

Supplementary Items

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Supplementary Materials and Methods

Immunofluorescence (IF) staining

Cultured cells were fixed and stained with primary antibodies according to standard protocols [1]. The antibodies used for IF are listed in Supplementary Table S1. For tissue staining, paraffin-embedded sections were deparaffinized and subjected to antigen retrieval in 10 mM citrate buffer (pH 6.0) using an autoclave. Sections were then blocked with 1% Normal Goat Serum Blocking Solution (Vector Laboratories, Burlingame, CA, USA) for 10 minutes at room temperature, followed by overnight incubation with primary antibodies at 4 °C. The primary and secondary antibodies used for IF are detailed in Supplementary Table S2. Images were acquired using a KEYENCE BZ-X810 microscope (KEYENCE, Osaka, Japan).

scRNA-seq analysis

scRNA-seq libraries were prepared using a Chromium Single Cell 3' Reagent Kits v3.1 (10x Genomics, Pleasanton, CA, USA) according to the manufacturer's instructions. The libraries were sequenced using the Novaseq 6000 system (Illumina, San Diego, CA, USA).

The fastq files were mapped to the reference genome provided by 10x Genomics (GRCh38). The read count was quantified using the Cell Ranger version 6.0.0 count pipeline (10x Genomics) with default parameters. The Seurat package [2] was used for downstream analysis (<https://doi.org/10.1016/j.cell.2021.04.048>). Low-quality cells with over 10% of mitochondrial RNAs and fewer than 400 or more than 6,000 features were excluded. For the filtered cells, normalization of the transcript count matrix was

performed according to the total count number per cell, and the counts were scaled by a factor of 10,000. Then natural-log transformations was applied using Seurat's "NormalizeData()" function, followed by a linear transformation using "ScaleData()". Principal component analysis was conducted utilizing the "RunPCA()" method while selecting the top 2,000 variable features identified by "FindVariableFeatures()" implementing the vst selection method. We conducted graph-based clustering using "FindNeighbors()" and "FindClusters()" with the top 20 PCs and a 0.4 resolution. To perform uniform manifold approximation and projection (UMAP), "RunUMAP()" was used with the same PCs to determine the clusters.

Publicly available single-cell RNA sequencing (scRNA-seq) data of human PDAC samples was acquired from the Genome Expression Omnibus, under the accession numbers GSE155698 (<https://www.nature.com/articles/s43018-020-00121-4>).

Single cell gene expression analysis by digital droplet PCR

Pancreas tissues from 12-week-old KPC mice were minced and dissociated using the Tumor Dissociation Kit (130-096-730, MACS Miltenyi Biotec, Bergisch Gladbach, Germany). Non-tumor-associated fibroblasts were depleted using the depletion cocktail from the Tumor-Associated Fibroblast Isolation Kit (130-116-474, MACS Miltenyi Biotec). The remaining cells were then incubated with a Thy1-A567 antibody (Supplementary Table S1) for 40 minutes, followed by fluorescence-activated cell sorting (FACS) using a FACS Melody system (BD Biosciences) to isolate Thy1-positive cells. Each single Thy1-positive cell was sorted into an individual well of 96 well plate containing lysis buffer. The cells were lysed and subject to reverse transcription using the iScript Advanced cDNA Synthesis Kit (BioRad). Droplet digital PCR (ddPCR) was performed using 4 ul of cDNA

from each well with three different primers (Acta2-FAM, Tagln-HEX, and Gapdh-FAM; BioRad, Supplementary Table S1) and analyzed using a QX200 Droplet Digital PCR system (Bio-Rad).

Cell lines

The BxPC-3 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was recently authenticated by short tandem repeat (STR) profiling. The Panc-1 cell line was obtained from the RIKEN BioResource Research Center (RIKEN BRC, Tsukuba, Japan). BxPC-3 was maintained in RPMI1640 medium (FUJIFILM WAKO PURE CHEMICAL, Osaka, Japan), while Panc-1 was maintained in DMEM, both containing 10% FBS (Nichirei Biosciences, Tokyo, Japan) and 1% antibiotic–antimycotic. Mouse PDAC cell (mT5) was generously provided by David Tuveson (Cold Spring Harbor Laboratory) and Chang-Il Hwang (UC Davis College of Biological Sciences, Davis, CA). These mouse PDAC cells were cultured in DMEM containing 5% FBS and 1% antibiotic–antimycotic. Cells were cultured at 37 °C in a humidified incubator with 5% CO₂.

