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METTL3-IGF2BP3-axis mediates the proliferation and migration of pancreatic cancer by regulating spermine synthase m6A modification

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Spermine synthase (*SMS*) is an enzyme participating in polyamine synthesis; however, its function and role in pancreatic cancer remains elusive. Here we report that *SMS* is upregulated in pancreatic cancer and predicts a worse overall survival and significantly promotes the proliferation and migration of pancreatic cancer cells. Excessive *SMS* reduces the accumulation of spermidine by converting spermidine into spermine, which activates the phosphorylation of serine/threonine kinase (AKT) and epithelial-mesenchymal transition (EMT) signaling pathway, thereby inhibiting pancreatic cancer cell proliferation and invasion. Moreover, *SMS* was identified as the direct target of both methyltransferase like 3 (*METTL3*) and insulin like growth factor 2 mRNA binding protein 3 (*IGF2BP3*), which directly bind to the m6A modification sites of *SMS* and inhibit mRNA degradation. Knockdown of *METTL3* or *IGF2BP3* significantly reduced the *SMS* protein expression and inhibited the migration of pancreatic cancer. We propose a novel regulatory mechanism in which the METTL3-IGF2BP3 axis mediates the mRNA degradation of SMS in an m6A-dependent manner to regulate spermine/spermidine conversion, which regulates AKT phosphorylation and EMT activation, thereby inducing tumor progression and migration in pancreatic cancer.

KEYWORDS

spermine synthase, polyamine, m6A, pancreatic cancer, EMT, AKT

Introduction

Pancreatic cancer is a highly malignant tumor with the highest annual mortality among all cancers (1). At present, the main treatment for pancreatic cancer is surgical removal (2); however, most pancreatic cancers have already metastasized at the time of diagnosis (3). The molecular mechanism of pancreatic cancer metastasis has not been clarified. Thus, it is important to uncover the mechanism of how pancreatic cancer metastasizes to provide potential therapeutic targets for more effective management of this deadly disease.

m6A is the most abundant epigenetic modification in eukaryotic mRNAs and is tightly and closely correlated to many fundamental biological processes (4) (5). Numerous studies have found that m^6A modification plays an important role in the occurrence and development of tumors including pancreatic cancer (4, 6, 7) (8–10). Xia et al. found that *METTL3* can promote the proliferation and invasion of pancreatic cancer (9). Y. Chen found that *ALKBH5* can mediate *PER1* m6A and then regulate the EMT of pancreatic cancer (11). It has also been suggested that *METTL3* inhibits tumorigenesis (12). In view of the multiple roles of m^6A in pancreatic cancer, more in-depth research is needed.

SMS is an enzyme involved in the synthesis of spermine, which converts spermidine into spermine and plays an important role in maintaining the homeostatic balance of polyamines in the cell (13). The native polyamines include putrescine, spermidine and spermine, which are small-molecule polar compounds containing two or more amino groups (14). Polyamines can regulate metabolism, intracellular DNA oxidative damage stress (15), and are important factors for cell growth and development. Studies have reported that polyamines are elevated to varying degrees in most tumor cells. Moreover, polyamines can promote the progression of pancreatic cancer (16). However, the effects of the changes in various components of polyamines are rarely studied in the context of pancreatic cancer. Studies have shown that extremely high levels of spermidine can inhibit the growth of myeloma (17). In addition, spermine plays an important role as an immune suppressant. Studies have shown that spermidine can affect the NADPH oxidase activation of neutrophils and thus inhibit the immune response (18, 19). Some researchers believe that exogenous spermidine can inhibit the phosphorylation of AKT causing inactivation, thereby promoting autophagy and ultimately leading to cell growth (20). Spermine synthetase can alter the ratio of polyamine components in the cell by synthesizing spermidine into spermine (21). Therefore, it is worthwhile to examine the role of spermine synthase in pancreatic cancer.

EMT is an important sign of tumor migration and invasion (22). As an important signal transduction pathway, the PI3K-AKT pathway plays a key role in tumor proliferation and migration (23). PI3K-AKT pathway can regulate the EMT pathway, leading to tumor cell migration and invasion (24). We speculate that a reduced spermidine to spermine ratio further leads to weakened inhibition of AKT phosphorylation. The phosphorylated AKT can promote the EMT transition of cells through the signal amplification mechanism, and ultimately lead to tumor metastasis.

However, there are only a few reports describing the role of SMS in pancreatic cancer (18, 25). In this study, we explored the clinical relationship between SMS and pancreatic cancer, investigated its biological functions and the molecular mechanism by which SMS promotes the progression of pancreatic cancer.

Abbreviations: METTL3, methyltransferase like 3; IGF2BP3, Insulin like growth factor 2 mRNA binding protein 3; AKT, Serine/threonine kinase; ME-RIP, Methylated RNA immunoprecipitation;M6A,N6- methyladenosine; SMS, Spermine Synthase; GEO, Gene Expression Omnibus; H&E, hematoxylin and eosin; IHC, immunohistochemistry; LC/MS, Liquid chromatography-tandem mass spectrometry; mTOR, mechanistic target of rapamycin; NC, negative control; ATCD, Actinomycin D; qRT-PCR, quantitative real-time polymerase chain reaction; TCGA, The Cancer Genome Atlas; GEPIA, Gene Expression Profifiling Interactive Analysis.

Materials and methods

Patients and specimens

Clinical tissue samples from 59 patients were obtained from The First Affiliated Hospital of Fujian Medical University (Fuzhou, China). All experiments involving human samples and clinical data were approved by the Accreditation Committee of The First Affiliated Hospital of Fujian Medical University.

Public datasets

The public datasets used in this study included four GEO datasets (http://www.ncbi.nlm.nih.gov/geo/, GSE15471, GSE16515, GSE71989 and GSE22780). GEPIA (http://gepia.cancer-pku.cn/),the m6A-ATLAS database (www.xjtlu.edu.cn/biologicalsciences/atlas).

Immunohistochemistry

Immunohistochemistry staining was performed using antibodies targeting SMS (Novus, 1:800), METTL3 (Abcam, 1:2000), IGF2BP3 (Abcam, 1:200), AKT (CST, 1:200), p-AKT (T308) (CST, 1:200) and p-AKT(S473) (CST, 1:200). The immunoreactivity was scored blindly according to the value of immunoreaction intensity (none = 0; weak = 1; intermediate = 2;

and strong = 3) and the percentage of tumor cell stained (none = 0; <10% = 1; 10–50% = 2; >50% = 3). The intensity and percentage values were added to provide immunoreactivity score ranging from 0 to 6. High expression of SMS was defined as an immunohistochemical score of ≥5, and low expression was defined as <5. (26)

Establishment of cell lines

Human pancreatic cancer cell lines ASPC-1, PANC-1, BxPC-3, SW1990, Mia-Paca2 were purchased from American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10% FBS. Cells were cultured in a 37 °C, 5% CO_2 incubator. Plasmids were transfected in HEK-293 T cells using Lipofectamine 3.0 (Invitrogen) following the manufacturer's instructions. Next, the pancreatic cancer cell lines with stable gene expression were selected in culture medium supplemented with puromycin (1 μg/ml; Sigma Aldrich). Small interfering RNA (siRNA) targeting METTL3/IGF2BP3 specific regions were synthesized by Gene Pharma (Shanghai, China). Transfections were carried out using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions. The siRNA sequence is summarized in Table S2.

Western blot analysis

For Western blot analysis, the nuclear and total cellular protein fractions were extracted with Western IP Lysis Buffer (Beyotime, China). The primary antibodies were incubated at 4°C overnight and then incubated with secondary antibodies (1:2000, CST) for 1h. Finally, they were washed using 1% TBST and detected by a chemiluminescence system.

