Macrophage-secreted granulin supports pancreatic cancer metastasis by inducing liver fibrosis

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Pancreatic ductal adenocarcinoma (PDAC) is a devastating metastatic disease for which better therapies are urgently needed. Macrophages enhance metastasis in many cancer types; however, the role of macrophages in PDAC liver metastasis remains poorly understood. Here we found that PDAC liver metastasis critically depends on the early recruitment of granulin-secreting inflammatory monocytes to the liver. Mechanistically, we demonstrate that granulin secretion by metastasis-associated macrophages (MAMs) activates resident hepatic stellate cells (hStCs) into myofibroblasts that secretes periostin, resulting in a fibrotic microenvironment that sustains metastatic tumour growth. Disruption of MAM recruitment or genetic depletion of granulin reduced hStC activation and liver metastasis. Interestingly, we found that circulating monocytes and hepatic MAMs in PDAC patients express high levels of granulin. These findings suggest that recruitment of granulin-expressing inflammatory monocytes plays a key role in PDAC metastasis and may serve as a potential therapeutic target for PDAC liver metastasis.

Pancreatic cancer is among the most lethal cancers in part owing to its aggressive metastatic nature.¹² Metastatic spreading is a multistage process, which starts with the dissemination of cancer cells from the primary tumour site and ends with clinically detectable metastatic outgrowth at distant organs. Tumours can release large numbers of cancer cells into the circulation, but only a small proportion of these cells are able to successfully survive and colonize the hostile environment at the distant metastatic site.³⁴ Thus, the successful outgrowth of metastatic tumour cells in this new distant environment is a severe rate-limiting step during metastasis. Emerging evidence indicates that the colonization of a new organ by metastatic tumour cells critically depends on the support of non-cancerous stromal partners.⁵⁻⁸ Multiple steps of the metastatic cascade are supported by stromal partners, particularly macrophages and, in many cancers, metastasis correlates with increased macrophages at the metastatic site.⁶⁻¹⁴ PDAC is characterized by its formation of ductal structures and a rich stromal compartment containing mainly fibroblasts, stellate cells and infiltrating immune cells. In response to the presence of tumour cells, quiescent fibroblasts and stellate cells become activated myofibroblasts that express alpha smooth muscle actin (αSMA). In non-pathological conditions, myofibroblasts are critical for wound healing.¹⁶ In cancer, the role of myofibroblasts is controversial at present. Previous studies have suggested that myofibroblasts support tumour growth and restrict the delivery of chemotherapeutic drugs to the tumour.¹⁷⁻¹⁹ However, recent reports have shown that genetic depletion of myofibroblasts results in tumour progression and metastasis.²⁰,²¹ Most pancreatic cancer studies have focused on the role of stromal partners at the primary site; however, the role of stromal cells at the secondary metastatic site, which for PDAC is most often the liver, remains poorly understood.

At the moment, the best treatment option for PDAC patients is resection of the pancreatic tumour, but, unfortunately, by the time PDAC patients are diagnosed, most (~80%) present with non-resectable metastatic cancer. Moreover, more than 60% of the patients whose tumours are resected, relapse with distant hepatic recurrence within the first 24 months after surgery.²²,²³ Thus, a better understanding of the mechanisms underlying the metastatic process in pancreatic cancer is critical to improve treatment and patient outcome.

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RESULTS
Metastatic PDAC cells trigger macrophage recruitment and an extensive stromal response in the liver

The most common route of PDAC metastasis is to the liver. To understand whether and how stromal cells influence liver PDAC metastasis, we first analysed liver biopsies from advanced metastatic PDAC patients and healthy volunteers by immunohistochemical and immunofluorescence techniques. We found that metastatic tumour cells (cytokeratin+) are surrounded by an abnormal stromal compartment rich in haematopoietic immune cells (CD45+), macrophages (CD68+), myofibroblasts (αSMA+; PDGFRα+) and connective tissue deposition (Fig. 1a and Supplementary Fig. 1a–c).
To further evaluate the type of immune cells accumulating at the metastatic site, we intrasplenically injected KPC-derived cells (FC1199), isolated from the genetically engineered mouse model of PDAC (KrasG12D, Trp53R172H, Pdx1–Cre mice)24. In this model, tumour cells migrate to the liver through the portal circulation (a common way of metastasis occurring in humans)25,26, and generate metastases restricted to the liver (Supplementary Fig. 1d). We analysed established metastatic tumours at day 12, by flow cytometry, and found that the percentages of CD45+ immune cells, B220+ B cells, CD3+ T cells, Nk1.1+ NK cells, CD11b+Ly6G+ neutrophils,
and F4/80<sup>+</sup> macrophages were increased in tumour-bearing livers compared with tumour-free livers (Supplementary Fig. 2a–c). However, among the CD45<sup>+</sup> immune cells, metastasis-associated macrophages (MAMs; CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6G<sup>neg</sup>Ly6C<sup>+</sup>Ly6G<sup>neg</sup>CCR2<sup>+</sup>) were the most predominant cell population (Fig. 1b, Supplementary Figs 2d and 3a,b). Interestingly, metastatic tumour cells initially induced a rapid accumulation of CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6G<sup>neg</sup>Ly6C<sup>+</sup>Ly6G<sup>neg</sup>CCR2<sup>+</sup> inflammatory monocytes (IMs) followed by an accumulation of MAMs (Supplementary Fig. 3a). In contrast, accumulation of αSMA<sup>+</sup> myofibroblasts and stromal expansion were detected only in established experimental and spontaneous metastatic lesions (Fig. 1c,d and Supplementary Fig. 3b–g).

Macrophages in the liver can be broadly categorized into two classes: embryonically derived tissue-resident macrophages known as Kupffer cells (KCs), and infiltrating macrophages derived from IMs that originate from the bone marrow (BM). To investigate whether the increased number of MAMs we observe is due to an expansion of resident KCs or to the recruitment of BM-derived cells, we generated BM chimaeras by engrafting tdTomato red (tdTomato) BM into irradiated wild-type mice (WT + tdTomato<sup>+</sup> BM) or WT BM into irradiated WT mice (WT + WT BM). As KCs in the liver are radio-resistant<sup>31</sup>, they remain of host origin and are therefore F4/80<sup>+</sup> tdTomato<sup>+</sup>, whereas BM-derived macrophages are of donor origin and F4/80<sup>+</sup> tdTomato<sup>+</sup> (Fig. 1e). After confirming successful BM reconstitution (Supplementary Fig. 3h), chimaeric mice were intrasplenically implanted with the murine PDAC cell line Panc02<sup>22</sup>. We found that MAMs were exclusively tdTomato<sup>+</sup>, and, thus, derived from BM (Fig. 1f and Supplementary Fig. 3i).