Western blot analysis

Cells and tissue were harvested and lysed with RIPA buffer. Protein lysates were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. A total of 100 µg of protein was separated on 10% SDS/PAGE gels, transferred to nitrocellulose membranes, and incubated with the primary antibodies. The antibodies used for western blotting are listed in Supplementary Table. S1. The band intensity was quantified by ImageJ software

(<https://imagej.nih.gov/ij/>).

RNA extraction and quantitative reverse transcription-PCR (qPCR)

Total RNA from cells was extracted using TRIzol (Thermo Fisher Scientific), followed by reverse-transcription using Prime Script RT Master Mix (Takara, Kusatsu, Japan). Expression levels of target genes were normalized to that of Mouse ACTB (Actin Beta) (MM04394036, Thermo Fisher Scientific). Oligonucleotide primers for SYBR Green assays are listed in Supplementary Table. S1.

Cell proliferation assay

To analyze short-term cell proliferation, cancer cells were seeded into 96-well plates at a density of 5,000 cells per well in 100 μ L of DMEM with 10% FBS or conditioned medium (CM) and incubated at 37 °C. Cell proliferation was measured daily for four days using Cell Count Reagent SF solution (Nacalai Tesque, Kyoto, Japan) following the manufacturer's instructions. For long-term cell proliferation analysis, cells were seeded into 6-well plates at a density of 20,000 cells per well with 2 mL of CM and incubated at 37 °C. The medium was changed every 2-3 days with fresh CM, and the cell number was counted after 2 weeks.

Public Data analysis

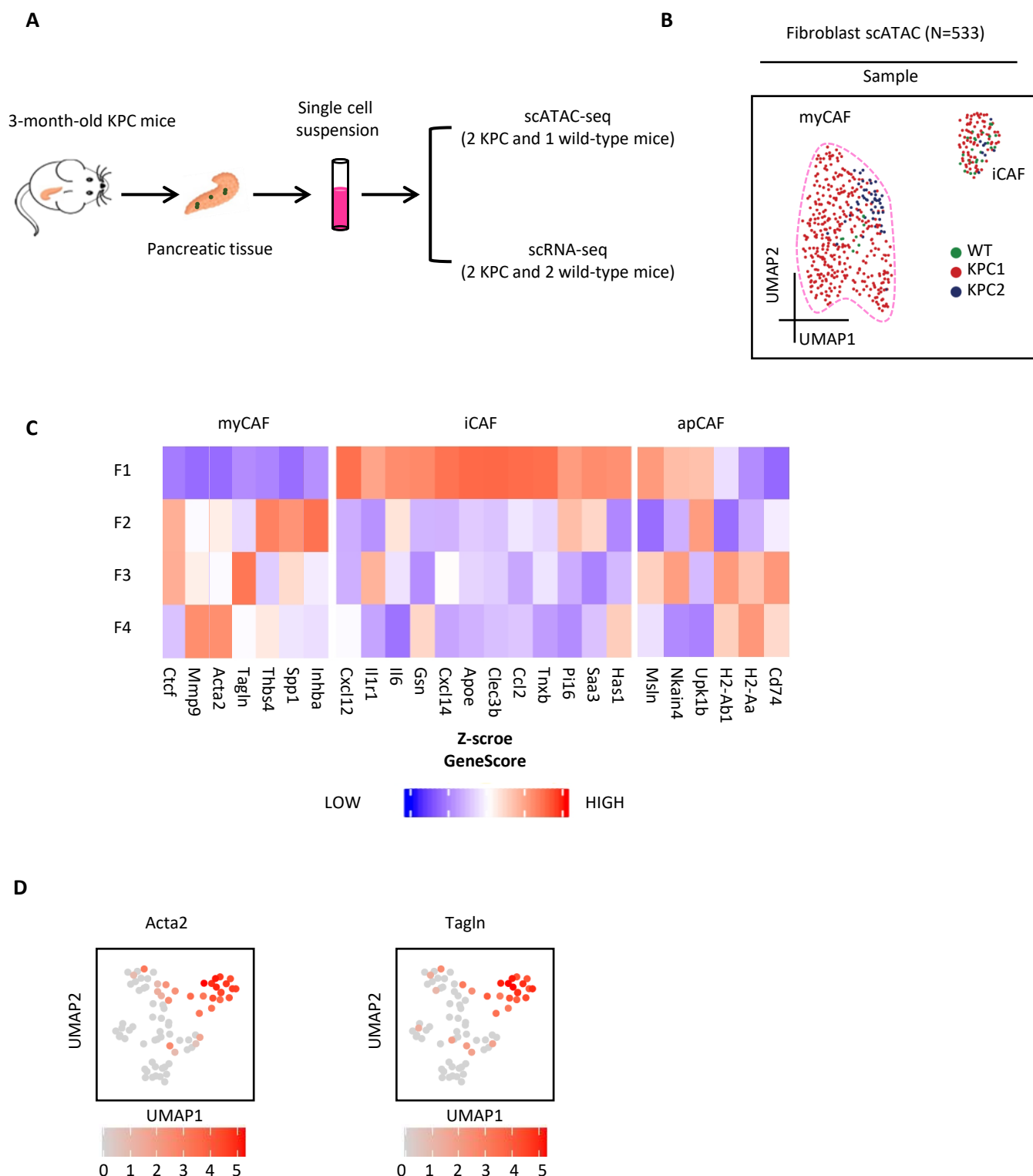
To analyze the relationship between the expression of genes and survival, survival analysis was performed using Kaplan-Meier Plotter (www.kmplot.com) [3]. A total of 177 PDAC samples from TCGA were analyzed to evaluate the overall survival (OS) and relapse-free survival (RFS). During the analysis, patient samples were split into two