Colony formation assay

Cells were seeded into 6-well plates at a density of 500 cells/well and the culture was replaced with new medium every 48 hours, then were cultured for 10-14 days. The colonies were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, China). Numbers of colonies in triplicate wells were counted for each treatment group.

Cell proliferation assay

1,000 cells suspended in 100 μL DMEM medium were seeded into 96-well plate. The cell proliferation was assessed by the CCK8 (Dojindo, Japan). 10 ul CCK8 solution CCK8 solution (10 μL) was added to each well of the plate after different incubation times. The absorbance was measured 2h later at 450 nm using a microplate reader after 2 hours.

Cell invasion assays

Cell invasion was assessed with transwell plates (BD Biosciences, USA) as previously described (27).

Animal experiments

Male BALB/c nude mice (5-week old) were purchased from Beijing Vital River Laboratory Animal Technology Co, Ltd. (Beijing, China), and cultured at the specific pathogen-free (SPF) facility. A total of 3×10^6 stably transfected cells with genetically altered expression of SMS were subcutaneously injected into the axillary fossa of the nude mice (eight mice per group). Tumor length (L) and width (W) were measured every 4 days, and tumor volume was calculated as $0.5\times L\times W^2$. Stably transfected Mia-Paca2 cells with SMS overexpression were then injected into the tail veins of nude mice at a dose of 5×10^6 cells/mouse to establish the lung metastasis model.

Measurement of intracellular polyamines

Chromatography was performed with a Shiseido nanospace SI-2 HPLC system (Shiseido Co., Tokyo, Japan) coupled to a Shiseido MG C18 column (5 μm, 150 × 1.5 mm i.d.). A gradient eluent (A, 0.2% acetic acid; B, 0.2% acetic acid in acetonitrile) at100 μL/min was used. All data were recorded on a Thermo LCQ advantage iontrap MS equipped with electrospray ionization (ESI; Thermo,San Jose, CA, USA) operated in the positive ionization mode. The operating conditions were set as follows: spray voltage, 6 kV; capillary voltage, 4 V; tube lens offset voltage,40 V; sheath gas flow rate, 30 units; and capillary temperature, 250°C. In tandem MS analysis, the protonated molecular ions were fragmented by helium gas collisions. Levels of polyamines were normalized to protein amount (28).

RNA extraction, reverse transcription and quantitative PCR

Total RNA was extracted by Trizol Reagent (Invitrogen) from cells. cDNA was obtained from total RNA with PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan). The mRNA expression was assessed by Real-time quantitative PCR. The primers for RT-qPCR are shown in Table S3.

mRNA stability assay

Cells were plated in 6-wells dish and incubated with actinomycin D (Santa Cruz) at 5 μM for the indicated time. The first time point (t = 0 h) was taken after 10 min, then 4 and 8 h. Total RNA extracted from each sample was used for reverse transcription and qRT–PCR analysis.

MeRIP-qPCR

M^6A RNA immunoprecipitation (MeRIP) was performed with Magna MeRIP m^6A kit (17–10, 499, Millipore) according to the manufacturer's instructions.

Luciferase reporter assay

The wild type (SMS-WT) and m^6A sites mutated SMS (SMS-MUTE 1, 2, 3) were constructed into luciferase reporter vector pmir-GLO. After 48 h transfection, the cells were lysed by passive lysis buffer. Firefly Luciferase and Renilla Luciferase of lysis were detected, respectively.

Statistical analysis

Statistical analyses were performed using the SPSS software (version 17.0). Differences between the indicated groups were compared using the t-tests and one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. The cumulative overall survival (OS) rates were calculated using the Kaplan–Meier method, and differences between curves were evaluated using the log-rank test. P value < 0.05 was considered statistically significant.

Results

SMS expression is increased in pancreatic cancer and is related to prognosis

We analyzed the GEO database to determine the differentially expressed genes in pancreatic cancer and took the intersection of differential genes in GSE15471, GSE16515, GSE22780, and GSE71989. The results showed that there were 65 overlapping genes (Figure 1A), with 46.15% up-regulated and 53.85% down-regulated (Figure 1). Among these genes, *SMS* was the only gene related to polyamine metabolism. We further analyzed the expression of *SMS* in pancreatic cancer tissues. By analyzing the results in the GEO database, we found that the expression of *SMS* in pancreatic cancer tissues was significantly higher than that in adjacent tissues (Figures 1C–F). Additionally, we analyzed The Cancer Genome Atlas (TCGA) database and the results showed that the expression of SMS in cancer tissue specimens was significantly higher than that in adjacent tissues, and the expression of SMS was related to the prognosis of pancreatic cancer. The survival time of patients with high *SMS* expression was much shorter than that of the low expression group after surgery ($P < 0.05$) (Figure 1G). To further verify the results of bioinformatics analysis, we performed immunohistochemical analysis on 59 cases of cancerous and adjacent tissues. The results showed that the expression of *SMS* in cancerous tissues was significantly higher than that in adjacent tissues (Figures 1H–K). Moreover, survival analysis showed that the postoperative survival time of the SMS high expression group was significantly shorter than that of the low expression group (Figure 1L). Based on clinical-pathological data of patients, chi-square test analysis showed that the expression level of *SMS* was correlated with lymph node metastasis and tumor stage of pancreatic cancer, but not with sex, age, tumor differentiation, and tumor size (Table S1). Therefore, the expression of *SMS* in pancreatic cancer is increased and represents an important factor in the progression of pancreatic cancer, which is in line with the findings of Phanstiel et al in their study the relative mRNA expression of SMS was significantly increased in both PanIN and PDAC samples of 223 human patients (25).

SMS can promote the growth, migration, and invasion of pancreatic cancer *in vitro*

Western blotting showed that compared to the immortalized pancreatic epithelial cells HPDE, *SMS* expression in other cell lines was increased to varying degrees, and PANC-1 was representative of high expression, while Mia-Paca2 expression increased slightly (Figure 2A). Therefore, we used lentiviral transfection technology to construct a stable transfected cell line (Figure 2B). The growth curve showed that over-expression of *SMS* can promote the growth of pancreatic cancer cells, while knockdown of *SMS* can inhibit their proliferation (Figure 2C). Colony formation experiments also showed that increasing the level of *SMS* expression can increase the number of clones, whereas reducing *SMS* expression can inhibit the formation of clones (Figures 2E–H).The results of the wound-healing experiment showed that over-expression of *SMS* can promote the migration of pancreatic cancer cells while knocking down *SMS* significantly inhibited the migration rate (Figures 2I–L). Additionally, the results of transwell assays demonstrated that overexpression of *SMS* can promote the migration and invasion of pancreatic cancer cells, while knockdown of *SMS* had the opposite effect (Figures 2M–R). Therefore, increased expression of *SMS* in pancreatic cancer cells promotes the proliferation, migration, and invasion of pancreatic cancer cells.

