LMO7 as an Unrecognized Factor Promoting Pancreatic Cancer Progression and Metastasis

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Pancreatic cancer (PC) is one of the most lethal human malignancies without effective treatment. In an effort to discover key genes and molecular pathways underlying PC growth, we have identified LIM domain only 7 (LMO7) as an under-investigated molecule, which highly expresses in primary and metastatic human and mouse PC with the potential of impacting PC tumorigenesis and metastasis. Using genetic methods with siRNA, shRNA, and CRISPR-Cas9, we have successfully generated stable mouse PC cells with LMO7 knockdown or knockout. Using these cells with loss of LMO7 function, we have demonstrated that intrinsic LMO7 defect significantly suppresses PC cell proliferation, anchorage-free colony formation, and mobility in vitro and slows orthotopic PC tumor growth and metastasis in vivo. Mechanistic studies demonstrated that loss of LMO7 function causes PC cell-cycle arrest and apoptosis. These data indicate that LMO7 functions as an independent and unrecognized druggable factor significantly impacting PC growth and metastasis, which could be harnessed for developing a new targeted therapy for PC.

Keywords: pancreatic cancer, LIM domain only 7 (LMO7), cell cycle, apoptosis, CRISPR-Cas9

INTRODUCTION

Pancreatic cancer (PC) is one of the most lethal malignancies with an average 5-year survival rate of <6% (Ferlay et al., 2010; Siegel et al., 2012; Chu et al., 2018). The American Cancer Society estimates that 57,600 people will be diagnosed with PC and 47,050 of them will die in the US in 2020 (Ren et al., 2018; Siegel et al., 2020). Surgery is a curable treatment but only eligible for 10%–20% of patients. Majority of patients are diagnosed at the late stage with tumor metastasis at which point surgical treatment is minimally effective (Kim et al., 2018). Therefore, developing a novel therapeutic strategy for PC treatment is urgently required.
Over the past 15 years, the therapeutic paradigm in cancer treatment has shifted from traditional cytotoxic chemotherapy to targeted therapy. Many of them have been approved by the Food and Drug Administration (FDA) to treat different cancers (Sawyers, 2004). Imatinib is one outstanding example which is used in the treatment of chronic myelogenous leukemia (CML) with a significant therapeutic effect by specifically inhibiting tyrosine kinases BCR-ABL, c-KIT, and PDGFRA (Iurlo et al., 2015). Previous studies have identified some critical signaling pathways mediating PC initiation and progression which significantly improve our understanding of PC (Tanaka, 2016). Targeting key factors involved in EGFR, RAS/RAF/MEK, PI3K/AKT, WNT, and HEDGEHOG signaling pathways has resulted in the generation of various targeted therapies (Thayer et al., 2003; Wong and Lemoine, 2009). However, the ongoing and completed targeted therapies focusing on these pathways failed to demonstrate efficacy in PC (di Marco et al., 2016) with a median overall survival of less than one year (Amanam and Chung, 2018). Thus, we propose to identify under-investigated molecules important for PC initiation, progression, and metastasis in order to develop new powerful molecularly targeted therapy (MTT) against PC (O’Brien, 1996).

In an effort to discover key genes important for PC progression or suppression, we have identified some under-investigated molecules significantly impacting PC tumorigenesis and metastasis (Qian et al., 2017; Liu et al., 2019). Here, we report another novel finding about LIM domain only 7 (LMO7). LMO7 is a member of PDZ and the LIM domain-containing protein family, implicated in cancer-related pathological conditions and the regulation of cell adhesion (Ooshio et al., 2004; Hu et al., 2011). While having only low or limited expression in a few tissues, LMO7 has been found to upregulate in colorectal, breast, lung, thyroid, and liver cancer and plays a critical role in cancer metastasis (Nakamura et al., 2005, 2011; Hu et al., 2011; He H. et al., 2018). Recent studies demonstrated that LMO7 may be involved in recurrent chromosomal rearrangements of FRK in inflammatory hepatocellular adenomas (Bayard et al., 2020) and serve as a biomarker in common chronic lung diseases (Magsoudloo et al., 2020). Last year, another group discovered that LMO7 is specifically localized in the cuticular plate and the cell junction. LMO7 knockout mice suffer multiple cuticular plate deficiencies, including reduced filamentous actin density and abnormal stereocilia rootlets (Du et al., 2019). The elevated levels of LMO7, also known as PCD1, have been reported in PC (Kang et al., 2000). However, no report has been found to describe the role of LMO7 in PC carcinogenesis, progression, and metastasis. In addition, the current studies about the role of LMO7 on cancers generated controversial information (Kainu et al., 2000; Rozenblum et al., 2002). We proposed to investigate the contribution of LMO7 on PC and elucidate the underlying mechanisms.