Conversely, in adjacent 'normal liver tissue', F4/80<sup>+</sup> macrophages remained tdTomato<sup>+</sup>, consistent with resident F4/80<sup>+</sup> KCs (Fig. 1f). In addition, αSMA<sup>+</sup> metastasis-associated myofibroblasts remained tdTomato<sup>+</sup>, suggesting that these cells are not of BM origin, but are locally activated mesenchymal cells, such as resident hepatic stellate cells (hStCs) or fibroblasts (Fig. 1f and Supplementary Fig. 3j).
Figure 4 Granulin is highly expressed in hepatic metastatic lesions and metastasis-associated macrophages are the main source of granulin secretion. (a,b) Quantification of Grn mRNA levels (a) and granulin protein levels (b) in intrametastatic pancreatic cancer cells, immune-cell-depleted stromal cells (zsGreenCD45neg), and MAMs (CD45F4/80+) isolated by fluorescence-activated cell sorting from established tumour-bearing livers 12 days after intrasplenic implantation of 1 × 10⁶ KPC4zsGreen cancer cells (a, data are from three pooled mice; one experiment; b, n=3 independent experiments; mean ± s.e.m.; two-tailed unpaired t-test). (c) Quantification of Grn and Tgfb mRNA levels in tissue resident macrophages (KCs) and MAMs sorted from established metastatic lesions from chimaeric WT mice harbouring tdTomatoRed+ BM as described in Fig. 1g (data are from three pooled mice; one experiment). (d) Spontaneous metastatic hepatic tumours derived from KPC mice were isolated and analysed. Representative images of immunohistochemistry staining for MAMs (CD68+) and granulin expression on serial tissue sections from metastatic lesions and healthy liver and quantification of the data (n=5 control mice, n=5 KPC mice, five fields assessed per sample; mean ± s.e.m.; two-tailed unpaired t-test). HL, healthy liver; LM, liver metastasis. (e) Representative images of immunohistochemistry staining for MAMs (CD68+) and granulin expression on serial tissue sections from human metastatic PDAC lesions and healthy liver and quantification of the data (n=5 healthy subjects, n=5 PDAC samples; five fields assessed per sample; mean ± s.e.m.; two-tailed unpaired t-test). Scale bars, 100μm; asterisks indicate matched tissue areas.

Together, our findings demonstrate that in PDAC liver metastasis, MAMs originate from IMs; MAMs represent the predominant immune cell population in metastatic liver lesions; and MAM accumulation in the liver precedes myofibroblast activation.

Macrophages promote myofibroblast activation and metastatic growth

To determine the functional role of MAMs in pancreatic cancer metastasis, we next intrasplenically implanted KPC cells into isogenic PI(3)Kγ−/− mice (p110γ−/−) and control WT mice. PI(3)Kγ is mainly expressed in the haematopoietic compartment, and PI(3)Kγ knockout mice have a defect in monocyte recruitment in response to inflammatory and tumour-derived signals. As we previously found that MAMs are IM-derived macrophages that are actively recruited from the BM to the metastatic liver (Fig. 1f), we reasoned that depletion of PI(3)Kγ will ablate IM trafficking to the metastatic site and consequently abolish MAM accumulation in the liver. Indeed, macrophage and IM numbers were significantly reduced in metastatic livers of PI(3)Kγ−/− mice compared with WT mice as measured by flow cytometry analysis (Fig. 2a). Importantly, PI(3)Kγ depletion markedly reduced metastatic frequency, average metastatic lesion size, and αSMA+ myofibroblast numbers, in both KPC (Fig. 2b–d and Supplementary Fig. 4a) and Panc02 (Supplementary Fig. 4b–e) liver-metastasis-bearing mice. Together, these results suggest that inhibition of BM-derived macrophage recruitment to the liver prevents pancreatic cancer metastasis to the liver and is accompanied by a decrease in myofibroblast activation.

As most (~80%) PDAC patients present with liver metastases at the time of diagnosis, or relapse with hepatic recurrence after surgical removal of primary pancreatic tumours, we next focused our
Figure 5 Granulin depletion prevents myofibroblast activation and PDAC metastasis. (a–l) Liver metastasis was induced by intrasplenic implantation of 5 × 10^5 KPC cells into WT and granulin-deficient (Grn−/−) mice (a–e) and chimaeric WT + WT BM and WT + Grn−/− BM mice (f–l). Entire livers were collected and analysed 12 days later. (a) Representative images of haematoxylin and eosin staining of liver sections (data are from 6 WT and 7 Grn−/− mice; one experiment). (b) Metastatic frequency (n = 6 WT mice, n = 7 Grn−/− mice; all metastatic nodules assessed from one section per sample; one experiment; individual data points, horizontal lines represent mean ± s.e.m.; two-tailed unpaired t-test). (c) Metastatic area (n = 6 WT mice, n = 7 Grn−/− mice; all metastatic nodules assessed from one section per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). (d) Representative immunofluorescent staining and quantification of MAM (F4/80+) and myofibroblast (αSMA+) cell frequency in tumour-bearing livers. Nuclei were counterstained with DAPI (n = 6 WT mice, n = 7 Grn−/− mice; four fields assessed per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). (e) qPCR analysis of multiple hStC activation markers in intrametastatic myofibroblasts isolated from established metastatic lesions (data are from three pooled mice per condition; one experiment). (f) Metastatic frequency (n = 5 WT + WT BM mice, n = 6 WT + Grn−/− BM mice; all metastatic nodules assessed from one section per sample; one experiment; individual data points, horizontal lines represent mean ± s.e.m.; two-tailed unpaired t-test). (g) Metastatic area (n = 5 WT + WT BM mice, n = 6 WT + Grn−/− BM mice; all metastatic nodules assessed from one section per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). (h) Representative immunofluorescent staining and quantification of MAM (F4/80+) and myofibroblast (αSMA+) cell frequency in tumour-bearing livers. Nuclei were counterstained with DAPI (n = 5 WT + WT BM mice, n = 6 WT + Grn−/− BM mice; four fields assessed per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). (i) qPCR analysis of multiple hStC activation markers in intrametastatic myofibroblasts isolated from established metastatic lesions (data are from five pooled mice per condition; one experiment). (j) qPCR analysis of multiple M2- and M1-macrophage-associated genes in MAMs isolated from metastatic tumours developed in WT + WT BM and WT + Grn−/− BM mice (data are from six pooled mice per condition; one experiment). (k,l) Representative immunohistochemical staining and quantification of Ki67+ tumour cell frequency (k) and cleaved caspase 3+ cell numbers (l) in metastatic livers (n = 5 WT + WT BM mice, n = 6 WT + Grn−/− BM mice; five fields assessed per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). Scale bars, 100 m; NS, not significant.
Figure 6 Myofibroblast-secreted peristin enhances pancreatic cancer cell growth. (a) Top gene ontology (GO) functions of secreted proteins enriched in human myofibroblasts (Mf) following in vitro education with macrophage-conditioned media (CM). (b) Fold change rank of identified proteins associated with ECM organization. Data were obtained from one experiment assessing two biologically independent samples (green, peristin). (c) Colony-formation assay of primary murine KPC cells, murine Panc02, and human Panc1 cells in the presence or absence of Mf CM and peristin-neutralizing antibody (anti-peristin). Representative images reflecting colonies and single cells are shown for KPC cells (n = 3 independent experiments; mean ± s.e.m.; two-tailed unpaired t-test). (d) Immunohistochemical (IHC) staining of peristin in human liver biopsies (n = 5 healthy subjects, n = 5 PDAC; five fields assessed per sample; mean ± s.e.m.; two-tailed unpaired t-test) and in spontaneous metastatic liver tumours collected from KPC mice (n = 5 mice per condition; five fields assessed per sample, mean ± s.e.m.; two-tailed unpaired t-test). Representative micrographs and quantification of the data. HL, healthy liver; LM, liver metastasis. Scale bars, 100 μm.

