Menin Coordinates C/EBPβ-Mediated TGF-β Signaling for Epithelial-Mesenchymal Transition and Growth Inhibition in Pancreatic Cancer

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Menin displays either tumor suppression or promotion functions in a context-dependent manner. Previously, we proposed that Menin acts as a tumor suppressor by inhibiting cell growth in pancreatic ductal adenocarcinoma (PDAC), whereas the relationship between the Menin expression and overall survival rate of PDAC patients has not been completely elucidated, indicating the complexity of Menin functions in PDAC progression. Here, we identify Menin as a promoter of epithelial-mesenchymal transition (EMT), which is largely associated with cell migration or metastasis, with modest activity in cell growth inhibition. Ectopic expression of Menin suppresses the expression of CCAAT/enhancer-binding protein beta (CEBPB) and epithelial-specific genes by histone deacetylation and further enhances the TGF-β signaling-related EMT process. We also demonstrate that CCAAT/enhancer binding protein (C/EBP) beta (C/EBPβ), encoded by CEBPB, acts downstream of Menin and TGF-β signaling for balancing growth inhibition and EMT, and C/EBPβ overexpression could restore the anticancer functions of Menin in pancreatic cancer by cooperatively activating CDKN2A/B genes and antagonizing EMT processes. Taken together, our results suggest that Menin functions as an oncogene for cancer metastasis upon C/EBPβ depletion or acts as a tumor suppressor by cooperation with C/EBPβ to activate CDKN2A transcription.

INTRODUCTION
Pancreatic cancer is one of the most lethal and metastatic cancers, with no effective prognosis markers and especially poor prognosis. The malignancies of pancreatic cancer are associated with the frequently occurring mutations in oncogenic genes and tumor suppressors, including K-RAS, p16, TP53, MADH4, and MEN1 (the protein product named as Menin).1–3 At the time of diagnosis, 30% of pancreatic cancer patients have locally advanced, metastatic, and unresectable tumors.4 Thus, metastasis is still a major cause of pancreatic cancer-associated mortality, and elucidating the mechanisms underlying metastasis will be helpful for understanding the pathogenesis of pancreatic carcinogenesis and developing effective therapeutic targets.

Although the role of the epithelial-to-mesenchymal transition (EMT) program in prompting metastasis for pancreatic cancer has been challenged,5 activation of the EMT program is still considered to be a major driver of metastasis.6,7 EMT is a process in which epithelial cells lose their cell junctions and polarity characterized by E-cadherin (CDH1) downregulation to gain a motile mesenchymal phenotype, induction of a series of mesenchymal-specific transcription factors, such as Snail1, Snail2, ZEB1, ZEB2 and Twist1.1 Accumulating evidences have demonstrated that EMT contributes to the invasion and metas-tasis of pancreatic ductal adenocarcinoma (PDAC).9–12 EMT is induced by an interplay of soluble growth factors, such as transforming growth factor β (TGF-β), downstream transcription factors, epigenetic regulators, and cytoskeleton reorganization events.8 TGF-β is a well-known potent inducer of EMT, which may promote EMT for cancer cell invasion and metastasis12 or induce cancer cell apoptosis and tumor suppression.13,14 Elucidating the mechanistic basis for the role of TGF-β in the EMT process is a meaningful and long-unsolved question. It has become clear that a common EMT response gene set is rare and that the downstream effectors of the EMT program are strongly dependent on the genetic context or on the expression of cell-specific coactivators or repressors in cancer cells.15

