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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.[8] Accumulating evidences have demonstrated that EMT contributes to the invasion and metastasis of pancreatic ductal adenocarcinoma (PDAC).[9–11] 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 metastasis[12] 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 tumorigenesis.[16–18] Mutation of the *MEN1* gene leads to an inherited tumor syndrome named multiple endocrine neoplasia type 1 (MEN1), and

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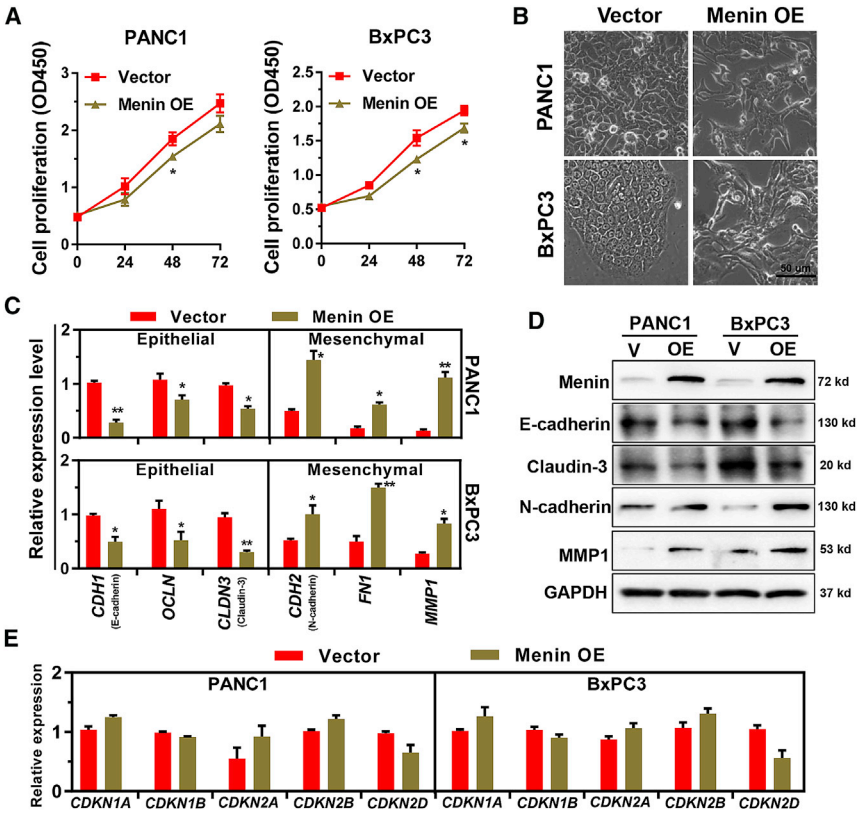

Figure 1. Menin Overexpression Induces Epithelial-Mesenchymal Transition and Modest Inhibition of Cell Growth

(A) Lentiviral mediated overexpression of Menin (Menin OE) in PANC1 and BxPC3 cells. The empty vector (FUW-GFP) served as a control. The cell proliferation rate was determined by cell counting kit-8 (CCK8) assays at 0, 24, 48, and 72 h, respectively. (B) Morphology of control (vector) or Menin-overexpressed (Menin OE) PANC1 and BxPC3 cells. (C) Quantitative real-time PCR analysis of the epithelial (*CDH1*, *OCLN*, and *CLDN3*) and mesenchymal gene expression in control and Menin OE PANC1 and BxPC3 cells. (D) Western blot analysis of Menin, E-cadherin, Claudin-3, N-cadherin, MMP1, and GAPDH in control and Menin OE PANC1 and BxPC3 cells. (E) Quantitative real-time PCR analysis of the relative expression of CDK inhibitors (*CDKN1A*, *CDKN1B*, *CDKN2A*, *CDKN2B*, and *CDKN2D*) in control and Menin OE PANC1 and BxPC3 cells.

its mutations are frequently identified in pancreatic neuroendocrine tumors (PanNETs).[19,20] Numerous studies propose that Menin displays as either a tumor suppressor or promotor 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.[21–23] 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.[19,24] 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.[25] However, the relationship between the Menin expression and overall survival rate or metastasis of cancer patients is not explicit as expected,[26,27] 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;[25,28] 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), which is highly similar to the EMT process.[29] 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 (p14ARF or p16INK4A), CDKN2B (p15INK4B), 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

