer (50 mM Tris HCl, pH 8.1, 10 mM EDTA, 1% SDS including protease inhibitors) and sonicated with seven 10 s bursts of a Bandelin Sonicator UW 2200 (Bandelin Electronic). After 10 min centrifugation at 14000 rpm (4°C), supernatant was saved and an aliquot of 200 µl was saved for ‘input control’. Lysates were diluted in buffer (16.7 mM Tris HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS), precleared with protein A agarose/salmon sperm DNA and immunoprecipitation was performed by adding antibodies against SIRT1, MBD1, Twist and incubated overnight at 4°C under rotation. Precipitated complexes were collected with protein Agarose/salmon sperm DNA and immunoprecipitation was performed by adding antibodies against SIRT1, MBD1, Twist and incubated overnight at 4°C under rotation. Precipitated complexes were washed with low salt, high salt and LiCl wash buffer. DNA complexes were eluted in 500 µl elution buffer (0.1 M sodium hydrogen carbonate, 1% SDS), heated at 65°C for 4 h and treated with proteinase K. DNA was recovered using the QIAEX-II Gel Extraction Kit (Qiagen) and dissolved in 60 µl water. Immunoprecipitated and input DNA were subjected to PCR analysis. For the Re-ChIP assay, bead elutes from the first round of ChIP were incubated with 10 mM DTT at room temperature for 30 min and then diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1) followed by Re-ChIP with the second antibody.

The following primers were used to detect E-cadherin promoter region:

Forward:5’-AGGGTCACCGCGTCTATG-3’
Reverse:5’-CTTCCGCAAGCTCACAGG-3’

**Statistical Analysis**

Results are expressed as mean ± standard deviation. Differences between groups were evaluated using the Student’s t-test and Fisher’s exact test. The association between immunoreactive markers and clinicopathologic variables was analyzed using the χ² test or Fisher’s exact test. Overall survival was calculated by the Kaplan-Meier method, and the differences between the survival rates were compared using the log-rank test. P-values less than 0.05 was considered significant.

**RESULTS**

Expression of MBD1 Correlates with Lymph Node Metastasis and Survival of PC Patients

To validate the over-expression of MBD1 in PC, the paired tumor tissues and surrounding benign tissues were examined using RT-PCR. Significantly higher MBD1 expression was observed in 12 PC tissues
compared with their surrounding benign tissues (Fig. 1A). TMA containing 58 PC tissues was used to examine the expression of MBD1 using immunohistochemical analysis. MBD1 was found to be expressed in both cytoplasm and nucleus with diffuse staining pattern and different intensity (Fig. 1B-E).

**Fig. (1). Over-expressed MBD1 in PC tissues correlates with lymph node metastasis and poor prognosis of PC patients.**

(A) Relative mRNA expression of MBD1 in PC tissues and surrounding benign tissues. Higher MBD1 expression was found in the tumor tissues compared with surrounding benign tissues among a total of 12 paired PC specimens analyzed by RT-PCR with normalization of GAPDH. (B-E) Immunohistochemical staining of MBD1 in TMA containing 58 PC specimens. B & C represent the high expression of MBD1 in PC tissues; D & E are examples of low MBD1 expression. Positive cells were stained brown while areas encompassed by frame dotted line were further enlarged (in the right). Magnification:×200. (F) Correlation between MBD1 expression levels and lymph node metastasis (LNM) status. Low MBD1 expression (+/-) is depicted as blue columns, whereas high MBD1 expression (+++/++) is depicted as red columns. (G) Kaplan-Meier plot of overall survival of 58 patients with PC stratified by MBD1 expression level. A log rank test was used to show differences between groups.
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Among all PC specimens, MBD1 was detected in 82.7% of the samples (48/58 cases). We also found that MBD1 expression significantly correlated with lymph node metastasis (Table 1), with higher level of MBD1 detected in patients with lymph node metastasis (Fig. 1F). Furthermore, Kaplan-Meier analysis revealed that higher MBD1 expression significantly correlated with markedly reduced overall survival of PC patients (Fig. 1G). Taken together, the above clinical data indicated that up-regulation of MBD1 was a critical event in PC tumor progression.