In this study, we have applied different strategies to generate stable PC cells with LMO7 knockdown or knockout. Using these cells and their formed orthotopic tumors, we evaluated how intrinsic LMO7 defect impacts PC tumor progression and metastasis as well as the underlying mechanisms. Our findings suggest that LMO7 plays an important role in PC and represents a novel and druggable molecule target for PC.

RESULTS

PC Progression in Human and Mice Is Associated With the Increased Expression of LMO7

To define the relationship between LMO7 expression and PC progression, the expression of LMO7 in primary and metastatic PC has been measured. In freshly harvested human pancreatic ductal adenocarcinoma (PDAC) and pancreatic neuroendocrine tumors (PNETs), we detected the enhanced LMO7 production in human PDACs and PNETs with IHC (Figures 1A,B) and Western blot (Figures 1C,D). Similar LMO7 expression patterns were observed in other 2 normal pancreas, 11 primary PDAC tumors, 2 metastatic PDAC in liver, and 1 metastatic PDAC in lung (Supplementary Figures 1A,B). Furthermore, qPCR detected the significantly increased expression of LMO7 mRNA in tumors from 45 human PC patients compared to that in relevant adjacent tissues (peritumoral tissues) (Figure 1E). Further analysis revealed that the increased LMO7 expression was mainly detected in tumors from the patients at stages I and II, but not stage III (Supplementary Figure 1C). Different levels of LMO7 mRNA expression were also detected in various types of human and mouse PC cells with expression levels higher in human Panc-1 cells (Figure 1F) and mouse Panc02-H7 cells (Figure 1G) than human Mia-Paca-2 cells and mouse Panc02 and UN-KPC-961 cells. Among them, Panc-1 and Panc02-H7 cells are more aggressive and able to induce tumor metastasis. By injecting cells into the pancreas of wild-type C57BL/6 mice (Figure 1H), we developed orthotopic PC-bearing mice with Panc02-H7, Panc02, or UN-KPC-961 cells. The formed PC tumors (yellow arrow) were detected in all recipient mice 17 days post cell injection (n = 5 for each group, Figure 1I), but tumor liver metastasis (green arrow) was only observed in the tumor-bearing mice developed with Panc02-H7 cells (middle panel in Figure 1I). Western blot detected the expression of LMO7 protein with a level that is higher in tumors developed with panc02-H7 cells than Panc02 cells or UN-KPC-961 cells (Figure 1J). Together, these results indicate that LMO7 mRNA and protein expression is consistently increased in human and mouse primary and metastatic tumors, suggesting their positive correlation with PC progression.

Generation of Stable PC Cell Lines With LMO7 Knockdown and Knockout

To study the role of LMO7 in PC tumor progression, genetic methods with siRNA, shRNA, and CRISPR-Cas9 technologies were used to establish PC cells with LMO7 knockdown and knockout with our established method (Liu et al., 2019). In this regard, three independent genome-wide siRNAs were designed and transfected to Panc02-H7 cells (Figure 2A). A significant decrease of LMO7 mRNAs was detected in all three siRNA-transfected cells compared to the cells receiving...
Sanger DNA sequencing demonstrated nucleotide depletion in cell clone 8, which was named as LMO7-CRISPR-Panc02-H7. Expression of LMO7 mRNA (clones. qPCR and Western blot detected the significantly reduced expression of LMO7 mRNA (Figure 2F) to develop LMO7-knockout cells with CRISPR-Cas9 (Liu et al., 2019). We designed crRNA which was annealed with tracrRNA to get a guide RNA duplex our successful protocol (Liu et al., 2019). We designed crRNA and inserted it into lentivirus vector pLL3.7. Co-transfection of this recombinant plasmid with scramble siRNA. These effects were verified in stable LMO7-shRNA-Panc02-H7 cells with LMO7 knockdown (Figures 3E–G) and stable LMO7-CRISPR-Panc02-H7 cells with LMO7 knockout (Figures 3H–J). These results indicate that either transient or stable LMO7 knockdown or knockout significantly damages PC cell growth and tumorigenesis, suggesting its critical role in PC development.

