Supplementary Information

Materials and Methods

Cells

Murine pancreatic cancer cells KPC FC1199, referred to as KPC, were generated in the Tuveson lab (Cold Spring Harbor Laboratory, New York, USA) isolated from PDA tumor tissues obtained from $Kras^{G12D/+}$; $p53^{R172H/+}$; Pdx1-Cre mice of a pure C57BL/6 background as described previously with minor modifications [24]. For some experiments, $KPC^{luc/zsGreen}$ cells were generated by using pHIV Luc-zsGreen (gift from Bryan Welm, Addgene plasmid # 39196) lentiviral particle infection. Infected cells were selected for high zsGreen expression levels using flow cytometry cell sorter (ARIA, BD).

Primary murine hepatic stellate cells were isolated from murine livers of 10-12 week old female C57BL/6 mice according to previously reported protocols [53] . The murine immortalized hepatic stellate cell line from livers of C57BL/6 mice was generously provided by Takao Sakai's Laboratory, University of Liverpool, UK) [53]. Primary murine dermal fibroblasts were generated as previously described [19]. All fibroblasts were used at an early passage (passage 2-4).

Human primary pancreatic fibroblasts were acquired from Vitro Biopharma, human peripheral blood monocytic cell line THP-1 and human pancreatic cancer cell line PANC1 were acquired from ATCC, and murine C57BL/6 Panc02 pancreatic ductal carcinoma cells were obtained from the NCI DCTD Tumor Repository, NIH.

All cells were cultured in DMEM+10% FBS and supplemented with 100 units Penicillin, 100 μg/mL Streptomycin and 2.5 μg/mL Amphotericin B, except human primary macrophages and THP-1 cells which were cultured in RPMI+10% FBS and supplemented with Penicillin/Streptomycin and Amphotericin B.

Primary murine macrophages were generated by flushing the bone marrow from the femur and tibia of C57BL/6 mice followed by incubation for 5 days in DMEM containing 10% FBS and 10 ng/mL murine M-CSF (Peprotech). For some experiments, differentiated macrophages were polarized into an M1, M2, or tumour educated phenotype using INFγ (20 ng/ml, Peprotech) and LPS (100 ng/ml, Sigma Aldrich); IL-4 (20 ng/ml, Peprotech); or tumour conditioned media, respectively. Primary human macrophages were generated by purifying CD14+ monocytes from human total blood followed by incubation for 5 days in RPMI containing 10% FBS and 50 ng/mL recombinant human M-CSF (Peprotech). THP-1 cells were incubated with 50 nM phorbol 12-myristate 13-acetate for 72 hours in RPMI + 10% FBS to generate THP-1 derived macrophages. For some experiments, differentiated primary macrophages or THP-1 derived human macrophages were polarized into an M1, M2, or tumour educated phenotype using INFγ (50 ng/ml, Peprotech) and LPS (10 ng/ml, Sigma Aldrich); IL-4 (40 ng/ml, Peprotech); or tumour conditioned media, respectively.

All cells were routinely tested negative for the presence of mycoplasma contamination. None of the cell lines used in this manuscript is listed in the ICLAC and NCBI Biosample database of misidentified cell lines. Cell lines have been directly purchased from ATTC and NCI DTCP respectively, as

described above.

Mice

We obtained transgenic mice lacking PI3-kinase γ expression (p110$\gamma^{-/-}$) on the C57BL/6 background [33] from Dr. Emilio Hirsch, Institute of Molecular Biotechnology Center, University of Torino, Italy. Grn$^{-/-}$ mice (B6(Cg)-*Grn*$^{tm1.1Aidi}$/J) and tdTomatoRed mice (B6.129(Cg)-Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J) both on the C57BL/6 genetic background were purchased from The Jackson Laboratory. C57BL/6 mice were purchased from Charles River. Kras$^{G12D/+}$; p53$^{R172H/+}$; Pdx1-Cre mice [24] were purchased from CRUK, Cambridge Research Institute, Cambridge.

All animal experiments were performed in accordance with current UK legislation under an approved project licence PPL 40/3725 (Dr M Schmid). Mice were housed under specific pathogen-free conditions at the Biomedical Science Unit at the University of Liverpool. In general, for animal studies the group size was calculated by power analysis using a significance level kept at 5% and the power at 80% (according to approved corresponding Home Office Project License Application). For tumour studies, female animals age 6-8 weeks were used. Animals were randomly assigned to experimental groups. The investigators were not blinded to allocation during experiments and outcome assessments.

