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Effects of ME3 on the proliferation, invasion and metastasis of pancreatic cancer cells through epithelial-mesenchymal transition

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Malic enzyme 3 (ME3) aberrant expression contributes to the development of human malignancies. ME3 expression was higher in pancreatic cancer tissues than that in non-tumor tissues, and patients with higher ME3 levels had significantly shorter survival than those with lower levels analyzed by of Badea and TCGA databases. Further, the abilities of proliferation, migration and invasion in pancreatic cancer cells were inhibited by ME3 knockdown and were promoted by ME3 overexpression. Meanwhile, ME3 can promote EMT in pancreatic cancer cells possibly by regulation of TGF-β/Smad2/3 signaling pathway. In conclusion, ME3 is extensively involved in carcinogenesis of pancreatic cancer and may become a new candidate target for diagnosis, treatment and prognosis of pancreatic cancer.

Key words: malic enzyme 3, epithelial-mesenchymal transition, TGF-β/Smad2/3, pancreatic cancer

Pancreatic cancer is one of the leading causes of malignancy-related death worldwide [1, 2]. The 5-year survival rate is less than 5% due to the lack of effective tools for early detection and treatment. Importantly, approximately 50% of patients with pancreatic cancer have metastasis at the time of diagnosis [3, 4]. A growing number of studies have shown that glycolysis, mitochondrial oxidation and energy metabolism of glucose are important for tumor cell growth and chemoresistance [5–9]. Although altered cellular metabolism is a hallmark of cancer, research is still lacking in terms of the specific gene(s) involved in the metabolic homeostasis and invasiveness of pancreatic cells.

Metastasis is an early event in pancreatic cancer progression and occurs after cells have undergone epithelial-mesenchymal transition (EMT). The activation of EMT allows for the dissemination of tumor cells, hence EMT has been considered a prerequisite for metastasis [10]. Transforming growth factor-β (TGF-β), a secreted anti-inflammatory cytokine, regulates apoptosis, cell growth and differentiation. TGF-β is associated with advanced tumor stages, in which it plays an antitumorigenic role by restricting cell growth and enhancing apoptosis [11, 12]. Defects in TGF-β receptors and mutations in small mothers against decapentaplegic (SMAD) have been observed in numerous pancreatic cancer cell lines [13]. These defects result in the emergence of an opposite role of TGF-β signaling, in which it promotes tumorigenesis by enhancing cancer cell growth, survival, invasion and metastasis, leading to reduced survival of patients with pancreatic cancer [14]. TGF-β is a major regulator of EMT via canonical SMAD-dependent pathways [15], and TGF-β also modulates the expression of other EMT regulators, such as Slug and Sail, through SMAD and mitogen-activated protein kinase (MAPK) activation in both normal and malignant mammary epithelial cells (MECs) [16, 17]. In addition, the TGF-β-TGF-βR-SMAD2 signaling axis is required to maintain epigenetic silencing of crucial EMT genes in breast cancer progression [17].

Recent studies have shown that malic enzymes might serve as targets for the suppression of tumor growth and invasiveness in several tumor cells, including lung cancer, glioma, hepatocellular carcinoma, cutaneous melanoma and nasopharyngeal carcinoma [18–25]. Targeting human malic enzymes could be an effective approach to inhibit tumor growth [22]. Malic enzymes catalyze the divalent metal ion-dependent (Mn^{2+} or Mg^{2+}) oxidative decarboxylation of malate to yield pyruvate and CO_2, accompanied by the production of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) [26]. There are three different isoforms of malic enzymes (MEs) in mammalian tissues: cytosolic $NADP^+$-dependent

(ME1), mitochondrial NAD(P)$^+$-dependent (ME2) and mitochondrial NADP$^+$-dependent malic enzyme (ME3) [27]. ME3 is a key member of the ME family and plays important roles in physiological and pathological functions, such as insulin release and EMT [28, 29]. However, the potential function of ME3 has not been thoroughly investigated in pancreatic cancer.

In this study, we aimed to investigate the role of ME3 in pancreatic cancer, the effects of ME3 on epithelial-mesenchymal transition and the underlying mechanisms of the effects of ME3 in pancreatic cancer. ME3 may become a potential target for pancreatic cancer therapy.

Materials and methods

Analysis of ME3 mRNA and protein expression in pancreatic cancer. Correlations between pancreatic cancer histology, patient survival and ME3 gene expression were determined through analysis of the Badea and TCGA databases, which are available through Oncomine (Compendia Biosciences, www.oncomine.org) and the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov). High and low groups were defined as above and below the mean, respectively. ME3 protein and mRNA expression were detected by RT-qPCR and western blotting in pancreatic cancer cell lines.

Immunohistochemistry. Between 2017 and 2018, 69 pancreatic cancer specimens and 21 adjacent noncancerous tissues were obtained by routine surgical procedures at the Jingzhou First People Hospital and the Wuhan People Hospital. Immunohistochemistry was performed using the labeled streptavidin-biotin immunoperoxidase technique to determine the expression of ME3. Tissues were fixed in 10% formalin overnight and embedded in paraffin. IHC was performed as described previously. Briefly, endogenous peroxidases were inactivated by 3% hydrogen peroxide. Non-specific signals were blocked using 3% BSA, 10% goat serum in 0.1% Triton X-100. Tumor samples were stained with the following primary antibodies ME3 (Abcam, ab172972). After overnight incubation, the slides were washed and incubated with secondary antibody (HRP-polymers, Biocare Medical) for 30 min at room temperature. The slides were washed three times and stained with DAB substrate (Zhongshan Corp. Beijing, China). The slides were then counterstained with hematoxylin and mounted with mounting medium.

Cell culture and transfection. The human pancreatic cancer cell lines (PANC1, PaTu8988 and SW1990) were obtained from the Chinese Academy of Sciences (Shanghai, China). Use of the cell lines was approved by the ethics committee of First Hospital of Yangtze University. The cell lines were cultured in DMEM (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 100 mg/l penicillin at 37 °C in a humidified incubator with 5% CO_2. Then, 3×Flag vector, 3×Flag ME3, shEGFP and shME3 were transfected into pancreatic cancer cells using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Plasmid construction. The complete ME3 sequence was amplified by RT-PCR using ME3-all-F (5'-CGGAATTCCGATGGAGCGGAAGAGCCCGAGCG-3') and ME3-all-R (5'-GGGGTACCCCCTAGACGTGCTCCATCTCCGGGT-3') primers from a cDNA library of PANC1 cells and were then inserted into the expression vector p3xFLAG-Myc-CMV™-24 (Sigma, USA). Four ME3 shRNAs were designed, and the most effective one was chosen. The ME3 and EGFP shRNA oligos (ME3-shRNA-F 5'-CCGGCCTCCGAATCATGAGATATTACTCGAGTAATATCTCATGATTCGGAGGTTTTTG-3', ME3-shRNA-R 5'-AATTCAAAAACCTCCGAATCATGAGATATTACTCGAGTAATATCTCATGATTCGGAGG-3', EGFP-shRNA-F 5'-CCGGTACAACAGCCACAACGTCTATCTCGAGATAGACGTTGTGGCTGTTGTATTTTTG-3', EGFP-shRNA-R 5'-AATTCAAAAATACAACAGCCACAACGTCTATCTCGAGATAGACGTTGTGGCTGTTGTA-3') were first annealed into double strands and then cloned into pLKO.1-puro (Sigma, USA).