Intrinsic LMO7 Defect Causes Suppression of Panc02-H7 Cell Proliferation, Colony Formation, and Motility in vitro

Using stable Panc02-H7 cells with LMO7 knockdown and knockout, we investigated the role of intrinsic LMO7 defect in PC cell growth and tumorigenesis in vitro (Figure 3A). MTT examination revealed that siRNA-induced LMO7 knockdown significantly reduced Panc02-H7 cell proliferation (Figure 3B) and colony formation (Figures 3C,D) compared to control cells transfected with scramble siRNA. These effects were verified in stable LMO7-shRNA-Panc02-H7 cells with LMO7 knockdown (Figures 3E–G) and stable LMO7-CRISPR-Panc02-H7 cells with LMO7 knockout (Figures 3H–J). These results indicate that either transient or stable LMO7 knockdown or knockout significantly damages PC cell growth and tumorigenesis, suggesting its critical role in PC development. Next, we investigated if LMO7 defect also influences PC cell motility. For this purpose, the LMO7-siRNA-transfected Panc02-H7 cells, stable LMO7-shRNA-Panc02-H7 cells, and control scrambled siRNAs (Figure 2B). Among three siRNAs, siRNA 2 and 3 showed a similar effect in strongly inducing LMO7 knockdown. Therefore, siRNA 3 was selected to use in the following experiments. In reference to siRNA 3, we designed LMO7-shRNA and inserted it into lentivirus vector pLL3.7. Co-transfection of this recombinant plasmid with helper vectors psPAX2 and pMD2G to HEK293T cells was used to package lentivirus particles with LMO7-shRNA. The resultant recombinant lentiviruses were infected into Panc02-H7 cells for establishing stable cells expressing LMO7-shRNA. A limited dilution method was used to generate single-cell clones (Figure 2C). qPCR and Western blot detected a significantly reduced expression of LMO7 at mRNA (Figure 2D) and protein (Figure 2E) levels in cell clone 3, which was named as LMO7-shRNA-Panc02-H7 cells. Using CRISPR-Cas9 technology, stable Panc02-H7 cells with LMO7 knockout were also generated with our successful protocol (Liu et al., 2019). We designed crRNA which was annealed with tracrRNA to get a guide RNA duplex which was transfected to the stable Cas9-Panc02-H7 cells in order to develop LMO7-knockout cells with CRISPR-Cas9 (Liu et al., 2019; Figure 2F). Limited dilution was used to isolate single-cell clones. qPCR and Western blot detected the significantly reduced expression of LMO7 mRNA (Figure 2G) and protein (Figure 2H) in cell clone 8, which was named as LMO7-CRISPR-Panc02-H7. Sanger DNA sequencing demonstrated nucleotide depletion in the LMO7 gene (Figure 2I). These results indicate that stable Panc02-H7 cells with LMO7 knockdown and knockout have been successfully established.
LMO7-CRISPR-Panc02-H7 cells were respectively seeded in 24-well plates with an inserter. On the second day, the inserters were removed. The cell-free gaps were measured microcopy over the indicated times. As shown in Figures 4A,B, siRNA-mediated LMO7 knockdown significantly inhibited cell motility. The more obvious suppression of cell motility was observed in both stable LMO7-shRNA-Panc02-H7 cells (Figures 4C–D) and stable LMO7-CRISPR-Panc02-H7 cells (Figures 4E–F). LMO7 defect-mediated suppression on cell proliferation, colony formation, and cell motility were also detected in UN-KPC-961 cells post knockdown of LMO7 with shRNAs (Supplementary Figure 2). Together, the results indicate that loss of LMO7 function inhibits not only PC growth but also cell motility.

**Intrinsic LMO7 Defect Causes Slowed Tumor Growth in vivo**

Next, we investigated whether intrinsic LMO7 defect in Panc02-H7 cells impacts tumor growth in vivo. LMO7-siRNA-transfected Panc02-H7 cells, LMO7-shRNA-Panc02-H7 cells, LMO7-CRISPR-Panc02-H7 cells, and corresponding control cells at a dose of $2.5 \times 10^5$ cells per mouse were injected to the pancreas of wild-type mice at eight weeks of age. Seventeen days post cell injection, all mice were euthanized. No significant difference for body weight was detected in each mouse among groups. However, the mean volume of the tumors developed with Panc02-H7 cells with siRNA-induced LMO7 knockdown was smaller than the tumors developed with the control cells transfected with scrambled siRNAs, but this difference was not statistically significant ($n = 5$ for each group, $p > 0.05$, Figures 5A,B). In contrast, the tumors developed with stable LMO7-shRNA-Panc02-H7 or LMO7-CRISPR-Panc02-H7 cells were significantly smaller than those induced with the relevant control cells ($n = 5$ for each group, $p < 0.01$, Figures 5D–E, and $n = 6$ for LMO7-CRISPR cells, $n = 7$ for relative control, $p < 0.01$, Figures 5G–H). Also, no liver metastatic tumor was detected in the mice receiving inoculation of the cells with LMO7 defect (data not shown). The reduced LMO7 protein expression was observed in the tumors developed with LMO7-siRNA-transfected Panc02-H7 cells (Supplementary Figure 3A), stable LMO7-shRNA-Panc02-H7 cells (Supplementary Figure 3B), and stable LMO7-CRISPR-Panc02-H7 cells (Supplementary Figure 3C).