The tumor suppressor Menin, which is encoded by the MEN1 gene, participates in many pancreas-related cellular processes, including cell growth, pancreatic islet growth, and pancreatic endocrine tumorogenesis.16–18 Mutation of the MEN1 gene leads to an inherited tumor syndrome named multiple endocrine neoplasia type 1 (MEN1), and
its mutations are frequently identified in pancreatic neuroendocrine tumors (PanNETs). Numerous studies propose that Menin displays as either a tumor suppressor or promoter in a context-dependent manner. For instance, Menin promotes cancer progression by enhancement of c-Myc-mediated transcription or cooperation with enhancer of zeste homolog 2 (EZH2) or switching JunD from a growth suppressor into a promoter. Mechanistically, Menin associates with epigenetic factors, such as histone methyltransferases (HMTs) and histone deacetylases (HDACs), to regulate the transcription of its target genes in tumor suppression or promotion. In our previous study, we have reported that the interaction between Menin and Dnmt1 reversibly regulates pancreatic cancer cell growth downstream of the Hedgehog pathways by activation of the expression of the cyclin-dependent kinase (CDK) inhibitors in pancreatic cancer cells. However, the relationship between the Menin expression and overall survival rate or metastasis of cancer patients is not explicit as expected, indicating the complexity of Menin functions in PDAC progression, possibly in a context-dependent manner.

Here, we reveal that Menin overexpression leads to epithelial-mesenchymal transition (EMT) and the downregulation of CCAAT/enhancer binding protein (C/EBP) beta (C/EBPβ) in a histone-deacetylation manner. We also demonstrate that Menin acts as an EMT promoter or growth suppressor and interferes TGF-β signaling for EMT process, depending on the absence or presence of C/EBPβ. These data indicate that the malfunction of TGF-β/Menin/C/EBPβ regulatory axis may trigger the metastatic response in pancreatic cancer.

RESULTS
Menin Overexpression Induces EMT and Modest Inhibition of Cell Growth
Menin is lowly expressed in a large percentage of pancreatic cancer tissues and pancreatic cancer cell lines; therefore, we overexpressed Menin in two pancreatic cancer cell lines (PANC1 and BxPC3) to address the functions of Menin for pancreatic carcinogenesis. Consistently, ectopic expression of Menin in these cells resulted in a modest although significant decrease of cell proliferation (Figure 1A). Intriguingly, the cell morphology of the epithelial-like pancreatic cancer cells changed into a disassociated state upon Menin overexpression (Figure 1B). To confirm this notion, we determined the expression of epithelial and mesenchymal genes in control and Menin-overexpressed cells and found that the epithelial-specific genes (CDH1, OCLN, and CLDN3) and mesenchymal-specific genes (CDH2, FN1, and MMP1) were significantly downregulated and upregulated at mRNA and protein levels, respectively (Figures 1C and 1D). However, the expression of multiple CDK inhibitors, including CDKN1A/1B, CDKN2A, CDKN2B, and CDKN2D, was not significantly altered in both PANC1 and BxPC3 cells (Figure 1E). These data demonstrate that Menin overexpression induces EMT program, with only modest inhibition of cell proliferation.

Menin Activates the TGF Signaling-Related EMT Process
To confirm the Menin function in the EMT process, we performed RNA-sequencing (RNA-seq) analysis to check the transcriptomic alterations in Menin-overexpressed PANC1 cells. A series of epithelial-specific genes were downregulated and
mesenchymal-specific genes were upregulated upon Menin overexpression (Figures 2A and 2B). Geno Ontology (GO) analysis showed that the upregulated genes were mainly enriched for cell proliferation, motility, migration, and mesenchymal development; the downregulated genes were mainly related to keratinocyte or epithelial differentiation and negative regulation of cell migration. Moreover, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis demonstrated that pathways in cancer, such as phosphatidylinositol 3-kinase (PI3K)-AKT, TGF-β, and p53 signaling pathways were activated in upregulated genes by Menin (Figure 2A). Consistently, the expression of some known Menin-regulated genes, such as TERT, ITGB2, SERPINE1, etc., was significantly changed. Gene set enrichment analysis (GSEA) further demonstrated that TGF-β signaling gene signature was enriched for Menin-upregulated genes (Figure 2C). We also compared these differentially expressed genes upon Menin overexpression and TGF-β treatment, and 156 upregulated genes (156/762 = 20.5%) and 181 downregulated genes (181/783 = 23.1%) were overlapped with TGF-β-upregulated or -downregulated genes, respectively (Figure 2D). Heatmapping analysis further showed that many TGF-β-responsive genes were significantly changed in Menin-overexpressed PANC1 cells (Figure 2E).

These results indicate that Menin overexpression could induce TGF-β signaling-related EMT process in pancreatic cancer cells.