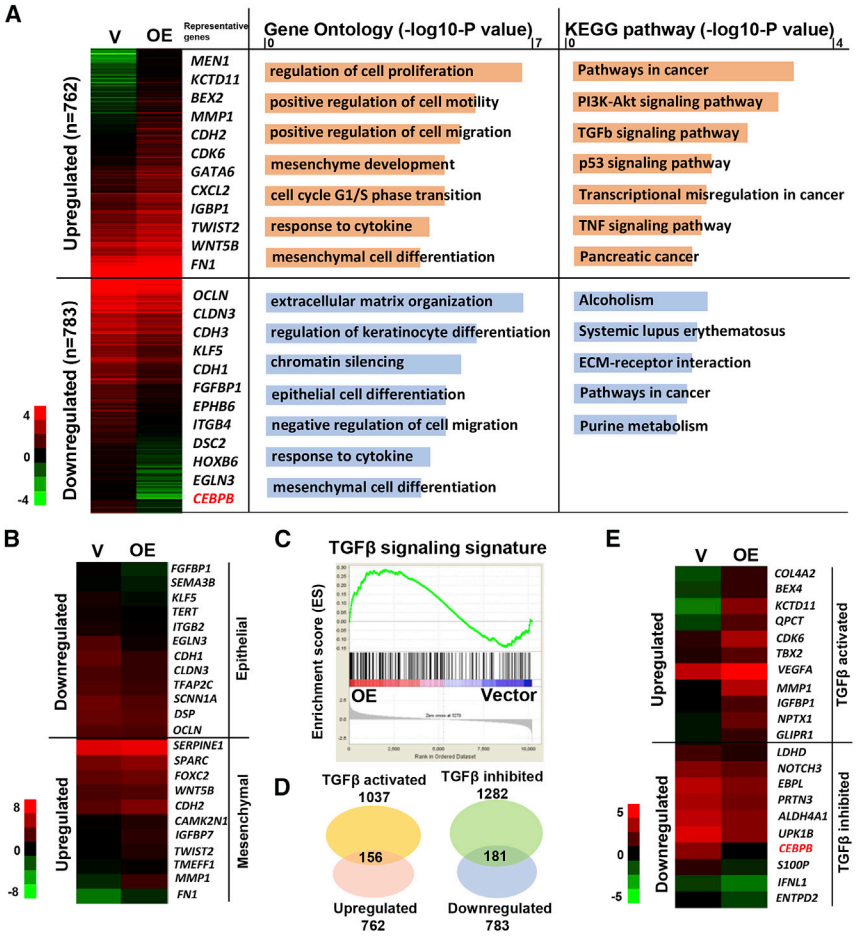

Figure 2. Menin Induces TGF-β Signaling-Related EMT Process

(A) Control (V) and Menin-overexpressed (OE) PANC1 cells were subjected to RNA-seq analysis. 762 upregulated and 783 downregulated genes were subjected to heatmapping, Gene Ontology (GO), and KEGG pathway analyses. (B) Heatmapping analysis of representative epithelial and mesenchymal genes that were deregulated upon Menin overexpression. (C) Gene set enrichment analysis (GSEA) of the deregulated genes upon Menin overexpression (OE), indicating that TGF-β signaling signature was enriched for the gene set in Menin-overexpressed PANC1 cells. (D) Venn diagram analysis of the deregulated genes upon Menin overexpression with previously reported TGF-β responsive genes.[32] (E) Heatmapping analysis of the representative deregulated genes upon Menin overexpression, which were altered in TGF-β-stimulated cells.[32]

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.,[19,30] was significantly changed. Gene set enrichment analysis (GSEA)[31] further demonstrate 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,[32] 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 3A). C/EBPβ has been proposed to be a key regulator for TGF-β signaling and metastasis in breast cancer.[33,34] 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.[35] 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,[19] 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 blockaded 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).