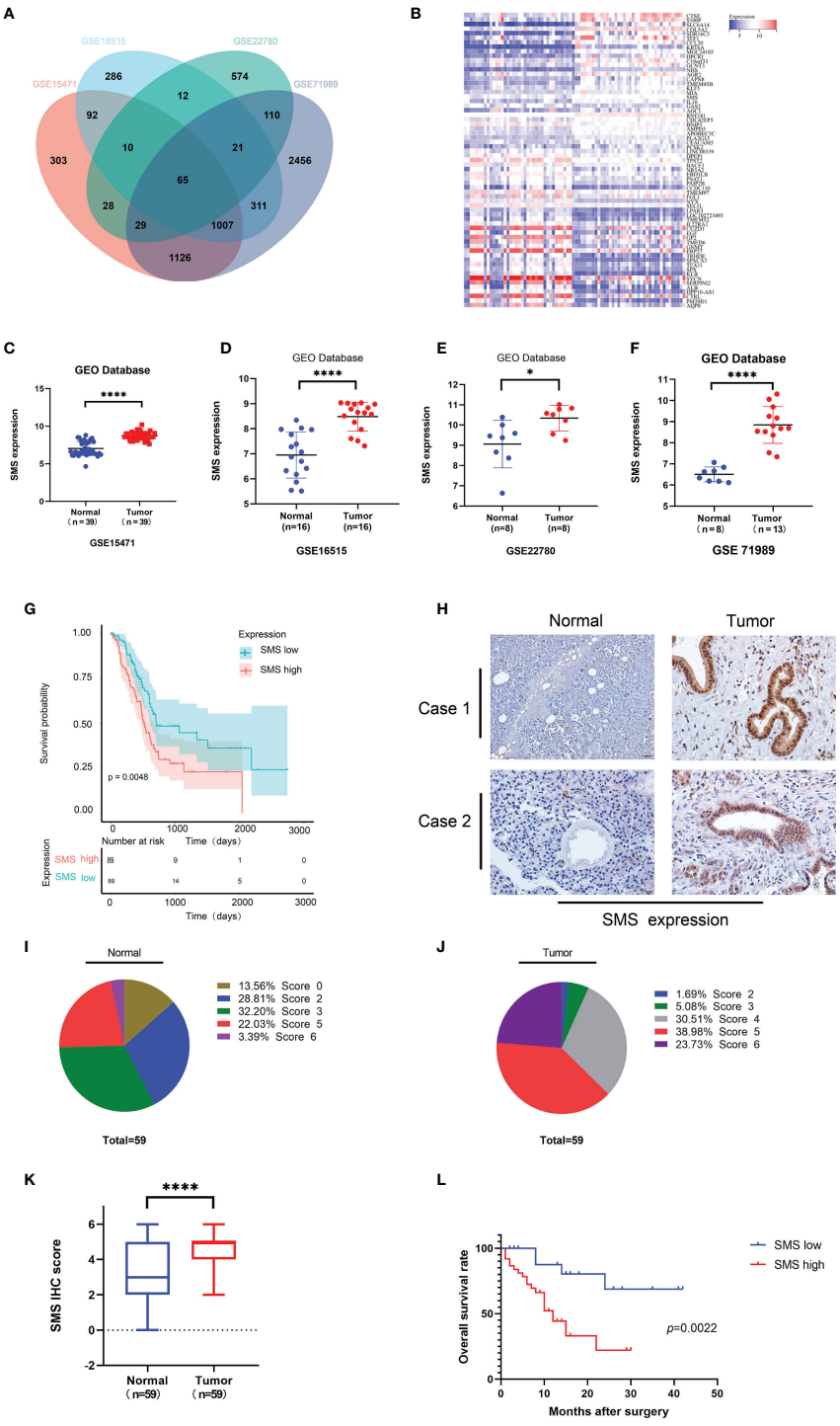

FIGURE 1
The expression and prognostic value of SMS in human pancreatic cancer. **(A)** Coincidence of differential Genes in GSE15471, GSE16515, GSE22780, and GSE71989 from GEO database. **(B)** The heat map of up-regulated genes and down-regulated genes in overlapping genes. **(C-F)** Expression level of SMS in pancreatic tumor and adjacent normal tissues from GEO database were analyzed. **(G)** Kaplan–Meier analyses of SMS high expression and low expression were analyzed from the TCGA database. **(H-K)** The expression of SMS in 59 paraffin embedded specimens from the internal cohort was determined by IHC staining. Representative IHC images are shown **(H)**, and the relative SMS staining intensity was scored **(I-K)**. Scale bar, 200 μm. **(L)** Kaplan–Meier analyses of the correlations between SMS expression and overall survival of all PDAC patients. *P < 0.05; ****P < 0.0001.