Real-time PCR. Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen). Two micrograms of RNA for each sample were reversed transcribed into cDNA by the First-Strand cDNA Synthesis System (Marligen Biosciences). Real-time PCR was performed using a 2×SYBR Green mix kit from Applied Biosystems. PCR was performed using the CFX-96 Sequence Detection System (Bio-Rad Laboratories) with GAPDH as an endogenous control. Relative gene expression levels were represented as ΔCt = Ct gene – Ct reference, and the fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: ME3 forward, 5'-CTTGGTTTCGCTTTGCCTGG-3', and ME3 reverse, 5'-CAGGTGCTCCCAAAGGGTTA-3', MMP2 forward, 5'-CACAGGAGGAGAAGGCTGTG-3', MMP2 reverse, 5'-GAGCTTGGGAAAGCCAGGAT-3', MMP9 forward, 5'-TTCAGGGAGACGCCCATTTC-3', MMP9 reverse, 5'-TGTAGAGTCTCTCGCTGGGG-3', GAPDH forward, 5'-GGTGAAGGTCGGTGTGAACG-3', and GAPDH reverse, 5'-CTCGCTCCTGGAAGATGGTG-3'. Samples were cycled once at 98 °C for 2 min and then subjected to 35 cycles of 95 °C, 56 °C and 72 °C for 30 sec each.

Western blotting. The cultured cells were extracted with cold PBS before being treated with RIPA lysis buffer at 100 °C for 5~10 min. Then, the mixture was centrifuged at 12000×g for 10 min at 4 °C. Approximately 25–30 μg of protein was loaded into each lane, separated by 10% SDS-PAGE and transferred to the PVDF membrane. The membrane was blocked with 5% nonfat milk powder for 1 h at room temperature, and then the membrane was incubated with primary antibodies at 4 °C overnight. The blots were incubated with primary antibodies against rabbit anti-ME3 (Abcam, ab172972), anti-TGF-β (Cell Signaling, CAT3709),

anti-MMP2 (Immunoway, CAT YT2798), anti-MMP9 (Immunoway, CAT YT1892), EMT kit (Cell Signaling, CAT 9782), Smad2/3 Antibody Sampler Kit (Cell Signaling, CAT 12747), mouse anti-Flag (SIGMA, CAT 6631), mouse anti-β-Tubulin (Cell Signaling, CAT4466), anti-CDC20 (Cell Signaling, CAT 4823S), E-cadherin antibody (Cell Signaling, CAT 14472), rabbit anti-Vimentin (Cell Signaling, CAT 5741), α-SMA antibody (Abbkine, Abm40185) and rabbit anti-Snail (Cell Signaling, CAT 3879). Membranes were washed in PBS plus 0.1% Tween 20 and probed with anti-rabbit or anti-mouse HRP-conjugated secondary antibody (both at 1:10 000 dilution), and proteins were detected using the ECL Plus chemiluminescence detection reagent (Cell Signaling, Western Blotting Application Solutions, CAT 12957). Densitometry of X-ray films was performed with Alpha View software (Cell Biosciences; Santa Clara, CA), and the expression levels were normalized according to the densitometry of β-Tubulin.

Cell proliferation assay. Cellular proliferation was measured using a Cell Counting Kit-8 (CCK8, Sigma-Aldrich, CAT 96992). Pancreatic cancer cells were treated with 3×Flag vector, 3×Flag ME3, shEGFP and shME3 for 24 h, then the cell suspensions containing 3000~4000 cells (100 µl) continued to incubate at 37 °C. At 24, 48, 72 and 96 h, 100 µl of serum-free culture medium and 10 µl of CCK8 solutions were added to each well, followed by incubation at 37 °C for 2 h. Optical density was determined at 450 nm using the ELX-800 spectrometer reader. Six independent samples were detected in each experimental group.

Transwell migration, invasion assay and Wound healing assay. Transwell and invasion assays were carried out using Matrigel chambers (BD Biosciences) according to the manufacturer's protocol. Transfected PANC1, PaTu8988 and SW1990 cells were harvested and resuspended in serum-free medium. Cell suspensions containing 10 000 cells/100 µl were plated into the upper chamber of the transwell, and conditioned medium (500 µl) containing 10% FBS was added to the lower chamber of the transwell. The system was incubated in 5% CO_2 at 37 °C for 24 h. The cells on the upper surface were scraped and washed away, whereas the migrated and invaded cells on the lower surface were fixed and stained with 0.05% crystal violet for 30 min. Finally, migrated and invaded cells were counted and the relative number was calculated. 30×10^4 cells were seeded in a 6 cm dish and grown until 90–95% confluence. A single scratch across the dish was then made using a sharp edge. Images of the wound were taken at 0 and 48 h at 8× magnification using a Nikon AZ100 Multizoom microscope. The scratch width was measured at 0 h and 48 h, respectively. The wound healing rate was calculated as (0 h scratch width – 48 h scratch width)/0 h scratch width × 100%. All data were obtained from 3 independent experiments.

Statistical analyses. Statistical analysis was conducted using SPSS software (version 19.0, IBM, USA). The categorical variables were described by frequencies and proportions and tested by the chi-square test or Fisher's exact test. Differences were analyzed by Student's t-test (two groups) or a one-way ANOVA (multiple groups). Survival curves were evaluated with the Kaplan–Meier method (log-rank test). A p-value <0.05 was considered significant.

Results

ME3 is upregulated in pancreatic cancer and is associated with overall survival. To confirm the clinical relevance of ME3 expression, we first analyzed the ME3 mRNA expression in clinical specimens. The ME3 mRNA level was higher in pancreatic cancer tissues than in normal pancreatic tissues (2.128±0.044 vs. 1.826±0.049, p<0.0001, n=78) in the Badea pancreas database [30] (Figure 1A). Due to the limitation of the Badea pancreas database information, we investigated more information in the TCGA database and evaluated the correlation of ME3 expression with patient outcomes. Patients with low expression of ME3 had a median survival of 430 days compared with 308 days for the patients with high expression of ME3 (HR=4.22, p=0.0386, Figure 1B). These data suggest that ME3 is upregulated in pancreatic cancer and is associated with poor prognosis. In addition, using real-time PCR and western blotting, we analyzed the expression of ME3 in three pancreatic cancer cell lines, PANC1, SW1990 and PaTu8988. The results showed that ME3 mRNA and protein levels were higher in PANC1 cells than in SW1990 and PaTu8988 cells (p<0.05, Figures 1C, 1D).