groups based on various quantile expressions of the proposed biomarkers. Hazard ratio (HR) with 95% confidence intervals and log-rank *P* values were then calculated.

RNA expression of PDAC sample in The Cancer Genome Atlas (TCGA) data base was analyzed by Gene Expression Profiling Interactive Analysis (GEPIA) [4]. A total of 179 tumor samples and 171 normal samples were included in this analysis.

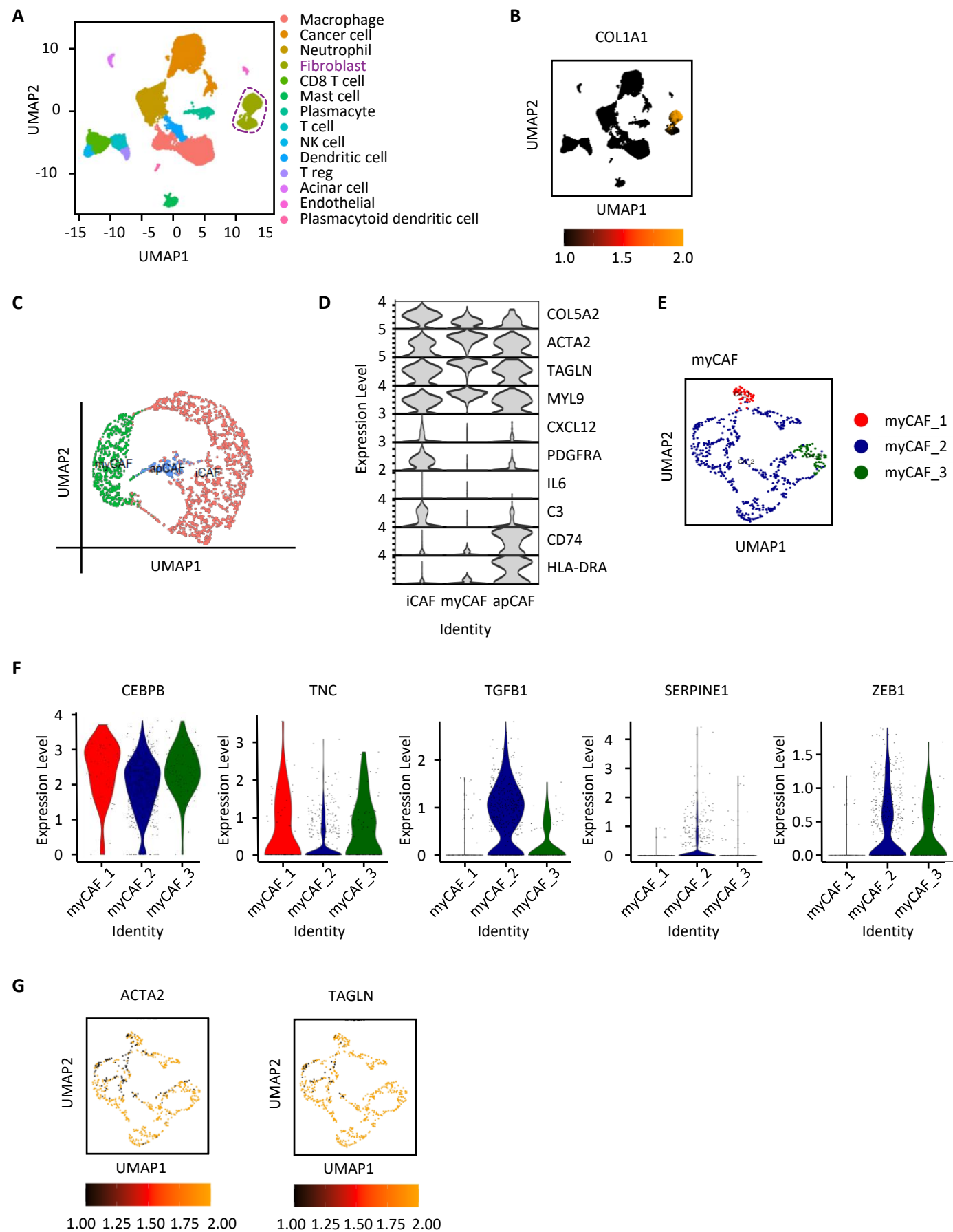
Reference

1. Muraoka A, Suzuki M, Hamaguchi T, et al. infection facilitates the development of endometriosis through the phenotypic transition of endometrial fibroblasts. *Sci Transl Med.