studies on investigating whether MAMs are required for supporting the metastatic growth of already disseminated cancer cells. To address this question, we chemically depleted MAMs in vivo, using clodronate liposomes (CLs). CL treatment was started at day 3 post intrasplenic injection of KPC cells, a time point in which initial seeding of the liver by KPC cells has already occurred (Fig. 2e and Supplementary Fig. 5a,b). As expected, in response to CL treatment, MAM numbers were significantly reduced (Fig. 2f and Supplementary Fig. 5c). Interestingly, we could barely detect any αSMA+ myofibroblasts in metastatic livers of CL-treated mice (Fig. 2f and Supplementary Fig. 5c). The ablation of MAMs and the prevention of αSMA+ myofibroblast accumulation was not cell line specific because similar results were observed using Panc02 cells (Supplementary Fig. 5d,e). Although the metastatic frequency was only modestly affected by CL treatment in both models, the size of the lesion area covered by metastatic cells was significantly reduced in response to macrophage depletion (Fig. 2g,h and Supplementary Fig. 5f,g). Together, these results suggest that depletion of MAMs prevents the activation of myofibroblasts and impairs the progression of metastatic lesions even after initial colonization of the metastatic site by cancer cells.

Granulin secreted by macrophages triggers myofibroblast activation

We next sought to understand how MAMs regulate myofibroblast activation at the metastatic site. In this respect, we found that macrophage conditioned media (CM) acts as a strong activator of quiescent primary fibroblasts in culture and enhances their invasion and proliferation (Supplementary Fig. 6a–c). To identify the macrophage-derived factors responsible for myofibroblast activation, we next exposed human THP-1 macrophages to the pancreatic cancer cell line Panc1 CM in vitro, and performed a secretome analysis. We identified several extracellular matrix (ECM) proteases associated with macrophage function (Supplementary Tables 1 and 2). Among the most highly secreted proteins we identified granulin (Grn), a secreted glycoprotein with a relative molecular mass of...
approximately 70,000 that has previously been shown to mediate wound healing, by stimulating fibroblast migration\(^40\), and to induce fibrosis in breast cancer\(^41\). As the main source of myofibroblasts in the liver is activated resident hStCs (Supplementary Fig. 3j)\(^42\), we next isolated primary hStCs from mice and stimulated them with CM generated from primary murine WT BM-derived macrophages or Grn\(^{-/-}\) (granulin deficient) murine BM macrophages (BMMs). We found that CM generated from WT, but not from Grn\(^{-/-}\), BMMs efficiently activated isogenic hStCs and promoted their migration (Fig. 3a,b). Importantly, addition of recombinant granulin to Grn\(^{-/-}\) BMM CM was sufficient to restore hStC activation and migration (Fig. 3a,b). Next, we isolated MAMs from tumour-bearing livers derived from chimaeric WT + WT BM and WT + Grn\(^{-/-}\) BM mice, and prepared MAM CM. We confirmed that only CM from WT MAMs efficiently induced hStC activation and migration, whereas CM from Grn\(^{-/-}\) MAM was unable to induce activation or migration of hStCs (Fig. 3c–e). In addition, we found that granulin expression and secretion is increased in tumour-educated (Fig. 3f,g) and alternatively (M2-like) activated macrophages (Supplementary Fig. 6d,e). Together, these data indicate that cancer-cell-derived factors induce the secretion of granulin in macrophages and that MAMs activate resident hStCs through granulin.

Granulin is highly expressed in pancreatic cancer metastatic lesions of mice and humans and MAMs are the main source of granulin in vivo.

In vivo, we found that within the metastatic tumour microenvironment, granulin is highly expressed in MAMs, and that MAMs are the main source of granulin secretion in metastatic lesions (Fig. 4a,b). When we analysed chimaeric mice harbouring...
Metastatic PDAC patients have increased circulating inflammatory monocytes that express high levels of granulin. (a) Peripheral mononuclear cells were isolated from healthy subjects and metastatic PDAC patients. Representative dot plot of inflammatory monocytes (IMs; CD14<sup>CD16<sup>dim</sup></sup>) and resident monocytes (RMs; CD14<sup>dim</sup>CD16<sup>hi</sup>) after gating for the CD45<sup>CD3<sup>neg</sup></sup> and Sytox<sup>neg</sup> cell population (data are from 6 different PDAC patients and 6 different healthy subjects). (b) Quantification of IMs. Percentage of monocyte populations in healthy subjects and metastatic PDAC patients (n = 6 different healthy; n = 6 different PDAC samples; individual data points, horizontal lines represent mean ± s.e.m.; two-tailed unpaired t-test). NS, not significant. (c) Quantification of Grn mRNA levels in IMs isolated from metastatic PDAC patients and healthy subjects (HSs) as described in a,b (n = 3 different healthy; n = 4 different PDAC samples; mean ± s.e.m.; two-tailed unpaired t-test). (d) Quantification of Grn mRNA levels in circulating IMs sorted from KPC mice with pathological confirmed liver metastasis or tumour-free littermates (data are from four pooled mice per condition; one experiment). (e) Schematic depicting the role of macrophage-derived granulin in activation of hStCs and in PDAC liver metastasis. Inflammatory monocytes are recruited to the liver by metastatic pancreatic tumour cells through a PI(3)K<sub>γ</sub>-dependent mechanism. Once in the metastatic tissue, differentiated macrophages stimulate the activation and recruitment of resident hStCs through granulin secretion, resulting in excessive accumulation of myofibroblasts. Granulin-induced myofibroblasts release high levels of the ECM protein periostin, thereby enhancing survival and growth of metastatic pancreatic cancer cells in a hostile environment. Interruption of this sequence, by either preventing macrophage accumulation or by abolishing granulin expression in recruited macrophages, limits metastatic growth of pancreatic cancer cells.

deTomatoRed BM, we found that BM-derived MAMs, but not resident KCs, express high levels of granulin (Grn) (Fig. 4c). Surprisingly, expression levels of Tgfb<sup>+</sup>, a common activator of hStCs<sup>42</sup>, were low in MAMs and KCs (Fig. 4c).

Finally, we further confirmed that granulin is highly expressed in the stroma of spontaneous hepatic and metastatic lesions of human and murine tissues<sup>24,43</sup> (Fig. 4d and Supplementary Fig. 6f). Taken together, our findings indicate that granulin is highly expressed by MAMs and that MAMs are the main source of granulin in PDAC liver metastasis.

