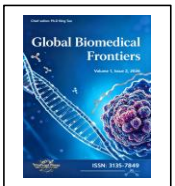
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Lentinus edodes polysaccharide MPSSS suppresses pancreatic cancer-associated fibroblast migration via HMGXB3

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Information

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a highly fibrotic tumor microenvironment (TME), which is dominated by cancer-associated fibroblasts (CAFs) that actively promote tumor progression and therapeutic resistance. Although the effects of MPSSS, a Lentinus edodes polysaccharide extract, on CAF immunosuppressive function and lymph angiogenesis have been reported, its impact on CAF migration and the role of HMGXB3 remain unknown. This study investigated the effects of MPSSS on CAF viability and migration and examined the potential involvement of HMGXB3. An in vitro CAF model was created by treating human pancreatic stellate cells with PANC-1-conditioned medium. The CAF phenotype was validated by the expression of α-smooth muscle actin (α-SMA), fibroblast activation protein-α (FAP) and interleukin-6 (IL-6). CAF viability was assessed using an MTT assay. HMGXB3 mRNA and protein expression were quantified using quantitative real-time PCR (qRT-PCR) and western blot analysis. CAF migration was evaluated using a Transwell assay. Normal fibroblasts served as controls. MPSSS reduced CAF viability in a dose-dependent manner ($IC_{50} \approx 0.95$ mg/ml), with significant inhibition occurring at concentrations of ≥0.8 mg/ml ($P < 0.05$). MPSSS significantly down-regulated HMGXB3 expression in CAFs at both the mRNA and protein levels, with no significant effect on normal fibroblasts. The Transwell assay showed that MPSSS markedly inhibited CAF migration, with a ~70% reduction observed at a concentration of 0.5 mg/ml ($P < 0.01$), which is below the cytotoxic threshold. HMGXB3 knockdown using small interfering RNA (siRNA) significantly inhibited CAF migration to a degree comparable to MPSSS treatment, and combined HMGXB3 knockdown with MPSSS exposure produced no additive effect, indicating that HMGXB3 is functionally required for CAF migration and mediates MPSSS-induced migratory inhibition. In conclusion, MPSSS suppresses CAF migration and viability through HMGXB3 downregulation. This suggests a novel strategy for TME remodeling in pancreatic cancer.

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Introduction

Pancreatic cancer is one of the malignancies with the poorest prognosis. It commonly develops as a result of germline mutations (e.g., BRCA1/2, PALB2 and CDKN2A) or acquired somatic mutations (e.g., KRAS, TP53, SMAD4 and CDKN2A). As pancreatic cancer typically exhibits no obvious symptoms in its early stages, around 80% of patients are diagnosed only after the disease has progressed to an advanced stage, resulting in a five-year survival rate of less than 10% (1,2). Pancreatic ductal adenocarcinoma (PDAC) accounts for over 90% of all pancreatic malignancies (3,4).

Current treatment strategies for pancreatic cancer include surgical resection, chemotherapy (e.g., gemcitabine, nab-paclitaxel and FOLFIRINOX) and radiotherapy. While these treatments can slow disease progression, their overall effectiveness is limited due to the aggressive nature of the cancer, its dense desmoplastic stroma and its therapeutic resistance. Postoperative recurrence is common, with rates exceeding 80% within two years of surgery, highlighting the urgent need for novel therapeutic approaches that target the tumor microenvironment (TME) (5).

The TME plays a critical role in pancreatic cancer progression and therapy resistance. The PDAC TME is characterized by a highly fibrotic and hypoxic stroma comprising the extracellular matrix (ECM), immune cells, vascular structures and cancer-associated fibroblasts (CAFs). CAFs constitute up to 80% of the tumor mass and are among the major stromal cell populations (6). They promote tumor progression by increasing ECM deposition, enhancing tumor cell invasion and metastasis, supporting angiogenesis, regulating immune responses and limiting drug penetration through physical barriers (7,8). CAF migration is a critical process in TME remodeling, as migrating CAFs contribute to ECM deposition, create tracks for cancer cell invasion and establish pre-metastatic niches (9,10). Pancreatic stellate cells (PSCs) are the main source of CAFs in PDAC and can be activated by tumor-derived signals to acquire a myofibroblastic phenotype (11).

CAFs are highly heterogeneous and can promote or suppress tumor progression depending on their activation state and subtype composition (12,13). Integrated transcriptomic analyses have identified distinct tumor and stroma-specific subtypes of PDAC (14). Transgelin (TAGLN) has been identified as a hallmark gene of pro-tumorigenic myofibroblastic CAFs (myCAFs) (15). As complete CAF ablation can paradoxically enhance tumor aggressiveness (16,17), functional reprogramming of CAFs is a more promising therapeutic strategy than elimination. This paradigm shift from CAF depletion to CAF re-education has received increasing attention (18).

Natural polysaccharides have attracted considerable attention as potential TME modulators due to their immunomodulatory, anti-inflammatory and anti-tumor properties (19,20). Lentinus edodes (shiitake mushroom) polysaccharides, particularly β-glucans such as lentinan, have been shown to activate macrophages, natural killer cells and dendritic cells (21,22).