* 2023;15(700).
2. Hao Y, Hao S, Andersen-Nissen E, et al. Integrated analysis of multimodal single-cell data. *Cell.* 2021;184(13):3573-87 e29.
3. Nagy A, Munkacsy G, Gyorffy B. Pancancer survival analysis of cancer hallmark genes. *Sci Rep.* 2021;11(1):6047.
4. Tang Z, Li C, Kang B, et al. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res.* 2017;45(W1):W98-W102.



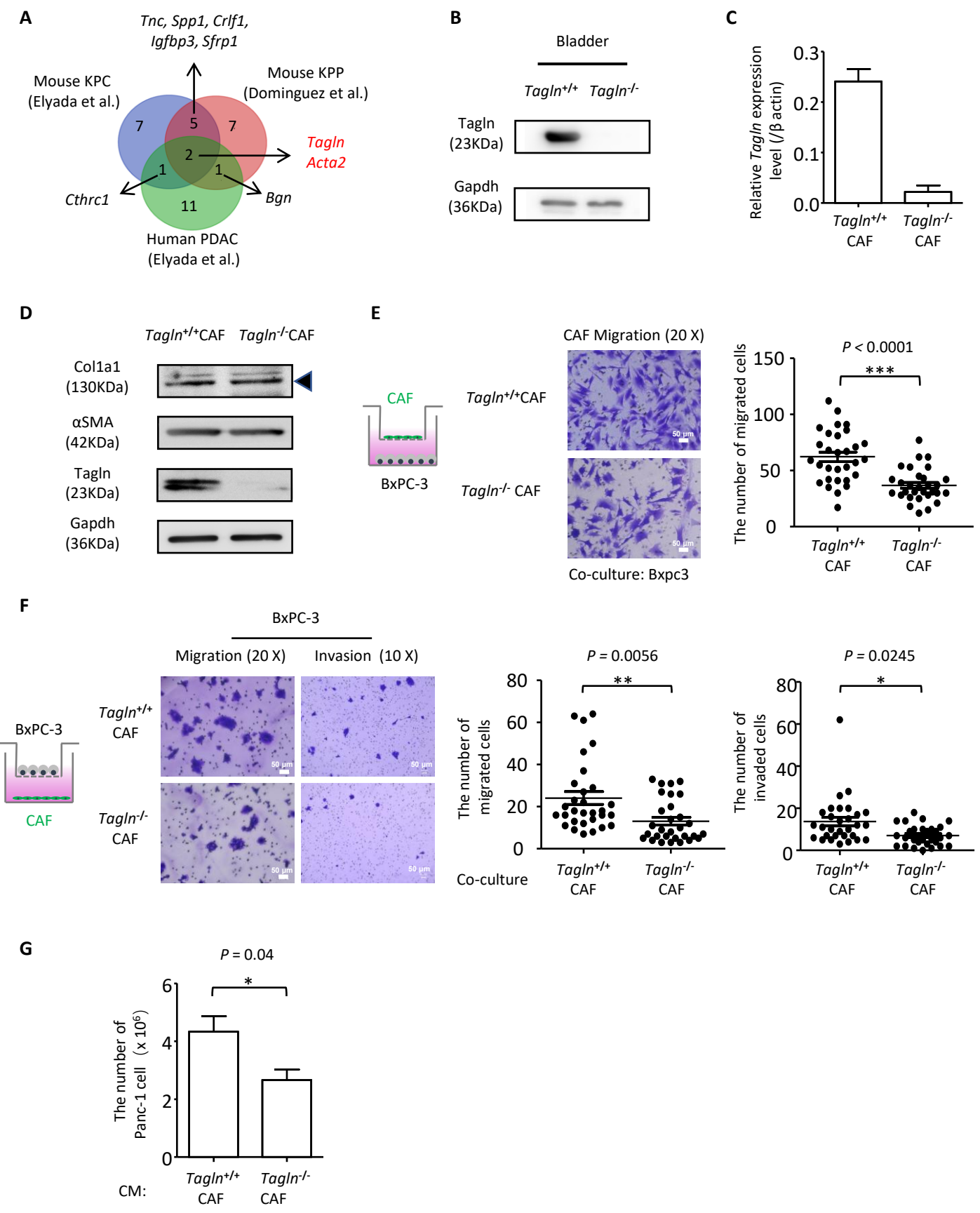
Supplementary Fig. S1. Single-cell analysis of the mouse PDAC