ME3 expression in pancreatic cancer tissues and adjacent noncancerous tissues. Rabbit anti-human ME3 monoclonal antibody was used to detect ME3 protein immunohistochemically. Positive staining of ME3 mainly located in cytoplasm and the color was yellow or brown (Figure 2). Adjacent noncancerous tissues showed positive staining for ME3 in only 1 out of 69 cases (1.4%). Overexpression of the ME3 in cancer was found in 63 (91.3%) of the 69 cholangiocarcinoma tissues. The expression of ME3 in tumor tissues was compared to the expression in adjacent noncancerous tissues. ME3 expression of the carcinoma tissues was remarkably higher than the adjacent noncancerous tissues and there was statistical significance (p<0.01, Figure 2).

The effect of the shME3 and 3×Flag ME3 plasmids is verified. To confirm the effects of the shME3 and 3xFlag ME3 plasmids, shEGFP and shME3 were transfected into PANC1 and PaTu8988 cells, and 3×Flag vector and 3×Flag ME3 were transfected into PaTu8988 and SW1990 cells, respectively. ME3 mRNA and protein levels were examined by real-time PCR and western blot (p<0.05, Figures 1E, 1F). Therefore, shME3 and 3×Flag ME3 could contribute to the role of ME3 knockdown and ME3 overexpression, respectively.

ME3 promotes the proliferation ability of pancreatic cancer cells. To determine the effect of ME3 on the proliferation of pancreatic cancer cells, PANC1, PaTu8988 and SW1990 cells were treated with shME3 and 3×Flag ME3 for 24 h, 48 h, 72 h, and 96 h. A CCK-8 assay was employed to

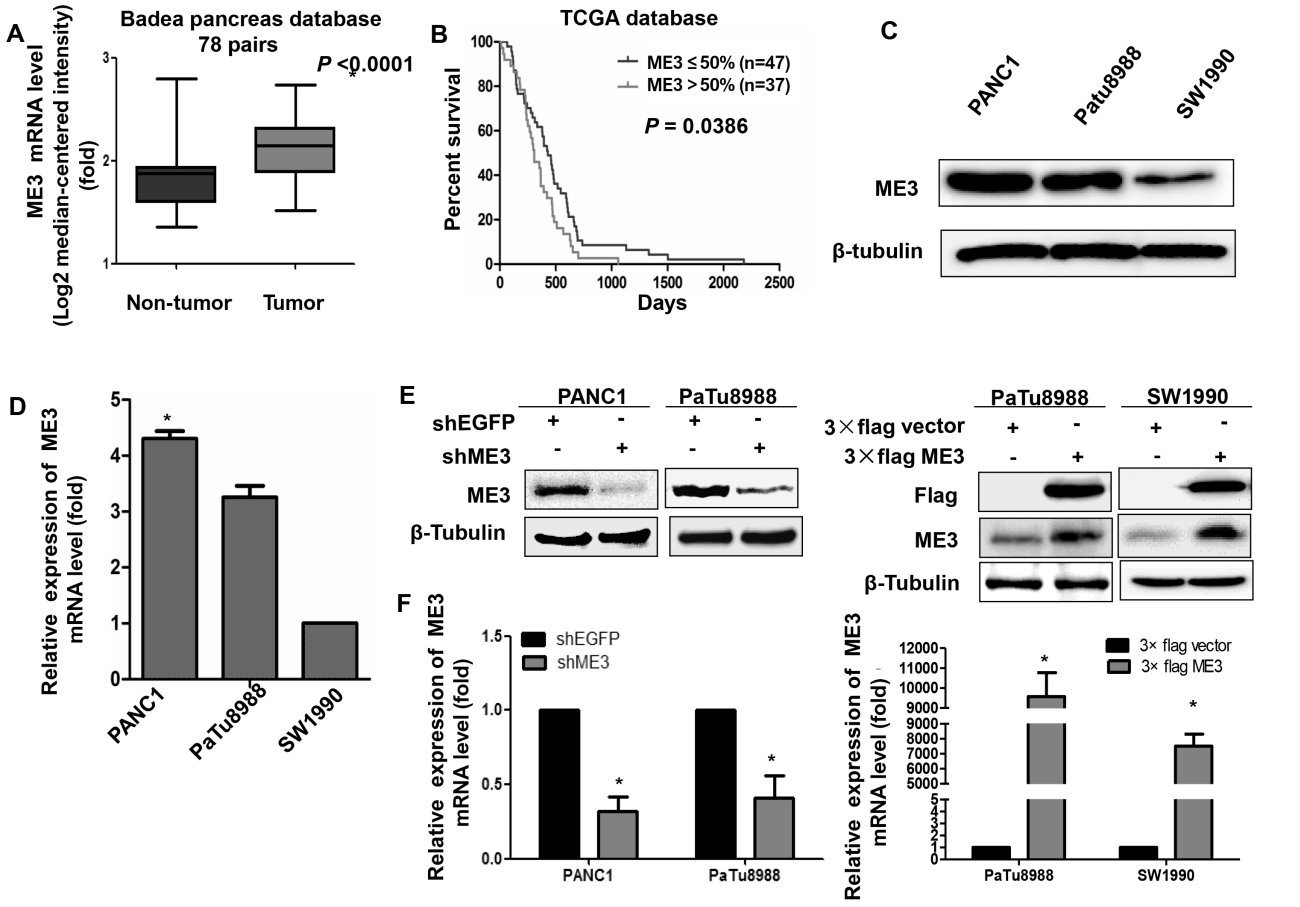

Figure 1. ME3 is upregulated in pancreatic cancer and is associated with overall survival. A) Analysis of ME3 mRNA levels in 78 pairs of pancreatic cancer and nontumor tissues in the Badea pancreas database. N=39 for the nontumor group and N=39 for the tumor group. p<0.001. B) Analysis of the TCGA database indicates that ME3 expression is correlated with overall survival. N=47 for the ME3-low group and N=37 for the ME3-high group. p=0.0386 was determined by a log rank test. C and D) Relative expression levels of ME3 protein and mRNA were assessed in SW1990, PaTu8988, and PANC1 cells. *p<0.05. E and F) ME3 protein and mRNA levels were reduced in shME3-PANC1 and PaTu8988 cells. ME3 protein and mRNA levels were increased in PaTu8988 and SW1990 cells transfected with 3×Flag-ME3. *p<0.05.