Menin Suppresses CEBPB Transcription and Enhances TGF-β-Induced EMT in a Histone-Deacetylation Manner

Among the Menin-regulated genes, CEBPB and its protein C/EBPβ were remarkably downregulated in RNA-seq (Figure 2A), quantitative real-time PCR, and western blot analysis (Figure 2B). C/EBPβ has been proposed to be a key regulator for TGF-β signaling and metastasis in breast cancer.63,34 We hypothesized that CEBPB might be a downstream target of Menin to mediate TGF-β signaling-induced EMT process. At first, we determined whether CEBPB was directly regulated by Menin in PANC1 cells. We analyzed the binding activity of Menin at the promoter of CEBPB from a ChIP-seq profiling of Menin and histone H3 lysine tri-methylation (H3K4me3) in breast cancer cell lines MCF7, t47d, and mcf10a. Interestingly, Menin was enriched in the transcriptional start site (TSS) region of CEBPB, which was overlapped with H3K4me3-enriched locus; CEBPB was expressed with a relatively low level in t47d cells, in which CEBPB was highly occupied by Menin (Figure 3B). It indicates that CEBPB is directly repressed by Menin. Considering the finding that Menin can act as a transcriptional repressor by interacting with HDACs to result in histone deacetylation, we tested whether the downregulation of CEBPB by Menin was depending on HDAC activity. As expected, the decrease of CEBPB mRNA and protein levels upon Menin overexpression was blocked by a HDAC inhibitor, Trichostatin A (TSA) (Figures 3C and 3D). To further confirm this observation, we performed chromatin immunoprecipitation (ChIP)-quantitative real-time PCR analysis to survey the enrichment for an active histone modification-histone H3 lysine 27 acetylation (H3K27ac) at the TSS region of CEBPB gene, and it clearly showed that the enrichment for H3K27ac was markedly reduced in Menin-overexpressed PANC1 cells (Figure 3E).
From the above results, we found that Menin overexpression activates the TGF-β signaling-related EMT process (Figures 1 and 2). Then we explored the functional and regulatory relationship between Menin, TGF-β signaling, and CEBPB. Consistent with previous reports, TGF-β treatment leads to the upregulation of multiple mesenchymal-specific genes (CDH2, FN1, and MMP1) and the downregulation of epithelial-specific genes (CDH1, OCLN, and CLDN3) as well as CEBPB. However, neither the expression of Menin mRNA nor protein levels was affected upon TGF-β stimulation (Figures 4A and 4B). Notably, Menin and H3K4me3 were occupied at the same locus of epithelial-specific but not mesenchymal-specific genes in MCF7 or t47d breast cancer cells. Moreover, the t47d cells with stronger Menin binding at CDH1 or CLDN3 promoter showed lower expression of these two genes than that MCF7 or mcf10a cells (Figures 4C and 4D), indicating that the expression of epithelial genes is inversely correlated with Menin binding activity. In our Menin overexpression and TGF-β treatment experiments, the downregulation of epithelial genes was relatively modest, although significant. When Menin-overexpressed PANC1 cells were treated with TGF-β, the downregulation of epithelial gene (CDH1 and CLDN3) expression was remarkably enhanced in quantitative real-time PCR and western blot analysis (Figures 4E and 4F), suggesting that Menin could enhance TGF-β-induced EMT program. Then, we explored the mechanism underlying epithelial gene downregulation by Menin and TGF-β. Similar to the case for CEBPB, the downregulation of CDH1 and CLDN3 by Menin and TGF-β was abolished by TSA co-stimulation (Figure 4G). Furthermore, ChIP-quantitative real-time PCR results showed that the enrichment for H3K27ac at CDH1 promoter was also restored by TSA even in Menin-overexpressed and TGF-β-stimulated cells (Figure 4H). Collectively, our data demonstrate that Menin represses CEBPB and epithelial gene transcription to enhance TGF-β-induced EMT in a histone-deacetylation manner.