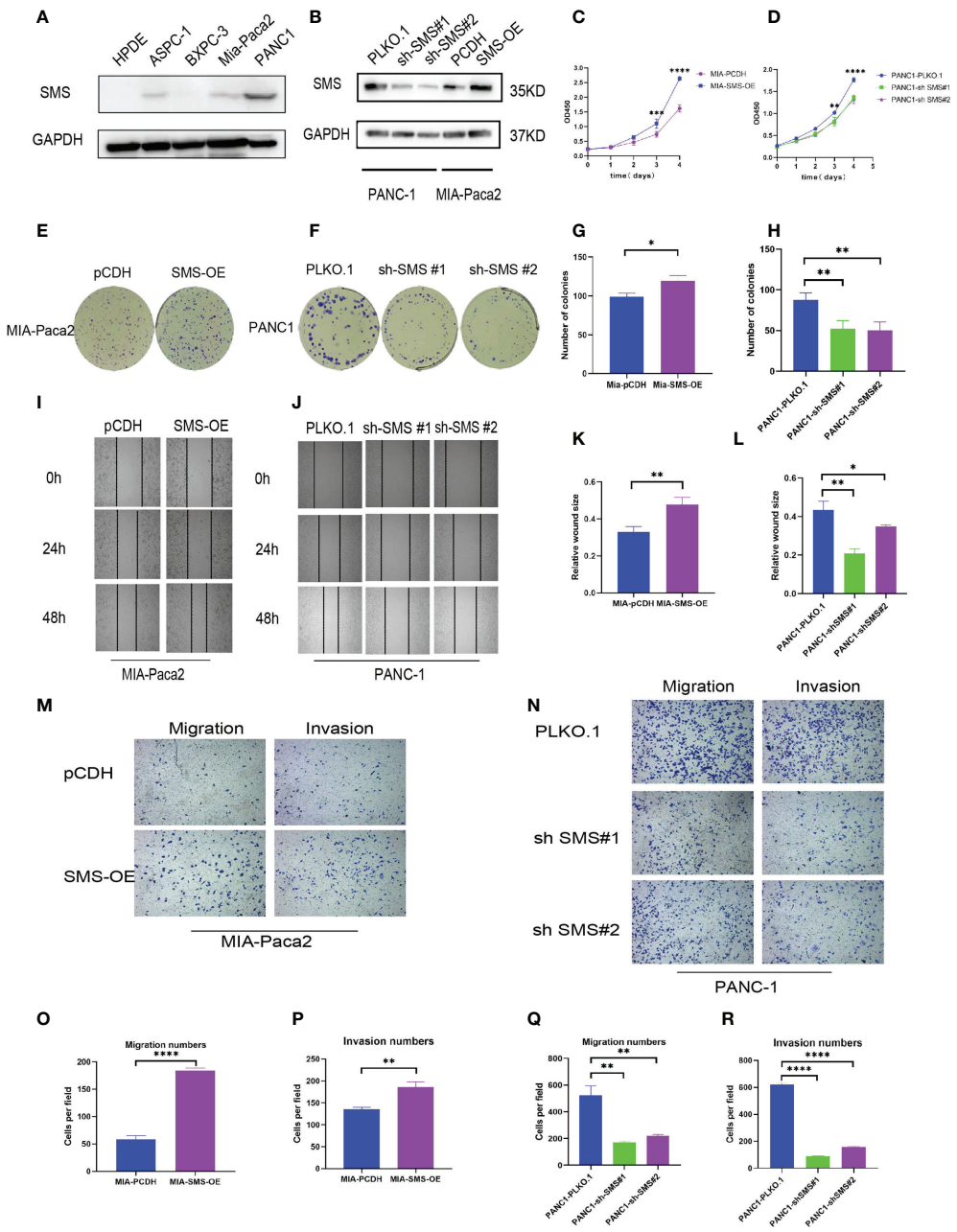

FIGURE 2
SMS can promote the growth, migration and invasion of pancreatic cancer. **(A)** The protein levels of *SMS* in normal human pancreatic duct epithelial (HPDE) cells and selected human pancreatic cancer cell lines were quantitated by western blot assays. **(B)** Mia-Paca2 cells with stable SMS overexpression PCDH-SMS-OE and PANC-1 cells with *SMS* knockdown PLKO.1-shSMS#1 and PLKO.1-shSMS#2 were generated. The changes in *SMS* expression were conformed using western blot. **(C, D)** The proliferative ability of stably transfected PANC-1 or Mia-Paca2 cells was investigated *via* CCK-8 assays. **(E-H)** Representative colony formation images are shown **(E, F)**, and the numbers of colonies were summarized **(G, H)**. **(I-L)** Wound-healing assays with stably transfected Mia-Paca2 **(I)** and PANC-1 **(J)** cells were performed. Representative images and quantifcation of wound closure are presented **(K, L)**. **(M-R)** Transwell assays with stably transfected Mia-Paca2 **(M)** and PANC-1 **(N)** cells were performed. Representative images and quantifcation of the results are presented. **(O-R)** *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

SMS promotes pancreatic cancer metastasis, which is mediated through the AKT/EMT signaling pathway

Treatment with spermidine *in vitro* can inhibit the phosphorylation of AKT, thereby inhibiting cell growth (20). We speculate that changes in the expression of SMS in pancreatic cancer could affect the levels of spermidine and spermine in cells, while changes in spermidine/spermine levels could affect the phosphorylation of AKT and ultimately affect the activation and inhibition of the AKT signaling pathway. Therefore, we detected spermine and spermidine levels in the cells. The results showed that the spermine level was significantly increased in cells overexpressing SMS, while after knocking down SMS, the spermidine level was significantly accumulated (Figures 3A, B). It is noteworthy that similar results of spermidine buildup have been seen in the fibroblasts of patients with Snyder Robinson Syndrome which have defective SMS (29). To further verify the relationship between *SMS* and *AKT*, western blot analysis was performed and showed that over-expression of *SMS* can up-regulate the level of p-AKT while knocking down *SMS* can reduce the expression of p-AKT (Figure 3C). We further verified the relationship between *SMS* content and the corresponding pathway. The results showed that *SMS* overexpression can promote the EMT pathway. As a key protein in EMT, E-cadherin was decreased whereas snail and Vimentin were increased. Inhibition of *SMS* expression had the opposite effect (Figure 3D). Moreover, we have also found that overexpression or knockdown of *SMS* expression can affect *ERK* and *mTOR* phosphorylation (Figures S2 A, B). Furthermore, we found that the addition of spermidine/spermine could reverse the expression of AKT phosphorylation (Figure 3E) and the migratory ability of *SMS*-overexpressed or knockdown -stable cells by Transwell assay (Figure 3F). Therefore, *SMS* promotes AKT phosphorylation and affects the EMT pathway by converting spermidine to spermine, ultimately leading to pancreatic cancer progression. It should be realized that SMS may also directly act on other oncogenes through protein-protein interactions. There are three domains in SMS protein (29, 30). By searching The Molecular INTeraction Database (MINT database), we found some proteins that may interact with SMS proteins, including IMMP2L, MAPK6, MAPKAPK3, MAPK8IP2, RPS6KA3. Furthermore, through Co-IP experiments we found that SMS protein may interact with MAPKAPK3 (data not shown), but this part of the mechanism needs to be verified in future studies.

METTL3 and *IGF2BP3* are involved in m6A modification of *SMS* and promote pancreatic cancer progression

We have demonstrated that expression of *SMS* is upregulated in pancreatic cancer, thereby promoting its progression. However, the underlying mechanism remains to be determined. By searching the GEO database, we found that knockout of both *METTL3* and *METTL4* significantly reduced the expression of *SMS* (Figure 4A). Through the GEO database, it was found that expression of *METTL3* and *IGF2BP3* in pancreatic cancer tissues were higher than those in adjacent tissues (Figures 4B, C). We also analyzed the expression of m6A readers in pancreatic cancer tissues from the TCGA database, and the expression of *IGF2BP3* was associated with the prognosis of pancreatic cancer (Figure S1A). Additionally, the GEPIA online prediction website showed that the expression of *IGF2BP3* mRNA in pancreatic cancer was positively correlated with the expression of *SMS* (Figure 4D). Therefore, we speculate that *METTL3* can regulate *SMS* mRNA m6A methylation and be recognized by *IGF2BP3* to further affect the prognosis of pancreatic cancer. Furthermore, the results of the immunohistochemical analysis showed that the expression of *METTL3* and *IGF2BP3* was consistent with that of *SMS* (Figure 4E). All results were scored and a chi-square test was performed, with the results showing that the expression of *METTL3/IGF2BP3* was significantly correlated with *SMS* expression (Figure 4F). These findings indicate that METTL3 may play a regulatory role in *SMS* expression. Moreover, it was found that the expression levels of *METTL3* and *IGF2BP3* were higher in cancer tissues than those in adjacent tissues (Figures 4G–J). Survival analysis showed that the expression of *METTL3* and *IGF2BP3* affected the prognosis of pancreatic cancer patients and that high expression of *METTLE/IGF2BP3* can promote the progression of pancreatic cancer (Figures 4K, L). Therefore, *METTL3* and *IGF2BP3* may play key roles in the m6A modification of SMS and pancreatic cancer progression.

METTL3 and *IGF2BP3* can promote the migration and invasion of pancreatic cancer

To further explore the role of *METTL3* and *IGF2BP3* in pancreatic cancer, we conducted western blot experiments by transfecting METTL3-siRNA and IGF2BP3-siRNA into PANC-1 and Mia-Paca2. The results showed that compared to the control group, interference with *METTL3* and *IGF2BP3* expression can reduce the expression of *SMS* (Figures 5A–D). Therefore, we further explored the role of *METTL3* and *IGF2BP3* on pancreatic cancer cell. In the migration and invasion experiments, interfering with the expression of *METTL3* and *IGF2BP3* inhibited the migration and invasion of pancreatic cancer (Figures 5E–L). Moreover, wound healing assay results showed that knockdown of *METTL3* expression significantly inhibited cell migration (Figure S1B). Furthermore, the overexpression of *METTL3* reversed the expression of *SMS* in *SMS* stable knockdown cells, while knockdown of *METTL3*