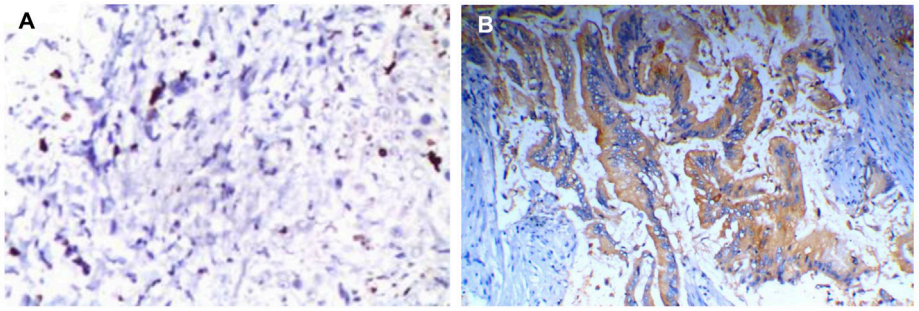

Figure 2. Immunohistological expression of ME3 in pancreatic cancer and adjacent noncancerous tissues. A) The negative stain in pancreatic cancer tissue (x 200). B) The positive stain in pancreatic cancer tissue (x 200).C) Comparison of ME3 expression in pancreatic cancer and adjacent noncancerous tissues. ME3 expression of the carcinoma tissues was remarkably higher than the adjacent noncancerous tissues and there was statistical significance (p<0.01).

C. ME3 expression in pancreatic cancer and adjacent noncancerous tissues

Group	N	Postive	Negtive
Pancreatic cancer tissues	69	63	6
Adjacent noncancerous tissues	69	1	68

analyze the cell activity in PANC1 and PaTu8988 cells. ME3 silencing by shME3 transfection significantly decreased cell activity ($p<0.05$. Figures 3A, 3B), while overexpression of ME3 by 3xFlag ME3 in PaTu8988 and SW1990 cells increased cell activity ($p<0.05$, Figures 3C, 3D). Additionally, ME3 knockdown led to decreased CDC20 protein levels in PANC1 and PaTu8988 cells (Figure 3E), while ME3 overexpression increased the expression of CDC20 in SW1990 and PaTu8988 cells (Figure 3F). These data show that ME3 promotes the proliferation of pancreatic cancer cells.

Effect of ME3 on the migration of pancreatic cancer cells. Next, we examined the migration ability of pancreatic cancer cells by transwell assays. The numbers of migrated cells were 28±3 and 20±5 after ME3 knockdown, and there were significant differences compared to the numbers of migrated PANC1 and PaTu8988 cells, which were 86±6 and 99±8, respectively (Figure 4A). This indicated that ME3 knockdown inhibited the migration ability of PANC1 and PaTu8988 cells. To confirm the above results, we assessed the change in migration ability in PaTu8988 and SW1990 cells after ME3 overexpression. The numbers of migrated cells were 126±9 and 184±12 with ME3 overexpression, compared to 54±5 and 74±8 in the control PaTu8988 and SW1990 cells, respectively (Figure 4B). PANC1 were transfected with shEGFP, shME3, 3×Flag vector and 3×Flag ME3 for 48 h, the wound healing rates were 53.72±5.03%, 34.34±4.83%, 56.56±4.77% and 89.86±5.83%, respectively (Figure 5). These data indicated that ME3 overexpression could promote the migration ability of pancreatic cancer cells.

ME3 promotes the invasion ability of pancreatic cancer cells. Subsequently, we examined the invasion ability using BD Matrigel invasion assays. PANC1 and PaTu8988 cells were transfected with shEGFP and shME3 plasmids, and PaTu8988 and SW1990 cells were transfected with 3×Flag vector or 3×Flag ME3 for 72 h. After ME3 silencing, the numbers of invasive cells decreased from 34±3 to 13±2 in PANC1 cells

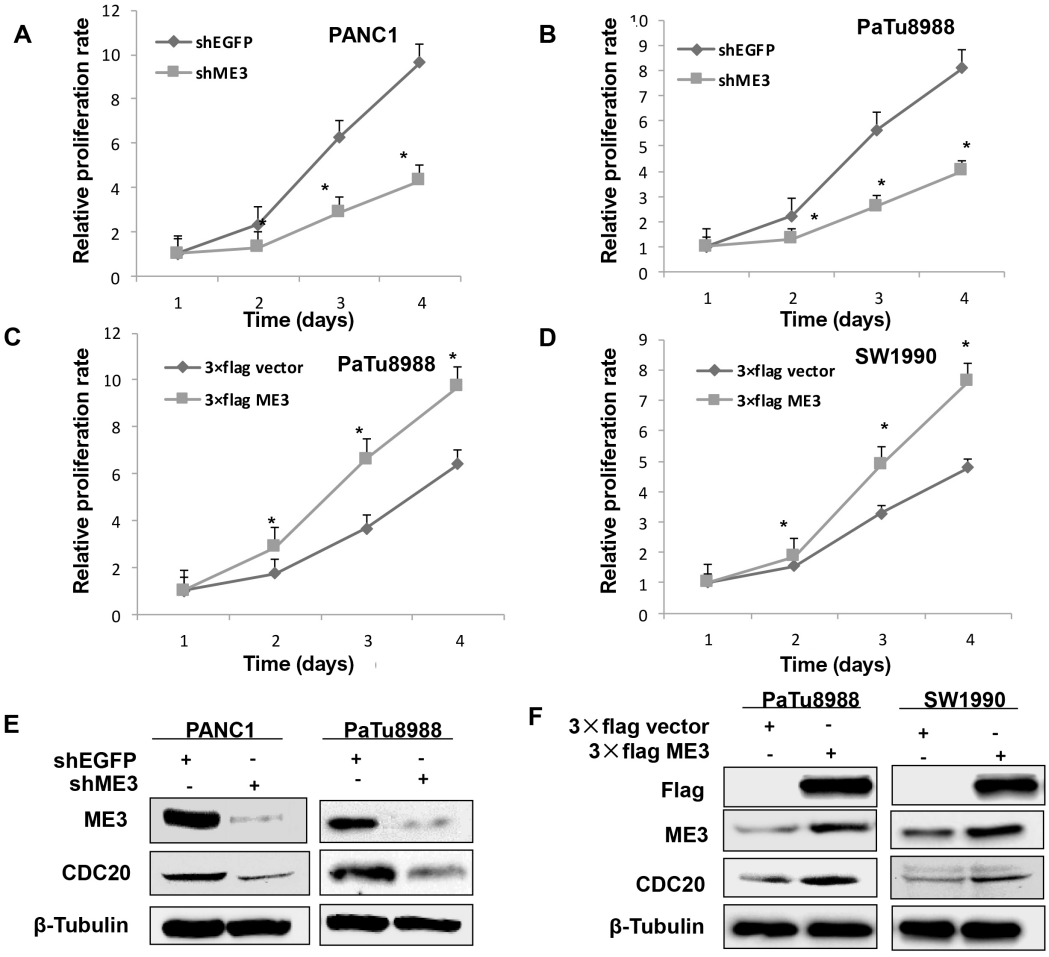

Figure 3. ME3 promotes the proliferation ability of pancreatic cancer cells. A and B) The ability of proliferation was examined using CCK-8 assay in PANC1 and PaTu8988 cells transfected with shME3 or shEGFP plasmids. Representative images of migrated cells were shown. *p<0.05. C and D) The ability of proliferation was examined using CCK-8 assay in SW1990 and PaTu8988 cells transfected with 3×Flag ME3 or Vector plasmids. *p<0.05. E and F) CDC20 and ME3 were determined using western blotting.