In a separate study, we examined whether intrinsic LMO7 knockdown or knockout impacts the lifespan of the recipient mice. After inoculation of the distinct cells with or without LMO7 knockdown and knockout, the development of ascites and impairment of gait and breathing were daily observed which is used to define the humane endpoint of the resultant
tumor-bearing mice. Kaplan–Meier curves showed that the median life span of the mice receiving LMO7-siRNA-transfected cells is approximately 1.4-fold longer than that of the mice receiving control cells transfected with scrambled siRNAs (n = 6 for each group, Figure 5C). Similarly, the extended life span was also observed in the mice receiving stable LMO7-shRNA-Panc02-H7 cells compared to the mice (n = 6 for experimental group, n = 7 for the control group, Figure 5F). Correspondingly, a median life span of 30 days was observed in mice receiving stable LMO7-CRISPR-Panc02-H7 cells, which is much longer than 22 days in mice receiving relevant control cells. The log-rank test of significance across the groups has a p value < 0.001 (n = 13 for the experimental mice receiving LMO7-CRISPR-Panc02-H7 cells, n = 9 for the control mice receiving the relevant control cells, Figure 5I). These results suggest that intrinsic LMO7 defect not only slows tumor growth but also suppresses tumor metastasis, resulting in the significantly extended lifetime.

**LMO7 Knockout Causing Cell-cycle Arrest at the G1/S Phase and PC Cell Apoptosis**

To elucidate the molecular pathways underlying LMO7-mediated effects on PC tumorigenesis, we investigated how LMO7 knockout impacts Panc02-H7 cell cycle and apoptosis. As shown in Figure 6A, LMO7-CRISPR-Panc02-H7 cells with LMO7 knockout caused a cell-cycle arrest at the G1/S phase which was demonstrated by flow cytometric analysis by staining DNAs with propidium iodide (PI), suggesting regulated effect of LMO7 on cell cycle. Furthermore, we detected the impact of LMO7 knockout on expression of the well-characterized signaling molecules relevant with the cell cycle. Western blotting showed that LMO7 knockout markedly reduced the expression of cyclin D3, but not cyclin D1, in the LMO7-CRISPR-Panc02-H7 cells in comparison to the control cells (shown in Figure 6B), implying that LMO7 knockout induces cell-cycle arrest possibly by suppressing cyclin D3. Cyclin D3 is a member of the cyclin D family, which regulates the initial G1 to S transition (Kato, 1999). Next, we investigated if LMO7 defect-mediated cell-cycle arrest is linked to cell apoptosis (Pucci et al., 2000). For this purpose, we detected a panel of apoptotic signaling molecules in LMO7-CRISPR-Panc02-H7 cells. Western blot detected markedly increased the protein expression of PARP, cleaved PARP, and Bax and decreased the expression of Bcl2 and Bcl-xL in the LMO7-CRISPR-Panc02-H7 cells compared to control cells (Figure 6B). IHC detected the decreased expression of Ki67 and CD31 and increased the expression of cleaved PARP in the tumors developed with stable LMO7-CRISPR-Panc02-H7 cells compared to the tumors induced with control cells (Figure 6C). These results suggested...
that intrinsic LMO7 knockout suppresses PC tumorigenesis through coupling of the cell-cycle arrest and programmed cell death (Figure 6D).

**DISCUSSION**

Our *in vitro* and *in vivo* studies have demonstrated that LMO7 as an under-investigated factor significantly promotes PC tumorigenesis through influencing cell cycle and programmed cell death. Genetic knockdown or knockout of LMO7 not only significantly suppresses PC cell proliferation, colony formation, and motility *in vitro* but also obviously slows tumor growth and metastasis *in vivo*. This novel finding suggests that LMO7 represents a druggable molecule, which could be harnessed to develop a novel molecularly targeted therapy for PC.