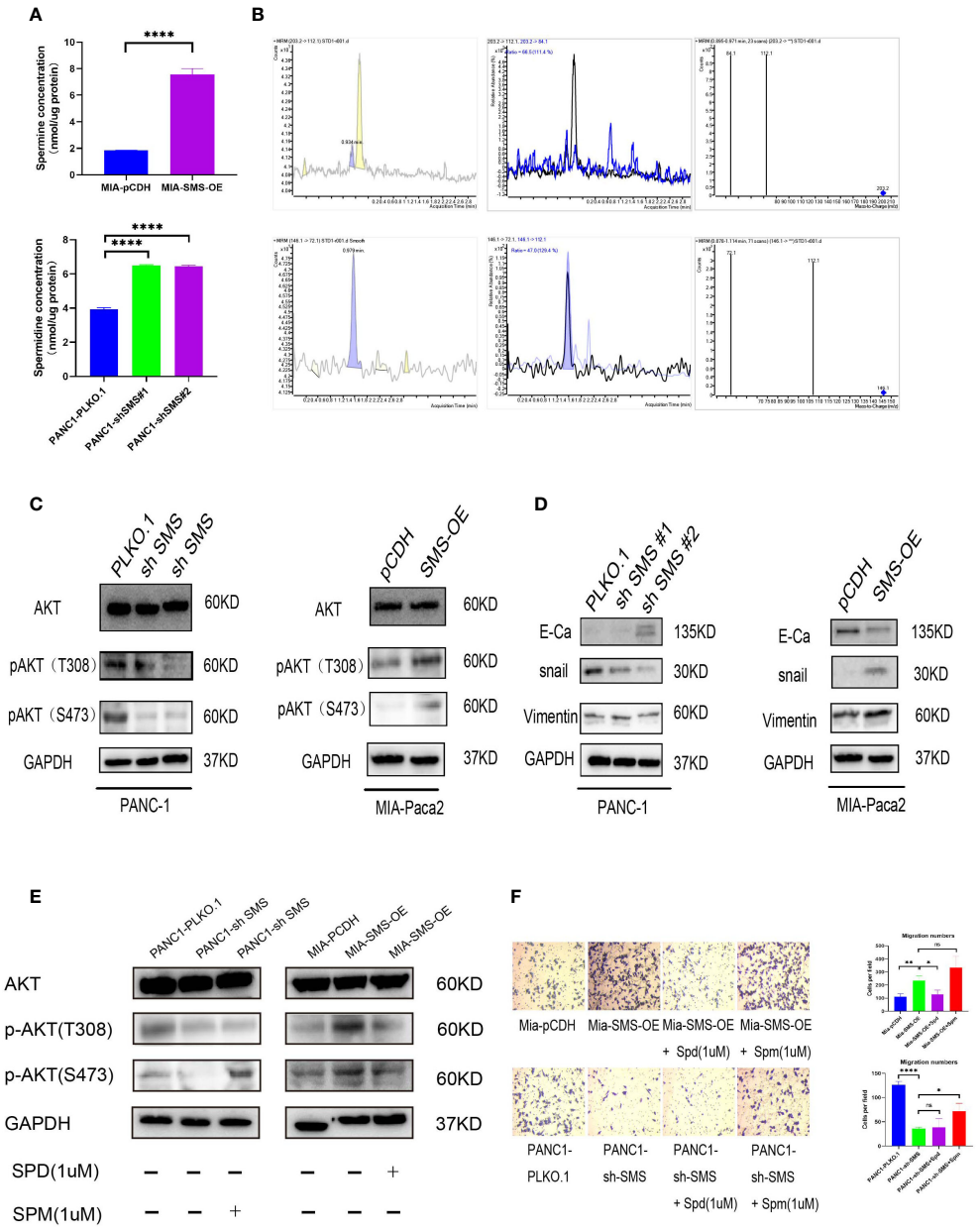

FIGURE 3
SMS promotes pancreatic cancer metastasis is mediated through the AKT/EMT signaling pathway. **(A)** SMS metabolizes spermidine to spermine. The levels of spermidine (SPD), and spermine (SPM) in SMS-OE or SMS-knockdown Mia-Paca2 and PANC1 cells were determined by LC-MS. **(B)** Mass spectrometry images of spermidine and spermine detection. **(C, D)** The protein expressions of AKT and EMT pathway were analyzed. **(E)** Spermidine/spermine could rescue the expression of AKT phosphorylation. **(F)** Spermidine/spermine could rescue the migration ability of overexpressed/knockdown SMS-stable cells by transwell assay. *P < 0.05; **P < 0.01; ****P < 0.0001. ns, no significance.

reversed the protein expression of *SMS* in overexpressed *SMS* (Figures 6M, N). The Transwell migration assay also demonstrated that *METTL3* could reverse the migration ability of overexpressed/knockdown SMS pancreatic cancer cells (Figures 5O, P). The results of the CCK-8 assay showed that *METTL3* could reverse the proliferation of stably transfected cells (Figure 5Q). Therefore, the highly expressed SMS protein in pancreatic cancer may be related to the expression of *METTL3* and *IGF2BP3*, and the expression of *METTL3* and *IGF2BP3* related to the migration and invasion of pancreatic cancer.

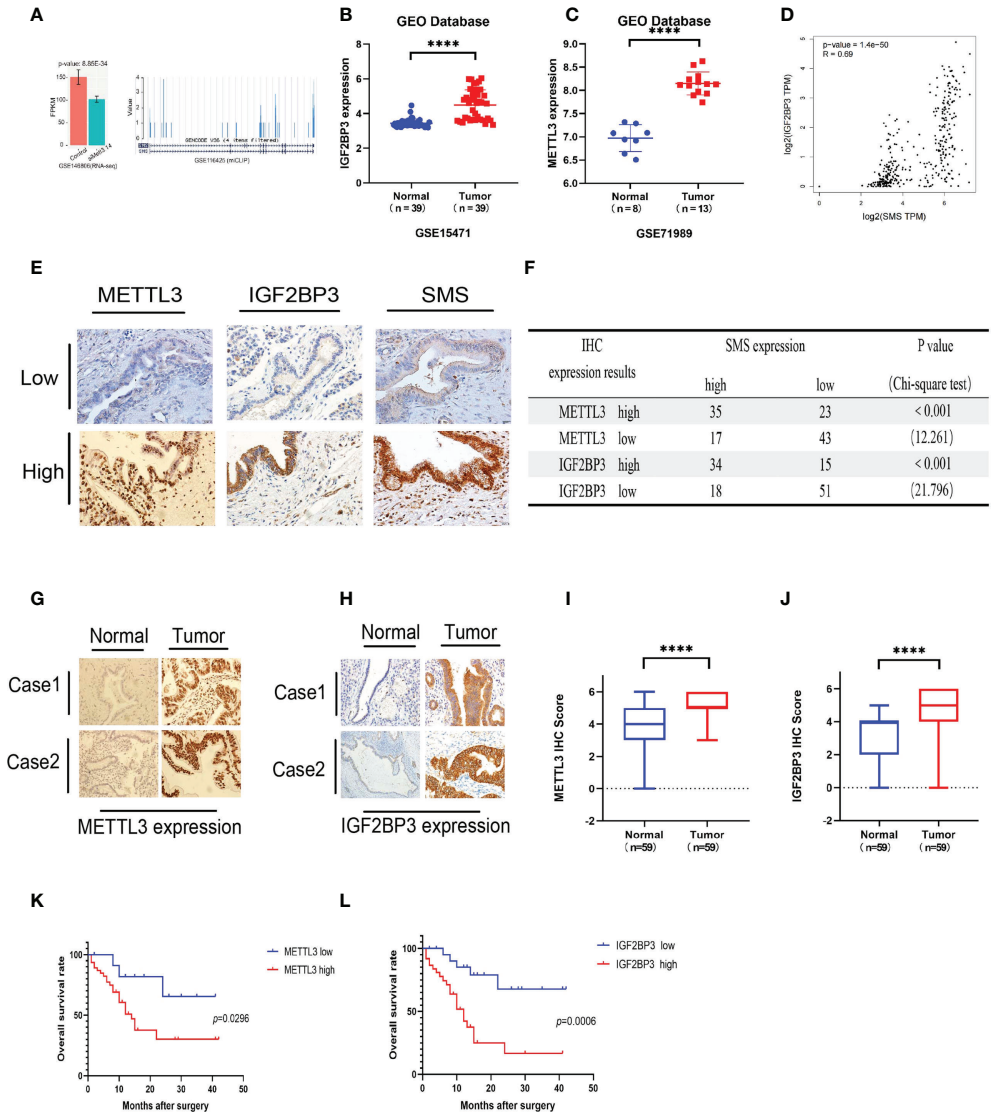

FIGURE 4
METTL3 and IGF2BP3 are highly expressed in pancreatic cancer and predicts poor prognosis of pancreatic cancer patients. **(A)** The expression of SMS in the METTL3 and METL14 double knock chips in the GEO database. **(B, C)** The expression of METTL3 and IGF2BP3 in cancer tissues and adjacent tissues. **(D)** The GEPIA database showed that IGF2BP3 was positively correlated with SMS mRNA expression. **(E)** The expressions of METTL3/IGF2BP3 and SMS in tissues of PDAC patients were evaluated by IHC. **(F)** The correlation between METTL3/IGF2BP3 and SMS levels in tissues was analyzed. **(G-J)** The expression of METTL3 and IGF2BP3 in pancreatic cancer tissues was higher than that in adjacent tissues by IHC, and the relative METTL3/IGF2BP3 staining intensity was scored. **(K, L)** Kaplan–Meier analyses of the correlations of METTL3/IGF2BP3 expressions and overall survival of PDAC patients. ****P < 0.0001.