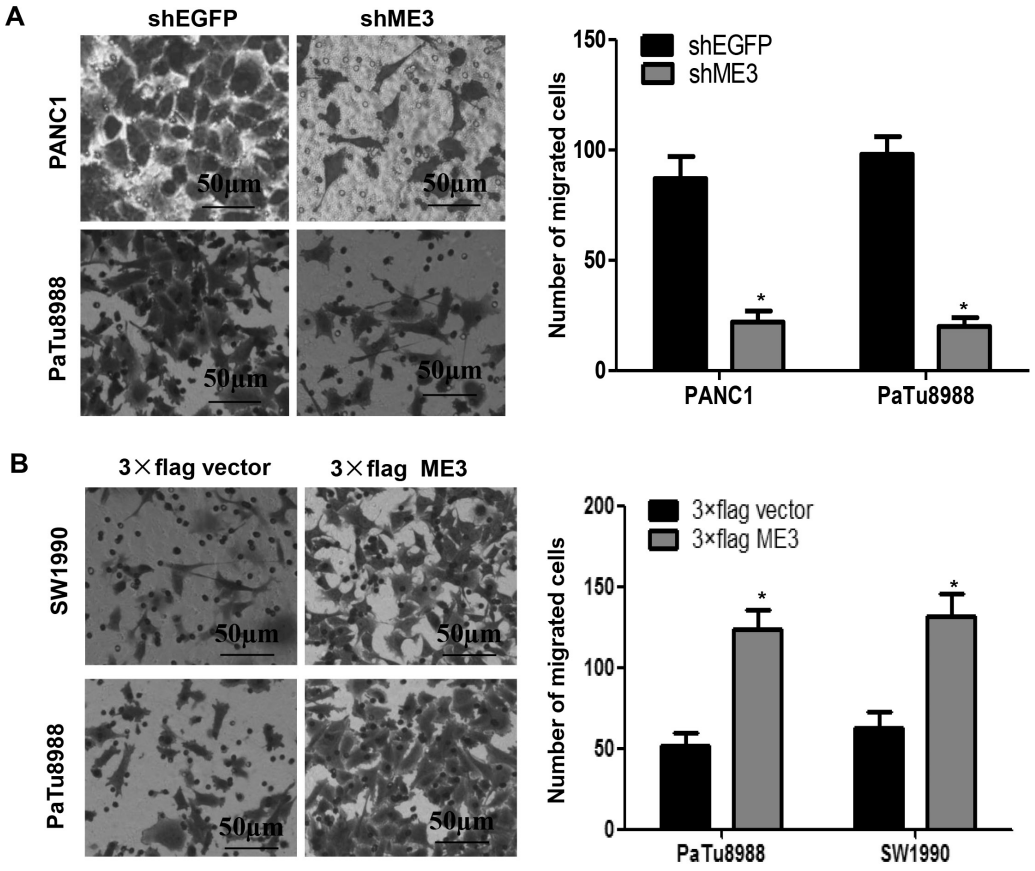

Figure 4. ME3 promotes the migration ability of pancreatic cancer cells. A and B) the abilities of migration were examined using transwell assay in PANC1, PaTu8988 and SW1990 cells transfected with shME3 or 3×Flag ME3 plasmids. Results were presented as the mean±SD of triplicate samples from representative data of three independent experiments. *p<0.05.

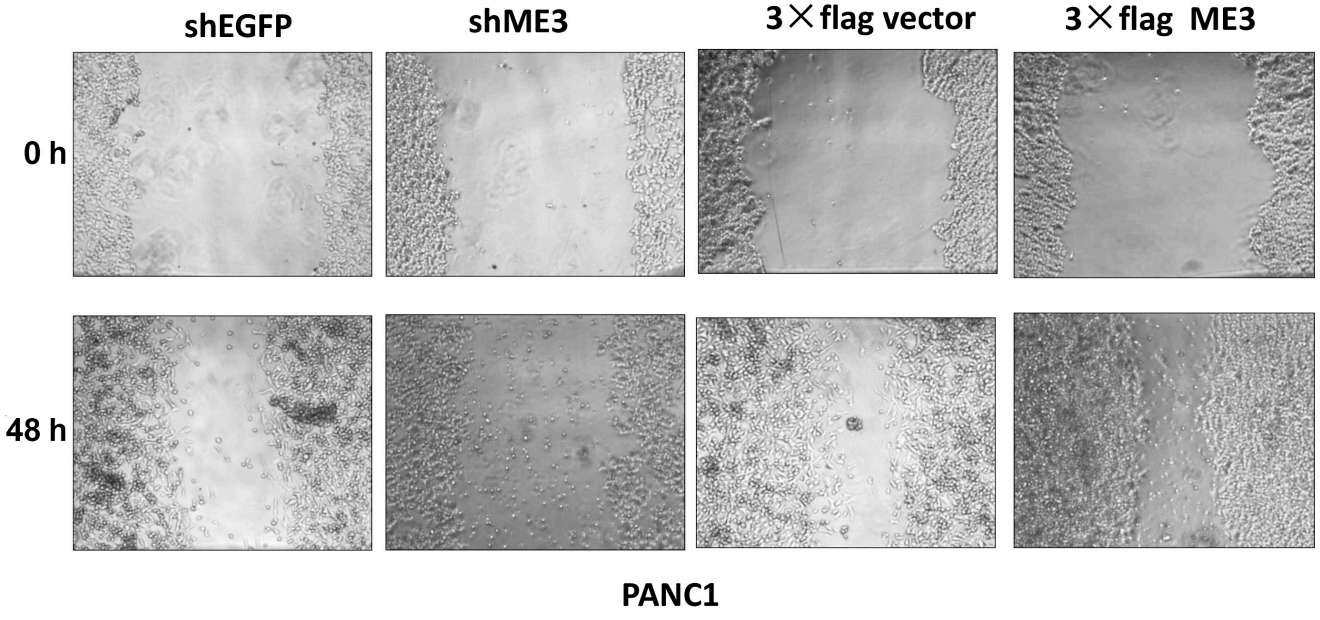

Figure 5. Wound healing assay. PANC1 were transfected with shEGFP, shME3, 3×Flag vector and 3×Flag ME3 for 48 h, the wound healing rates were 53.72±5.03%, 34.34±4.83%, 56.56±4.77% and 89.86±5.83%, respectively.