Bone Marrow Transplantation

Bone marrow transplantation was performed by reconstituting the bone marrow of lethally irradiated (10Gy) female, 6-weeks old C57BL6 mice by tail vein injection of 5 x 10^6 total bone marrow cells isolated from tdTomatoRed mice or Grn-/- mice [35]. After 4 weeks successful engraftment of tdTomatoRed bone marrow was assessed by flow cytometry. WT recipient mice reconstituted with tdTomatoRed bone marrow showed exclusively tdTomatoRed+ hematopoietic cells, while WT recipient mice reconstituted with unlabelled WT bone marrow did not show any tdTomatoRed+ signal. After 4 weeks engraftment of Grn-/- bone marrow was assessed by genomic DNA PCR according to The Jackson Laboratory protocol on peripheral blood cells from fully recovered bone marrow transplanted mice. After confirmation of successful bone marrow reconstitution mice were enrolled in tumour studies.

Metastasis studies

Experimental liver metastasis was performed by implanting 1x10^6 KPC cells or 1x10^6 Panc02, respectively, unless otherwise stated, in 20 µl PBS into the spleen of immunocompetent isogenic C57BL/6 mice using a Hamilton 29 G Syringe as previously described [32, 52]. Experimental lung metastasis was performed by injecting 5x10^5 KPC cells in 200 µl PBS into the tail vein of immunocompetent isogenic C57BL/6 mice using an insulin syringe. At indicated time points, mice were euthanized and metastatic tumour burden was assessed by quantifying the frequency and size of metastatic lesions in hematoxylin and eosin-stained paraffin-embedded liver or lung sections by microscopy using ZEN imaging software.

Clodronate Liposome treatment

Macrophages were depleted *in vivo* by intraperitoneal injections of 200 µl PBS Liposomes (Control) or Clodronate Liposomes (5 mg of clodronate per 1 ml) from ClodronateLiposomes.com [54].

Flow Cytometry

Single cell suspensions from murine livers were prepared by mechanical and enzymatic disruption in Hanks Balanced Salt Solution (HBSS) with 1 mg/mL Collagenase P (Roche) as previously described [55] with some modifications. Briefly, cells suspension were centrifuged for 5 min. at 1200 rpm, resuspended in HBSS and filtered through a 500 µm polypropylene mesh (Spectrum Laboratories). Cell suspension was resuspended in 1mL 0.05%Trypsin and incubated at a 37°C for 5 minutes. Cells were filtered through a 70 µm cell strainer and resuspended in PBS + 5% BSA. Cells were blocked for 10 minutes on ice with FC Block (BD Pharmingen, Clone 2.4G2) and then stained with Sytox viability marker (Life Technologies) and fluorochrome-conjugated antibodies (Supplementary Table 5). Flow Cytometry was performed on a FACSCanto II (BD Biosciences) and FACS performed on a FACSAria (BD Biosciences). For FACS experiments, cells were sorted directly into RLT buffer + β-Mercaptoethanol according to the manufacturer's instruction for RNA isolation.

Magnetic bead isolation of cells

Samples for magnetic bead isolation were prepared from livers as described above for preparation of flow cytometry samples. Then samples were stained and PDGFRα+ or F4/80+ cells were isolated according to the manufacturer's instruction (Miltenyi).

Conditioned Medium Preparation

Conditioned medium from all cells were generated according to previous reports (Kaplan et al., 2005). Briefly, the medium was removed from 70% confluent cells and the cells were washed three times with PBS before addition of serum-free medium**.** Cells were incubated for 18-24 hours in serum-free medium then harvested and filtered through 0.45µm filters before use or stored at -20°C.

Proteomics

To analyse the secretome of cancer educated macrophages, THP-1 derived human macrophages were stimulated for 24 hours with conditioned medium from the human pancreatic cancer cell line PANC1. Cells were then washed three times with PBS and incubated for 24 hours in serum-free DMEM. After 24 hours of incubation, over 100 ml of cell culture supernatant was collected and filtered through 0.45µm filters for mass spectrometry. The samples were spiked with 10µg of human serum albumin (Sigma-Aldrich) as an internal reference and all proteins in each sample were concentrated using MWCO 3kDA Amicon Ultra Centrifugal Filtration Tubes (Millipore) according to the manufacturer's instructions. The protein mixtures were reduced, alkylated, and tryptic-digested as described previously[56]. Each sample was individually labelled with a TMT kit (Thermo Scientific), and then aqueous hydroxylamine solution (5% w/v) was added to quench the reaction. After combined, the labelled peptide samples were vacuum-dried, and then dissolved in 0.1% formic acid for LC-