The METTL3-IGF2BP3 axis promotes increased *SMS* mRNA stability and protein expression by mediating *SMS* m6A modification

We next performed a methylated RNA immunoprecipitation (ME-RIP) analysis to further explore the regulatory relationship between the METTL3-IGF2BP3-axis and *SMS*. The results showed that knockdown of METTL3 significantly reduced m6A level of SMS mRNA (Figures 6A, D). The RNA stability experiment results showed that compared to the control group, interference with *METTL3* and *IGF2BP3* led to an increase in the degradation rate of *SMS* mRNA (Figures 6B, C, E, F). We then constructed three mutant SMS CDS plasmids for the luciferase reporter assay to determine the specific modification sites (Figure 6G). The results of the dual-luciferase reporter experiment showed that the transcriptional level of wild-type, MUT1 or MUT2, but not MUT3, was significantly decreased with

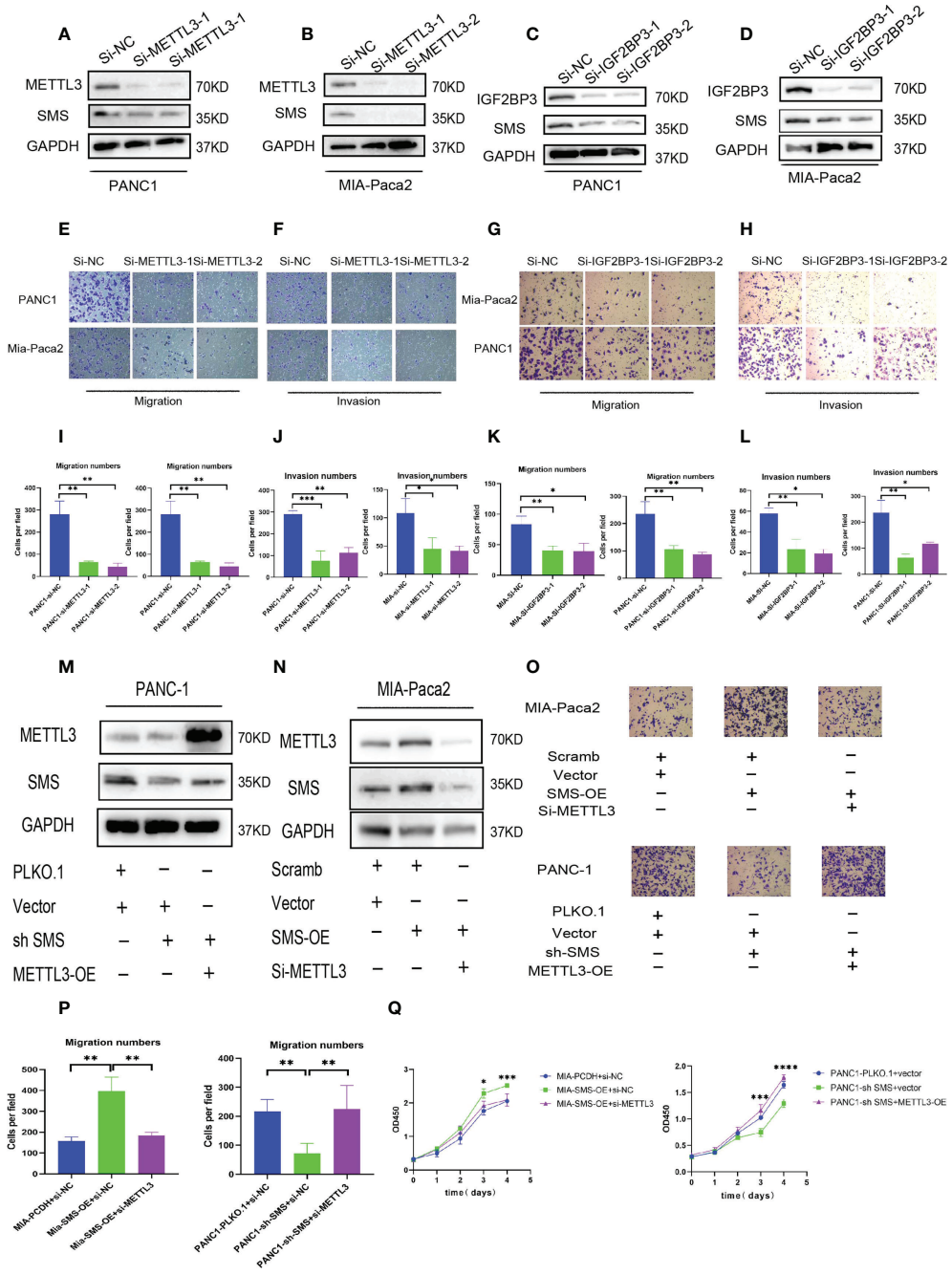

FIGURE 5
METTL3 and *IGF2BP3* regulate *SMS* expression and promote pancreatic cancer metastasis. **(A-D)** The expression level of *SMS* interferes with the expression of *METTL3* and *IGF2BP3* in Mia-Paca2 and PANC1 cells. **(E-L)** Transwell assays of SMS interferes with the expression of *METTL3* and *IGF2BP3* in Mia-Paca2 and PANC1 cells were performed. Representative images and quantifcation of the results are presented. **(M, N)** *METTL3* reversed the expression of *SMS* in stable SMS overexpression/knockdown PDAC cell. **(O, P)** Transwell experiments found that *METTL3* could reverse the migration ability of stable SMS overexpression/knockdown PDAC cell. **(Q)** CCK-8 experiments found that *METTL3* could reverse the proliferation of stable SMS overexpression/knockdown PDAC cell. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