Down-Regulation of MBD1 Inhibits the Proliferation and Migration of PC Cells In Vitro

We then evaluated the effects of MBD1 on cell proliferation and invasion in vitro. We generated a series of MBD1-shRNAs to silence the expression of MBD1 in highly aggressive PANC-1 cells. Our results showed that shRNA-MBD1-3 was the most effective shRNA to knock down the expression of MBD1 in PANC-1 cells (Fig. 2A). Thus shRNA-MBD1-3 was used in the subsequent studies. Cells stably expressing shRNA-MBD1-3 led to potent suppression of proliferation, invasion and migration of PANC-1 cells (Fig. 2C-E). To confirm that the observed inhibition was not due to an off-target effect of the MBD1-shRNA, we reintroduced MBD1 with MBD1-cDNA engineered to be insensitive to shRNA-3 (sh-MBD1-res) (Fig. 2B) into the MBD1 silenced cells to examine whether exogenous MBD1 caused MBD1-silenced PC cells to regain the malignant potency. We confirmed that reintroducing MBD1 into the cells rescued the cell proliferation, invasion and migration (Fig. 2C-E). Furthermore, the morphology of the MBD1 silenced PANC-1 cells was compared with that of the control cells using a confocal microscopy. The wild type cells without MBD1-knockdown showed one or more pseudopodia-like membrane protrusions compared with MBD1-silenced cells, which may explain the difference in their migration ability (Fig. 2F). These genetic loss-of-function studies using shRNA-mediated knockdown indicated that MBD1 functionally promoted PC cell proliferation and migration.

### Table 1. Clinicopathologic Characteristics of Patients with Low and High Expression of MBD1 in 58 PC Patients

<table>
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<tr>
<th>Variable</th>
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<th>High Expression (Score ++/+++ n=31)</th>
<th>P Value</th>
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<tr>
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<tr>
<td>Poor (n=14)</td>
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<td>Tumor Size (no. of patients)</td>
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<td>Median survival (in months)</td>
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*P values were derived with Pearson chi-square tests.

**P values was derived with log rank test.
All statistical tests are two sided. Abbreviations: MBD1, Methyl-CpG binding domain protein 1.
Fig. (2). Effects of down-regulation MBD1 on the in vitro proliferation and migration of PC cells. (A) PANC-1 cells were infected with lentivirus carrying different shRNA for MBD1 and subjected to Western-blot assay to detect the knockdown effect. The sh-MBD1-3 plasmid was found to be the most effective silencer. (B-E) PANC-1 cells infected with lentivirus carrying sh-MBD1 were re-introduced with MBD1 by shRNA resistant MBD1 (sh-res) (B). The growth inhibition assay (C), transwell migration assay (D) and scratch test (E) were further investigated. Cells stably expressed shRNA against MBD1 displayed a proliferation, migration and invasion inhibition. Cells regained a malignant phenotype with re-introduced MBD1. Each data point is representative as mean of triplicate experiments. *P < 0.05. (F) The cellular morphology was altered in cells with down-regulated MBD1 expression for resuming an epithelial phenotype. PANC-1-sh-MBD1 and PANC-1-sh-SCR were fixed and stained for F-actin (green). Nuclei were counterstained with DAPI (blue). Scale bars, 50μm.
MBD1 Shifts the EMT Status in PC Cells