(A) Schematic representation of the experimental design for scATAC-seq and scRNA-seq analysis of mouse PDAC. (B) Different colors represent scATAC-seq samples, each derived from an individual mouse. (C) Heatmap displaying the gene scores of CAF subtype marker genes across CAF subclusters (Fig. 1C). (D) Visualization of Acta2 and Tagln expression across the myCAF cluster in scRNA-seq data using feature plots.



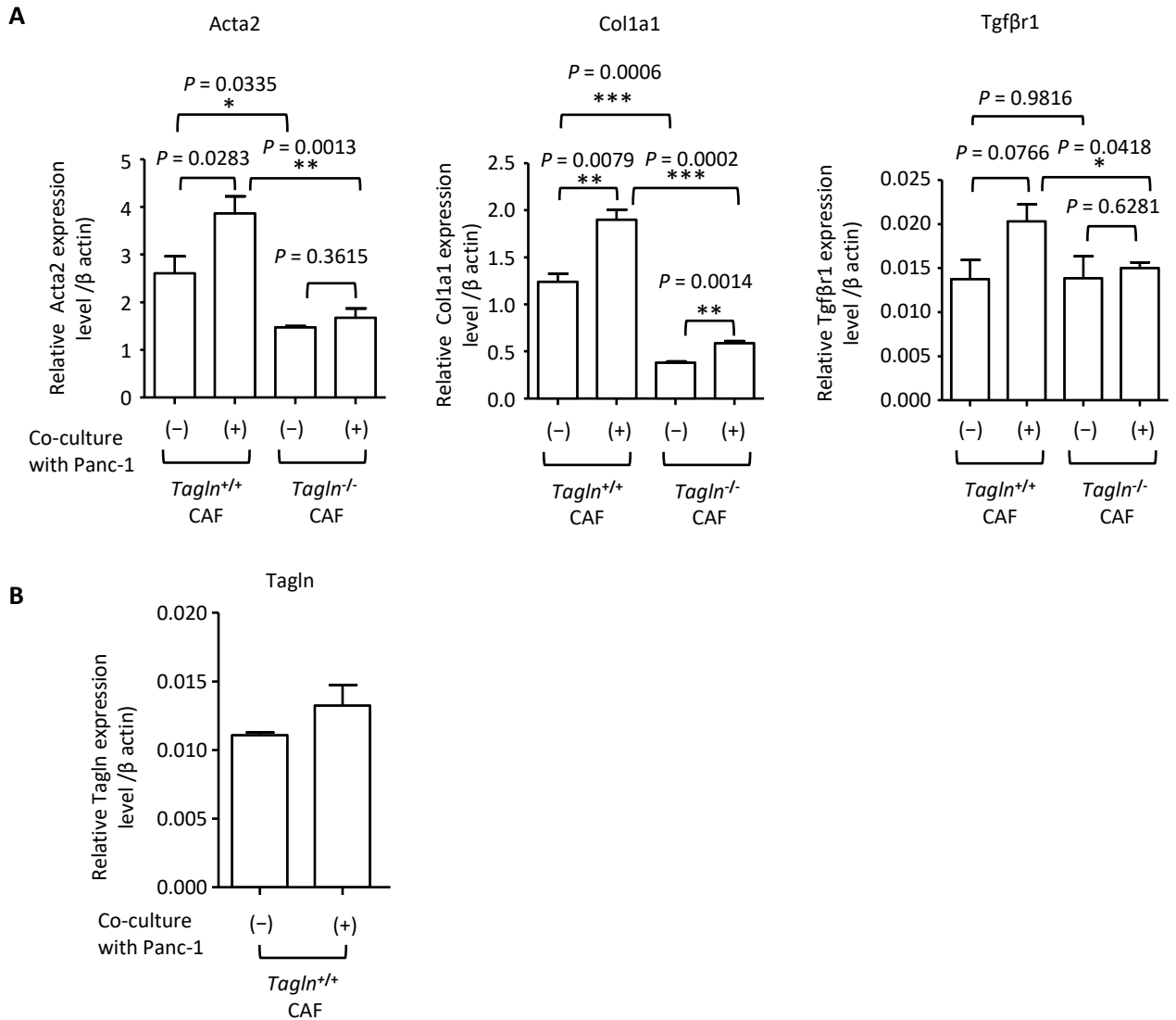
Supplementary Fig. S2. Single-cell RNA-seq profiles of the human samples (GSE155698).