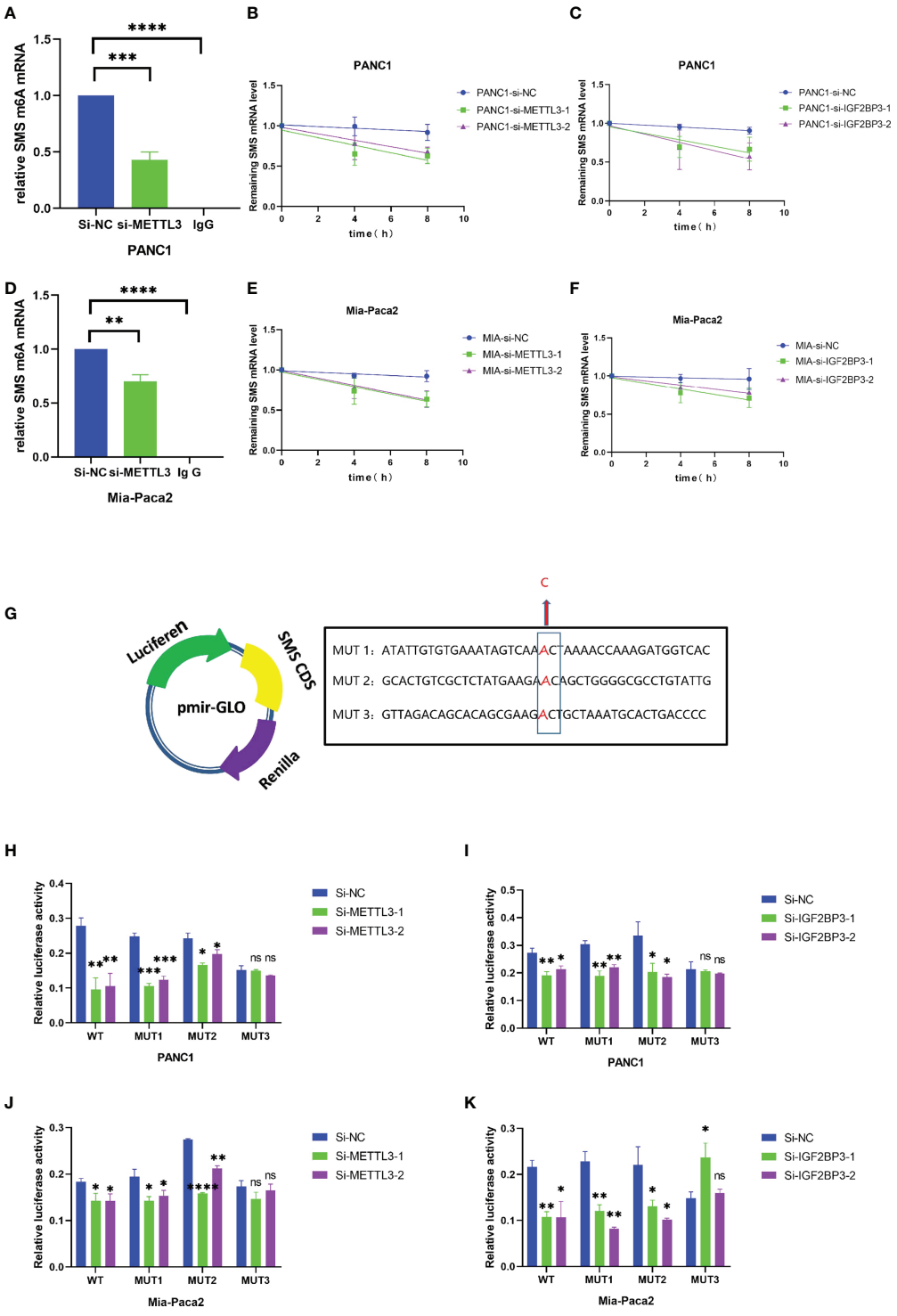

FIGURE 6
The METTL3-IGF2BP3 axis promotes increased *SMS* mRNA stability and protein expression by mediating *SMS* m6A modification. **(A, D)** ME-RIP assays showed the relative percentage of *SMS* mRNA with methylation. **(B, C, E and F)** The mRNA stability and degradation halftime of *SMS* in Mia-Paca2 and PANC1 treated by Actinomycin D. **(G)** Wild-type or mutant 1-3 sites m6A consensus sequence was fused with firefly luciferase reporter, respectively. **(H-K)** Mutation of m6A sites in *SMS* (constructed in firefly reporter) repressed the luciferase expression of reporter. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, no significance.

METTL3 or IGF2BP3 knockdown (Figures 6H–K), assuming that the regulation of SMS expression was under control of METTL3 associated m6A modification, which was acted mainly through the MUT3 site since SMS expression in the presence of intact Site 3 as in wild-type, MUT1 or MUT2 correlated with METTL3 status.

SMS can promote tumor growth and metastasis *in vivo*

A subcutaneous tumor model was established by subcutaneously injecting the pancreatic cancer cells into nude mice. Compared to the control group, overexpression of *SMS* can promote tumor growth, while knockdown of *SMS* can significantly inhibit tumor formation and slow the rate of tumor growth (Figure 7A–F). The immunohistochemistry results showed that knockdown of *SMS* resulted in decreased phosphorylation of AKT, while overexpression of *SMS* resulted in increased phosphorylation of AKT (Figure 7). Stably transfected Mia-Paca2 cells with SMS overexpression were then injected into the tail veins of nude mice to establish the lung metastasis model. Additionally, the anatomy of the lung metastasis model mice showed that the number of lung metastases in the *SMS* overexpression group was greater than that in the control group (Figure 7I). H-E staining showed similar results (Figure 7). Therefore, these results indicate that SMS can promote the growth and metastasis of pancreatic cancer *in vivo*.

Discussion

At present, the incidence of pancreatic cancer is increasing annually (31). As a grave disease with high mortality, research into pancreatic cancer has achieved widespread attention. (1, 32) Despite extensive research, the specific molecular mechanism underlying the metastasis of pancreatic cancer has not yet been elucidated.

SMS is an enzyme in the pathway of polyamine synthesis, which can specifically convert spermidine to spermine and plays an important role in polyamine synthesis and metabolism (13). At present, there are relatively few studies on *SMS* in tumors, although studies have shown that overexpression of *SMS* can promote the progression of colon cancer (21). Ja Ladanki et al. demonstrated that the use of *SMS* inhibitors can inhibit the level of polyamines, thereby inhibiting tumor cell proliferation (33). Numerous studies have shown that the level of polyamines in tumors is significantly increased and that polyamines play a critical role in the growth of tumor cells (13, 16, 34). However, there remains little research on the role of each component in polyamines. Additionally, studies have shown that spermidine can inhibit the proliferation of myeloma cells, and exogenous spermidine can inhibit cell proliferation; this effect is caused by inhibiting the phosphorylation process of AKT (35). In this study, we confirmed that overexpression of *SMS* can promote the increase of spermine levels in pancreatic cancer cells, knockdown of SMS can lead to increased spermidine, and the level of *SMS* protein is positively correlated with AKT phosphorylation and the PI3K-AKT/EMT pathways. Moreover, at both cellular and animal levels, we also verified that SMS can promote the proliferation, migration, and invasion of pancreatic cancer.

The development of epigenetics has led to a deeper understanding of gene expression regulation (36, 37). m6A modification has been extensively studied as an important regulatory mechanism (38, 39). At present, the role of m6A in tumorigenesis and development has been proven (11, 40; Wei 38, 41). The role of m6A in pancreatic cancer has also been extensively studied, and some studies have shown that it can regulate pancreatic cancer progression by affecting alternative splicing of *METTL14* (42). In view of the multiple effects of m6A modification on tumors, further research is needed. By searching the m6A-ATLAs database, we found that *SMS* mRNA has many m6A modifications, with three m6A-specific sites predicted in *SMS* mRNA. Therefore, m6A modification plays a key role in the regulation of SMS expression. Through further exploration, we found that *METTL3* and *IGF2BP3* can significantly inhibit the migration and invasion of pancreatic cancer cells. Additionally, interference with the expression of *METTL3* and *IGF2BP3* increased the degradation rate of *SMS* mRNA. These results well explain why the expression of *SMS* in pancreatic cancer is elevated, as well as the main cause of polyamine anabolic disorder in pancreatic cancer.

In summary, our findings verified the effects of *SMS* on the proliferation, migration, and invasion of pancreatic cancer *in vitro* and *in vivo*. Moreover, overexpression of *SMS* could change the spermidine/spermine levels and further regulated the phosphorylation process of AKT and the state of PI3K-AKT/EMT signaling pathways. Finally, regarding the SMS regulation mechanism, we verified that the METTL3-IGF2BP3-axis could promote the migration and invasion of pancreatic cancer and that *METTL3* and *IGF2BP3* further increased the stability of *SMS* mRNA by modifying *SMS* mRNA by m6A. This led to increased *SMS* protein expression, the regulation of which relied on m6A modification of its mRNA (Figure 8).