To better understand the regulatory mechanisms of MBD1 on PC progression, we investigated the expression profile of MBD1 and EMT related proteins: E-cadherin, c-myc, Twist, Snail, N-cadherin, Vimentin and p- β-catenin by Western blotting in four PC cell lines including AsPC-1, BxPC-3, PANC-1 and SW1990 (Fig. 3A). We found that E-cadherin expression varied from low to high in four cell lines. The moderately or poorly differentiated cell lines such as PANC-1 and AsPC-1 had a low expression of E-cadherin and abundant Vimentin, which exhibited mesenchymal-dominated phenotype [22]. The well differentiated cells such as SW1990 and BxPC-3 showed a higher expression of E-cadherin with a lower level of Vimentin; accordingly, these cells led to an epithelial-dominated phenotype. The mesenchymal cells PANC-1 and AsPC-1 had higher expression of MBD1 compared with epithelial cells BxPC-3 and SW1990, indicating that MBD1 may suppress the expression of E-cadherin and promote the EMT of PC cells. Furthermore, silencing of MBD1 by shRNA in PANC-1 resulted in up-regulation of E-cadherin and down-regulation of Vimentin at the mRNA and protein level (Fig. 3B, C). We also found that introducing MBD1 into cells that had a low expression of MBD1 such as SW1990 decreased E-cadherin and increased Vimentin expression (Fig. 3D). Moreover, phase-contrast microscopy revealed that after treatment with MBD1-shRNA, PANC-1 cells regained an epithelial cell type morphology compared with controls (Fig. 3E, a and b). Immunofluorescent staining also showed that knockdown of MBD1 led to an elevated level of E-cadherin and a decrease of Vimentin expression, which would reverse EMT in PC cell lines (Fig. 3E, c, d, e and f). Given these data, we supposed that down-regulating E-cadherin expression in PC by MBD1 and shifting the EMT phenotype in PC cells played an important role in PC invasion and metastasis.

MBD1 Interacts with Twist and SIRT1 in CDH1 Promoter Region

In order to further explore the molecular mechanism of MBD1 in the negative regulation of E-cadherin expression and tumor progression, we performed a co-immunoprecipitation assay to identify the possible interaction proteins with MBD1 which may be involved in EMT and PC metastasis. We found that MBD1 could physically interact with Twist, a well-known transcriptional repressor of E-cadherin, both in 293T cells and in PANC-1 cells (Fig. 4A, C). In addition, we found that MBD1 interact with SIRT1 using co-immunoprecipitation both in 293T and PANC-1 cells (Fig. 4B, C). We also validated this interaction in PANC-1 cells endogenously (Supplementary Fig. 1A). Furthermore, the interaction between SIRT1 and Twist was further strengthened in the presence of MBD1 (Fig. 4D), therefore we hypothesized that MBD1 might function as a bridge to connect SIRT1 with the classical transcriptional repressor Twist to suppress the expression of E-cadherin. We did not detect any direct interaction between MBD1 and E-cadherin (data not shown). Thus, the above findings further suggest that MBD1 couples with SIRT1 and Twist and might act as a complex to epigenetically down-regulate E-cadherin expression during the EMT status in PC.

To test this hypothesis, we performed ChIP and Re-ChIP assay to examine the binding of MBD1 and SIRT1 in the promoter region of CDH1 in PANC-1 cells (Fig. 4E). ShRNA-mediated knockdown of MBD1 in PANC-1 cells decreased binding of SIRT1 and HP1, which is the marker of heterochromatin in CDH1 promoter region, while the occupancy of AcH3, the substrate of SIRT1, which is a marker of euchromatin, was increased (Fig. 4F). Furthermore, over-expression of MBD1 and SIRT1 led to reduced occupancy of AcH3 and increased occupancy of HP1 in the CDH1 promoter region. The most dramatic change appeared when we transfected MBD1 and SIRT1 simultaneously (Fig. 4G). These results indicate that MBD1 cooperates with SIRT1 to establish a tightened chromatin structure of CDH1 promoter therefore inhibiting the transcription of E-cadherin.