and from 35±4 to 10±3 in PaTu8988 cells (Figure 6A). These results indicate that knockdown of ME3 notably inhibited the invasion ability of PANC1 and PaTu8988 cells. Moreover, ME3 overexpression increased the number of invasive cells from 22±4 to 58±5 in 3 PaTu8988 cells and from 16±3 to 37±4 in SW1990 cells. These results suggested that upregulation of ME3 significantly enhanced the invasion ability of PaTu8988 and SW1990 cells (Figure 7A). Additionally, ME3 knockdown downregulated the expression of MMP2 and MMP9 (Figures 6B, 6C), while ME3 overexpression resulted in the upregulation of MMP2 and MMP9 (Figures 7B, 7C). These data indicated that ME3 could promote the invasion ability of pancreatic cancer cells.

ME3 promotes EMT by activating TGF-β/Smad signaling in pancreatic cancer cells. To further investigate how ME3 affects pancreatic cancer cell proliferation, migration and invasion, we detected the effect of ME3 on epithelial and mesenchymal markers. Western blot analysis revealed that ME3 knockdown induced the expression of the epithelial cell marker E-cadherin and decreased the expression of the mesenchymal markers Vimentin, N-cadherin and Snail (Figure 8A); ME3 overexpression repressed the expression of E-cadherin and increased the expression of Vimentin, N-cadherin and Snail (Figure 8B). The characteristics of EMT include downregulation of epithelial markers, such as E-cadherin, desmoplakin and cytokeratins, and upregulation of mesenchymal markers, such as N-cadherin, fibronectin, Vimentin and α-smooth muscle actin (α-SMA). Therefore, we deduced that ME3 could promote EMT. We also explored the relationship between EMT and the TGF-β/Smad signaling pathway. When EMT was inhibited through ME3 knockdown, the expression of TGF-β and Smad2/3 phosphorylation were decreased (Figure 8C). In contrast, when EMT was promoted through ME3 overexpression, the levels of TGF-β and phosphorylated-Smad2/3 were elevated (Figure 8D). However, neither knockdown nor overexpression of ME3 affected total Smad2/3 expression. These results suggested that ME3 could be involved in EMT and that this effect might be related to its activation of the TGF-β/Smad2/3 signaling pathway.

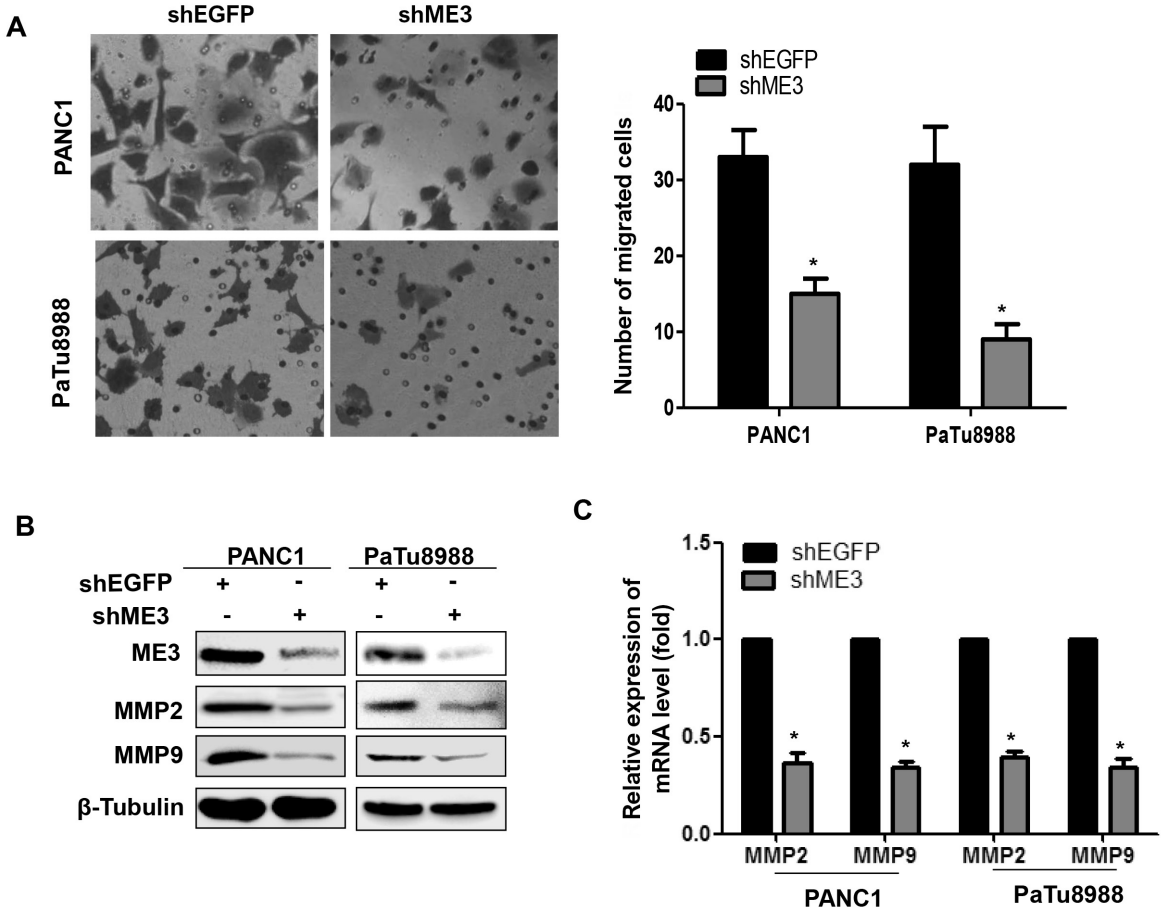

Figure 6. Downregulation ME3 inhibits the invasion ability of pancreatic cancer cells. A) The invasion ability was examined using BD Matrigel invasion assay in PaTu8988 and PANC1 cells transfected with shEGFP or shME3 plasmids. Invasive cells were counted and analyzed. *p<0.05. MMP2 and MMP9 were identified using western blotting (B) and real-time PCR (C) in above cells. *p<0.05.