**Depletion of granulin prevents liver fibrosis and PDAC metastatic growth**

To investigate whether granulin is required for PDAC metastatic progression in vivo, we next inoculated Grn<sup>−/−</sup> and WT mice with isogenic KPC cells through intrasplenic implantation. We observed that lack of granulin expression did not alter metastatic frequency because WT and Grn<sup>−/−</sup> mice showed similar numbers of metastatic nodules (Fig. 5a,b). However, the area covered by metastatic tumour cells in Grn<sup>−/−</sup> mice was significantly smaller compared with WT, resulting in an overall significant decrease of hepatic metastatic tumour burden (Fig. 5a,c). Whereas depletion of granulin did not reduce the total number of MAMs (Fig. 5d), nor their polarization at the metastatic site (Supplementary Fig. 7a), granulin deficiency abolished the accumulation of activated myofibroblasts in metastatic liver lesions (Fig. 5d). Quantitative gene expression analysis of myofibroblast activation markers (Acta2, Fn, Colla1)<sup>16</sup> further confirmed that lack of granulin prevents the activation of intrametastatic hStCs into myofibroblasts in vivo (Fig. 5e).

Next, we characterized metastatic pancreatic tumour growth in WT mice transplanted with BM from Grn<sup>−/−</sup> or WT animals. We found that depletion of granulin in the haematopoietic compartment was sufficient to suppress metastatic growth of KPC cells (Fig. 5f,g) and to prevent myofibroblast activation in vivo (Fig. 5h,i), while MAM numbers and polarization remained unchanged (Fig. 5j) and Supplementary Fig. 7b). Interestingly, ablation of granulin in the haematopoietic compartment markedly reduced the number of proliferating (Ki67<sup>+</sup>) metastatic pancreatic tumour cells (Fig. 5k), but apoptotic (cleaved caspase 3<sup>+</sup>) tumour cell numbers remained unchanged (Fig. 5l). Taken together, these results suggest that granulin secretion by MAMs is required for hStCs activation and for metastatic growth of pancreatic tumours in the liver.

Granulin induces periostin expression in hStCs thereby allowing tumour growth

Next, we sought to gain a better understanding of how MAM-induced myofibroblast activation promotes pancreatic cancer cell growth. To address this question, we stimulated human fibroblasts with macrophage CM and performed a mass spectrometry quantitative analysis of the secretome from fibroblasts exposed to macrophage CM compared with unexposed fibroblasts. We found that macrophage CM induces fibroblast secretion of proteins associated with ECM remodelling (Fig. 6a and Supplementary Table 3), and in particular the secretion of the ECM component periostin (Fig. 6b). Periostin has been reported to enhance metastatic growth of breast and colon cancer cells through activation of Wnt and αvβ3-Akt/PKB signalling pathways, respectively. Thus, we next tested whether periostin expression by activated myofibroblasts is necessary to promote pancreatic cancer cell survival and growth. We found that myofibroblast CM markedly promoted colony formation and proliferation of PDAC cancer cells, and that addition of a periostin-neutralizing antibody completely abolished these effects (Fig. 6c and Supplementary Fig. 7c). We confirmed that periostin acts in a paracrine myofibroblast–tumour cell loop because we did not detect any periostin expression in PDAC cells (Supplementary Fig. 7d). Moreover, we found that periostin was markedly upregulated in metastatic lesions, especially in areas rich in CD68+ macrophages (Fig. 7a). Consistent with this finding, we found that MAMs isolated from metastatic PDAC patients secrete high levels of granulin-expressing IMs in blood from metastatic subjects. This observation suggests that granulin could also potentially be used as a predictive biomarker of PDAC metastasis.
and migration during wound healing\textsuperscript{40}. In addition, in breast cancer, granulin expression correlates with increased fibrosis and poor survival\textsuperscript{41}. In agreement with a role for granulin in fibrosis, we found that macrophage-secreted granulin plays a critical role in PDAC metastasis by activating resident hStCs and stimulating the secretion of periostin. The precise signalling pathway by which granulin activates hStCs and primary fibroblasts remains unknown, as the cognate cell-surface receptor to which granulin binds is still controversial\textsuperscript{49-51}. Interestingly, depletion of granulin in the haematopoietic compartment did not change macrophage recruitment to the liver, macrophage polarization, or CD8+ T cell infiltration (Fig. 5 and Supplementary Fig. 7).

In agreement with ref. 52, we found that macrophages play a key role in PDAC metastasis. Whereas ref. 52 describes a role for resident hepatic macrophages in the establishment of a pre-metastatic niche that facilitates initial tumour cells seeding, our work focuses on understanding the subsequent step of metastatic growth. In this respect, we found that once tumour cells have reached the metastatic site, their survival and outgrowth capacity critically depends on the further recruitment of IMs that secrete granulin.

Unfortunately, current imaging approaches are unable to detect micro-metastases and by the time pancreatic cancer patients are diagnosed, micro-metastatic spreading has already occurred in most cases\textsuperscript{1}. Our findings suggest that recruitment of IMs that express granulin plays a key role in pancreatic cancer metastasis and may serve both as a prognostic marker, and a potential target for PDAC liver metastasis.

\section*{METHODS}

Methods and any associated references are available in the online version of the paper.

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\section*{AUTHOR CONTRIBUTIONS}

S.R.N. designed and performed most of the experiments, analysed and interpreted the data, and contributed to the preparation of the manuscript. V.Q. performed qPCR experiments, immunofluorescence staining and immunoblotting. A.L. performed human immune cell analysis and immunohistochemistry. V.Q., P.E., A.S. and L.I. helped with in vivo experiments. C.R. performed flow cytometry and cell sorting. A.S. performed immunohistochemistry. T.S. and K.S. provided primary and immortalized hStCs. Y.-S.K. and J.H.K. performed proteomic analysis. D.E. and D.A.T. provided primary murine KPC-derived pancreatic cancer cells. F.C. helped with the analysis and interpretation of tumour biopsies. D.P. provided patient samples. E.H. provided the PI(3)K<sup>−/−</sup> mouse colony. A.M. provided conceptual advice, designed experiments, and wrote the manuscript. M.C.S. conceived and supervised the project, interpreted data, and wrote the manuscript. All authors critically analysed and approved the manuscript.

\section*{COMPETING FINANCIAL INTERESTS}

The authors declare no competing financial interests.