Conclusion

SMS promotes pancreatic cancer progression *in vitro* and *in vivo*. Overexpression of SMS could change the spermidine/

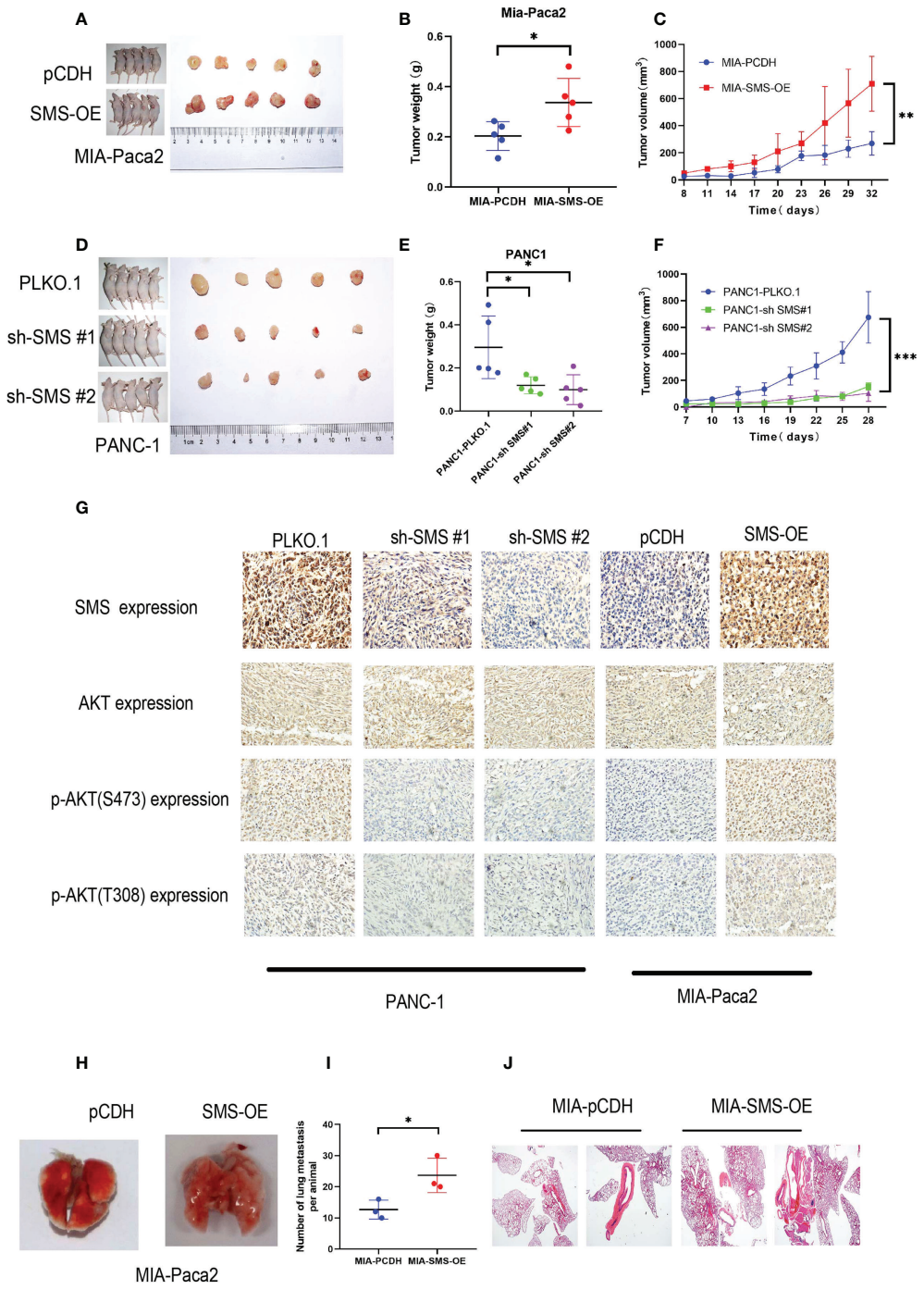

FIGURE 7
SMS can promote tumor growth and metastasis *in vivo*. **(A, D)** Tumor xenograft models were constructed with stable SMS-overexpressing (n = 5) or SMS-knockdown (n = 5) Mia-Paca2 cells and PANC1 corresponding negative control cells. **(B, E)** Then tumors were collected from sacrificed mice and tumor weights were measured. **(C, F)** The size of the tumors was measured at the indicated time points. **(G)** Tumors from mice were analyzed by IHC staining, Scale bar, 200 μm. **(H-J)** Representative images of lung metastasis and hematoxylin and eosin staining are shown. Metastatic nodules were counted with or without a microscope and recorded. Spd, spermidine;Spm, spermine.*P < 0.05; **P < 0.01; ***P < 0.001.

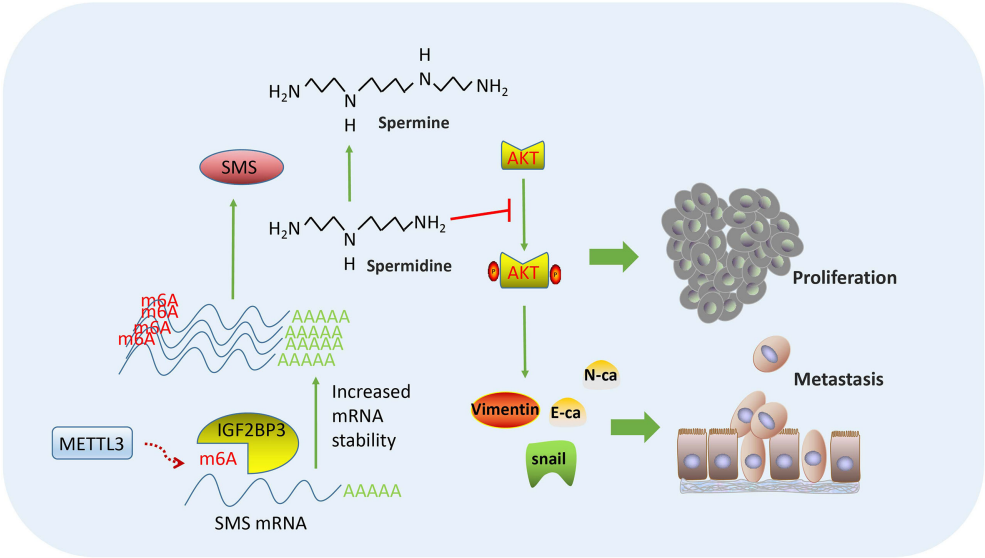

FIGURE 8
Flowchart of our experiments: METTL3-IGF2BP3 axis promotes pancreatic cancer progression by mediating *SMS* m6A modification.

spermine levels and further regulate the phosphorylation of AKT and the state of PI3K-AKT/EMT signaling pathways.The METTL3-IGF2BP3-axis could increase the stability of SMS mRNA in a m6A-dependent manner.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE15471, GSE16515, GSE71989 and GSE22780. The data used to support the findings of this study are available from the corresponding author upon request.

Ethics statement

The animal study was reviewed and approved by The animal experiments were approved by Animal Welfare Committee of Fujian Medical University (Fuzhou, China).

Author contributions

Conception and design: ZG, SW. Development of methodology: ZG, XZ, CL, YH, YZ, HG, ZZ. Acquisition of data (acquired and managed patients, provided facilities, etc.): ZG, XZ, CL, YH, YZ, HG, ZZ. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): ZG, XZ, CL. Writing, review, and/or revision of the manuscript: ZG, XZ, CL, SW. Study supervision: SW. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.962204/full#supplementary-material

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