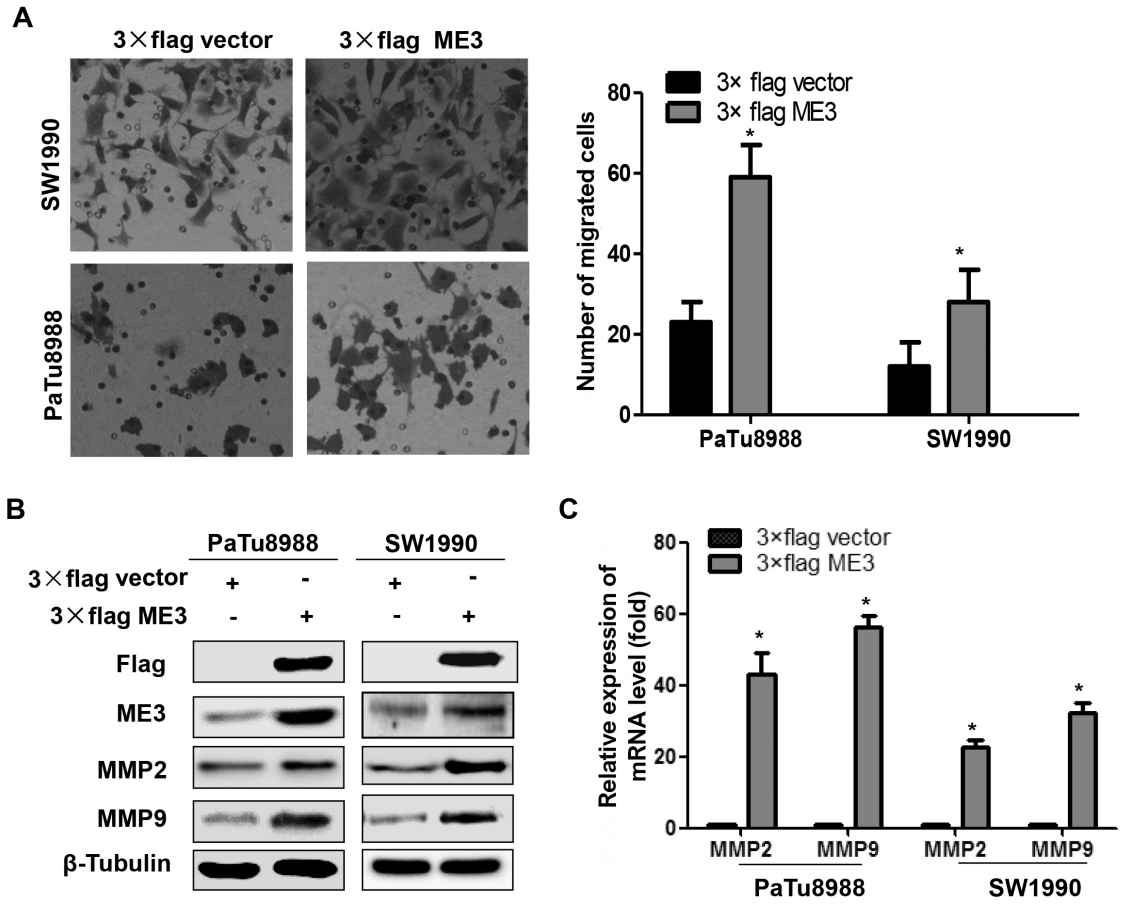

Figure 7. Upregulating ME3 promotes the ability of invasion in pancreatic cancer cells. A) The invasion ability was examined using BD Matrigel invasion assay in PaTu8988 and SW1990 cells transfected with 3×Flag Vector or 3×Flag ME3 plasmids. Invasive cells were counted and analyzed. *p<0.05. MMP2 and MMP9 were determined using western blotting (B) and real-time PCR (C) in above cells. *p<0.05.

Discussion

Pancreatic cancer is one of the leading causes of cancer mortality and one of the most lethal malignant neoplasms worldwide [31]. The causes of pancreatic cancer are still insufficiently understood, so the pathogenesis of pancreatic cancer is still in the focus of current research.

Cancer-associated metabolic reprogramming affects gene expression, cellular differentiation and the tumor microenvironment, and these characteristics were recently summarized by Pavlova et al. [32] as the six hallmarks of cancer metabolism. One hallmark corresponds to the use of glycolysis and tricarboxylic acid (TCA) intermediates for biosynthesis and nicotinamide adenine dinucleotide phosphate (NADPH) production [33, 34]. MEs regulate cellular energy, redox balance and biomolecular synthesis by converting the TCA cycle intermediate malate into the TCA carbon source pyruvate and NADPH [35]. Mitochondrial malic enzyme 3 is an oxidative decarboxylase that catalyzes malate to pyruvate and is essential for NADPH regeneration and reactive oxygen species (ROS) homeostasis [34–36]. ROS can serve as both signaling molecules and cell death mediators to promote proliferation or induce cell death in response to chemotherapy in cancer [37, 38]. ME3 has been shown to play an important role in the development of pancreatic cancer. The newest study focused on ME3 depletion, which selectively kills ME2-null PDAC cells in a manner consistent with an essential function of ME3 in ME2-null pancreatic cancer cells; highly specific ME3 inhibitors could provide an effective therapy for a meaningful fraction of cancer patients [29]. Therefore, ME3 levels may be used as a prognostic and predictive marker for radiation therapy in cancer. Through analysis of the Badea and TCGA databases and UCSC, we found that ME3 expression was higher in pancreatic cancer tissues than in normal pancreatic tissues. This finding indicated that the increased ME3 expression might play an important role in the development of pancreatic cancer and act as a biomarker of cancer development and progression. In addition, ME3 expression was directly associated with clinicopathological features. Additionally, patients with higher