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METHODS

Cells. Murine pancreatic cancer cells KPC FC1199, referred to as KPC, were generated in the Tuveson laboratory (Cold Spring Harbor Laboratory, New York, USA) isolated from PDA tumour tissues obtained from Kras<sup>G12D/+</sup>; p53<sup>F9</sup>; Pdx1-Cre mice. The tumour was cryopreserved and transplanted into lethally irradiated (10 Gy) female C57BL/6 mice by tail vein injection of 5 x 10<sup>6</sup> KPC cells or 1 x 10<sup>5</sup> Panc02, respectively, in 20 μl PBS into the spleen of immunocompetent isogenic C57BL6 mice using a Hamilton 29 G Syringe as previously described.<sup>21,22</sup> Experimental lung metastasis was performed by injecting 5 x 10<sup>5</sup> KPC cells in 200 μl PBS into the tail vein of immunocompetent isogenic C57BL6 mice using an insulin syringe. At the indicated time points, mice were euthanized and metastatic tumour burden was assessed by quantifying the frequency and size of metastatic lesions in haematoxylin 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<sup>44</sup>. 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<sup>−1</sup> collagenase P (Roche) as previously described<sup>45</sup> with some modifications. Briefly, cells in suspension were centrifuged for 5 min at 1 200 r.p.m., resuspended in HBSS and filtered through a 500 μm polypropylene mesh (Spectrum Laboratories). The cell suspension was resuspended in 1 ml 0.05% trypsin and incubated at 37 °C for 5 min. Cells were filtered through a 70 μm cell strainer and resuspended in PBS + 5% BSA. Cells were blocked for 10 min on ice with FC Block (BD Pharamingen, 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 FACSCan II (BD Biosciences) and FACS performed on a FACSaria (BD Bioscience). For FACS experiments, cells were sorted directly into RLT buffer + β-mercaptoethanol according to the manufacturer’s instructions 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<sup>+</sup> or F4/80<sup>+</sup> cells were isolated according to the manufacturer’s instructions (Miltenyi).

Conditioned medium preparation. Conditioned medium from all cells were generated according to previous reports<sup>18</sup>. Briefly, the medium was removed from 70% confluent cell cultures and the cells were washed three times with PBS before addition of serum-free medium. Cells were incubated for 18–24 h in serum-free medium and then collected and filtered through a 0.45 μm 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 h with conditioned medium from the human pancreatic cancer cell line Panc1. Cells were then washed three times with PBS and incubated for 24 h in serum-free DMEM. After 24 h of incubation, over 100 ml of cell culture supernatant was collected and filtered through 0.45 μm filters for mass spectrometry. The samples were spiked with 10 μg 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<sup>54</sup>. 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 being combined, 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 two-dimensional 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<sup>54</sup>. Briefly, 5 μl aliquots of samples were resolved on a strong cation exchange (5 μm, 3 cm) column, trapped on a C<sub>4</sub> trap column, and then separated on a 200 mm microcapillary C<sub>4</sub> 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 2 kV with acquisition parameters 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 x. Each selected parent ion was first fragmented by CID and then by HCD. MS/MS spectra were analysed against the Uniprot human database (released 18 March 2013). ProLumid<sup>53</sup> 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. For better peptide identification...
identifications and quantifications, six reporter ions were extracted from the HCD spectrum within 0.02 mass tolerance using home-made software and inserted into the corresponding CID spectrum with the same precursor ions\(^6\). The output data files were filtered and sorted to compose the protein list using DTASelect\(^6\) with ≥2 peptides assignments for a protein identification and a false positive rate ≤0.01.

For quantitative analysis, Cenbus 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\(^7\). Two different TMT reagents were used to control the quality in quantification, and data with ≥30% variation were 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 was analysed using the gene ontology consortium web page for the GO term 'Extracellular Vesicular Exosome' (Gene ontology: tool for the unification of biology\(^8\)) and then referenced to the Uniprot database (www.uniprot.org) for confirmation.

Dermal fibroblast/hepatic stellate cell activation. Primary PDGFrβ+ murine dermal fibroblasts or hepatic stellate cells were isolated as previously described\(^16\). 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 a control and incubated for 24 h. 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 a control and incubated for 24 h. To rescue the phenotype from Grn-/- macrophage-conditioned medium, Grn-/- MCM were supplemented with recombinant murine protein\(^3\). ELISA. Hepatic stellate cells were stimulated with macrophage-conditioned medium (supplemented with 2% FBS) from WT or Grn-/- macrophages or DMEM + 2%FBS as a control and incubated for 24 h. After 24 h, the hepatic stellate cells were washed three times with pre-warmed PBS and then incubated for 48 h in serum- and phenol red-free DMEM. At 48 h, the medium was collected and used for ELISA to measure periostin according to the manufacturer's instructions (R&D Systems, MOSF20). For murine macrophages, cells were stimulated with KPC- or Panc02-conditioned medium for 24 h, then washed three times with PBS and incubated in serum- and phenol red-free DMEM. The conditioned medium was collected after 48 h and used for ELISA to measure granulin according to the manufacturer's instructions (LS Bio, LS-F264).

Transwell migration assay. Transwell inserts with 8 μm pore size (Corning) were coated with 50 μl growth factor-reduced Matrigel (BD Biosciences) and allowed to solidify. Macrophages were seeded in DMEM + 10%FBS in a 24-well plate. Growth medium was removed, cells were washed three times 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 h. The inserts were removed, and the inside was swabbed thoroughly with cotton swabs and then fixed in methanol containing 0.05% crystal violet. The 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 to the manufacturer's 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 2,000 cells per 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 2,000 cells per well in 96-well plates in DMEM + 10% FBS. Cells were washed three times with PBS and then stimulated with serum-free DMEM as a control or hepatic stellate cell-conditioned medium with or without a neutralizing antibody against periostin (R&D Systems, AF2955). Cell viability was determined as described by measuring at 48 and 72 h.

Multicellular colony formation assay. Tumour cells were strained through a 70 μm cell filter to ensure a single-cell suspension. The cells were then embedded at a concentration of 2,000 cells per well in a 0.3% agar mix consisting of either DMEM + 5%FBS (control) or myofibroblast-conditioned medium + 5%FBS. A neutralizing anti-periostin antibody (Abcam, 1:500; human: Abcam14041) was incubated for 10 min 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 per well was 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 instructions 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 instructions. Total RNA (500 ng) was used to generate cDNA. RT-qPCR assays were performed using LightCycler-480 SYBR Green 1 Master Mix (Roche) on a LightCycler 480 (Roche). Relative expression levels were normalized to Gapdh expression according to the formula 2−ΔΔCT method \(2^\Delta\Delta CT\) (ref. 63).

Primers are listed in Supplementary Table 4.

Immunofluorescence. For human tissue sections, antigen retrieval was carried out 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 inOptimal 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 theZsGreen fluorescence\(^6\). All tissue sections were imaged using an Alexa Observer Light Microscope with the Apotome.2 (Zeiss) and quantified using the Zen Software (Zeiss). Antibodies are listed in Supplementary Table 5.

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

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

Human studies. Human studies using blood samples were approved by the National Research Ethics (NRES) Service Committee North West—Greater Manchester North 08/H1101/36 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 individuals 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 (Ficoll-Paque 1077, Sigma-Aldrich) according to the manufacturer's protocol.