To our knowledge, this is the first study demonstrating that LMO7 functions as an independent tumor-promoting factor, highly expressed in PC. We detected the increased expression of LMO7 in different types of human and mouse PC tumors in comparison with peritumoral and normal pancreas tissues. This increase is reflected in both LMO7 mRNA and protein levels, which has been validated by different methods including IHC, Western blot, and qPCR (Figure 1 and Supplementary Figure 1). These data indicate that PC progression is significantly accompanied by the enhanced production of LMO7. This finding is consistent with the studies on other cancers, where investigators identified highly expressed LMO7 in a significant percentage of colon, breast, liver, lung, pancreas, stomach, and prostate tumor tissues but very few in normal tissues (Kang et al., 2000). Human Protein Atlas showed that all human PC tumors have medium or high LMO7 expression and high
expression is unfavorable. LMO7 as a member of the PDZ-LIM protein family is characterized by their PDZ and LIM domains; the combination of two functional domains in one protein has wide-ranging and multi-compartmental cell functions during development and homeostasis (Krcmer et al., 2010). Misregulation can lead to cancer formation and progression. Using genetic methods with the siRNA, shRNA, or CRISPR-Cas9 approach to induce loss of LMO7 function, we investigated the effect of LMO7 on PC tumorigenesis. Our in vitro experiments demonstrated that intrinsic LMO7 defect significantly suppresses PC cell proliferation (Figure 3 and Supplementary Figure 2); this effect has been verified by various types of human and mouse PC cell lines. By intrapancreatic injection of stable cells with LMO7 knockdown or knockout into wild-type C57BL/6 mice, we demonstrated that intrinsic LMO7 suppression slowed orthotopic PC tumor growth and metastasis (Figure 5). These in vitro and in vivo results imply that LMO7 functions as an intrinsic tumor-promoting factor in PC which provides a novel therapeutic target for PC treatment. Different from our findings in PC, Miyoshi et al. demonstrated that LMO7-deficient mice developed irregular and protruding epithelial lesion in the terminal and respiratory bronchioles at younger ages and tended to develop naturally occurring lung cancer at older ages. These results suggest that intrinsic and extrinsic LMO7 might exert different roles in tumorigenesis in a cancer type-dependent way (Tanaka-Okamoto et al., 2009). To address this question, we will use LMO7-knockout mice to investigate whether extrinsic LMO7 similarly affects PC and elucidate the underlying mechanisms.

Our studies suggest that LMO7 as a critical factor is involved in PC metastasis. The Panc02-H7 cell line is derived from non-metastatic Panc02 cells by continuously passaging the cells to wild-type mice as selection pressure (Wang et al., 2003). Using qPCR and Western blot, we detected the higher expression of LMO7 at either mRNA or protein level in Panc02-H7 cells compared to Panc02 cells (Figure 1G). Intrapancreatic
inoculation of both Panc02-H7 cells and Panc02 cells into wild-type mice induces orthotopic PC tumors, but tumor liver metastasis was only detected in the mice receiving Panc02-H7 cells (Figures 1H,1). These results reveal the positive correlation between LMO7 expression and PC tumor metastasis. Next, we used different Panc02-H7 cells with LMO7 knockdown or knockout to investigate whether LMO7 is critical to mediate PC metastasis. As described in Figure 4, LMO7 defect induced with siRNA, shRNA, and CRISPR-Cas9 approaches suppresses Panc02-H7 cell motility in vitro. Consistent with our finding, one group previously reported that the expression of LMO7 was detected in metastatic breast cancer cell line MDA-MB-231, but not in non-metastatic MCF7 cells (Hu et al., 2011). Using transwell migration assays, they demonstrated that LMO7 knockdown markedly reduced the migration of MDA-MB-231 breast cancer cells by specifically regulating MRTF in a cell-specific manner (Hu et al., 2011). Mechanistic studies revealed that LMO7 enhances cell migration by activating Rho and MRTF. These data suggest that LMO7 as an independent factor drives tumor metastasis in different cancers including PC (Ooshio et al., 2004; Pipes et al., 2006). The mechanisms underlying LMO7-mediated PC metastasis remain to be understood.