MPSSS (23) is a purified polysaccharide extract derived from Lentinus edodes, characterized by a molecular weight of approximately 577.2 kDa and a monosaccharide composition of glucose (75.0%), galactose (11.7%), mannose (7.8%) and xylose (0.4%). Notably, MPSSS has been reported to attenuate CAF immunosuppressive function via TLR4-NF-κB (24), reduce CAF-derived TGF-β1 to prevent chemoresistance (25), and inhibit CAF-mediated lymph angiogenesis through TLR4/JNK (26). These findings establish MPSSS as a multifunctional CAF modulator. However, it is unclear whether MPSSS can regulate CAF migration. Furthermore, the molecular mechanisms underlying MPSSS-mediated CAF modulation, particularly the potential involvement of transcription factors such as HMGXB3, remain to be investigated.

High-mobility group (HMG) proteins are a family of non-histone chromosomal proteins that regulate DNA architecture and gene transcription. HMGB family members, including HMGB1, HMGB2, HMGB3 and HMGXB3, have been associated with various cellular processes such as proliferation, differentiation and migration (27). While HMGB3 has been extensively studied in PDAC, HMGXB3—a structurally related but functionally distinct HMG-box transcription factor—remains under-explored in cancer biology. Emerging evidence links HMGXB3 to cell migration in breast, colorectal and renal cancers (28-30). In colorectal cancer, the DICER/miR-324-5p axis regulates HMGXB3 and WASF-2, a key component of the Arp2/3 complex that drives actin polymerization and lamellipodium formation. This suggests that HMGXB3 may function as a conserved transcriptional regulator of cell motility.

Given the critical role of CAFs in PDAC progression and the emerging paradigm of CAF re-education therapy, the present study aimed to investigate: (i) the effects of MPSSS on CAF viability and migration; (ii) changes in HMGXB3 expression in CAFs following MPSSS treatment; and (iii) whether HMGXB3 is functionally required for CAF migration and mediates MPSSS-induced migratory inhibition using siRNA-mediated knockdown.

Materials and methods

Preparation and characterization of MPSSS. MPSSS was extracted and purified from crude Lentinus edodes polysaccharides (supplied by Zhongzhikanggu Company, Hangzhou, China) in our laboratory. The polysaccharides were dissolved in deionized water at 4 °C with gentle stirring overnight. The solution was then centrifuged at 8,000×g for 15 minutes at 4 °C and the supernatant collected. Anhydrous ethanol was then added in steps at volume ratios of 1:1, 2:1 and 3:1 (ethanol: water) to precipitate the polysaccharides. The precipitate was collected by centrifugation at 10,000×g for 20 min at each step. The final precipitate was then washed sequentially with anhydrous ethanol and anhydrous ether before being lyophilized to produce a grey-white MPSSS powder. Purity was confirmed by UV spectrophotometry (no protein or nucleic acid contamination was present at 280 nm and 260 nm,

respectively). The molecular weight (577.2 kDa) and monosaccharide composition (glucose 75.0%, galactose 11.7%, mannose 7.8%, xylose 0.4%) were characterized by high-performance gel permeation chromatography and high-performance anion-exchange chromatography, respectively. These results are consistent with previously reported data (23). For experiments, MPSSS was dissolved in DMEM to prepare a 5 mg/ml stock solution. This was then filter-sterilized through a 0.22 μm membrane and stored at -20°C.

Cell culture and establishment of the CAF model. This study used human pancreatic stellate cells (hPSCs, ScienCell Research Laboratories, Carlsbad, CA, USA, Cat# 3830), normal human fibroblasts (NFs, ATCC CRL-2522) and the human pancreatic cancer cell line PANC-1 (ATCC CRL-1469). All cell lines were authenticated by short tandem repeat profiling and tested negative for mycoplasma contamination. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured under standard conditions of 37 °C, 5% CO_2 and saturated humidity. Once the cells had reached 80–90% confluence, they were passaged every two to three days using 0.25% trypsin-EDTA (Gibco, USA).

To establish a CAF-like model, hPSCs were treated with 20% PANC-1-conditioned medium (CM) for two weeks. The CM was prepared by culturing PANC-1 cells in DMEM supplemented with 10% FBS for 48 hours at 37 °C. The supernatant was then collected, centrifuged at 1,000 × g for 10 minutes to remove cell debris, and diluted with fresh DMEM to a final concentration of 20%. The hPSCs were supplied with fresh 20% PANC-1 CM every two days. During the induction period, the hPSCs exhibited the characteristic morphological changes associated with CAF activation, such as enlargement of the cell body, elongation of the cytoplasmic processes and loss of lipid droplets. To validate the CAF phenotype, quantitative reverse transcription PCR (qRT-PCR) and western blot analysis were performed to detect established CAF markers, including α-smooth muscle actin (α-SMA), fibroblast activation protein-α (FAP) and interleukin-6 (IL-6), as well as the quiescent PSC marker desmin.

MTT cell viability assay. CAFs and NFs were seeded into 96-well plates at a density of 1,000 cells per well in 100 μl of complete medium. After 24 hours, the medium was replaced with fresh, serum-free DMEM containing various concentrations of MPSSS: 0 mg/ml (control), 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1.0 mg/ml. Each treatment group included six replicate wells, and the experiment was independently repeated three times.

After 24 hours, 10 μl of MTT solution (5 mg/ml in PBS, Sigma-Aldrich, USA) was added to each well. The cells were then incubated at 37 °C for 4 hours.