MBD1 Cooperates with SIRT1 and Twist to Promote PC Metastatic Potential

In order to further clarify the physical interaction and explore the functional connection between MBD1 and SIRT1, we investigated the role of this complex in the EMT stage of PC metastasis. We analyzed the expression of E-cadherin in low MBD1 expression PC cells SW1990, which were transfected with MBD1, SIRT1 and Twist individually or in combination (Fig. 5A). We found that the most suppression of E-cadherin occurred when all three transcriptional factors were co-transfected (Fig. 5A). This was also accompanied by enhanced invasiveness compared to controls (Fig. 5B). Meanwhile, when MBD1 or SIRT1 was silenced in PANC-1 cells, the expression of E-cadherin was up-regulated. Conversely, a decrease of E-cadherin expression was seen only when MBD1 and SIRT1 were re-introduced into these cells simultaneously (Fig. 5C). Likewise, transwell assay was performed to test the change in invasiveness of these cells. We found that the cells with silenced MBD1 and SIRT1 expression showed an insufficient invasive ability while the reintroduction of both MBD1 and SIRT1 could significantly increase the cell invasion (Fig. 5C). In addition, we further tested the correlation of MBD1, SIRT1 with E-cadherin in clinical samples. We found that patients with higher expression levels of MBD1 and SIRT1 often led to a lower expression pattern of E-cadherin (Fig. 5D, b), while patients displayed a higher expression of E-cadherin were coupled with lower MBD1 and SIRT1 expression (Fig. 5D, a). These results were consistent with the above mentioned cell line data, indicating that MBD1 in combination with SIRT1 and Twist played an important role in down-regulating E-cadherin in PC cells which regulated EMT status as well as invasion in PC patients.
Fig. (3). MBD1 mediates the EMT status in PC cells. (A) Expression of MBD1 as well as EMT-related factors: E-cadherin, N-cadherin, Vimentin, p-β-catenin and transcriptional repressor proteins SIRT1, c-myc, Twist and Snail were analysed by immunoblot with specific antibodies in different PC cell lines PANC-1, AsPC-1, BxPC-3 and SW1990. (B, C) Silencing MBD-1 in vitro reversed EMT status in a mesenchymal phenotype PC cell line PANC-1. RT-PCR analyses of the epithelial (E-cadherin) and mesenchymal (Vimentin) mRNA expression (B) and western-blot examination of their proteins (C) in PANC-1 cells. MBD1 depletion led to an elevation or a reduction in E-cadherin or Vimentin protein and gene level in PANC-1 cells, respectively. (D) The protein expression of MBD1 and EMT-related factors was analysed by western-blot using specific antibodies in SW1990 cells introduced with MBD1, or their respective controls. (E) PANC-1 cells infected with MBD1-shRNA (a), or their respective controls (b), and the morphological alterations of cells were observed by phase-contrast microscopy. Immunofluorescence staining of epithelial (E-cadherin, Green, c, d) and mesenchymal (Vimentin, Red, e, f) markers was visualized by confocal microscopy. DAPI staining was included to visualize the cell nucleus (Blue).
Fig. (4). MBD1 interacts with Twist and SIRT1. (A-C) MBD1 interacts with Twist and SIRT1. The indicated constructs (FLAG-MBD1, myc-MBD1, FLAG-SIRT1, HA-SIRT1, FLAG-Twist, HA-Twist) were transiently expressed in either 293T or PANC-1 cells and the whole cell lysates were immunoprecipitated (IP) with anti-FLAG antibody. FLAG-tagged MBD1 as indicated were co-expressed with HA-tagged Twist (A) and SIRT1 (B) in 293T cells and immunoprecipitated with the relevant antibody. The presence of associated MBD1 protein was assessed by immunoblotting using anti-MBD1 antibody. In PANC-1 cells, FLAG-tagged fragments of MBD1 as indicated were also co-expressed with HA-tagged SIRT1 and Twist and immunoprecipitated with the relevant antibody (C). (D) The interaction of Twist and SIRT1 was strengthened by MBD1. Co-immunoprecipitation of Twist and SIRT1 in the presence of MBD1 was followed by immunoblot analysis for Twist and SIRT1 in 293T cells. (E) ChIP with MBD1 antibody and Re-ChIP with SIRT1 antibody followed by PCR with CDH1 promoter-specific primers showed both MBD1 and SIRT1 could bind to the same region of CDH1 promoter in PANC-1 cells. (F) ChIP results further showed that in MBD1-silenced cells the occupancy of SIRT1 and HP1γ in the CDH1 promoter was lost or attenuated, but the occupancy of Ach3 was increased. (G) The occupancy of Ach3 was reduced in CDH1 promoter region with increased occupancy of HP1γ in cells with elevated level of MBD1 and SIRT1, and the most dramatic change appeared when MBD1 and SIRT1 were introduced simultaneously.
Targeting MBD1 Affects Gemcitabine Resistance in PC Cells