C/EBP Coordinates TGF Signaling and Restores the Anti-cancer Functions of Menin in Pancreatic Cancer

Given that CEBPB can be regulated by both Menin and TGF-β signaling, we next tested whether C/EBPβ could mediate TGF-β signaling and Menin functions in the EMT process. PANC1 cells was transfected with a CEBPB short hairpin RNA (shRNA)-expressing vector, resulting in significant downregulation of CEBPB expression (Figure 5A). The control or CEBPB knockdown (KD) cells were then subjected to TGF-β treatment and quantitative real-time PCR analysis showed that the downregulation of epithelial genes (CDH1 and CLDN3) was enhanced by CEBPB knockdown (Figure 5B). In contrast, overexpression of liver-enriched activator protein 2 (LAP2), one of the three CEBPB isoforms (LAP1, LAP2, and liver-enriched inhibitory protein [LIP]), antagonized the downregulation of epithelial genes induced by TGF-β (Figure 5C).
same time, LAP2 overexpression enhanced the growth inhibition effect of TGF-β in PANC1 cells (Figure 5D). It demonstrates that CEBPB could balance the functions of TGF-β signaling for growth inhibition and EMT induction.

Next, we asked whether C/EBPb is a functional downstream target of Menin in cell proliferation and EMT. Strikingly, the growth inhibition of Menin overexpression was obviously reinforced by co-overexpression of LAP2 (Figure 5D), indicating that the anti-proliferation function of Menin depends on the activation of C/EBPb. Coincidently, C/EBPb turned Menin from a suppressor into an activator for the expression of CDH1 (E-cadherin) and CLDN3 (Figures 5E and 5F). More importantly, the expression of CDK inhibitors, such as CDKN2A (p14ARF or p16INK4A) and CDKN2B (p15INK4B), was strongly induced by co-overexpression of Menin and C/EBPb (Figures 5E and 5F). Morphological analysis also showed that co-overexpression of Menin and C/EBPb results in decreased number of cells with perfect epithelial cell features (Figure 5G). To verify the coordinated functions of Menin and C/EBPb, we performed xenograft tumor growth assays and found that consistent with their function in vitro culture, co-expression of Menin and C/EBPb displayed much more severe inhibition for tumor growth (Figure 5I). Based on these observations, we conclude that C/EBPb is an essential mediator for TGF-β signaling response and anti-cancer functions of Menin in pancreatic carcinogenesis.

Menin Cooperates with C/EBPB to Directly Activate CDKN2B Transcription
To investigate the mechanism underlying CDKN2A activation and CDH1 re-activation by cooperation of Menin and C/EBPb, we searched for the binding motif of C/EBPb in the Menin-enriched regions at the promoter of CDKN2A and CDH1 genes, and at least one conserved binding element of C/EBPb was identified for both genes. ChIP-quantitative real-time PCR analysis further showed that C/EBPb indeed binds to the motif-containing regions at the promoter of CDKN2A and CDH1 genes (Figures 6A–6D). It was worthy to notice that Menin overexpression could enhance the binding activity of C/EBPb on CDKN2A but not CDH1 gene promoter when LAP2 is co-transfected into PANC1 cells (Figures 6B and 6D), suggesting that there might be a difference between regulation of CDK inhibitors and epithelial genes by Menin and C/EBPb cooperation. In accordance with this hypothesis, CEBPB knockdown disrupted the binding
Figure 5. C/EBPβ Coordinates TGF-β Signaling and Restores the Anti-cancer Functions of Menin in PANC1 Cells