MS/MS analysis. The labelled samples were analysed using a 2D-LC-MS/MS system consisting of a nanoACQUITY UltraPerformance LC System (Waters) and an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific) equipped with a nano-electrospray source[56]. Briefly, five µL aliquots of samples were resolved on a strong cation exchange (5 µm, 3 cm) column, trapped on a C_{18} trap column, and then separated on a 200 mm microcapillary C_{18} column (particle size 3 µm) packed into 100 µm silica tubing. During the chromatographic separation, the mass spectrometer was operated in a data-dependent mode at the voltage of 2kV with an acquisition parameter as follows: five data-dependent CID-HCD dual MS/MS scans per full scan; CID scans acquired with two-microscan averaging; full scans and HCD scans acquired at resolution 60,000 and 15,000, respectively, with two-microscan averaging; 35% normalized collision energy (NCE) in CID and 45% NCE in HCD; ±1 Da isolation window. Previously fragmented ions were excluded for 60 sec. Each selected parent ion was first fragmented by CID and then by HCD. MS/MS spectra were analysed against the Uniprot human database (released Mar. 18. 2013). ProLucid [57] was used to identify the peptides with a precursor mass error of 25 ppm and a fragment ion mass error of 600 ppm. TMT modification (+229.1629), carbamidomethylation at cysteine, and oxidation at methionine were considered. The CID and HCD tandem MS spectra from the same precursor ion were combined to allow better peptide identification and quantification [58]. We used homemade software where reporter ions from HCD spectrum were inserted into CID spectrum with the same precursor ion at the previous scan. Reporter ions were extracted from small windows (±20 ppm) in the HCD spectrum. The output data files were filtered and sorted to compose the protein list using the DTASelect [59] with ≥2 peptides assignments for a protein identification and a false positive rate ≤0.01.

For quantitative analysis, Census in IP2 pipeline (Integrated Proteomics) was used. Protein abundance was calculated from the average of a reporter ion's intensities from all constituent peptides from a protein[60]. Two different TMT reagents were used to control the quality in quantification, and the data with ≥30% variation was excluded. Relative protein abundance was calculated from the ratios of reporter ion intensities

To identify secreted proteins in the THP-1 macrophages, the full list of identified proteins were analysed using the gene ontology consortium webpage for the GO term "Extracellular Vesicular Exosome " (Gene ontology: tool for the unification of biology [61]) and then referenced to the Uniprot database (www.uniprot.org) for confirmation.

Dermal Fibroblast/Hepatic Stellate Cell Activation

Primary PDGFRα+ murine dermal fibroblasts or hepatic stellate cells were isolated as previously described [19, 53]. On the day before stimulation, dermal fibroblasts were washed three times with PBS and then incubated overnight in DMEM+1%FBS. The next morning, dermal fibroblasts were stimulated with macrophage conditioned medium (supplemented with 1% FBS) or DMEM + 1%FBS as control and incubated for 24 hours.

Murine hepatic stellate cells were washed three times with PBS and then incubated overnight in

DMEM+2%FBS. The next morning, hepatic stellate cells were stimulated with macrophage conditioned medium (supplemented with 2% FBS) from WT or Grn-/- macrophages or DMEM + 2%FBS as control and incubated for 24 hours. To rescue the phenotype from Grn-/- macrophage conditioned medium, Grn-/- MCM were supplemented with recombinant murine Progranulin (R&D Systems, 2557-PG-050) at 1µg/mL for activation experiments.

ELISA

Hepatic stellate cells were stimulated with macrophage conditioned medium (supplemented with 2% FBS) from WT or Grn-/- macrophages or DMEM + 2%FBS as control and incubated for 24 hours.. After 24 hours the hepatic stellate cells were washed three times with pre-warmed PBS and then incubated for 48 hours in serum- and phenol red-free DMEM. After 48 hours the medium was collected and used for ELISA to measure Periostin according to the manufacturer's instruction (R&D Systems, MOSF20). For murine macrophages, cells were stimulated with KPC or Panc02 conditioned medium for 24 hours, then washed three times with PBS and incubated in serum- and phenol red-free DMEM. The conditioned medium was collected after 48 hours and used for ELISA to measure Granulin according to the manufacturer's instruction (LS Bio, LS-F284)

