Systemic ablation of MMP-9 triggers invasive growth and metastasis of pancreatic cancer *via* deregulation of IL-6 expression in the bone marrow.

Barbara Grünwald[1], Jennifer Vandooren[2], Michael Gerg[1], Kaarin Ahomaa[1], Annique Hunger[1], Sonja Berchtold[1], Sophia Akbareian[3], Susanne Schaten[1], Percy Knolle[1], Dylan Edwards[3], Ghislain Opdenakker[2] and Achim Krüger[1,#]

[1]Klinikum rechts der Isar der Technischen Universität München, Institut für Molekulare Immunologie und Experimentelle Onkologie, Ismaninger Str. 22, 81675 München, Germany

[2]Rega Institute for Medical Research, KU Leuven, University of Leuven, Department of Microbiology and Immunology, Minderbroedersstraat 10, 3000 Leuven, Belgium

[3]University of East Anglia, School of Biological Sciences, Norwich Research Park, Norwich, Norfolk, NR4 7TJ, United Kingdom

#Corresponding Author

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Contact Information: Achim Krüger

Klinikum rechts der Isar der TU München

Institut für Molekulare Immunologie und Experimentelle Onkologie

Ismaninger Str. 22

81675 München, Germany

Tel.: +49 (0)89 4140 4463

Fax: +49 (0)89 4140 4476

Email: achim.krueger@tum.de

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Abstract

Matrix metalloproteinase-9 (MMP-9)/gelatinase B is over-expressed in pancreatic ductal adenocarcinoma (PDAC) and plays a central role in tumour cell invasion and metastasis. We here complemented mechanistic insights in the cancer biology of MMP-9 and investigated the effects of specific long-term loss-of-function of MMP-9 on PDAC initiation and progression by genetic ablation in a well-established mouse model of spontaneous PDAC. Tumour growth and progression were analysed by histopathology and immunohistochemistry. Invasive growth of PDAC cells was analysed *in vitro* (proliferation, survival, migration, invasion assays) and *in vivo* (experimental metastasis assays). Retroviral shRNAi was used to knock-down target genes (MMP-9, IL-6R). Gene expression was analysed by qRT-PCR, Western Blot, ELISA, *in situ* hybridization and zymography. PDAC tumours from MMP-9-deficient mice were dramatically larger, more invasive and contained more stroma. Yet, ablation of MMP-9 in PDAC cells did not directly promote invasive growth. Interestingly, systemic ablation of MMP-9 led to increased IL-6 levels resulting from abrogation of MMP-9-dependent SCF-signalling in the bone marrow (BM). IL-6 levels in MMP-9$^{-/-}$ mice were sufficient to induce invasive growth and STAT3 activation in PDAC cells *via* IL-6 receptor (IL-6R). Interference with IL-6R blocked the increased invasion and metastasis of PDAC cells in MMP-9-deficient hosts. In conclusion, ablation of systemic MMP-9 initiated fatal communication between maintenance of physiological functions of MMP-9 in the BM and invasive growth of PDAC *via* the IL-6/IL6R/STAT3 axis. Thus, the beneficial effects of host MMP-9 on PDAC are an important caveat for the use of systemic MMP-9 inhibitors in cancer.

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MMP-9 ablation-induced invasive growth of pancreatic cancer

Introduction

Pancreatic cancer is a lethal malignancy, characterized by aggressive local invasion and metastatic spread (1). Pancreatic ductal adenocarcinoma (PDAC) is the predominant form of pancreatic cancer accounting for >90% of the diagnosed cases (1). Currently, treatment options for PDAC are scarce (1) and new therapeutic targets are subject to intensive research (2,3). Matrix metalloproteinases (MMPs) are zinc-dependent proteases that contribute to development and tissue homeostasis, and modulate cancer progression by digesting a variety of substrates (4). The functions of MMPs in extracellular matrix (ECM) degradation, invasive growth and angiogenesis led to the assumption that inhibition of catalytic MMP activity *in vivo* would have an anti-tumourigenic effect (5). However, clinical trials on MMP inhibitors failed to demonstrate clinical benefit and in some cases even showed adverse effects on the patient´s outcome (5). One explanation for this unexpected failure was that broad-spectrum inhibition of MMPs gave rise to a metastasis-promoting environment in the liver (6). Later it was discovered that some MMPs even have protective, anti-tumourigenic functions (7) from which it was concluded that target MMPs have to be inhibited selectively to inhibit cancer growth and metastasis (7,8). Within the whole MMP family, MMP-9 is considered an especially compelling target, given a large body of evidence demonstrating a key role for MMP-9 in tumourigenesis and metastasis (9). Recently, inhibition of MMP-9 was again suggested as a potential therapeutic approach (10) and novel, selective MMP-9-inhibitory antibodies are currently tested in clinical trials (11). However, it is important to note that MMP-9 is also involved in physiological processes so that the functional role of MMP-9 needs to be carefully defined for the individual disease context (12), before a final conclusion about its precise function can be drawn. In addition, MMP-9 also has non-catalytic activities in tumour biology (13).