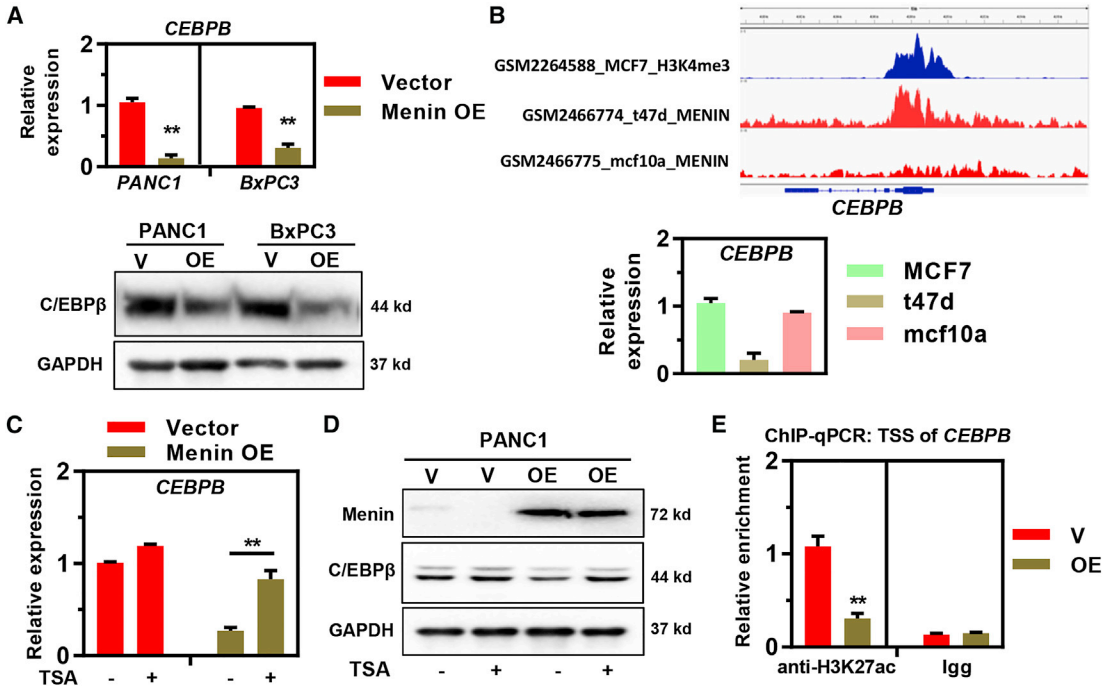

Figure 3. Menin Suppresses *CEBPB* Transcription by Histone Deacetylation

(A) Quantitative real-time PCR and western blot analysis of *CEBPB* expression in control (vector) and Menin-overexpressed (OE) PANC1 and BxPC3 cells. (B) The genome browser snapshot of H3K4me3 enrichment in MCF7 cells and Menin enrichment in t47d and mcf10a cells at the loci of *CEBPB* promoter from previously published data.[35] The relative expression of *CEBPB* in MCF7, t47d, and mcf10a cells were determined (below). (C and D) Quantitative real-time PCR and western blot analysis of *CEBPB* expression in control (vector) and Menin-overexpressed (OE) PANC1 cells in the absence or presence of TSA (200 nM) for 24 h. V, vector; OE, Menin overexpression. (E) ChIP-quantitative real-time PCR analysis of H3K27ac enrichment in the TSS region of *CEBPB* gene with an anti-H3K27ac antibody. Mouse IgG served as a negative control.

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,[13] 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.[35] 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 reversely 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,[33] 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 contrary, 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). At the