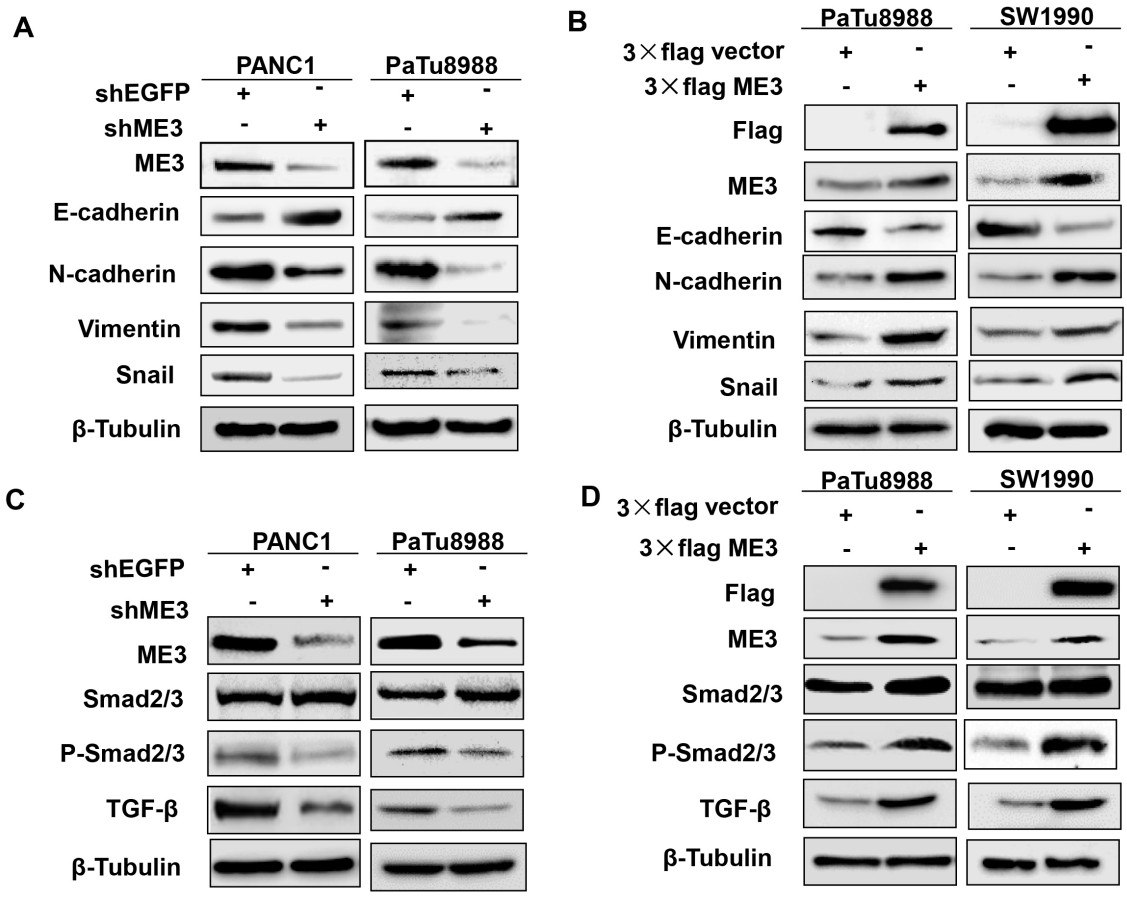

Figure 8. ME3 promotes EMT through TGF-β/Smad signaling in pancreatic cancer cells. A) ME3 knockdown in PANC1 and PaTu8988 cells reversed EMT, as detected by increase in E-cadherin and decrease in Vimentin, α-SMA and Snail. B) Treatment of PaTu8988 and SW1990 cells with 3×Flag Vector or 3×Flag ME3 induced EMT, as defined by an increases in E-cadherin and decrease in Vimentin, α-SMA and Snail. C) Immunoblotting of Smad2/3, p-Smad2/3, TGF-β in PANC1 and PaTu8988 cells treated with shME3 or shEGFP. β-Tubulin was used as a loading control. D) Immunoblotting of Smad2/3, p-Smad2/3/3, TGF-β in SW1990 and PaTu8988 cells transfected with 3×Flag Vector or 3×Flag ME3. β-Tubulin was used as a loading control.

ME3 levels had a significantly shorter survival time than those with lower levels of ME3, suggesting that upregulation of ME3 expression was associated with poor outcome in patients. These results implied that ME3 may be a promoter in pancreatic cancer progression. Furthermore, we focused on the functions of ME3 in PANC1 cells, PaTu8988 cells and SW1990 cells, based on the positive correlation between the expression level of ME3 and prognosis. Hence, through downregulation and upregulation of ME3 gene expression, we found that proliferation, invasion and metastasis of pancreatic cancer cells changed based on the expression of ME3. We confirmed that ME3 might be involved in pancreatic cancer cell growth, proliferation, metabolism and invasion. Therefore, ME3 may be considered as a potential candidate for the diagnosis and treatment of pancreatic cancer and has been evaluated as such.

It is well known that EMT is associated with tumor cell invasion leading to metastatic dissemination by promoting mesenchymal cell phenotypic characteristics, including enhanced migratory properties and invasiveness [39]. In general, EMT is a complex process and is strictly controlled both temporally and spatially by the binding of several transcriptional repressors, including Vimentin, N-cadherin and Snail, to the promoters of E-cadherin and a variety of EMT-related genes [40, 41]. At the molecular level, the characteristics of EMT include downregulation of epithelial markers, such as E-cadherin, desmoplakin, and cytokeratins, and upregulation of mesenchymal markers, such as N-cadherin, fibronectin, Vimentin and α-SMA [42]. Our results showed that ME3 knockdown enhanced E-cadherin expression and inhibited Vimentin, N-cadherin and Snail expression, while ME3 overexpression resulted in decreased E-cadherin and upregulation of Vimentin, N-cadherin and Snail. These data suggest that ME3 might drive typical epithelial phenotype cells to transform into spindle-shaped mesenchymal phenotype cells, resulting in the promotion of proliferation, migration and invasion of human pancreatic cancer cells.

TGF-β is an important cytokine that is involved in a wide range of biological processes. It has been reported that TGF-β signaling is associated with regulating the initiation of malignancy, progression and metastasis in mammary carcinoma, pancreatic cancer, colon carcinoma and hepatocellular carcinoma [43]. Under resting conditions, Smad2 is unphosphorylated and is retained in the cytoplasm. When TGF-β is activated, Smad2 is phosphorylated and undergoes dimerization with Smad3, thus allowing its translocation into the nucleus [40, 41, 44–47]. TGF-β has emerged as a potent secreted factor that drives cancer progression not only through its immunosuppressive and proangiogenic roles but also, perhaps more importantly, as a potent inducer of epithelial plasticity leading to EMT [48, 49]. EMT is now considered to play fundamental roles in the initiation and progression of carcinomas. Epithelial cell plasticity manifested by TGF-β-induced EMT and its reversibility appears to play pivotal roles in the control of many aspects of cancer progression. The roles and molecular mechanisms of TGF-β signaling in EMT have been extensively studied in cell culture but also in mouse models [50]. More importantly, our results provide evidence to confirm that ME3 promotes growth and EMT by driving TGF-β/Smad signaling. In our results, EMT was inhibited through ME3 knockdown, while the expression of TGF-β and Smad2/3 phosphorylation were decreased. In contrast, EMT was promoted through ME3 overexpression, while the levels of TGF-β and phosphorylated-Smad2/3 were elevated. These results showed that there was a correlation between EMT and TGF-β/Smad signaling. By combining the literature and our own experimental data, we deduced that ME3 could be involved in EMT, which might be related to its activation of the TGF-β/Smad2/3 signaling pathway.

ME3 is extensively involved in the carcinogenesis of pancreatic cancer and can promote EMT in pancreatic cancer cells, which may be caused by the regulation of the TGF-β/Smad2/3 signaling pathway. Therefore, ME3 may become a new candidate target for the diagnosis, treatment and prognosis of pancreatic cancer.

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