We hypothesize that MBD1 is involved in the chemo-sensitivity of PC, and might be a novel target for clinical intervention. First we examined the chemosensitivity in the four PC cell lines PANC-1, AsPC-1, BxPC-3 and SW1990, and found that cells with a higher MBD1 level (PANC-1 and AsPC-1) were chemo-resistant to Gemcitabine compared with BxPC-3 and SW1990 cells with a lower-expression of MBD1 (Fig. 6A). Moreover, the sensitivity of PANC-1 cells with stable expression of MBD1-shRNA to

Fig. (5). Functional interplay of MBD1 with SIRT1 and Twist in promoting EMT and metastatic potential in PC. (A) Western blot analysis of MBD1, SIRT1, Twist and EMT-related factors as E-cadherin, Vimentin in SW1990 cells with MBD1, SIRT1 and Twist transfected individually or together indicated that all the three transcriptional factors would suppress the expression of E-cadherin and the greatest lost of E-cadherin occured when the three transcriptional factors were introduced at the same time. (B) SW1990 cells were transfected with MBD1, SIRT1 or Twist shRNA either alone or in combination with each other, as indicated and subjected to transwell migration assay. (C) Western blot analysis of MBD1, SIRT1, Twist, E-cadherin and Vimentin in PANC-1 cells with silenced of MBD1 or SIRT1 and re-introduced with these factors shows the corresponding changes of E-cadherin. Transwell assay was performed to testify the change of invasiveness of these cells. The fold difference represents the mean of triplicate experiments compared with control cells. *P < 0.05. (D) Clinical relevance between the expression of E-cadherin with MBD1 and SIRT1 in different PC specimens. Representative high expression of E-cadherin related to the low expression of MBD1 or SIRT1 (a) and low expression of E-cadherin accompanied with a high expression of MBD1 or SIRT1 (b) were shown, frame dotted line was further enlarged (in the right).
Fig. (6). Targeting MBD1 affects Gemcitabine resistance in PC cells. (A) Inhibition of cell growth by Gemcitabine in PC cell lines. Various concentrations of Gemcitabine were applied to PANC-1, AsPC-1, BxPC-3 and SW1990 cells for 24h, and cell viability was determined using the MTT assay. The results were expressed as the percentage of growth with 100% representing control cells treated with 0.05% ethanol, showing that a high expression of MBD1 as PANC-1 and AsPC-1 also displayed a higher Gemcitabine resistance. The differences between PANC-1 and BxPC-3 were statistically significant. *$P<0.05$. (B) Down-regulation MBD1 increased chemosensitivity to Gemcitabine in PANC-1 cells. PANC-1-sh-MBD1 cells showed increased sensitivity to Gemcitabine at different dosages. *$P<0.05$. (C) Up-regulation of MBD1 in BxPC-3 cells decreased chemosensitivity to Gemcitabine. BxPC-3-LV-MBD1 cells showed decreased sensitivity to Gemcitabine at different dosages. *$P<0.05$. (D) PANC-1 cells with silenced MBD1 or not were either received 24 h treatment with Gemcitabine or not with different dosages. Apoptosis related proteins as bcl-2, bax, and Cleaved Caspase-3, 9 et al. were determined by Western blot assay, GAPDH was used to verify loading equivalency. (E) Schematic representation depicts the effects on MBD1 binding to SIRT1 and Twist, targets to the promoter of CDH1, then suppress the expression of E-cadherin and results in enhancement of the malignancy capacity as metastasis and drug resistance of PC.
Gemcitabine was significantly enhanced compared with the control cells (Fig. 6B). On the other hand, BxPC-3 cells as well as MBD1 over-expressed BxPC-3 cells were also treated with Gemcitabine to determine their chemo-sensitivity. Compared with wild type BxPC-3, cells that had been introduced with MBD1 displayed enhanced chemo-resistance (Fig. 6C). Total protein was extracted from PC cells with or without silencing of MBD1 for subsequent analysis of drug-resistance-related (p-Erk, Erk) and apoptosis-related signaling proteins (bax, bcl-2, Cleaved Caspase-3, 9) after treatment with different dosages of Gemcitabine (Fig. 6D). The total Erk level was not significantly changed by Gemcitabine treatment. However, p-Erk was significantly down-regulated in PANC-1-sh-MBD1 cells after treatment of Gemcitabine, whereas in PANC-1-sh-SCR cells, p-Erk level was significantly increased. Moreover, the expression level of pro-apoptosis-related proteins Cleaved Caspase-3, 9 and bax were significantly elevated in PANC-1-sh-MBD1 after treatment with Gemcitabine compared to controls, while anti-apoptosis-related bcl-2 protein expression was down regulated in the PANC-1-sh-MBD1 cells. These findings suggest that inhibition of MBD1 expression may increase the chemo-sensitivity in PC.