Mononuclear cells were resuspended in PBS + 2% BSA and blocked using human TrueStain FcX (Biologend) following by staining with antibodies and the viability marker SYTOX Green (Life Technologies). Flow cytometry analysis was performed on a FACSSeq 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 instructions 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 immunofluorescence analyses of human healthy and metastatic liver biopsies (Figs 1a, 4e, 6d and Supplementary Fig. 1a–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 five different visual fields from each tissue section. For immunohistochemical and immunofluorescence analysis of murine tissue sections, the following numbers of different visual fields from each tissue section were analysed: Supplementary Fig. 4c (3 fields); Supplementary Fig. 3d (3 fields); Figs 1c,d, 2d, 5d,h and 7d,e (4 fields); Figs 2f, 4d, 5k and 6d, and Supplementary Figs 5e and 8a,c–e (5 fields), Supplementary Figs 6f and 7c (8 fields). For Figs 2b,c,g,h and 5b,c,f,g, and Supplementary Figs 4c,d, 5f,g and 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 the 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 the mean from *n* = 3 independent experiments; total number of colonies per cell type per experiment was counted. qPCR analysis (Fig. 3a,c,f and Supplementary Figs 6d,e and 7d) are shown as the mean from *n* = 3 independent experiments. qPCR analyses of in vivo-derived murine cells are shown as the mean from *n* = 3 healthy and *n* = 4 PDAC patients (Fig. 8c). ELISA analyses (Figs 3g, 4b and 7c) are shown as the mean from *n* = 3 independent experiments, whereby two (Figs 4b and 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 the public repository MassIV database, accession ID MSV000079491 with the title ‘THP_1 and human fibroblast secretomes’.

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

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Corrigendum: Macrophage-secreted granulin supports pancreatic cancer metastasis by inducing liver fibrosis

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In the version of this Article originally published, in the last sentence of the 'Acknowledgements' section, 'Pancreas Biomedical Research Unit' should have read 'National Institute for Health Research Biomedical Research Unit funding scheme through a NIHR Pancreas BRU'. This has been corrected in all online versions of the Article.
**Supplementary Figure 1** Morphological characterisation of metastatic PDAC lesions in human and mouse. (a) Lower magnification of representative micrographs shown in Fig. 1a. Identification of pan-cytokeratin (CK)\(^+\) metastatic pancreatic cancer cells, hematopoietic cells (CD45\(^+\)), macrophages (CD68\(^+\)) and myofibroblasts (αSM\(^+\)) as predominant cell types at the hepatic metastatic microenvironment of pancreatic cancer by immunohistochemical analysis of human biopsies (data are from 5 PDAC patients and 5 healthy subjects; five fields assessed per sample). HL = healthy liver, LM = liver metastasis. (b) Identification of cytokeratin (CK) 19\(^+\) metastatic pancreatic cancer cells, tumour associated macrophages (CD68\(^+\)) and myofibroblasts (PDGFR\(\alpha\)\(^+\)) as predominant cell types at the hepatic metastatic microenvironment of pancreatic cancer by immunofluorescence analysis of human biopsies. Representative micrographs are shown and quantification of data (n = 5 PDAC, n = 5 healthy subjects; five fields assessed per sample; mean ± s.e.m; two-tailed unpaired t-test). HL = healthy liver, LM = liver metastasis. (c) Representative Masson’s trichrome staining and quantification of area occupied by fibrotic stroma in human biopsies (n= 5 PDAC patients, n = 5 healthy subjects; five fields assessed per sample; mean ± s.e.m; two-tailed unpaired t-test). HL = healthy liver, LM = liver metastasis. (d) Experimental metastasis model by intrasplenic implantation of 1x10\(^6\) Kras\(^{G12D}\); Trp53\(^{R172H}\); Pdx1-Cre (KPC) mice derived pancreatic cancer cells expressing a luciferase/zsGreen lentiviral reporter plasmid (KPC\(^{luc/zsGreen}\)). Liver tissues were isolated at day 5 and day 12 post implantation. (Upper panel) Liver tissue sectioned stained by Hematoxylin and Eosin (HE) showing initial micrometastatic lesions of disseminated cancer cells at day 5 post implantation followed by the generation of an excessive stromal microenvironment surrounding disseminated cancer cells at day 12 post implantation. (Lower panel) Representative immunofluorescence staining of disseminated KPC\(^{luc/zsGreen}\) (zsGreen) cells. Data are from 6 mice per time point; four fields assessed per sample; data combine two independent experiments. Scale bars = a, 200 µm; b, c, d, 100 µm.
Supplementary Figure 2. FACS-based quantification of immune cell infiltrating the metastatic site in PDAC. Experimental metastasis model by intrasplenic implantation of 1x10^6 KPC-derived pancreatic cancer cells. After 12 days, liver tissues were isolated, enzymatically digested and resulting single cell suspensions were stained for flow cytometry analysis. Naïve livers were used as controls (healthy).