In this study, we have successfully developed in vitro and in vivo cancer models to study the role and mechanisms of LMO7 in PC. Using siRNA, shRNA, and CRISPR-Cas9 technologies, we generated stable PC cells with LMO7 knockdown or knockout (Figure 2). These cells have been used to induce orthotopic PC tumors in wild-type C57BL/6 mice (Figure 5). These cells and animal models complementarily provide us ideal platforms to mechanistically study LMO7’s important role in PC. We have demonstrated that LMO7 knockout causes not only cell-cycle arrest (Figure 6A) but also cell apoptosis (Figure 6B), suggesting that LMO7 has dual but related functions by linking cell-cycle progression and programmed cell death (Figure 6D). Single protein-acting dual-signal models have been found in many other molecules such as tumor-suppressor genes p53 and Rb, the dominant oncogenes c-Myc, and several cyclin-dependent kinases (Cdks) and their regulators (Pucci et al., 2000). For example, p53 is recognized as one of widely studied tumor suppressors. As a nuclear DNA-binding phosphoprotein, p53 influences cell proliferation by acting predominately in the G1 phase of the cell-cycle progression. In addition, p53 also plays an important role in triggering apoptosis in certain cell types. It has been reported that LMO7 as an emerin-binding protein regulates its transcription (Holaska et al., 2006). One group has demonstrated that emerin is a nuclear protein gene important for maintaining cell-cycle timing (Fairley et al., 2002). Together, LMO7 induces cell-cycle arrest likely through regulation of cyclin D3 and emerin. We will test this hypothesis in the future studies.
In this study, we have established stable PC cells with LMO7 knockdown and knockout. Using these cells as a platform, we have demonstrated that intrinsic LMO7 defect causes significant suppression of tumor growth and metastasis by inducing PC apoptosis and cell-cycle arrest at the G1/S phase. These results suggest that LMO7 functions as an oncogene in PC. Targeting this under-investigated molecule offers us an opportunity to develop a new molecularly targeted therapy for PC.

**MATERIALS AND METHODS**

**Antibodies, Reagents, and Plasmids**

Antibodies against PARP, cleaved PARP, Bcl-2, Bcl-XL, Bax, β-actin, Cyclin D1, Cyclin D3, Ki67, and CD31 were bought from Cell Signaling (Danvers, MA, United States). All immunohistochemistry (IHC) reagents from Cell Signaling (Danvers, MA, United States). Mouse β-Antibodies against PARP, cleaved PARP, Bcl-2, Bcl-XL, Bax, Antibodies, Reagents, and Plasmids

**MATERIALS AND METHODS**

**Cell Lines, Medium, and Culture**

Murine Panc02-H7 cells were a generous gift from Dr. Keping Xie in MD Anderson Cancer Center, which is an invasive cell line derived from Panc02 (Wang et al., 2003). The UN-KPC-961 cell line was purchased from ATCC (Manassas, VA); this cell was generated from pancreatic tumors in 17-week-old Pdx1-Cre (KPC) mice with mutation of Kras (G12D) and Trp53 (R172H). Human Panc-1 and MIA PaCa-2 cells were purchased from ATCC (Manassas, VA, United States). The cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Cellgro, Manassas, VA, United States) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, 10 mmol/L HEPES, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ humidified atmosphere (Qi et al., 2016).

**Mice**

Six-week-old male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed under standard conditions with a 12-h light/12-h dark cycle with room temperature 21 ± 2°C in the first floor of the Medical Sciences Building at the University of Missouri-Columbia. This space is an AAALAC- and USDA-accredited state-of-the-art facility and administered by the Animal Care Unit at the University of Missouri. Mice were maintained in individually ventilated cages (IVC) with free access to water and standard mouse diet (PicoLab Rodent Diet 20, 21% protein, 11.3% fat). All experiments with mice were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at University of Missouri. All mice received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals.” Animals are free of clinical signs of disease before surgery. After surgery, mice were immediately kept on a warm pad in a dry area and vital signs will be monitored during recovery. After recovery from anesthesia, the mice were returned to routine housing. Animals are monitored daily, and mice that became ill during these experiments will be euthanized.

**Human PC Tumor Samples**

The protocol (IRB2010166) for freshly harvesting human PC tumors and adjacent tissues in Ellis Fischel Cancer Center was approved by the Institutional Review Board (IRB) at the University of Missouri School of Medicine. All subjects gave their informed consent for inclusion before they participated in the study. Presence of tumor was confirmed by a pathologist with hematoxylin and eosin (H&E) staining. Other 45 human PC tumor tissues and corresponding adjacent tissues were collected at the Pancreas Center, the First Affiliated Hospital of Nanjing Medical University in Nanjing, China. The protocol (2017-SR-171) was approved by the Ethics Committee in this hospital. All of these patients underwent pancreatic resection and promised to provide their tumors with the informed consent. None of them received chemotherapy and/or radiotherapy before surgery. The tissues were frozen at −80°C within 10 min after tumor excision (He Y. et al., 2018; Hu et al., 2018).