Previous studies addressed the role of MMP-9 in pancreatic cancer in *xenograft* models, but after failure of the clinical trials, it was suggested that these previous data may have been biased (5).

Genetic models that recapitulate the heterogeneity and multistage nature of tumour progression are believed to be more predictive than *xenograft* models (14). Interestingly, genetic mouse models of neuroendocrine pancreatic cancer recently demonstrated that the ablation of MMP-9 gave rise to more invasive tumours (15). However, neuroendocrine tumours of the pancreas are a relatively rare condition, accounting for only 1-5% of the diagnosed pancreatic cancers, while the large majority of tumours are PDACs, arising from the exocrine cellular compartment (1). A well-characterized genetic mouse model of PDAC is the LSL-Kras^{G12D}-LSL-Trp53^{R172H}-Pdx1-Cre (KPC) mouse (16). Here, we crossed KPC mice with non-leaky MMP-9 knock-out mice in order to investigate the functional role of MMP-9 in the context of PDAC in avoidance of the complexities of pharmacological agents, such as inhibition of off-targets, incomplete inhibition, and toxicity. We report that systemic ablation of MMP-9 has detrimental effects in PDAC because abrogation of MMP-9-mediated SCF-signalling in the bone marrow led to increased IL-6 levels in the host which activated STAT3 signalling in tumour cells and thereby promoted invasive growth and metastasis of PDAC.

Material and Methods

Animal experiments. Mice were kept at the animal facility of Klinikum rechts der Isar (Munich, Germany) under specific and opportunistic pathogen-free conditions. Animal experiments were performed in compliance with the guidelines of the *Tierschutzgesetz des Freistaates Bayern* and approved by the *Regierung von Oberbayern*. During animal experiments investigators were unaware of the identity of animals and samples. The following transgenic mouse models were described in detail elsewhere: Pdx-1+/Cre;Kras+/LSL-G12D;Trp53+/LSL-R172H ('KPC') (16), and pure C57BL/6 non-leaky MMP-9 knock-out mice ('MMP-9$^{-/-}$') mice (17). Female or male KPC mice were used at 5-6 weeks ('CP stage'), 8-10 weeks ('PanIN stage') or 12-14 weeks age ('PDAC stage') or 14-16 wks age ('Advanced PDAC stage'); healthy littermates (Pdx-1+/Cre or Trp53+/LSL-R172H) served as controls. MMP-9$^{-/-}$ mice were used at 8-10 weeks age; wild-type littermates (MMP-9$^{+/+}$) served as controls. To study the role of MMP-9 in PDAC, we cross-bred KPC mice with MMP-9$^{-/-}$ mice (17) to obtain C57BL/6-MMP9$^{-/-}$/Pdx-1+/Cre;Kras+/LSL-G12D;Trp53+/loxP ('KPCxMMP-9$^{-/-}$') mice. To test the impact of pharmacological MMP-9 inhibition, female 8-10 weeks old DBA/2 mice were obtained from Charles River and treated daily for 14 days with inhibitor or vehicle control as described previously (8,18). *In vivo* invasive growth of PDAC cells in MMP-9$^{-/-}$ mice and MMP-9-kompetent littermates was tested by intravenous inoculation of 1×10^6 syngeneic KPC-derived 9801 or Panc02 pancreas carcinoma cells (19). Mice were sacrificed at the indicated time-points after tumour cell inoculation (*in vivo* invasion: 24 h; out-growth: 14 d), and tumour cells on the median liver lobe and the right lung lobe were X-Gal-stained and quantified as described previously (19).

Cell lines, cell culture*, in vitro* **assays.** Bone marrow cells were harvested from femurs by flushing the bone cavity with PBS. Erythrocytes were lysed and BM cells were re-suspended in BBMM (Lonza) and seeded in uncoated tissue culture dishes. Where indicated, BM cell cultures were supplemented with 2.5 ng/ml recombinant murine SCF (R&D Systems) and mRNA was extracted 2 h later. KPC-derived murine 9801 pancreas carcinoma cells (19) and the Panc02 cell line (20) were cultured as described previously. Cells were regularly tested for mycoplasma contamination (MycoTrace, PAA). Genetic

tagging, retroviral knock-down of MMP-9 (21) and lentiviral knock-down of IL-6R (19) in cells were achieved by lentiviral transduction as described previously. Where indicated, 20 ng/ml recombinant IL-6 was added to the medium for 24 h prior to and during analysis. Motility and invasion were analysed through 3 µm Corning®TransWell inserts (Sigma Aldrich). For invasion assays, inserts were coated with coated with 1 mg/mL BD Matrigel^{TM} Basement Membrane Matrix (BD Biosciences). $5x10^5$ cells per well were added in FCS-free medium and allowed to migrate or invade towards the 10% FCS-containing medium in the lower well for 24 h or 48 h, respectively. Cells were removed from the upper side of the membrane using a cotton stick. Cells migrated/invaded to the lower side were fixed with methanol, stained with DAPI, and counted under a microscope. Proliferation (in 10% FCS) and viability (in 0.1% FCS) over 96 h were determined by AlamarBlue Assays (ThermoFisher) according to the manufacturer´s instructions.