(A) Lentiviral-mediated knockdown of CEBPB in PANC1 cells. The knockdown (KD) efficiency was determined by quantitative real-time PCR and western blot analysis. The shRNA targeting luciferase served as a control (Ctrl knockdown). (B) Control or CEBPB knockdown PANC1 cells co-cultured with or without TGF-β (5 ng/mL) for 24 h were subjected to quantitative real-time PCR analysis of CDH1 and CLDN3 expression. (C) Control (Ctrl) or LAP2-overexpressed (OE) PANC1 cells treated with or without TGF-β (5 ng/mL) for 24 h were subjected to quantitative real-time PCR analysis of CDH1 and CLDN3 expression. (D) CCK8 assay was performed to analyze cell proliferation of control (Ctrl) or LAP2-overexpressed (OE) PANC1 cells treated with or without TGF-β (5 ng/mL). (E) CCK8 assay was performed to analyze cell proliferation of control (Ctrl), LAP2-overexpressed (LAP2 OE), Menin-overexpressed (Menin OE), or Menin and LAP2 co-overexpressed (Menin OE+LAP2 OE) PANC1 cells at 0, 24, 48, and 72 h. (F) The cells in (E) were collected for western blot analysis of E-cadherin, p15, Menin, C/EBPβ, and GAPDH expression. (G) The cells in (E) were collected for quantitative real-time PCR analysis of CDH1, CLDN3, CDKN2A, and CDKN2B expression. (H) The representative morphology images for cells in (E). (I) The mean volume of tumors burdened in mice receiving PANC1 cells expressing Menin and/or C/EBPβ, respectively, or simultaneously. In brief, the manipulated PANC1 cells were subcutaneously injected with 1 × 10^6 cells in nude mice. The tumor size was measured by using a digital caliper every 7 days, and the tumor volume was determined with the following formula: tumor volume [mm³] = (length [mm]) × (width [mm])^2 × 0.52. All the measurements were compared using unpaired Student’s t test. **p < 0.01, ***p < 0.001.
Figure 6. Menin Cooperates with C/EBPβ to Directly Activate CDKN2B Transcription

(A) The genome browser snapshot of H3K4me3 enrichment in MCF7 cells and Menin enrichment in t47d and mcf10a cells at the loci of CDKN2B gene promoters from previously published data.35 A potential C/EBPβ binding site around the TSS of CDKN2B gene was shown. (B) ChIP-qPCR analysis of C/EBPβ binding activity on the promoter of CDKN2B in control (V), LAP2-overexpressed (LAP2 OE), Menin-overexpressed (Menin OE), or Menin and LAP2 co-overexpressed (M+L) PANC1 cells. (C) The genome browser snapshot of H3K4me3 enrichment in MCF7 cells and Menin enrichment at the loci of the CDH1 gene promoter. A potential C/EBPβ binding site around the TSS of CDH1 gene was shown. (D) ChIP-qPCR analysis of C/EBPβ binding activity on the promoter of CDH1 in control (V), LAP2-overexpressed (LAP2 OE), Menin-overexpressed (Menin OE), or Menin and LAP2 co-overexpressed (M+L) PANC1 cells. (E) The genome browser snapshot of H3K4me3 enrichment in MCF7 cells and Menin enrichment at the loci of the CDKN2B gene promoter. A potential C/EBPβ binding site around the TSS of CDKN2B gene was shown. (F) ChIP-qPCR analysis of C/EBPβ binding activity on the promoter of CDKN2B in control (V), LAP2-overexpressed (LAP2 OE), Menin-overexpressed (Menin OE), or Menin and LAP2 co-overexpressed (M+L) PANC1 cells.

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activity of Menin on CDKN2A but not CDH1 gene promoter (Figure 6E). Then, the luciferase reporter assays showed that the promoter activity of the CDKN2A gene was strongly induced by LAP2 or co co-overexpression of Menin and LAP2 (Figure 6F); however, the promoter activity of the CDH1 gene was repressed by Menin overexpression but enhanced by LAP2 overexpression, and LAP2 could bypass the inhibitory effect of Menin over CDH1 promoter activity (Figure 6G). We also analyzed the relationship between overall survival rates and CEBPB or MEN1 expression and found that the high expression of CEBPB predicted a low survival rate (Figure 6H), which was consistent with previously documented role of C/EBPβ in skin cancer.28 Although we have revealed that MEN1 is locally expressed in pancreatic cancer tissues,25 there was no significant correlation between high or low MEN1 expression and overall survival rate (although with a favorable tendency for MEN1-high patients) (Figure 6H). It is possible that Menin develops to be a tumor suppressor or oncogenic gene, depending on C/EBPβ presence or absence. Together, Menin integrates with C/EBPβ to cooperatively activate CDK inhibitors and epithelial-specific genes.