After incubation, the culture medium was removed and 150 μl of dimethyl sulfoxide (Sigma-Aldrich, USA) was added to each well to dissolve the purple formazan crystals. The plate was then shaken gently for 10 minutes at room temperature in the dark. Absorbance was measured at 570 nm using a microplate reader (BioTek Epoch 2, USA). Cell viability was calculated as follows: Absorbance (treatment)/Absorbance (control) × 100%. The half-maximal inhibitory concentration (IC_{50}) was calculated using nonlinear regression analysis (GraphPad Prism 9.0).

Quantitative real-time PCR (qRT-PCR). qRT-PCR was performed to determine the effect of MPSSS on the expression of HMGXB3, α-SMA, FAP and IL-6. CAFs, NFs and hPSCs were seeded into 6-well plates at a density of 2×10^5 cells per well. After 24 hours of culture, the medium was replaced with MPSSS-containing medium at the following concentrations: 0 mg/ml (control), 0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml. Each well received 3 ml of the treatment medium.

Once the cells were 80–90% confluent, total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). A260/A280 ratios between 1.8 and 2.0 were considered acceptable. The RNA was then reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara, Japan), following the manufacturer's protocol.

qRT-PCR was performed using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA) and PowerUp SYBR Green Master Mix (Applied Biosystems, USA). The thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. A melting curve analysis was performed after each run to confirm amplification specificity. Melting curve analysis confirmed single-peak amplification for all primers.

GAPDH was used as the internal reference gene. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (31). The primer sequences used in this study were synthesized by Sangon Biotech (Shanghai, China) and are listed in Table I. Primer efficiency was validated by standard curve analysis ($R^2 > 0.98$; efficiency 90–110%).

Western blot analysis. Total protein was extracted from CAFs, NFs and hPSCs using RIPA lysis buffer (Beyotime, China), to which a protease inhibitor cocktail (Sigma-Aldrich, USA) was added. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, USA). Equal amounts of protein (30 μg per lane) were separated by SDS-PAGE using a 10% gel and transferred onto PVDF membranes (Millipore, USA) at 100 V for 90 minutes at 4 °C. The membranes were then blocked with 5% non-fat milk in TBST at room temperature for 1 h and incubated overnight at 4 °C with the following primary antibodies: HMGXB3 (1:1000, Abcam, ab198506); α-SMA (1:1000, Abcam, ab5694); FAP (1:1000, R&D Systems, AF3715); IL-6 (1:1000, Cell Signaling Technology, #12153); desmin (1:1000, Abcam, ab32362); and GAPDH (1:5000, Abcam, ab9485). After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (1:5000, Jackson ImmunoResearch, USA) for 1 h at room temperature. Protein bands were visualised using an enhanced chemiluminescence detection system (Bio-Rad, USA) and quantified using ImageJ software (NIH, USA). Protein expression was normalized to GAPDH.

Transwell migration assay, A 24-well Transwell chamber system containing 8.0 μm polycarbonate membranes (Corning, USA) was used to evaluate the effect of MPSSS on the migration of CAFs and NFs. Uncoated Transwell inserts were used to assess cell migration (invasion was not evaluated). The CAFs and NFs were pretreated with various concentrations of MPSSS (0 mg/ml, 0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml) in complete medium for 24 hours.

After pretreatment, the cells were harvested, washed with PBS and resuspended in serum-free DMEM at a density of 5×10^4 cells/ml. A total of 200 μl of the cell suspension was seeded into the upper chamber. The lower chamber was filled with 600 μl of DMEM containing 10% FBS as a chemoattractant. After 24 h of incubation at 37℃ in 5% CO_2, cells remaining on the upper surface of the membrane were gently removed with a cotton swab. The cells that had migrated to the lower surface were then fixed with 4% paraformaldehyde for 15 minutes and stained with a 0.1% crystal violet solution for 20 minutes at room temperature. Excess stain was removed by washing with PBS. Images were captured using an Olympus IX73 inverted microscope (Japan) at 200× magnification. Three random fields were selected from each insert for quantitative analysis using ImageJ software (NIH, USA).

HMGXB3 siRNA transfection. To assess the functional requirement of HMGXB3 for CAF migration, two independent HMGXB3-specific small interfering RNAs (siRNAs) and a non-targeting negative control siRNA (NC-siRNA) were synthesized by GenePharma (Suzhou, China). The siRNA sequences were as follows: si-HMGXB3-1, 5'-GCAGCUCAAGAAGAUCAAUTT-3'; si-HMGXB3-2, 5'-CCAGAAGAUCUACGAGAAUTT-3'; and NC-siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3'. CAFs were seeded into 6-well plates at a density of 2×10^5 cells per well and cultured for 24 h to reach 60–70% confluence. Transfection was performed using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol. Briefly, 50 nM siRNA was diluted in Opti-MEM medium and mixed with Lipofectamine 3000 reagent. After incubation for 15 minutes at room temperature, the mixture was added to the cells. After 6 hours, the transfection medium was replaced with complete DMEM. At 48 hours post-transfection, cells were harvested for qRT-PCR analysis to confirm knockdown efficiency or subjected to Transwell migration assay and MTT viability assay as described above. For the combined treatment group, cells were transfected with HMGXB3 siRNA for 24 hours, then treated with 0.5 mg/ml MPSSS for an additional 24 hours before functional assays.