**RNA Extraction and Quantitative PCR (qPCR)**

Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) in terms of the manufacturer’s instruction. Reverse transcription of RNA to cDNA was conducted with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, United States). qPCR was performed with QuantStudio 3 Detection System (ABI, Thermo Fisher) in a 20-µL reaction mixture containing SYBR Green I (Applied Biosystems, Foster City, CA, United States). The expression level of LMO7 was normalized to housekeeping gene of 18s rRNA and was further analyzed using the 2^−ΔΔCT method. The sequences of forward and reverse primers for human LMO7 are 5’-AATCAGCATAAACCAGACGCC-3’ and 5’-CTGGGCTACCTGCTTCAACT-3’; for 18S rRNA: 5’-AATCAGGTTGCATTGCGGA-3’ and 5’-CCAAGATCCACTACGAGCT-3’.

**Orthotopic PC Murine Model, Tumor Weight, and Mouse Lifespan Analysis**

PC cells grown to 90% confluence were harvested and suspended in 15% Matrigel in PBS. The suspended cells were seeded into the pancreas to make orthotopic PC models with our established protocol which was conducted in the biosafety cabinet of mouse vivarium (Liu et al., 2019). Briefly, male C57BL/6 mice (22.1 ± 1.8 g, aged 8 weeks) were anesthetized with VetEquip IMPAC by inhalation of 2% isoflurane (VetOne® Fluriso™, Boise, IL) mixed with oxygen at a flow of 1.5 L/min. Isoflurane caused faster induction of anesthesia and quick recovery as well as relative sparing effect on cardiovascular function. Adequate anesthesia was checked by the toe-pinch

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method. The anesthetized mice were placed on a temperature-controlled heating pad to induce vasodilatation in a ventilated restrainer immobilization for surgery. A 1.5-cm incision was made in the left flank to expose the pancreas. Mice were randomized into two groups for each experiment; each group contained 5–13 mice. 40-µl suspended cells or relevant control cells were injected into the head of the pancreas at a dose of 2.5 × 10^5 cells/mouse. After cell injection, two layers of the flank incision were sutured and the skin was clipped. Intraperitoneal injection of 100 µl Carprofen at a dose of 5 mg/kg was applied for mouse pain alleviation after surgery. Pain and distress of mice were monitored daily. Development of ascites and impairment of gait and breathing were used to define a humane endpoint of the resultant tumor-bearing mice. At the endpoint, mice were euthanized by CO₂ exposure with a flow of 3 L/min followed by cervical dislocation. A survival curve was constructed with the Kaplan–Meier method using GraphPad Prism software. Statistical significance was determined by single-factor analysis of variance and validated using the log-rank test. p values of <0.05 was considered significant.

siRNA Transfection
Three predesigned siRNAs for mouse LMO7 were bought from Integrated DNA Technologies, Inc., (IDT, Coralville, IA). siRNA-mediated LMO7 knockdown in PC cells was conducted with our established method (Liu et al., 2019). Briefly, PC cells grown to 50% confluence in a 6-well plate received transfection of 5 pmol of siRNAs with RNAi-MAX Lipofectamine reagent (Invitrogen, Carlsbad, CA). Eight hours post transfection, the medium was changed to complete DMEM medium. The cells were cultured for another 36 h then harvested for the subsequent assay.

Generating Stable LMO7-Knockdown Cells With shRNA
We used our reliable method to generate a stable LMO7 knockdown cell line (Liu et al., 2019). LMO7-shRNA or scrambled shRNA was designed, synthesized, annealed to form double-chain DNA fragments, then inserted into a vector plasmid pLL3.7 between HpaI and XhoI sites. The recombinant plasmids with LMO7-shRNA or scramble shRNA were transformed into competent Escherichia coli cells and identified with PCR and DNA sequencing. The expanded recombinant plasmids pLL-shRNA-LMO7 or control pLL-shRNA-CTR, together with helping plasmids psPAX2 and pMD2G, were co-transfected into HEK 293T cells to package the lentiviruses. The supernatants containing the relevant lentivirus were harvested 48 h and 72 h post transfection and concentrated using the Lenti-X Concentrator (Clontech, Fremont, CA, United States). The recombinant lentiviruses were first transfected to the Panc02-H7 cells or UN-KPC-961 cells grown to 50% confluence. The medium was changed to the normal culture medium 12 h post infection. A limited dilution method was used to generate single-cell clones. Each clone was amplified to measure the mRNA expression level of LMO7 with qPCR. The resultant stable cell clone with a significant LMO7 suppression was named as LMO7-shRNA-Panc02-H7 cells or LMO7-shRNA-UN-KPC-961 cells.