Transwell Migration Assay

Transwell inserts with 8µm pore size (Corning) were coated with 50µl growth factor-reduced Matrigel (BD Biosciences) and allowed to solidify. Macrophages were seeded in DMEM+10%FBS in a 24-well plate. Growth media was removed, cells were 3 times washed with PBS and DMEM + 1% FBS was added. Next, fibroblasts were added in DMEM + 1% FBS into the inserts and allowed to migrate through the insert membrane for 48 hours. The inserts were removed, and the inside was swapped thoroughly with cotton swaps and then fixed in Methanol containing 0.05% Crystal Violet. Number of migrated fibroblasts was counted by bright field microscopy. For transwell migration of hepatic stellate cells, the hepatic stellate cells were labelled with Vybrant Dil (Life Technology, emission 565) according manufactures protocol before addition to the inserts. Relative migration was evaluated by the amount of pixels from each condition relative to the pixels from the control using Zen Software.

Proliferation Assay

Human primary pancreatic fibroblasts were seeded at a concentration of 2000 cells pr. well in 96-well plates in DMEM + 10% FBS. Cells were washed three times with PBS and then stimulated with conditioned medium (supplemented with +1% FBS) prepared from primary human or THP-1 macrophages or DMEM+1% FBS as a control.

Cell viability was determined by measuring the conversion of water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan. The concentration of formazan was determined by optical density at 595 nm.

KPC cells were seeded at a concentration of 2000 cells pr. well in 96-well plates in DMEM + 10% FBS. Cells were washed three times with PBS and then stimulated with serum-free DMEM as control or hepatic stellate cell conditioned medium with or without a neutralising antibody against Periostin

(R&D Systems, AF2955). Cell viability was determined as described by measuring after 48 and 72 hours.

Multicellular Colony Formation Assay

Tumour cells were strained through a 70µm cell filter to ensure a single cell suspension. The cells were then embedded at a concentration of 2000 cells/well in a 0.3% agar mix consisting of either DMEM + 5%FBS (control) or myofibroblast conditioned medium + 5%FBS. A neutralising anti-Periostin antibody (Murine: R&D Systems, AF2955; Human: Abcam14041) was incubated for 10 minutes with the myofibroblast conditioned medium before embedding into the agar matrix. A layer of control medium or myofibroblast conditioned medium was added on top of the agar matrix with or without the anti-Periostin antibody. The colonies were fixed and stained with 0.05% Crystal Violet in Methanol/PBS and the number of colonies pr. well were counted at day 14 after cell seeding by bright field microscopy.

RT-qPCR

Cells were lysed in RLT buffer + β-Mercaptoethanol according to the manufacturer's instruction for RNA isolation. Total RNA purification was performed with the RNeasy kit and cDNA was generated using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instruction. 500 ng of total RNA was used to generate cDNA. RT-qPCR assays were performed with LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480 (Roche). Relative expression levels were normalized to *gapdh* expression according to the formula 2^− (Ct *gene of interest* – Ct *gapdh*). Fold increase in expression levels were calculated by comparative Ct method 2^- (ddCt) [62].
Primers are listed in Supplementary Table 4.

Immunofluorescence

For human tissue sections, antigen retrieval was done in high pH-buffer using the Dako PT-Link System. Tissue sections were blocked in PBS + 0.1% bovine serum albumin and 8% normal goat serum prior incubation with primary antibodies. Tissue sections were stained with secondary antibodies including DAPI (Life Technologies) and mounted using Dako Fluorescent Mounting Medium.

Murine liver tissues were embedded in Optimal Cutting Temperature (OCT) medium and stored at -80°C. Tissue sections were fixed in ice-cold acetone, permeabilized with PBS + 0.1% Triton X-100, blocked with PBS + 8% normal goat serum, and incubated with primary antibodies. Next, tissue sections were washed in PBS, stained with secondary antibodies including DAPI (Life Technologies, 1:100) and mounted using Dako Fluorescent Mounting Medium. Goat anti-rat or goat anti-rabbit secondary antibodies conjugated to AlexaFluor488 and AlexaFluor594 were used (Abcam, 1:500).

Livers from experiments involving ZsGreen/Luciferase-transfected cells were fixed using a sucrose gradient method to preserve the zsGreen fluorescence [63]. All tissue sections were imaged using an Axio Observer Light Microscope with the Apotome.2 (Zeiss) and quantified using the Zen Software (Zeiss). Antibodies are listed in Supplementary Table 5.