Statistical analysis. All experiments were performed in triplicate and repeated independently at least three times. Data are presented as the mean ± standard deviation (SD). The normality of the data distribution was assessed using the Shapiro–Wilk test. One-way analysis of variance was used to determine statistical significance, followed by a Tukey's post-hoc test for multiple comparisons, using GraphPad Prism 9.0 software (GraphPad Software, USA). A p-value of less than 0.05 was considered statistically significant. Pearson correlation analysis was performed to assess the relationship between HMGXB3 mRNA expression and the number of migrated cells.

Results

Establishment and validation of the in vitro CAF model, and MPSSS selectively inhibits CAF viability. Human pancreatic stellate cells were treated with 20% PANC-1-conditioned medium for two weeks to induce a CAF-like phenotype. Morphological observation revealed that hPSCs underwent characteristic activation changes, transforming from a quiescent star-shaped morphology to an activated spindle-shaped morphology resembling CAFs.

To validate the CAF phenotype at the molecular level, qRT-PCR was performed to detect established CAF activation markers. As shown in Fig. 1A, CAF-activated hPSCs exhibited significantly upregulated mRNA expression of α-SMA (4.2±0.3-fold, $P<0.001$), FAP (3.8±0.4-fold, $P<0.001$) and *IL-6* (5.1±0.5-fold, $P<0.001$) compared with quiescent hPSCs. In contrast, the quiescent PSC marker desmin was significantly downregulated (0.3±0.1-fold, $P<0.01$) in CAFs. Western blot analysis further confirmed elevated α-SMA protein levels in CAFs compared with hPSCs (Fig. 1B). These results confirm successful activation of hPSCs into a CAF-like phenotype.

The MTT assay was performed to evaluate the effect of MPSSS on CAF and NF viability. As shown in Fig. 1C, MPSSS reduced CAF viability in a dose-dependent manner within the concentration range of 0–1.0 mg/ml. At concentrations of 0.4 and 0.6 mg/ml, CAF viability was moderately decreased to 85.3±4.2% and 78.6±5.1% of the control group, respectively ($P>0.05$). Significant inhibition was observed at 0.8 mg/ml, with cell viability reduced to 62.4±3.8% ($P<0.05$) and further decreased to 48.7±4.5% at 1.0 mg/ml ($P<0.01$). The IC_{50} was estimated to be approximately 0.95 mg/ml (indicated by dashed line in Fig. 1C).

To assess the selectivity of MPSSS for CAFs over normal fibroblasts, CAFs and NFs were treated with 0.5 mg/ml MPSSS, and cell viability was compared. As shown in Fig. 1D, CAF viability was reduced to 68.5±4.2% at 0.5 mg/ml, whereas NF viability remained at 94.3±3.1% ($P<0.001$ for CAF vs. NF). These results indicate that MPSSS effectively inhibits CAF viability while sparing normal fibroblasts, suggesting selectivity for activated CAFs.

MPSSS significantly inhibits CAF migration with minimal effect on NFs. The Transwell migration assay was performed to evaluate the effect of MPSSS on CAF and NF migration. As shown in Fig. 2A and B, MPSSS significantly inhibited CAF migration in a dose-dependent manner. The number of migrated cells decreased from 285±18 cells/field in the control group to 198±15 cells/field at 0.1 mg/ml ($P<0.05$), 124±12 cells/field at 0.25 mg/ml ($P<0.01$) and 86±9 cells/field at 0.5 mg/ml ($P<0.001$). Compared with the control group, migrated cells in the 0.5 mg/ml MPSSS treatment group were reduced by approximately 70%. Notably, the concentration of 0.5 mg/ml used in the migration assay was substantially below the cytotoxic IC_{50} (0.95 mg/ml), suggesting that migratory inhibition is not merely a secondary consequence of reduced cell viability but may involve direct interference with CAF migratory machinery.

To assess the selectivity of MPSSS for CAF migration, CAFs and NFs were treated with 0.5 mg/ml MPSSS and migration capacity was compared. As shown in Fig.2C, MPSSS significantly inhibited CAF migration (86±9 cells/field vs. 285±18 in control, $P<0.001$) but had minimal effect on NF migration (178±14 cells/field vs. 192±16 in control, $P>0.05$). These findings indicate that MPSSS selectively inhibits CAF migration without significantly affecting normal fibroblast motility.

MPSSS downregulates HMGXB3 expression in CAFs at both mRNA and protein levels. qRT-PCR analysis was performed to determine the effect of MPSSS on HMGXB3 mRNA expression in CAFs and NFs. As shown in Fig. 3A, MPSSS dose-dependently downregulated HMGXB3 mRNA expression in CAFs. Compared with the control group, HMGXB3 expression was reduced to 72.4±6.3% at 0.125 mg/ml ($P<0.05$), 58.7±5.1% at 0.25 mg/ml ($P<0.01$) and 41.2±4.8% at 0.5 mg/ml ($P<0.001$). Western blot analysis confirmed that HMGXB3 protein levels were also reduced in a dose-dependent manner. Densitometric quantification revealed that HMGXB3 protein expression was reduced to 45.6±5.3% of control at 0.5 mg/ml ($P<0.01$), consistent with the mRNA data (Fig. 3B).