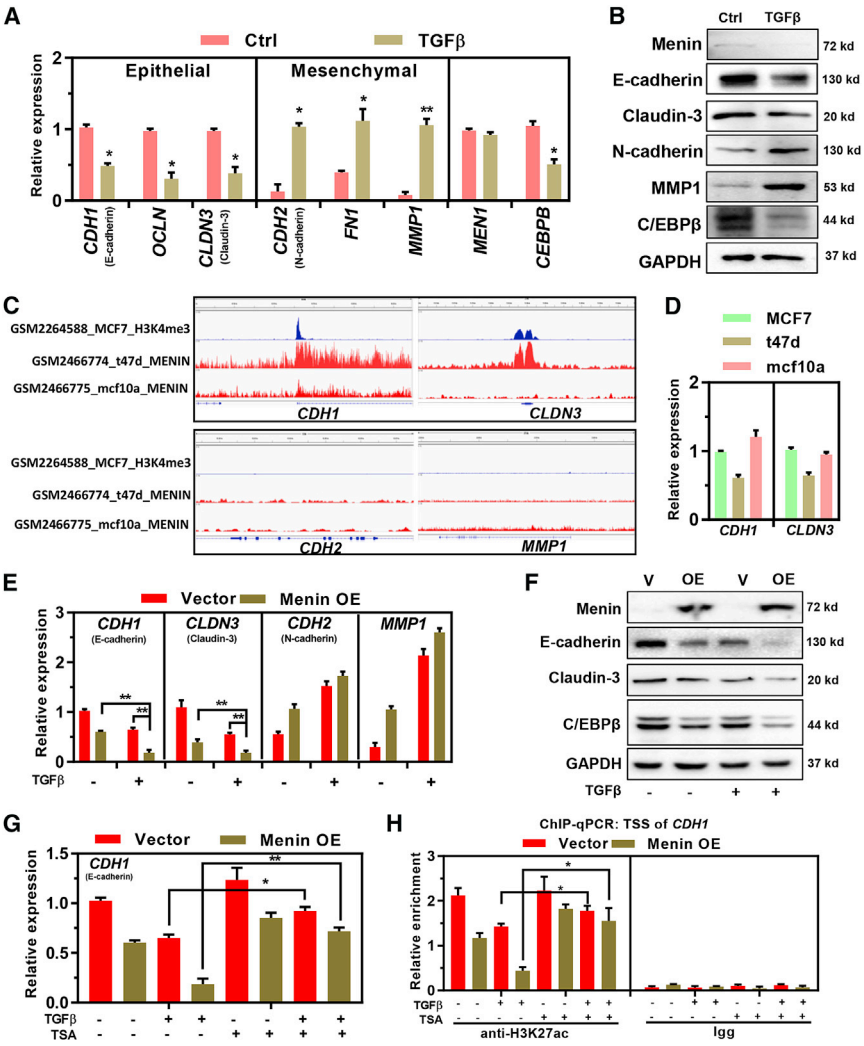

Figure 4. Menin Enhances TGF-β-Induced EMT in a Histone-Deacetylation Manner

(A) Quantitative real-time PCR analysis of epithelial, mesenchymal genes, *MEN1*, and *CEBPB* in control (Ctrl) or TGF-β-treated PANC1 cells. TGF-β (5 ng/mL) was supplemented to the culture medium for 24 h, and cells were collected for analysis. (B) Western blot analysis of Menin, E-cadherin, Claudin-3, N-cadherin, MMP1, CEBPβ, and GAPDH in control and TGF-β-treated PANC1 cells. (C) The genome browser snapshot of H3K4me3 enrichment in MCF7 cells and Menin enrichment in t47d and mcf10a cells at the loci of *CDH1*, *CLDN3*, *CDH2*, and *MMP1* gene promoters from previously published data.[35] (D) The relative expression of *CDH1* and *CLDN3* in MCF7, t47d, and mcf10a cells were determined by quantitative real-time PCR analysis. (E) Control (vector) and Menin-overexpressed (OE) PANC1 cells were treated with TGF-β (5 ng/mL) for 24 h, and then the cells were subjected to quantitative real-time PCR analysis of *CDH1*, *CLDN3*, *CDH2*, and *MMP1* expression. (F) Control (V) and Menin-overexpressed (OE) PANC1 cells in the presence or absence of TGF-β (5 ng/mL) for 24 h were collected for western blot analysis. (G) Control (vector) and Menin-overexpressed (OE) PANC1 cells were treated with TGF-β (5 ng/mL), in the absence or presence of TSA (200 nM) for 24 h. Then, the cells were collected for quantitative real-time PCR analysis of *CDH1* expression. (H) The cells in (G) were collected for ChIP-quantitative real-time PCR analysis of H3K27ac enrichment at the TSS region of *CDH1*. Mouse IgG served as a control.

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/EBPβ 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/EBPβ. Coincidently, C/EBPβ 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/EBPβ (Figures 5E and 5F). Morphological analysis also showed that co-overexpression of Menin and C/EBPβ results in decreased number of cells with perfect epithelial cell features (Figure 5G). To verify the coordinated functions of Menin and C/EBPβ, we performed xenograft tumor growth assays and found that consistent with their function *in vitro* culture, co-expression of Menin and C/EBPβ displayed much more severe inhibition for tumor growth (Figure 5I). Based on these observations, we conclude that C/EBPβ 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/EBPβ, we searched for the binding motif of C/EBPβ in the Menin-enriched regions at the promoter of *CDKN2A* and *CDH1* genes, and at least one conserved binding element of C/EBPβ was identified for both genes. ChIP-quantitative real-time PCR analysis further showed that C/EBPβ 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/EBPβ on *CDKN2A* but not *CDH1* gene promoter when LAP2 is co-transfected into PANC1 cells (Figuress 6B and 6D), suggesting that there might be a difference between regulation of CDK inhibitors and epithelial genes by Menin and C/EBPβ cooperation. In accordance with this hypothesis, *CEBPB* knockdown disrupted the binding