(A) Uniform Manifold Approximation and Projection (UMAP) plot of the human samples (GSE155698). Different colors represent different cell types. The purple circle highlights fibroblast clusters. (B) UMAP plots showing the expression of pan-fibroblast marker genes (COL1A1) across all clusters. (C) UMAP plot of fibroblasts (derived from Supplementary Fig. S7A), color-coded by different fibroblast subclusters. (D) Violin plots displaying the normalized expression of marker genes across different fibroblast subclusters. (E) UMAP plots of myCAFs (derived from Supplementary Fig. S7C) color-coded by distinct myCAF subclusters. (F) Violin plots showing the normalized expression of transcription factors and their associated target genes across myCAF subclusters. (G) UMAP plots showing the expression of ACTA2 and TAGLN in myCAF clusters.



Supplementary Fig. S3. Tagln-positive fibroblasts promote cell migration, invasion and proliferation in human PDAC cell, Bxpc3.

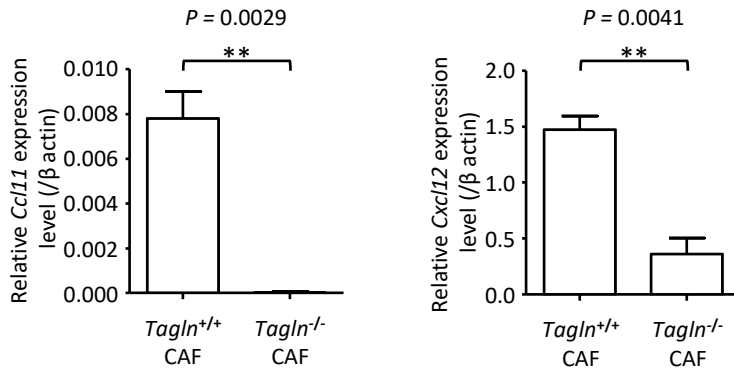
(A) Venn diagram of top representative genes in myCAF derived from the public data. The top 15 differentially expressed genes in KPC (LSL-KrasG12D/+; LSL-Trp53+/R172H; Pdx1cre/+), KPP (Pdx1cre/+; LSL-KrasG12D/+; p16/p19flox/flox), and human PDAC were compared. Tagln and Acta2 were consistently overexpressed across all these gene sets. (B) Western blot analysis of Tagln in bladder samples from wild-type (*Tagln*^{+/+}) and homozygous *Tagln*^{-/-} mice. Gapdh was used as a loading control. (C) mRNA levels of TAGLN in *Tagln*^{+/+} CAFs and *Tagln*^{-/-} CAFs (n = 3). Error bars indicate the SEM. (D) Western blot analysis of Col1a1, α SMA, and Tagln in *Tagln*^{+/+} CAFs and *Tagln*^{-/-} CAFs. Gapdh was used as a loading control. (E) Transwell migration assay of CAF cells. A schematic illustration of the experiment setup (left), representative images (middle) and quantification (right) of migrated cells. CAF migration was assessed in co-culture with or without BxPC-3 cells (n=3, 7 images per group). The y-axis indicates the number of migrated cells. Scale bar: 50 μ m. (F) Migration and invasion assay of BxPC-3 cells co-cultured with *Tagln*^{+/+} CAFs or *Tagln*^{-/-} CAFs: A schematic illustration of the experiment setup (left), representative images (middle) and quantification (right) of migrated cells and invaded cells (n=3, 7 image per group). The y-axis indicates the number of migrated and invaded cells. Scale bar: 50 μ m. (G) Effects of conditioned medium (CM) derived from *Tagln*^{+/+} CAFs or *Tagln*^{-/-} CAFs on human PDAC cell proliferation. The number of Panc-1 cells counted after two weeks of culture in CM from either *Tagln*^{+/+} CAFs or *Tagln*^{-/-} CAFs. Error bars indicate the SEM***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.