(a) Representative flow cytometry dot plots showing gating strategy used to quantify intrametastatic B cells (CD45^+ B220^+), T cell (CD45^+ CD3^+), NK cells (CD45^+ NK1.1^+ CD3^−/B220^−), Neutrophils (CD45^+ CD11b^+ F4/80^+ Ly6G^+ Ly6C^+), inflammatory monocytes (CD45^+ CD11b^+ F4/80^+ Ly6G^- Ly6C^+) and macrophages (CD45^+ CD11b^+ F4/80^+). Only viable cells (SYTOX^−) were used (data are from 5 healthy livers or 8 liver metastasis; repeated two times with similar results). (b) Quantification of CD45^+ hematopoietic cells detected in healthy control livers (Ctrl) and metastatic tumour bearing livers (LM) (n = 5 Ctrl mice; n = 8 LM mice; data combine two independent experiments; individual data points, horizontal lines represent mean ± s.e.m; two-tailed unpaired t-test). (c) Percentage of intrametastatic immune cells among total viable cells gated according to (a) in healthy control livers (Ctrl) and metastatic tumour bearing livers (LM) (individual data points, horizontal lines represent mean ± s.e.m; two-tailed unpaired t-test). Data shown combine two independent experiments; total mice: n = 5 Ctrl mice, n = 6 LM mice (B220, NK1.1, CD3); n = 5 Ctrl mice; n = 9 LM mice (Ly6G, Ly6C, F4/80). (d) Representative flow cytometry dot plots showing gating strategy to analyze CCR2 (CD192) expression levels. Intrametastatic inflammatory monocytes (CD45^+ CD11b^+ F4/80^+ Ly6G^+ Ly6C^+) and macrophages (CD45^+ CD11b^+ F4/80^+ Ly6G^- Ly6C^+) express CCR2 (data are from 5 Ctrl mice or 8 LM mice; repeated two times with similar results). ns, not significant.
Supplementary Figure 3 Liver PDAC metastasis induces the recruitment of monocyte-derived macrophages, followed by the activation of resident hStCs. (a, b) Experimental metastasis model by intrasplenic implantation of 1x10^6 KPC cells. (a) Intrametastatic CD11b^+ cells showing a marked expansion of the macrophage population (F4/80^+; blue) in established macro-metastatic lesions (day 12, D12), while inflammatory monocytes (Ly6C^+Ly6G^−, red) predominantly increased during early micro-metastatic spreading (day 5, D5). Ctrl= 5 mice; Day 5 = 2 mice, Day 12 = 5 mice; data combine two independent experiments. (b) Representative immunofluorescence staining showing increased CD11b^+ cells (upper) in micro-metastatic livers, followed by excessive accumulation of αSMA^+ myofibroblasts in livers with macro-metastatic lesions. Macrophages (lower) are equally distributed in healthy livers (Kupffer cells) and show increase clustering in tumour bearing livers. Data are from 6 mice per time point; two independent experiments. (c) Representative Masson’s trichrome staining (MTS) of healthy control liver indicating absence of fibrotic stroma (lack of blue colour). Data are from 6 mice; two independent experiments. (d) Statistical comparison showing a positive correlation (Pearson) between increased numbers of αSMA^+ myofibroblasts and area occupied by metastatic cancer cells in tumour bearing murine livers. solid line = best fit, dashed lines, 95% confidence intervals. Total n = 20 mice; four independent experiments. (e - g) Primary tumours and spontaneous metastatic hepatic tumours derived KPC mice. (e) Representative HE images showing the presence of an excessive stromal compartment at both sites (data are from 5 mice per condition, four fields assessed per sample). (f) Representative images of liver tumour sections stained for MAMs (F4/80^+), pancreatic cancer cells (CK19^+), or myofibroblasts (αSMA^+) showing the presence of an excessive metastatic microenvironment mainly consisting of MAMs and myofibroblasts surrounding the tumour cells (data are from 5 mice per condition, four fields assessed per sample). (g) Flow cytometry analysis showing a marked expansion of the macrophage population (F4/80^+) in metastatic livers (ML) compared to healthy livers (HL) (n = 5 mice per condition, data combine five independent experiments; mean ± s.e.m.; two-tailed unpaired t-test). (h - j) Chimeric WT + tdTomatoRed BM (WT+tdTR BM) mice. (h) Successful BM reconstitution was confirmed by flow cytometry analysing total circulating CD11b^+Gr1^+ cells. (i, j) Representative images showing co-localization of tdTomatoRed signal with F4/80^+ MAMs (i), but not with αSMA^+ myofibroblasts in the metastatic lesion (below white line). Data are from 6 mice per condition; one experiment. Scale bars, b, c, f, 100 µm; e, 50 µm; i, j, 25 µm.
Supplementary Figure 4 The recruitment of monocyte derived macrophages is necessary for efficient pancreatic cancer metastasis. 1x10^6 KPC-derived cells (a) or 1x10^6 Panc02 cells (b - e) were intrasplenically injected into age and sex matched WT and PI3Kγ-/- (-/-) mice. After 12 days, total livers were harvested and analysed by flow cytometry, immunohistochemistry, and immune fluorescence based methods. (a) Representative HE staining of liver tissue sections showing a marked decreased size of metastatic tumours in livers of PI3Kγ deficient (-/-) mice (data are from 7 WT and 9 PI3Kγ-/- (-/-) mice; data combine two independent experiments). (b) Percentage of intrametastatic macrophages among CD45+ cells quantified by flow cytometry. PI3Kγ deficiency (-/-) results in a marked reduction of MAMs compared to WT (n = 6 mice per condition; one experiment; individual data points, horizontal lines represent mean ± s.e.m; two-tailed unpaired t-test). (c, d) Quantification of metastatic frequency (c) and average metastatic lesion size (d) in WT and PI3Kγ knockout mice (-/-) by hematoxylin and eosin (HE) stained liver sections (n = 6 mice per condition; all metastatic nodules assessed from one section per sample; one experiment; c, individual data points, horizontal lines represent mean ± s.e.m; d, mean ± s.e.m; two-tailed unpaired t-test). (e) Representative immunofluorescence staining and quantification of myofibroblasts (aSMA+) cell frequency in livers in WT and PI3Kγ knockout mice (-/-). Nuclei were counterstained with DAPI (n = 6 mice per condition; two fields assessed per sample; one experiment; mean ± s.e.m; two-tailed unpaired t-test).
Supplementary Figure 5 Disseminated PDAC cells depend on MAMs to sustain growth after colonization of the metastatic site. (a, b) In vivo bioluminescence imaging to monitor metastatic colonization of the liver (a) Representative image showing radiance of a mouse two days post intrasplenic implantation of 1 x 10^6 PDAC cancer cells (KPC_luc/zsGreen) and tumour free control mouse. Main signal detected originates from the liver area, while some residual signal is measured from the injection site, the spleen. (b) Quantification of radiance (total flux) measure two days post implantation confirming colonization of the liver by implanted cancer cells has occurred (data are from 2 control or 10 KPC_luc/zsGreen mice; one experiment, individual data points, horizontal lines represent mean). (c) Representative flow cytometry dot plots and quantification showing a marked reduction of macrophages (F4/80^+) and myofibroblasts (PDGFRα^+) in KPC cell induced tumour bearing livers of mice treated with Clodronate Liposomes (CL) compared to control mice treated with PBS Liposomes (PL) (n = 3 mice per condition; one experiment; individual data points, horizontal lines represent mean ± s.e.m; two-tailed unpaired t-test). (d) Percentage of macrophages (F4/80^+) among viable cells in Panc02 cell induced tumour bearing livers of mice treated with Clodronate Liposomes (CL, data from 2 mice) compared to control mice treated with PBS Liposomes (PL, data from 2 mice) (one experiment; individual data points, horizontal lines represent mean). (e) Representative immunofluorescence staining and quantification of MAMs (F4/80^+) and myofibroblasts (αSMA^+) cell frequency in Panc02 cell induced liver tumours of mice treated with liposomes containing control PBS (PL) or clodronate (CL). Nuclei were counterstained with DAPI (n = 4 mice per condition; five fields assessed per sample; one experiment; mean ± s.e.m; two-tailed unpaired t-test). (f, g) Evaluation of metastatic frequency (f) and lesion area covered by metastatic cells (g) in Panc02 tumour bearding livers of mice treated with liposomes containing control PBS (PL) or clodronate (CL). Nuclei were counterstained with DAPI (n = 5 mice per condition; all metastatic nodules assessed from one section per sample; one experiment; individual data points, horizontal lines represent mean ± s.e.m; two-tailed unpaired t-test). Scale bars, 100 μm.