To assess the cell type specificity of MPSSS-mediated HMGXB3 regulation, CAFs and NFs were treated with 0.5 mg/ml MPSSS and HMGXB3 mRNA expression was compared. As shown in Fig. 3C, MPSSS significantly downregulated HMGXB3 expression in CAFs (41.2±4.8% of control, $P<0.001$) but had no significant effect on HMGXB3 expression in NFs (98.5±5.2% of control, $P>0.05$). These results suggest that MPSSS specifically regulates HMGXB3 expression in CAFs at both transcriptional and translational levels.

HMGXB3 is functionally required for CAF migration and mediates MPSSS-induced migratory inhibition. To determine whether HMGXB3 is causally involved in CAF migration and mediates the inhibitory effect of MPSSS, siRNA-mediated knockdown experiments were performed. CAFs were transfected with non-targeting control siRNA (NC-siRNA) or two independent HMGXB3-specific siRNAs (si-HMGXB3-1 and si-HMGXB3-2). As shown in Fig. 4A, both siRNAs achieved efficient knockdown of HMGXB3 mRNA ($P<0.001$ vs. NC-siRNA). Importantly, HMGXB3 knockdown alone significantly inhibited CAF migration to a degree comparable to MPSSS treatment ($P<0.001$ vs. NC-siRNA; ns between si-HMGXB3 groups and MPSSS-treated groups) (Fig. 4B). Furthermore, combined HMGXB3 knockdown with MPSSS exposure produced no additive or synergistic effect compared with either treatment alone (ns), indicating convergence on a common pathway. MTT cell viability assay confirmed that the observed migratory inhibition was not attributable to cytotoxicity, as HMGXB3 knockdown alone did not significantly affect cell viability (ns vs. NC-siRNA), whereas MPSSS treatment reduced viability to approximately 68% ($P<0.001$ vs. NC-siRNA) (Fig. 4C). These results demonstrate that HMGXB3 is functionally required for CAF migration and mediates MPSSS-induced migratory inhibition.

Discussion

The present study investigated the effects of MPSSS on the viability, migration and HMGXB3 expression of CAFs. The results demonstrated: (i) successful establishment of a CAF-like phenotype; (ii) dose-dependent inhibition of CAF viability with selectivity over NFs; (iii) downregulation of HMGXB3 at both the mRNA and protein levels; (iv) marked suppression of CAF migration; and (v) siRNA-mediated knockdown confirming that HMGXB3 is functionally required for CAF migration and mediates MPSSS-induced migratory inhibition. These findings identify HMGXB3 as a molecular target mediating MPSSS-induced CAF migration inhibition, extending the known bioactivities of MPSSS beyond immunomodulation and lymph angiogenesis.

The present findings complement and extend previous reports on MPSSS bioactivities. Earlier studies have shown that MPSSS can: attenuate CAF immunosuppressive function via TLR4-NF-κB (24); reduce CAF-derived TGF-β1 to prevent chemoresistance (25); and inhibit CAF-mediated lymph angiogenesis through TLR4/JNK (26). Beyond these immunomodulatory and angiogenic effects, our study has identified a previously unrecognized effect of MPSSS on CAF migration mediated through HMGXB3 downregulation.

The TLR4-NF-κB pathway has been implicated in MPSSS-mediated CAF immunomodulation and lymph angiogenesis. Notably, NF-κB can transcriptionally regulate HMG family members in certain contexts. It remains to be elucidated whether HMGXB3 downregulation is a direct consequence of TLR4-NF-κB inhibition or occurs through an independent mechanism. Future studies using TLR4 knockout CAFs or NF-κB inhibitors would help to clarify this relationship.

Importantly, MPSSS's selectivity for CAFs over NFs (94.3% NF viability retained at 0.5 mg/ml) suggests that it may preferentially target activated CAFs while sparing normal fibroblasts—a desirable property for TME-targeting therapies. The reduction in CAF migration by ~70% at 0.5 mg/ml MPSSS, substantially below the cytotoxic IC_{50} (0.95 mg/ml), suggests that migratory inhibition involves active modulation of cellular migratory machinery rather than cell death. CAF migration is a critical process in TME remodeling, as migrating CAFs contribute to ECM deposition, create tracks for cancer cell invasion and establish pre-metastatic niches (9,10). The minimal effect on NF migration further supports the selectivity of MPSSS. This distinction is clinically important because it implies that MPSSS may exert anti-tumor effects at sub-cytotoxic concentrations by reprogramming CAF function rather than simply eliminating them, consistent with the emerging concept of CAF re-education therapy (18).

Furthermore, combining MPSSS with conventional chemotherapy could improve drug penetration into the tumor core by reducing CAF-mediated physical barriers. The dense desmoplastic stroma of PDAC limits nanomedicine delivery; therefore, stromal remodeling strategies, such as inhibiting CAF migration, may improve therapeutic outcomes (32).

The siRNA-mediated knockdown experiments provide the first functional evidence that HMGXB3 is required for CAF migration. HMGXB3 knockdown alone significantly inhibited CAF migration to a degree comparable to MPSSS treatment, and combined

HMGXB3 knockdown with MPSSS exposure produced no additive effect, indicating convergence on a common pathway. This strongly suggests that HMGXB3 is a downstream mediator of MPSSS-induced migratory inhibition, rather than acting through an independent pathway.