Generating Stable LMO7-Knockout Cells With the CRISPR-Cas9 Approach
We used our reliable method to generate stable LMO7 knockout cells (Liu et al., 2019). The tracrRNAs and crRNAs specific for LMO7 were designed by CRISPR DESIGNI and synthesized by IDT (Coralville, IA). 1 µl crRNA (100 µM) and 1 µl tracrRNA (100 µM) were mixed with 18 µl nuclease-free water and heated to 95°C for 5 min, then cooled down to room temperature in order to make a guide RNA (gRNA) duplex. The gRNA duplex was transfected to the established Cas9-Panc02-H7 stable cells via the RNAi-MAX Lipofectamine reagent (Liu et al., 2019). Single-cell clones were generated with the limited dilution as in the above description. Each single clone was expanded, and genomic DNA for Sanger DNA sequencing was extracted. LMO7 knockout was verified with qPCR and Western blotting at the mRNA and protein level. The resultant cells with confirmed LMO7 knockout were named as LMO7-CRISPR-Panc02-H7 cells.

Cell Proliferation Assay
Different PC cells with or without LMO7 knockdown and knockout were seeded in 96-well plates at a dose of 2 × 10^3 per well. At the indicated time, cell proliferation was measured with Proliferation Assay Kit (Promega, Madison, WI, United States) according to the manufacturer’s instructions.

Colony Formation Assay
Different PC cells with or without LMO7 knockdown and knockout were seeded in 6-well plates at a dose of 2 × 10^2 per well. Seven to ten days later, the cells were rinsed with phosphate-buffered saline (PBS), then stained with 0.05% crystal violet for photography and colony counting.

Wound Healing Assay
Different PC cells with or without LMO7 knockdown and knockout were seeded in 24-well plates with an insert at a dose of 2 × 10^3 per well. The inserts were moved carefully on the second day, and cells were continued to culture. The cell-free gaps were measured many times with an optical microscope (Zeiss).

Western Blotting Analysis
Cell lysate and tumor lysates were respectively prepared with lysis protein extraction reagent (Thermo Fisher Scientific, Inc.) and M-PERTM mammalian protein extraction reagent (Thermo Fisher Scientific, Inc.). After quantitating, an equal amount of protein was loaded to perform Western blotting as previously described (Liu et al., 2019).

Cell-cycle Analysis
Cells with or without LMO7 knockout were harvested from the cultured dishes and suspended in PBS containing 50 μg/mL propidium iodide (PI) for flow cytometry. CellQuest software (BD Biosciences, San Jose, CA, United States) was used to determine cell-cycle distribution.

1http://crispr.mit.edu/
Immunohistochemistry (IHC)
As described in our literature (Liu et al., 2019), tumor tissues harvested from tumor-bearing mice were sequentially fixed, dehydrated, and embedded to make 4-µm tissue sections. Tissue sections were used to conduct IHC by sequential de-paraffinization, incubation with primary antibody and secondary antibody, and final color development with DAB substrate. Three random fields in each slide were photographed under the microscope.

Statistics Analysis
Paired data were analyzed using a 2-tailed paired Student’s t-test. A p value of <0.05 was considered significant.

DATA AVAILABILITY STATEMENT
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT
This study was performed in accordance with the Declaration of Helsinki. All experiments with mice were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at University of Missouri. All mice received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals.” Informed consent was obtained from all patients, and this study was approved by the Ethics Committee of the hospital (2017-SR-171) at The First Affiliated Hospital of Nanjing Medical University, China.

AUTHOR CONTRIBUTIONS
XL, HY, and JZ performed study design, acquisition of data, analysis and interpretation of data, and statistical analysis. QW, XQ, and CB performed study design and data analysis. DA and JK performed study design and critical revision of the manuscript for important intellectual content. KJ, KC, and YM performed study design and critical revision of the manuscript for important intellectual content. PT performed pathologist to review the H&E and IHC slides and critical revision of the manuscript for important intellectual content. KJ performed study concept and design, analysis and interpretation of data, drafting of the manuscript, and critical revision of the manuscript for important intellectual content. GL performed study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, obtained funding, and study supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.647387/full#supplementary-material

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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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