**Supplementary Figure 6** High levels of granulin are specifically expressed by tumour educated macrophages in vitro and in vivo. (a) Quantification of aSMA (Acta2) and collagen 1α (Col1α) mRNA levels in primary murine dermal fibroblasts stimulated with isogenic macrophage conditioned media (CM) as determined by qPCR. Bar graph show fold up regulation compared to unstimulated and are displayed as mean (data are from a single experiment, repeated four times with similar results; Supplementary Table 6). (b) Quantification of human (green) and mouse (red) myofibroblast that migrated towards human and mouse macrophages, respectively, in a matrigel-coated transwell assay (n = 4 independent experiments; mean ± s.e.m.; two-tailed unpaired t-test). (c) Quantification of human myofibroblast proliferation in the presence of control media, THP-1 derived macrophage CM, or primary macrophage CM (n = 4 independent experiments; mean ± s.e.m.; two-tailed unpaired t-test). (d) Quantification of granulin mRNA expression levels by qPCR in human primary unstimulated macrophages (M0) or primary macrophages stimulated with either interleukin (IL) -4 (M2-like phenotype), interferon (IFN) γ / LPS (M1-like phenotype) or educated with tumour conditioned media generated from human Panc1 cells (n = 3 independent experiments; mean ± s.e.m.; two-tailed unpaired t-test). (e) Quantification of granulin mRNA expression levels by qPCR in murine primary macrophages stimulated with either interleukin (IL) -4 (M2-like phenotype), interferon (IFN) γ / LPS (M1-like phenotype) or educated with tumour conditioned media generated from murine KPC and Panc02 PDAC cancer cells (n = 3 independent experiments; mean ± s.e.m; two-tailed unpaired t-test). (f) Metastatic hepatic tumours derived from the spontaneous mouse pancreatic cancer model Pdx1Cre-ERT;KrasG12D;Trp53R172H; (PdxCre-ERT KP mice) were isolated and morphometrically analysed. Representative images of immunohistochemistry staining for MAMs (CD68+) and granulin expression on serial tissue sections from metastatic lesions and healthy liver and quantification of the data (n = 1 mouse per condition; eight fields assessed per sample; bars represent means). ns, not significant.
**Supplementary Figure 7** Depletion of granulin does not affect the recruitment of macrophage to the metastatic site, their activation, or intrametastatic effector T cell numbers, but markedly reduces stromal expansion. 5x10^5 KPC-derived cells were intrasplenically injected into WT and Grn^-/- mice (a), and chimeric WT+ WT BM and WT + Grn^-/- BM mice (b). After 12 days, total livers were harvested and analysed by flow cytometry. (a) Quantification of intrametastatic total MAMs (F4/80^+), CD206^+ MAMs, and CD8^+ effector T cells by flow cytometry isolated from WT and Grn^-/- mice (n = 6 mice per condition; one experiment; individual data points, horizontal lines represent mean ± s.e.m; two-tailed unpaired t-test). (b) Quantification of intrametastatic total MAMs (F4/80^+), CD206^+ MAMs, and CD8^+ effector T cells by flow cytometry isolated from WT + WT BM and WT + Grn^-/- BM mice mice (n = 6 mice per condition; one experiment; individual data points, horizontal lines represent mean ± s.e.m; two-tailed unpaired t-test). (c) Quantification of murine KPC cancer cell proliferation in the presence or absence of MF CM and periostin neutralising antibody (anti-Periostin) (n = 4 independent experiments; mean ± SEM; two-tailed unpaired t-test). (d) Quantification of periostin expression by qPCR in primary activated murine myofibroblasts and KPC cells. Periostin expression was undetectable in KPC cells while myofibroblasts expressed high levels of periostin (n = 3 independent experiments; mean ± s.e.m). (e) Immunohistochemical analysis of periostin in healthy liver (HL) and spontaneous metastatic livers (ML) collected from Pdx1-CreERT KP mice, respectively. Representative micrographs and quantification of the data are shown (n = 1 mouse per condition; eight fields assessed per sample; bars represent means). (f, g) Representative images of the evaluation of periostin deposition and fibrotic stroma formation (MTS) in metastatic livers of control WT, WT mice treated with clodronate liposomes, PI3Kγ^-/-, Grn^-/-, and WT + Grn^-/- BM mice 12 days after intra-splenic implantation of KPC cells (data are from 4 mice per condition; four fields assessed per sample; data combine five independent experiments). Scale bars = 100mm. NS, not significant.
Supplementary Figure 8 Depletion of granulin in the hematopoietic compartment reduces pulmonary metastasis and myofibroblast activation in the lung. (a - e) 5x10^5 KPC-derived cells were intravenously injected into age and sex matched chimeric WT + WT BM and WT + Grn^-/- BM mice. After 12 days, total lungs were harvested and analysed. (a) Representative images of immunohistochemistry staining for MAMs (CD68+) and granulin expression on serial tissue sections from lung metastatic lesions and quantification of the data (data are from 5 WT + WT BM mice or 4 WT + Grn^-/- BM mice; four fields assessed per sample; one experiment). (b) Representative images and quantification of WT BM and Grn^-/- BM derived lung tissue stained with HE showing a marked reduction of area covered by metastatic cancer cells in Grn^-/- BM mice compared to WT BM mice (mean ± s.e.m.; two-tailed unpaired t-test), while frequency of metastatic lesions (metastatic foci) remained unchanged (n = 5 WT + WT BM mice, n = 4 WT + Grn^-/- BM mice; four fields assessed per sample; one experiment). (c) Representative immunofluorescence staining and quantification of MAMs (F4/80+) and myofibroblasts (αSMA+) cell frequency in tumour bearing lungs of WT + WT BM and WT + Grn^-/- BM mice. Nuclei were counterstained with DAPI (n = 5 WT + WT BM mice, n = 4 WT + Grn^-/- BM mice; five fields assessed per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). (d) Representative immunofluorescence staining and quantification of periostin expression in tumour bearing lungs of WT + WT BM and WT + Grn^-/- BM mice. Nuclei were counterstained with DAPI (n = 5 WT + WT BM mice, n = 4 WT + Grn^-/- BM mice; five fields assessed per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). (e) Representative Masson’s trichrome staining and quantification of area occupied by fibrotic stroma in tumour bearing lungs of WT + WT BM and WT + Grn^-/- BM mice (n = 5 WT + WT BM mice, n = 4 WT + Grn^-/- BM mice; five fields assessed per sample; one experiment; mean ± s.e.m.; two-tailed unpaired t-test). (f) Immunohistochemical analysis of periostin and granulin expression in human primary PDAC tumours. Representative micrographs are shown (data are from 4 different patients per condition). (g) IM from blood samples from healthy subjects and metastatic PDAC patients expressed high levels of the chemokine receptor CCR2 (CD192). Representative histogram shown from 6 different samples per condition. Scale bars a, b, c, e, f, 100 µm; d, e, 200 µm; inset, 20 µm.
Supplementary Tables

**Supplementary Table 1.** Identified proteins in tumour educated macrophage secretome.
Top 20 identified proteins secreted by tumour educated macrophages are listed according to their abundance.

**Supplementary Table 2.** Secretome macrophages.
Complete list of secreted proteins associated with GO term “Extracellular Vesicular Exosome” identified by mass spectrometry in human macrophages educated with Panc1 conditioned media.

**Supplementary Table 3.** Secretome myofibroblasts.
Complete list of secreted proteins associated with GO term “Extracellular Matrix Organization” identified by mass spectrometry as differentially expressed in human fibroblasts educated with primary macrophage conditioned media compared to unstimulated fibroblasts.

**Supplementary Table 4: Primers.**
List of primers used for qPCR analysis in this study.

**Supplementary Table 5: Antibodies.**
List of antibodies used in this study.

**Supplementary Table 6: Statistics source data.**
Activation assays primary dermal fibroblasts.