HMGXB3 has been implicated in cell migration across multiple cancer types. In breast cancer, HMGXB3 is overexpressed and has been shown to influence cell proliferation and migration (28). In colorectal cancer, the DICER/miR-324-5p axis regulates HMGXB3 and WASF-2, thereby affecting cytoskeletal remodeling and cell migration (29). In renal cell carcinoma, the circ-EGLN3/miR-1224-3p/HMGXB3 axis promotes tumor progression (30). The convergence of HMGXB3 and WASF-2 regulation in colorectal cancer is particularly notable, given that WASF-2 (also known as WAVE2) is a vital component of the Arp2/3 complex responsible for driving actin polymerization and lamellipodia formation. In CAFs, WASF-2-dependent cytoskeletal remodeling may be essential for migration through dense desmoplastic stroma. Furthermore, HMGXB3 may regulate the expression of matrix metalloproteinases (MMPs), given their established role in CAF-mediated ECM remodeling. These hypotheses warrant direct testing in future studies.

The fact that these findings converge across different malignancies suggests that HMGXB3 may function as a conserved transcriptional regulator of cell motility, potentially by modulating cytoskeletal components such as WASF-2, MMPs, integrins and Rho GTPases. The present study extends these observations to CAFs, demonstrating that HMGXB3 is functionally required for CAF migration and serves as a mediator of MPSSS action.

Several limitations of this study should be acknowledged. Firstly, while the siRNA experiments establish a functional requirement for HMGXB3 in CAF migration, the precise molecular mechanisms by which HMGXB3 regulates migration—including its downstream transcriptional targets—remain to be elucidated. Future studies should employ RNA sequencing or chromatin immunoprecipitation assays to identify HMGXB3 target genes in CAFs. Secondly, although we have demonstrated HMGXB3 downregulation at mRNA and protein levels, its function as a transcription factor depends on nuclear localization and DNA binding. Future studies should use immunofluorescence to assess whether MPSSS affects HMGXB3 subcellular localization. Thirdly, this study was based entirely on in vitro cell experiments. Future studies should establish co-culture models of CAFs and pancreatic cancer cells, as well as in vivo xenograft models, to determine whether MPSSS can inhibit CAF migration and pancreatic cancer progression in vivo. While the use of hPSC-derived CAF models is established in the field, it may not fully recapitulate the heterogeneity of patient-derived CAFs. Finally, the upstream mechanism by which MPSSS downregulates HMGXB3 remains unclear. It requires further investigation to establish whether MPSSS affects HMGXB3 expression through TGF-β signaling, TLR4 pathways or transcriptional regulation.

In conclusion, this study demonstrates that MPSSS significantly reduces CAF viability and inhibits CAF migration in an in vitro model, with minimal effects on normal fibroblasts. HMGXB3 expression decreases in a dose-dependent manner after MPSSS treatment at both the mRNA and protein levels. Critically, siRNA-mediated knockdown experiments establish that HMGXB3 is functionally required for CAF migration and mediates MPSSS-induced migratory inhibition. These findings identify HMGXB3 as a functional mediator of MPSSS action and suggest that MPSSS-mediated TME modulation may represent a novel therapeutic strategy for PDAC. While additional mechanistic studies and in vivo validation are required, the present findings expand the known bioactivities of MPSSS and open new avenues for research into the use of natural polysaccharides to intervene in the pancreatic cancer TME.

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CRediT authorship contribution statement

Zimeng Tu: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing.

Boyuan Hu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Supervision, Writing – original draft.