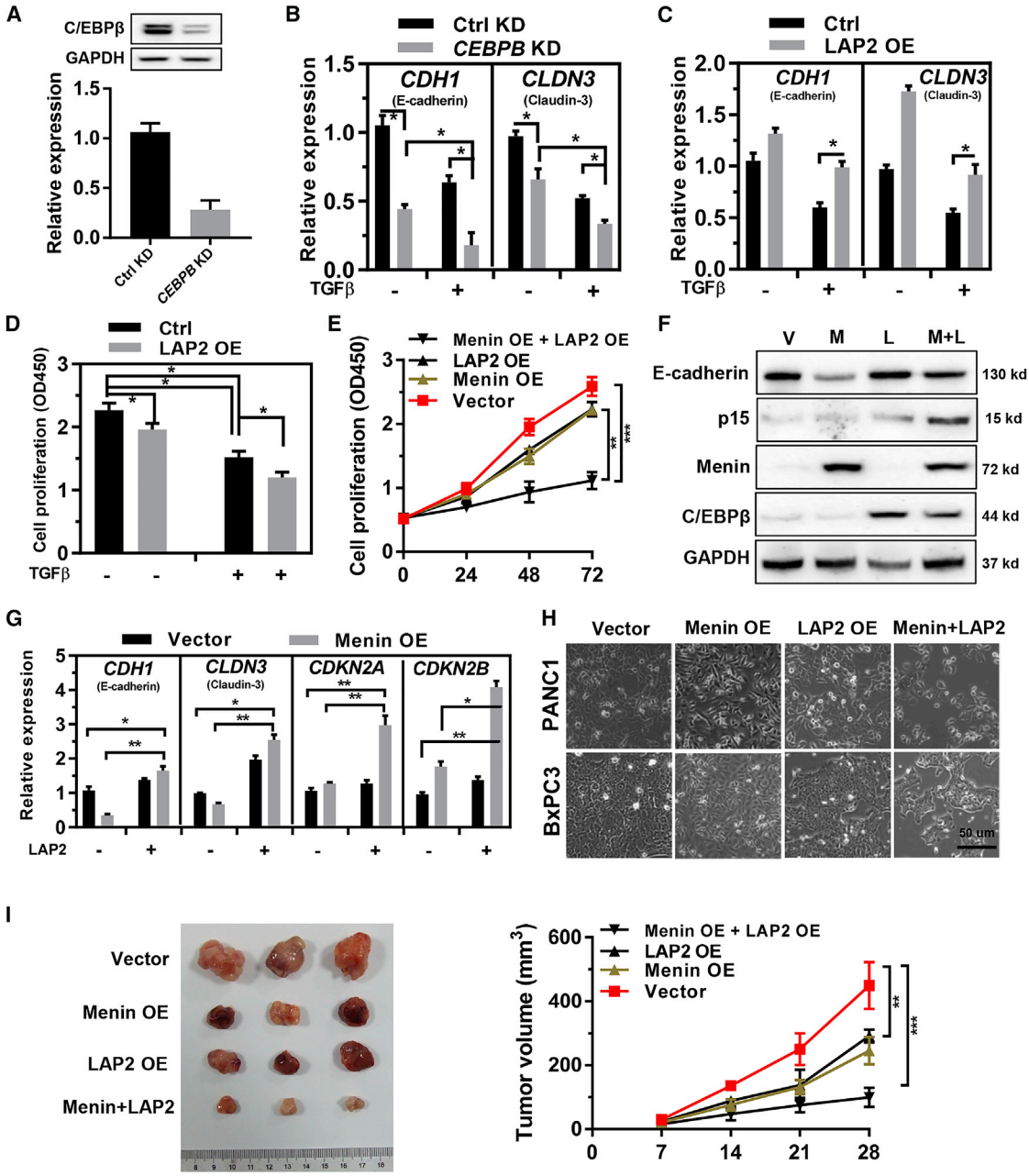

Figure 5. C/EBPβ Coordinates TGF-β Signaling and Restores the Anti-cancer Functions of Menin in PANC1 Cells

(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 analyzed 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 [mm^3] = (length [mm])*(width [mm])^2*0.52. All the measurements were compared using unpaired Student's t test. **p < 0.01, ***p < 0.001.