DISCUSSION

Tumor metastasis is the main cause of cancer-related death. Identifying the molecular basis of cell migration and metastasis, especially from the epigenetic regulation point of view, is crucial for understanding this deadly disease. EMT is a key step during embryonic morphogenesis and is involved in the progression of primary tumors including PC toward metastasis and drug resistance [25]. Previous studies [12] have demonstrated that MBD1 may contribute to tumorigenesis by binding to hypermethylated promoter CpG islands of tumor suppressor genes in cancer cells, for example, the prostate cancer [26] and leukemia [27]. In the present study, we found MBD1 promoted the EMT phenotype through recruitment of SIRT1 and Twist to increase the metastatic, invasive and drug-resistant potential of PC cells (Fig. 6E).

Loss of E-cadherin is one of the most common indicators of EMT onset [28], which can be caused by somatic mutations, chromosomal deletions, proteolytic cleavage and silencing of the CDH1 promoter by DNA hypermethylation or through the action of transcription factors such as Slug, Snail and Twist [3, 12, 29]. Previously, we found that the significant increase of MBD1, a methyl-CpG binding domain protein, in PC was associated with a simultaneous decrease in the expression of CDH1 [14]. Recent studies have also shown that MBD1 may play an important role in silencing tumor suppressor genes that are hypermethylated in their promoter CpG islands in cancer cells such as PC [13, 30, 31]. Our findings support the published data that expression of MBD1 is significantly increased in PC compared to normal pancreatic tissue. More importantly, up-regulation of MBD1 correlated with lymph node metastasis and short survival times of PC patients. Silencing of MBD1 caused a morphologic transition from fibroblastic-like to the epithelial shape, which represents the phenotypic hallmark of EMT [3, 32], implying that MBD1 might be involved in EMT. Meanwhile, we found that the proliferative and invasive capacity of PC cells in vitro was significantly suppressed upon silencing of MBD1, which further indicated that silencing of MBD1 contributed to the reversion of the EMT phenotype in PC cells by up-regulating E-cadherin.

Our data suggests that MBD1 epigenetically regulates E-cadherin expression through recruitment of SIRT1 on the promoter of CDH1, to influence EMT status in PC. SIRT1 was involved in epigenetic silencing of hypermethylated tumor suppressor genes in cancer cells [33]. In PC tissue, up-regulation of HDAC1, HDAC2, HDAC3 and HDAC7 has recently been reported [34, 35]. Recently, Zhao et al. [18] found that SIRT1 may promote cell proliferation and tumor formation by suppressing E-cadherin expression in PC. Physiologically, SIRT1 works on recruiting and deacetylating Suv39h1, which is the principal enzyme responsible for the accumulation of histone H3 [20]. Meanwhile, MBD1 links to histone deacetylases through Suv39h1, resulting in methylation and deacetylation of histones for gene inactivation [21], indicating that MBD1 and SIRT1 might interact with each other in gene transcription regulation.