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Figures and Figure Legends

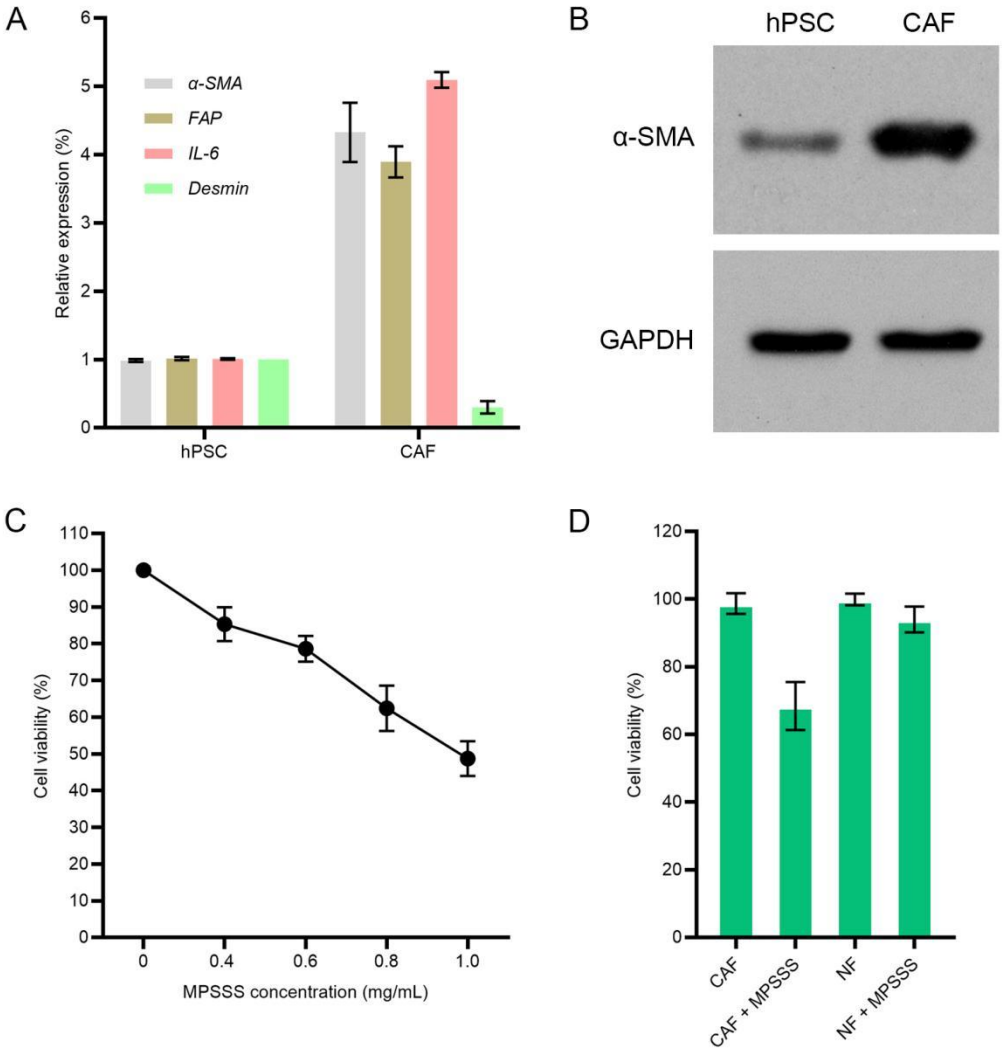


Figure 1. MPSSS selectively inhibits CAF viability. (A) Establishment and validation of the in vitro CAF model by qRT-PCR analysis of CAF activation markers (α-SMA, FAP and IL-6) and the quiescent marker desmin in hPSCs and CAFs. Data are presented as the mean ± SD (n=3). **P<0.01, ***P<0.001 vs. hPSC group. (B) Western blot analysis of α-SMA protein expression in hPSCs and CAFs, with GAPDH as the loading control. (C) Dose-dependent effect of MPSSS (0-1.0 mg/ml) on CAF viability after 24 h treatment, assessed by MTT assay. The IC_{50} was approximately 0.95 mg/ml (indicated by dashed line). (D) Comparison of cell viability between CAFs and NFs at 0.5 mg/ml MPSSS. Data are presented as the mean ± SD (n=6). *P<0.05, **P<0.01 vs. control group; ###P<0.001 vs. NF group.

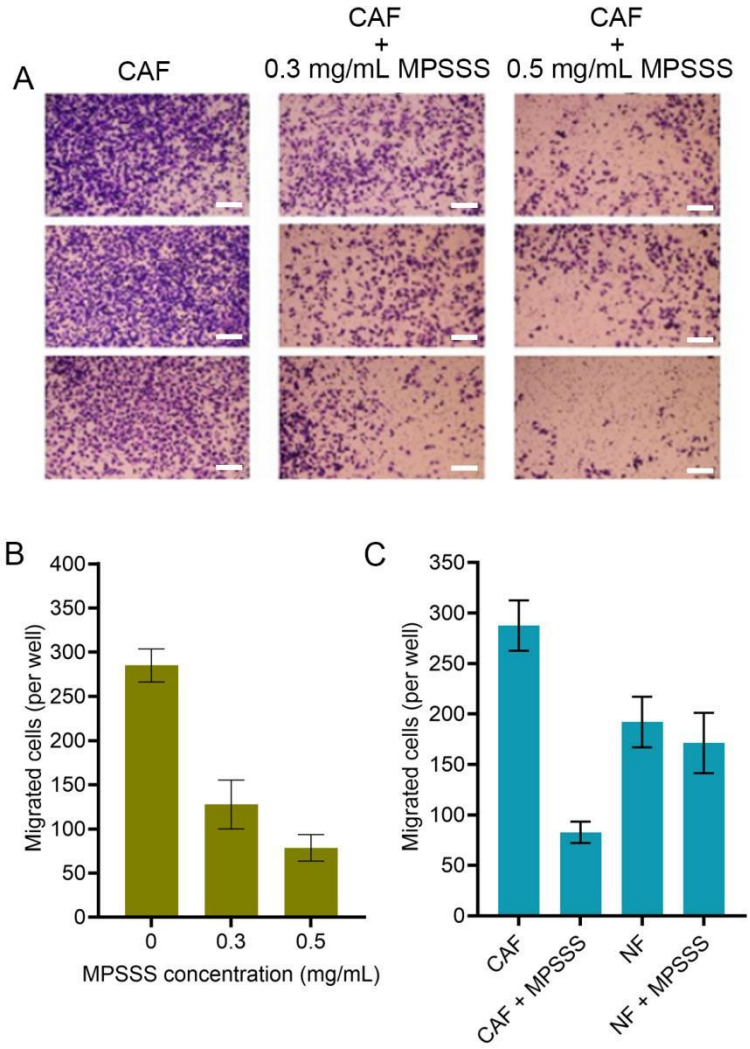


Figure 2. MPSSS suppresses CAF migration. (A) Representative images of migrated cells stained with crystal violet (200× magnification). Scale bar = 100 μm. (B) Quantitative analysis of migrated CAFs following MPSSS treatment. (C) Comparison of migrated cells between CAFs and NFs after treated by 0.5 mg/ml MPSSS. Data are presented as the mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001 vs. control group; ###P<0.001 vs. NF group.