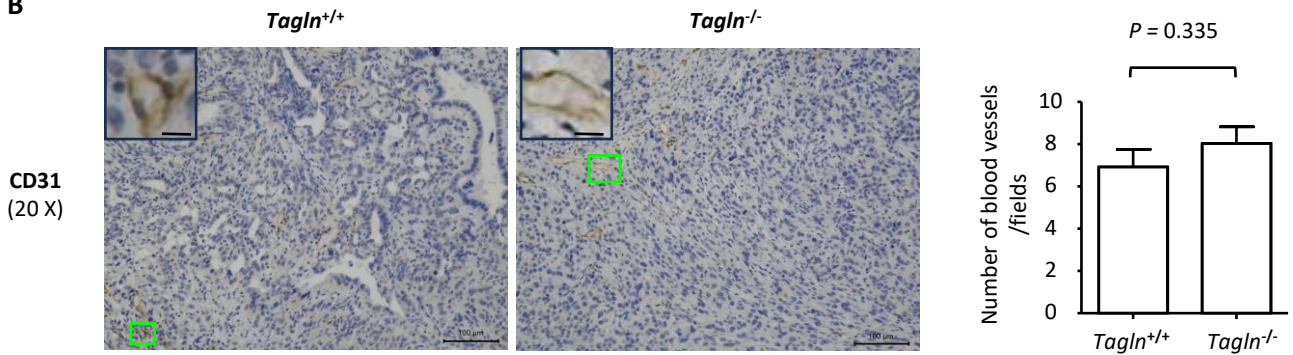
Supplementary Fig. S4. mRNA expression levels of *Tagln*^{+/+} CAFs or *Tagln*^{-/-} CAFs after co-cultures with Panc-1 cells

(A) Relative expression of *Acta2*, *Col1a1*, and *Tgfβr1* in *Tagln*^{+/+} CAFs compared with *Tagln*^{-/-} CAFs after co-culture with Panc-1 cells, as measured by qPCR (n = 3). Error bars represent SEM. (B) mRNA levels of *Tagln* in *Tagln*^{+/+} CAFs after co-culture with Panc-1 cells (n = 3). Error bars indicate the SEM.

A



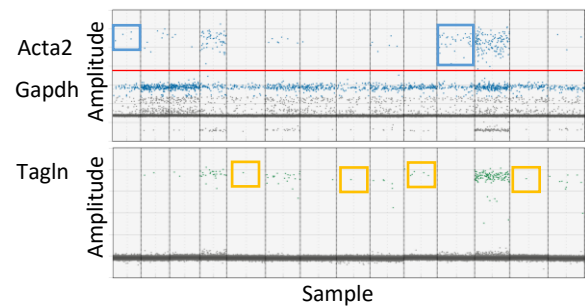
B



Supplementary Fig. S5. Changes in tumor microenvironment in Orthotopic mouse model

(A) mRNA levels of Ccl11 and Cxcl12 in *Tagln*^{+/+} CAFs and *Tagln*^{-/-} CAFs (n = 3). Error bars indicate the SEM. (B) Representative images of CD31 IHC staining (left) and quantification (right) in tumor sections from *Tagln*^{+/+} or *Tagln*^{-/-} orthotopic mouse models. The number of blood vessels was counted in each group (n=5). Scale bar: 100 μm. The magnified images (scale bar: 10 μm) in the upper left corner correspond to the green boxed areas. Data were analyzed using an unpaired Student's *t*-test. Results are presented as mean ± SEM.

Magnification (20 X)

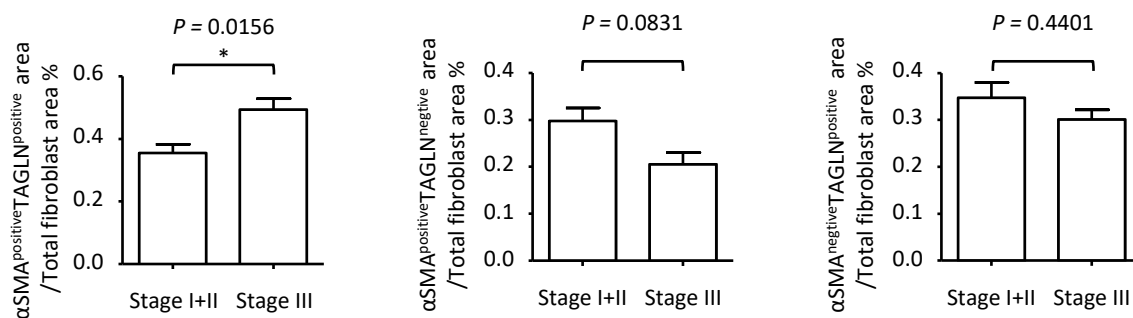


Cell number analyzed by ddPCR (total 38 cells)			
	Acta2(+)/Tagln(-)	Acta2(-)/Tagln(+)	Acta2(+)/Tagln(+)
cell number	6	8	24
%	16.7	22.2	66.7

Supplementary Fig. S6. Heterogeneity of expression Single-cell ATAC-seq profiles of the mouse PDAC