In the present study, we showed that MBD1 physically interacts with SIRT1 and targets the CDH1 promoter which may further suppress the transcription of E-cadherin by deacetylating the histone of the CDH1 promoter. We found that MBD1 and SIRT1 were functionally interdependent in triggering the alterations in the expression of EMT markers as well as the migration ability of different PC cell lines. Moreover, we showed that MBD1 played an important role in the recruitment of SIRT1 on the CDH1 promoter. This was confirmed by the ChIP assay which demonstrated that, in the absence of MBD1, SIRT1 alone cannot bind to the CDH1 promoter, which seems to be insufficient for the induction of E-cadherin expression in these carcinoma cells. Meanwhile, evidence from clinical samples suggested that the patients with both higher expression of MBD1 and SIRT1 displayed lower E-cadherin expression. These present findings together with published data suggest the possibility that MBD1 modulates the SIRT1-activated down-regulation of E-cadherin and EMT of PC.

We also found that MBD1 and SIRT1 may interact with Twist to trigger EMT. Twist, an important transcriptional factor of the basic helix-loop-helix (bHLH) and the zinc finger protein family, has been shown to induce EMT through the repression of E-cadherin expression by binding to the CDH1 promoter region in different cancers including PC. Recent studies have shown that up-regulation of Twist was associated with malignant transformation of PC [36, 37]. We found in the present study that the interaction between Twist and SIRT1 was further strengthened by MBD1. Thus we hypothesize that MBD1 might serve as a bridge to
connect Twist with SIRT1, and consequently regulate the expression of E-cadherin in PC to influence the status of EMT.

Recent results showed that the EMT phenotype was associated with drug resistance [38, 39]. EMT regulators may contribute to the development of chemo-resistance through up-regulation of key proteins that mediate drug resistance. For example, over-expression of Twist activates the expression of AKT2 and induces resistance to paclitaxel in breast cancer cells [40]. Clinically relevant HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), can restore E-cadherin expression and sensitivity to Gemcitabine and other agents [41]. In our study, we have shown that increased E-cadherin expression after silencing of MBD1 was also accompanied with increased sensitivity to Gemcitabine. We attributed this phenomenon partially to the activation of WNT-dependent signaling because activated WNT signaling associated with down-regulation of E-cadherin has been widely accepted [42]. It has been hypothesized that one of the most important determinants of cancer cell resistance toward chemotherapy or radiation might be an overall resistance to apoptosis [43]. WNTs prevent apoptosis in a variety of tissues during embryonic development [44]. Activation of the canonical β-catenin/TCF pathway, a synergistic action between kinases and β-catenin-mediated transcription, and activation of PI3K/Akt independently of β-catenin have been proposed to confer the pro-survival effect of WNTs in various cell types [45]. Almeida et al. [46] has also shown that WNT proteins prevent apoptosis by increasing the expression of the anti-apoptotic protein bcl-2 in an Erk-dependent manner. In this study, after treatment with MBD1-shRNA, the expression levels of anti-apoptotic factors like bcl-2, as well as p-Erk were reduced, while the expression levels of pro-apoptotic factors like bax were raised. Thus, MBD1-shRNA may restore Gemcitabine sensitivity in human PC cells by raising the expression of E-cadherin and disrupting WNT signaling to reverse the EMT phenotype and enhance Gemcitabine-induced apoptosis in human PC cell lines.

In conclusion, our study revealed a novel role for MBD1 in PC invasion and metastasis, providing a molecular mechanism underlying MBD1-promoted EMT and suggesting that MBD1 may be a potential target for both metastasis and chemosensitivity. Co-expression of MBD1, SIRT1 and Twist may be used as a valuable marker to predict the metastatic potential of PC tumors and prognosis for such cancers. Overall, our study may lead to new therapeutic options in the treatment of PC.

CONFLICT OF INTEREST
The authors confirm that this article content has no conflicts of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is available on the Publisher’s web site along with the published article.

REFERENCES