DISCUSSION

Based on our data, we propose a regulatory model that integrates Menin and C/EBPβ downstream of TGF-β signaling to balance the cell growth inhibition and EMT induction in pancreatic cancer cells (Figure 6I). In brief, Menin overexpression promotes the EMT program by inhibiting C/EBPβ expression in a histone deacetylation manner. Ectopic expression of C/EBPβ expression could restore the anti-cancer functions of Menin to antagonize EMT by epigenetically silencing epithelial genes and to suppress cell proliferation by activation of CDK inhibitors. Thus, Menin acts as a tumor suppressor or oncogenic genes in a C/EBPβ-dependent manner.

MEN1 is essential for development of endocrine pancreatic cells, the disruption of which is correlated with multiple tumor-related diseases.19,20,24,37 Therefore, identification of the content of the “context” for essential cancer regulators is of great importance. Here, we find that Menin can inhibit the expression of C/EBPβ and epithelial-specific genes by histone deacetylation, possibly by interaction with HDACs,35 to induce EMT, which is highly similar to the result of TGF-β treatment (Figures 1 and 2). Downregulation of C/EBPβ has been reported to be observed in triple-negative breast cancers,44 while high expression of C/EBPβ was correlated with bad prognosis (Figure 6), suggesting that C/EBPβ might be an oncogene in cases. Considering the negative regulatory relationship between C/EBPβ and Menin (Figure 3), it is reasonable that the loss of Menin in pancreatic cancer cells releases its inhibitory effect on C/EBPβ expression. Notably, it seems that C/EBPβ is the major downstream target of Menin for EMT and growth inhibition. Overexpression of C/EBPβ can restore the anti-cancer functions of Menin to antagonize EMT and cell proliferation and inactivation of epithelial genes can be achieved by inactivation of transcriptional activator C/EBPβ. Therefore, the imbalance between C/EBPβ and Menin will lead to the disrupted signaling transduction of TGF-β signaling during EMT process, and interfering with the functional relationship between C/EBPβ and Menin might be a possible therapy target for pancreatic carcinogenesis.

Interestingly, Menin overexpression transactivates and inactivates a series of downstream target genes of TGF-β signaling (Figure 2). It has been reported that Menin interacts with Smad3 to induce transcriptional activity at specific transcriptional regulatory sites.38 It is possible that the ectopic expression of Menin can directly interact with Smad3 and transactivate the downstream signaling pathways of TGF-β signaling, although we did not observe the upregulation upon TGF-β treatment (Figure 4). Considering the fact that Menin is lowly expressed in pancreatic cancer cells,29 the transcriptional response of Menin might be different with that in GH4C1 cells.38 We further reveal that Menin represses the transcription of C/EBPβ and epithelial-specific genes by directly binding to the promoter of these genes, leading to the downregulation of histone acetylation (Figures 3 and 4). We postulate that the Smad3/Menin complex can interact with the HDAC complex to regulate the histone acetylation levels for their target genes guided by Smad3 binding specificity, just like Smad3/HDAC complex regulation on Runx2 function.39 On the other hand, Menin can also act as a transcriptional activator by interacting with HMTs to activate the transcription of CDK inhibitors, depending on the guidance of C/EBPβ by its pre-binding activity on the CDK2B promoter (Figure 6). Thus, combination of C/EBPβ and Menin produces a super tumor suppressor, which is able to maintain epithelial-like features, to strikingly suppress cancer cell growth and to bypass the EMT induction by TGF-β signaling. It will be of interest in the future to elucidate how C/EBPβ and Menin cooperatively suppress the expression mesenchymal genes, although Menin cannot bind to mesenchymal genes (Figure 4).

TGF-β signaling is a well-known EMT inducer for multiple cancers,13 whereas it can be pro-tumorigenic or tumor suppressive also in a context-dependent manner.14 EMT is a developmental program to promote the acquisition of malignant traits for advanced tumors, and a high level of TGF-β signaling activity in PDA is associated with poor prognosis,45 although it has been proposed to be associated with tumor suppression in PDAC.14 Thus, the dual nature of TGF-β and related transcription factors, such as C/EBPβ and Menin, is regulated by multiple co-activators or co-repressors at multiple layers. Our results provide a cross-linked master transcriptional regulator for the TGF-β signaling-related EMT program. Re-activation of
epithelial-promoter factors, such as C/EBPβ, Menin, and other factors, to promoter epithelial differentiation might represent a more differentiated, benign tumor phenotype to restrict carcinogenesis. And manipulation of the downstream effectors to restore the TGF-β tumor-suppressive functions will be especially attractive.