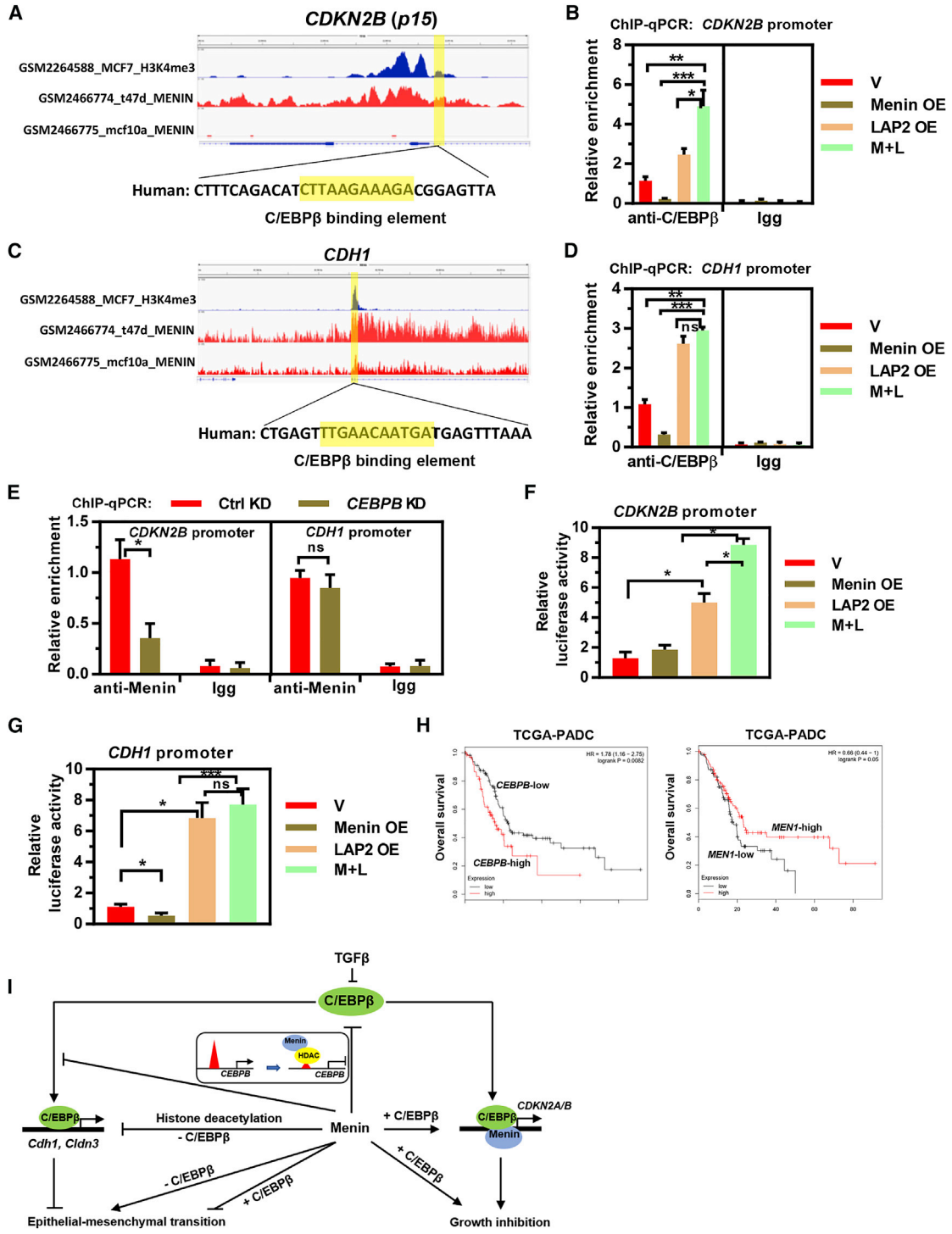

Figure 6. Menin Cooperates with C/EBPβ to Directly Activate *CDKN2B* Transcription

(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-quantitative real-time PCR 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 in t47d and mcf10a cells at the loci of the *CDH1* gene promoter. A potential C/EBPβ binding site around the TSS of *CDH1* gene was shown. (D) ChIP-quantitative real-time PCR analysis of C/EBPβ binding activity on the promoter of *CDH1* in