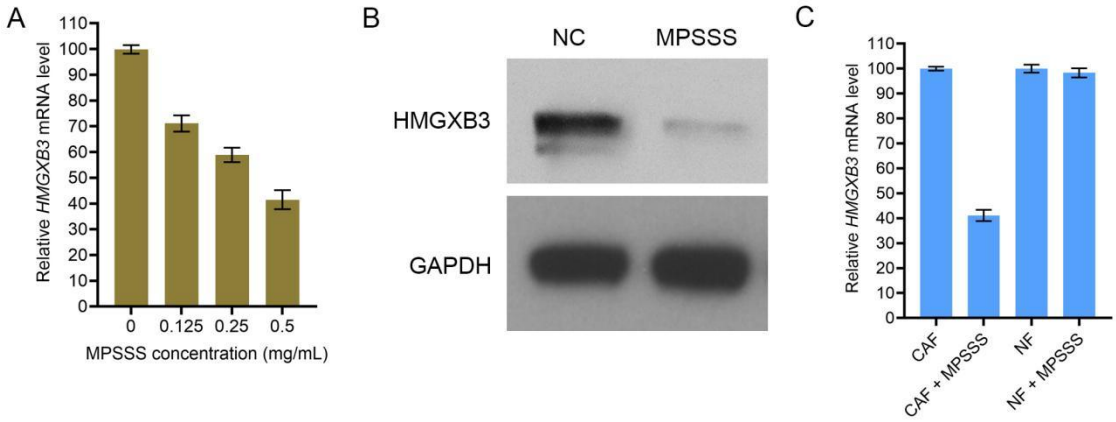


Figure 3. MPSSS downregulates HMGXB3 expression in CAFs. (A) qRT-PCR analysis of HMGXB3 mRNA expression in CAFs following MPSSS treatment (0-0.5 mg/ml). (B) Western blot analysis of HMGXB3 protein expression in CAFs, with GAPDH as the loading control. Densitometric quantification is shown below. (C) Comparison of HMGXB3 mRNA expression between CAFs

and NFs at 0.5 mg/ml MPSSS. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control group; ###P<0.001 vs. NF group.

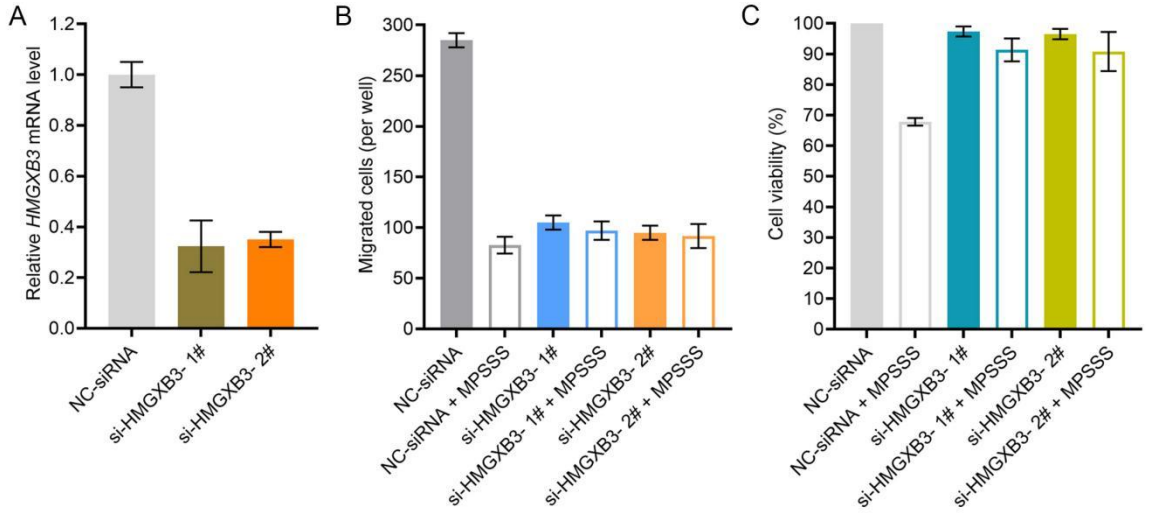


Figure 4. HMGXB3 is functionally required for CAF migration and mediates MPSSS-induced migratory inhibition. (A) qRT-PCR validation of HMGXB3 knockdown efficiency in CAFs transfected with NC-siRNA, si-HMGXB3-1 or si-HMGXB3-2. (B) Transwell migration assay showing that HMGXB3 knockdown alone inhibited CAF migration comparably to 0.5 mg/ml MPSSS, with no additive effect upon combined treatment (ns), indicating pathway convergence. (C) MTT assay confirming that migratory inhibition was not due to cytotoxicity; HMGXB3 knockdown did not affect viability, whereas MPSSS reduced viability to ~68%. Data are mean ± SD (n=3 for A and B; n=6 for C). ***P<0.001 vs. NC-siRNA; ns, not significant.

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