MATERIALS AND METHODS

Cell Culture and Treatment
Pancreatic cancer cell lines (PANC1 and BxPc-3) and breast cancer cell lines (MCF7, T47D, and MCF10a) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (CAS, Shanghai, China). These cells were cultured as per the manufacturer’s instructions. The following products were purchased for the present study: TSA (HDAC inhibitor, MCE, Monmouth Junction, NJ, USA) and TGF-β (Sigma-Aldrich, MO, USA).

Quantitative Real-Time PCR
Total RNA was extracted from cultured cells in 6-well plates using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers instructions. Total RNA was quantified by using Nanodrop, and a total of 1 μg was used for cDNA synthesis using oligo (dT) primers; quantitative real-time PCR was performed using a SYBR green premix Ex Taq (Takara Bio, Shiga Prefecture, Japan) in an Applied Biosystems 7900 machine. The relative expression level of target gene mRNA was normalized to that of GAPDH. The sequences of all used primers were shown below:

- MEN1-forward, 5'-GGGCTTGCTGAGCAGCTTTTCT-3'; MEN1-reverse, 5'-GGGCTTGCTGAGCAGCTTTTCT-3';
- CDH1-forward, 5'-GGGCTTGCTGAGCAGCTTTTCT-3'; CDH1-reverse, 5'-GGGCTTGCTGAGCAGCTTTTCT-3';
- CDH2-forward, 5'-TCAGGGCTCAGTCTAGGCTT-3'; CDH2-reverse, 5'-ATGACATCCTCTGCTATAAGCTG-3';
- CEBPB-forward, 5'-CTCCAGGCTGACATTGAG-3'; CEBPB-reverse, 5'-GGAGAGGAAATGTGGTGC-3';
- OCLN-forward, 5'-ACAAGGGGATTTTATCCAGAGT-3'; OCLN-reverse, 5'-GTCATCAGGAGCAAGTATT-3';
- CLDN3-forward, 5'-AACACCTTATCCGGGACTTT-3'; CLDN3-reverse, 5'-GCGGAGTGACAGCC-3';
- FN1-forward, 5'-GGGCTTGCTGAGCAGCTTTTCT-3'; FN1-reverse, 5'-AACACCTTGCTGAGCAGCTTTTCT-3';
- MMP1-forward, 5'-AAAGGAAATCCAGGACTTG-3'; MMP1-reverse, 5'-GGGCTTGCTGAGCAGCTTTTCT-3';
- CDKN1A-forward, 5'-TGGCTGCTGAGGAGAAATCCAGGACTTG-3'; CDKN1A-reverse, 5'-AAAGGAAATCCAGGACTTG-3';
- CDKNIB-forward, 5'-AACAGGCTGAGGAGAAATCCAGGACTTG-3'; CDKNIB-reverse, 5'-AACAGGCTGAGGAGAAATCCAGGACTTG-3';
- CDKN2A-forward, 5'-GATCCAGGACTTG-3'; CDKN2A-reverse, 5'-GATCCAGGACTTG-3';
- CDKN2B-forward, 5'-CACCGTGGCGGTAATACCTTACG-3'; CDKN2B-reverse, 5'-TAATGAGCCTGAGCCACCTTACG-3';
- CDKN2D-forward, 5'-AGTCCAGTGTCAATCGC-3'; CDKN2D-reverse, 5'-ATCAGGCAGTGGACATCG-3';
- GAPDH-forward, 5'-GGAGCGGAGATCCCTCAAAAT-3'; GAPDH-reverse, 5'-GGCCTGGTTGCTCATACTTCTCATGG-3';

Western Blot Analysis
Western blot was performed as we previously described. The following antibodies were used: anti-Menin (Santa Cruz Biotechnology, CA, USA), anti-E-cadherin (BD Biosciences, Oxford, UK), anti-claudin-3 (Invitrogen, CA, USA), anti-N-cadherin (BD Biosciences, Oxford, UK), anti-MMP1 (ab8480, Abcam, MA, USA), anti-C/EBPβ (Santa Cruz Biotechnology, Dallas, TX, USA), anti-p15 (ab3034, Abcam, MA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, MA, USA). GAPDH was used as a loading control.