(A) Representative images of H&E staining and IF for α SMA and Tagln in PDAC sections from KPC. The pink arrowhead indicates α SMA-positive/TAGLN-positive CAF, the gray arrowhead denotes α SMA-positive/TAGLN-negative CAF, and the yellow arrowhead represents α SMA-negative/TAGLN-positive CAF. Mouse 2 was used for ddPCR (Supplementary Fig. S5B). (B) Representative ddPCR plots of pancreatic tissue from KPC mice (right panel). Each sample represents a single cell sorted by FACS based on Thy1 expression. The blue box indicates Acta2-positive/Tagln-negative CAFs, and the yellow box indicates Acta2-negative/Tagln-positive CAFs. Right panel: The number of Acta2-positive/Tagln-positive, Acta2-positive/Tagln-negative, Acta2-negative/Tagln-positive CAFs was quantified by ddPCR, ($n = 2$).

A



Supplementary Fig. S7. IF staining of human PDAC section

(A) Bar graphs showing the proportional distribution of CAF subsets expressing both α SMA and TAGLN, α SMA only, or TAGLN only among total CAFs in PDAC patients across different clinical stages (Stage I, n = 1 ; Stage II, n = 14 ; Stage III, n = 5).

Supplementary Table S1. Antibody Details and qPCR Primer Sequences.

Target protein	Catalog number	Company	Dilution	Application
Transgelin/TAGLN	ab14106	Abcam	1:200	Immunohistochemistry (IHC)
α SMA	M0851	DAKO	1:200 (for mouse sample)	
			1:500 (for human sample)	
CD31	ab281583	Abcam	1:1000	
CD206/MRC1	24595	Cell Signaling Technology	1:200	
CD4	100511	BioLegend	1:50	
CD8a	100727	BioLegend	1:100	
Transgelin/TAGLN	ab14106	Abcam	1:1000	Western blot
α SMA	M0851	DAKO	1:500	
Col1a1	ab6308	Abcam	1:1000	
HRP-linked anti-mouse IgG (Secondary Antibodies)	7076S	Cell Signaling	1:1000	
HRP-linked anti-rabbit IgG (Secondary Antibodies)	7074S	Cell Signaling	1:1000	
Transgelin/TAGLN	ab14106	Abcam	1:100 (for cell)	Immunofluorescence (IF)
			1:150 (for tissue)	
Phalloidin/F-actin	20549	Cayman Chemical	1:1000	
α SMA	M0851	DAKO	1:150	
Goat anti-Rabbit IgG (Secondary Antibodies)	A-11071	Invitrogen	1:500	

Goat anti-Mouse IgG (Secondary Antibodies) for tissue	A-11029	Invitrogen	1:500	
DAPI	88-19000-99	Invitrogen	1:100 (for cell)	
			1:50 (for tissue)	
Thy1-A549	Ab317533	Abcam	1:500	Fluorescence-activated cell sorting (FACS)

Gene symbol	Primer sequences	
<i>Tagln</i>	Forward	5'-GCGGCCTTTAAACCCCTCAC-3'
	Reverse	5'-GCTCATGCCGTAGGATGGAC-3'
<i>Col1a1</i>	Forward	5'-GCTCCTCTTAGGGGCCACT-3'
	Reverse	5'-ATTGGGGACCCTTAGGCCAT-3'
<i>Acta2</i>	Forward	5'-ATTGTGCTGGACTCTGGAGATGGT-3'
	Reverse	5'-TGATGTCACGGACAATCTCACGCT-3'
<i>Tgfbr1</i>	Forward	5'-TGCTCCAAACCACAGAGTAGGC-3'
	Reverse	5'-CCCAGAACACTAAGCCCATTGC-3'
<i>Cxcl12</i>	Forward	5'-CGCCAAGGTCGTCGCCG-3'
	Reverse	5'-TTGGCTCTGGCGATGTGGC-3'

Primers for ddPCR

Tagln: dMmuCPE5113593

Acta2: dMmuCPE5117282

Gapdh: dMmuCPE5195282

Supplementary Table S2. Clinical background of human PDAC samples

Patient background			
Sample	Age	Gender	Clinical stage (UICC)
Case1	63	Male	IA
Case2	66	Male	IIA
Case3	73	Male	IIA
Case4	76	Male	IIA
Case5	69	Male	IIA
Case6	69	Male	IIA
Case7	59	Male	IIB
Case8	67	Male	IIB
Case9	60	Male	IIB
Case10	64	Female	IIB
Case11	65	Male	IIB
Case12	78	Male	IIB
Case13	76	Male	IIB
Case14	65	Male	IIB
Case15	63	Female	IIB
Case16	62	Male	III
Case17	57	Male	III
Case18	53	Female	III
Case19	68	Female	III
Case20	65	Male	III