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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.[36] Although we have revealed that *MEN1* is lowly 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] Whereas the activity of Menin in transcriptional activation and suppression makes its function complicit for pancreatic carcinogenesis.[19,24,37] 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,[32] 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,[34] 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,[25] 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,[40] 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

cells in (B). (E) ChIP-quantitative real-time PCR analysis of Menin enrichment on the promoters of *CDKN2B* and *CDH1* in control or *CEBPB* knockdown (*CEBPB* KD) PANC1 cells. (F) Luciferase assay was performed to determine the *CDKN2A* promoter activity in cells in (B). (G) Luciferase assay was performed to determine the *CDH1* promoter activity in cells in (B). (H) The overall survival rates of pancreatic cancer patients for *CEBPB* and *MEN1* were determined by an online tool (http://xvm145.jefferson.edu/proggene/). (I) The hypothetic model for Menin and C/EBPβ functions downstream of TGF-β signaling to regulate growth inhibition and EMT process.

epithelial-promoter factors, such as C/EBPβ, Menin, and other factors, to promoter epithelial differentiation might represent a faithful approach to preserve 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 manufacturer's 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 as below:

MEN1-forward, 5′-GGGCTTCGTGGAGCATTTTCT-3′; *MEN1*-reverse, 5′-GCGGCGATGATAGACAGGTC-3′.

CDH1-forward, 5′-CGAGAGCTACACGTTCACGG-3′; *CDH1*-reverse, 5′-GGGTGTCGAGGGAAAAATAGG-3′.

CDH2-forward, 5′-TCAGGCGTCTGTAGAGGCTT-3′; *CDH2*-reverse, 5′-ATGCACATCCTTCGATAAGACTG-3′.

CEBPB-forward, 5′-CTTCAGCCCGTACCTGGAG-3′; *CEBPB*-reverse, 5′-GGAGAGGAAGTCGTGGTGC-3′.

OCLN-forward, 5′-ACAAGCGGTTTTATCCAGAGTC-3′; *OCLN*-reverse, 5′-GTCATCCACAGGCGAAGTTAAT-3′.

CLDN3-forward, 5′-AACACCATTATCCGGGACTTCT-3′; *CLDN3*-reverse, 5′-GCGGAGTAGACGACCTTGG-3′.

FN1-forward, 5′-CGGTGGCTGTCAGTCAAAG-3′; *FN1*-reverse, 5′-AAACCTCGGCTTCCTCCATAA-3′.

MMP1-forward, 5′-AAAATTACACGCCAGATTTGCC-3′; *MMP1*-reverse, 5′-GGTGTGACATTACTCCAGAGTTG-3′.

CDKN1A-forward, 5′-TGTCCGTCAGAACCCATGC-3′; *CDKN1A*-reverse, 5′-AAAGTCGAAGTTCCATCGCTC-3′.

CDKN1B-forward, 5′-AACGTGCGAGTGTCTAACGG-3′; *CDKN1B*-reverse, 5′-CCCTCTAGGGGTTTGTGATTCT-3′.

CDKN2A-forward, 5′-GATCCAGGTGGGTAGAAGGTC-3′; *CDKN2A*-reverse, 5′-CCCCTGCAAACTTCGTCCT-3′.

CDKN2B-forward, 5′-CACCGTTGGCCGTAAACTTAAC-3′; *CDKN2B*-reverse, 5′-TAATGAAGCTGAGCCCAGGTCT-3′.

CDKN2D-forward, 5′-AGTCCAGTCCATGACGCAG-3′; *CDKN2D*-reverse, 5′-ATCAGGCACGTTGACATCAGC-3′.

GAPDH-forward, 5′-GGAGCGAGATCCCTCCAAAAT-3′; *GAPDH*-reverse, 5′-GGCTGTTGTCATACTTCTCATGG-3′.

Western Blot Analysis

Western blot was performed as we previously described.[25] 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 (ab53034, Abcam, MA, USA), and anti-gyceraldehyde-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) were 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-plate well for 60 min, and the number of cells were 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 *CEBPB* shRNA target sequence was as follows: 5′-ATCCATGGAAGTGGCCAAC-3′. Two complementary oligonucleotides of small hairpin RNAs targeting *CEBPB* 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 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,[35] 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.-l.H., G.J., and Y.-j.Z. conceived and designed the study. P.C., Y.C., and T.-l.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.-l.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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