ChIP
A ChIP assay kit was used (Upstate Biotechnology, NY, USA). In brief, control or Menin-overexpressing PANC1 cells were cross-linked by 1% formaldehyde in PBS for 10 min at room temperature. Then the reaction was neutralized by glycine. The cell lysates were sonicated for fragmentation in a Bioruptor (Diagenode, NJ, USA), yielding DNA fragments of 200–1,000 bp. The chromatin was incubated with antibodies (2 μg for each reaction) against H3K27ac, Menin, and C/EBPβ, respectively. The following antibodies were used: anti-Menin (Santa Cruz, CA, USA), anti-H3K27ac (39133, Active Motif, CA, USA), anti-C/EBPβ (Santa Cruz Biotechnology, Dallas, TX, USA). Immunoglobulin G (IgG) (sc-2027; Santa Cruz Biotechnology, CA, USA) was used as a control.

Cell Proliferation Assay
Cell proliferation was quantified using the cell counting kit-8 (CCK8; Dojindo Molecular Technologies, Shanghai, China) according to the manufacturer’s instructions. In brief, 10 μL of CCK8 solution was supplemented to each 96-well plate for 60 min, and the number of cells was counted every 24 h by measuring the absorbance at 450 nm (OD450) using Microplate Spectrophotometer (BioTek, VT, USA).

Lentivirus-Mediated Overexpression and Knockdown
A full-length Menin and LAP2 cDNA coding sequence was cloned from human cDNA library. Menin or LAP2 was then constructed into an adenovirus vector (FUW-GFP), which was subjected to lentivirus package in HEK293T cells. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer’s instructions. The CEBPβ shRNA target sequence was as follows: 5'-ATCCATGGAAGTGGCCAC-3'. Two complementary oligonucleotides of small hairpin RNAs targeting CEBPβ were chemically synthesized and subcloned into the psilencer lentiviral vector.
RNA-Seq Analysis and Bioinformatic Analysis

The control or Menin-overexpressing PANC1 cells were collected for RNA-seq analysis, and data processing and analysis were performed by Novel Biotechnology (Shanghai, China). The poly(A) containing mRNA from the total RNA was purified by using poly(T) magnetic beads. Then, the purified mRNA was subjected to first- and second-strand synthesis. Amplified cDNA was then used to construct Illumina sequencing libraries using Illumina’s Nextera DNA sample preparation kit (New England Biolabs, MA, USA). The constructed libraries were sequenced on an Illumina HiSeq2000 machine (Berry Genomics, Beijing, China). Raw sequencing reads were mapped to hg19 using the TopHat version 2.0.13 program and assigned FPKM values (fragments per kilobase of transcript per million) for each gene that were subjected to identification of differentially expressed genes between control and Menin-overexpressed samples. The heatmaps for differentially expressed genes were clustered by hierarchical clustering and visualized by using Java TreeView software. ChiP-seq data was downloaded from the NCBI database, and the enrichment for H3K4me3 and Menin in breast cancer cells were captured in IGV software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0. Significant differences between the groups was evaluated using Student’s t test. All experiments were repeated for at least three times. Values are reported as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significance.

AUTHOR CONTRIBUTIONS

P.C., Y.C., T.-I.H., G.J., and Y.-j.Z. conceived and designed the study. P.C., Y.C., and T.-I.H. performed the experiments; and C.W., H.H., and C.-m.N. analyzed the data. S.-w.G. provided clinical samples and clinical information. P.C., Y.C., and T.-I.H. wrote the manuscript. P.C., G.J., and Y.-j.Z. supervised the research.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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