Immunohistochemical analysis

Deparaffinization and antigen retrieval was performed using an automated DAKO PT-link. Paraffin-embedded human and mouse PDA tumors were immunostained using the DAKO envision+ system-HRP. Tissue sections were incubated with primary antibodies followed by secondary-HRP conjugated antibody (from DAKO envision kit). Staining was developed using diamino-benzidine and counterstained with hematoxylin. Antibodies are listed in Supplementary Table 5.

Connective Tissue Staining (Masson trichrome staining)

Masson trichrome staining was performed according to the manufacturer's instruction (Abcam ab150686).

Human Studies

Human studies using blood samples were approved by the National Research Ethnics (NRES) Service Committee North West – Greater Manchester North 08/H1011/36 and NRES Committee North West –Cheshire 11/NW0083 and REC15/NW/0477. Human blood samples were obtained from advanced PDAC patients with liver metastasis (all pathologically confirmed) or from control healthy subjects. All individual provided informed consent for blood donation on approved institutional protocols. Blood was collected in sodium heparin (NaH) tubes and immediately processed for mononuclear cell isolation using gradient centrifugation (Histopaque 1077, Sigma Aldrich) according to manufactures protocol.

Mononuclear cells were resuspended in PBS + 2% BSA and blocked using human TrueStain FcX (Biolegend) following staining with antibodies and the viability marker SYTOX Green (Life Technologies). Flow Cytometry analysis was performed on a FACSCanto II (BD Biosciences) and flow cytometry based cell sorting was performed on a FACSAria (BD Biosciences). For cell sorting, cells were sorted directly into RLT buffer + β-Mercaptoethanol according to the manufacturer's instruction for RNA isolation (see qPCR section for further details).

Paraffin embedded human tissue sections from control healthy subjects and advanced PDAC patients with liver metastasis were obtained from the Liverpool Tissue Bank, University of Liverpool, UK or approved by NRES Committee North West –Cheshire REC15/NW/0477. All samples were pathologically confirmed.

Statistics and reproducibility

The level of significance was determined by a two-tailed unpaired Student's t-test and analysed with GraphPad Prism5 software. $p<0.05$ was considered significant.

Immunohistochemical and immunoflurorescence analysis of human healthy and metastatic liver biopsies (Fig.1a, 4e, 6d and Supplementary Fig. 1a,b,c) are shown from n = 5 healthy subjects and n = 5 metastatic patients, and human primary PDAC tumour (Supplementary Fig. 8f) n = 4. Images were acquired and analysed using 5 different visual fields form each tissue section. For

immunohistochemical and immunofluorescence analysis of murine tissue sections following numbers of different visual fields from each tissue section were analysed: Supplementary Fig. 4e (2 fields); Supplementary Fig. 3d (3 fields); Fig. 1c,d,f, 2d, 5d,h, 7d,e (4 fields); Fig. 2f, 4d, 5j,k, 6d, and Supplementary Fig. 5e, 8a,c,d,e (5 fields), Supplementary Fig. 6f, 7e (8 fields). For Fig. 2b,c,g,h, 5b,c,f,g and Supplementary Fig. 4c,d, 5f,g, 8b all metastatic nodules were counted and measured (size) within one liver section. Numbers of different samples used to acquire images are reported in the corresponding figure legends. Transwell migration assays (Fig. 3b,d) are shown as mean from n = 3 independent experiments. For each experiment, 10 different visual fields per condition were counted. Colony formation assays (Fig. 6c) are shown as mean from n = 3 independent experiments, total number of colonies per cell type per experiment were counted. QPCR analysis (Fig. 3a,c,f and Supplementary Fig. 6d,e, 7d) are shown as mean from n = 3 independent experiments. QPCR analysis of *in vivo* derived murine cells are shown as mean from three technical replicates whereby samples were pooled from several animals from each condition: Fig. 4a,c, 5e (3 pooled mice); Fig. 8d (4 pooled mice) Fig. 5i (5 pooled mice), Fig. 5l, 7f (6 pooled mice); QPCR analysis of *in vivo* derived human cells are shown as mean from n = 3 healthy and n = 4 PDAC patients (Fig. 8c). ELISA analysis (Figs 3g, 4b, 7c) are shown as mean from n = 3 independent experiments, whereby two (Figs 4b, 7c) and three (Fig. 3g) technical replicates per condition per experiment were used.

Access to public repository for mass spectrometry data:

Mass Spec data have been placed in public repository MassIV database, accession ID MSV000079491 with a title of "THP_1 and human fibroblast secretomes".

Direct link: ftp://massive.ucsd.edu/MSV000079491

References associated with methods only:

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