Immunohistochemistry (IHC). IHC staining was previously described in detail (19). Briefly, sections were de-paraffinized, blocked and incubated with the following antibodies: αSMA (Abcam), PCNA (Santa Cruz Biotechnology), CK19 (Developmental Studies Hybridoma Bank) or phosphor-STAT3 (Cell Signalling Technology). Detection was done *via* polyclonal biotinylated secondary antibodies (anti-rabbit, Dako; anti-goat, Dako; anti-rat, Abcam), Streptavidin/HRP (Dianova) and DAB substrate (Dako). Counterstaining was done using Haematoxylin.

RNA isolation, reverse transcription, and quantitative RT-PCR. Total RNA from cells was isolated using TRIzol (Life Technologies) according to the manufacturer's instructions. Reverse transcription to cDNA was performed using High Capacity Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using Universal TaqMan Mastermix (Roche), primers were designed using Probefinder v2.45 (http://qpcr.probefinder.com/roche3.html) and used in combination with the respective probes from Universal Probe Library (Roche).

Protein Isolation, Immunoblotting, and ELISA. Total protein was isolated by sonification of cells in RIPA buffer followed by three freeze-thaw cycles. For collection of extracellular BM fluid, femurs were flushed with 1 ml PBS before cells and extracellular fluid were separated by centrifugation. Membrane

protein from the cells was isolated as previously described (22). The method used for Western Blotting was described previously (23). Briefly, 50 µg of total cell lysate, 40 µg of membrane fraction or 15 µg protein from BM extracellular fluid were electrophoretically separated, blotted onto nitrocellulose and probed with antibodies against α-Tubulin (Merck), SCF (Santa Cruz Biotechnology), phospho-p85, STAT3, phospho-STAT3, phospho-ERK1/2 (all from Cell Signalling Technology). Detection was done using Clarity ECL substrate (BioRad) in a ChemiDoc MP imaging system (BioRad). IL-6 levels were determined using the DuoSet ELISA Kit (R&D Systems) according to the manufacturer´s instructions.

Zymography analysis. Tissues were weighted, placed into extraction tubes (Precellys Lysing Kit) and 500 ml of buffer (150 mM NaCl, 50 mM Tris, 5 mM $CaCl_2$, 0.05% Tween20, pH 7.5-HCl) was added to the samples. Tissues were homogenised with the Precellys 24 and supernatants were recovered (15 min, 20000 g, 4°C). Prior to zymography analysis, the total protein content of the samples was determined with the method of Bradford and the samples were prepurified by gelatin affinity chromatography. For each sample, the equivalent of 120 µg protein was loaded onto a Micro Bio-Spin Chromatography Column (bio-rad) containing 40 µl of Gelatin-Sepharose® 4B beads (GE Healthcare) in equilibration buffer (50 mM Tris, 0.5 M NaCl, 10 mM $CaCl_2$, 0.01% Tween 20, pH 7.5-HCl). As an internal reference, 800 pg of proMMP-9 ΔOGHem was added and this mixture was incubated for 20 min at room temperature. Next, the samples were washed 3 times with 500 µl washing buffer 1 (50 mM Tris, 0.5 M NaCl, 10 mM $CaCl_2$, 0.05% Tween 20, pH 7.5-HCl) and once with washing buffer 2 (50 mM Tris, 10 mM $CaCl_2$, 0.05% Tween 20, pH 7.5-HCl). Finally, the samples were eluted in 20 µl non-reducing loading buffer (0.125M Tris-HCl pH 6.8, 4% SDS, 0.01% bromophenol blue, 20% glycerol) (24). The prepurified samples were loaded onto 7.5% polyacrylamide gels which contain 0.1% gelatin. After electrophoresis the gels were washed twice for 20 minutes with 2.5% Triton X-100 to remove SDS, and incubated overnight at 37°C in incubation buffer (50mM Tris-HCl, pH 7.5, 10mM $CaCl_2$, 0.02% NaN_3, 1% Triton X-100) for gelatin degradation. Finally, the gels were stained with the PhastGel Blue R-350 staining kit (GE Healthcare) and the densities of the bands were analysed with the ImageQuant TL software (GE Healthcare) (25). The concentrations of MMP-9 and MMP-2 were

calculated based on the density of the bands, the recovery of the internal reference after co-prepurification and a dilution series of recombinant wild-type MMP-9 of known concentration. MMP-9ΔOGHem and wild-type MMP-9 have been described in detail in previous publications (26). Gelatin gel zymography was performed twice for each sample.

Statistical analysis: The number of biological replicates (number of animals per group or number of independent repetitions of *in vitro* experiments, respectively) is listed in the figure legends. Each biological replicate consisted of at least three technical replicates. Data sets were tested for normality of distribution and compared by one-way analysis of variance (One-way ANOVA), in case of multiple groups followed by Bonferroni *post hoc* analysis. Values of $p < 0.05$ were considered significant.

Results

MMP-9 is expressed in murine PDAC

First, we tested whether MMP-9 is expressed in the KPC mouse model of PDAC. Pancreatic proMMP-9 protein levels in C57BL/6J-LSL-**K**ras^{G12D}-LSL-Tr**p**53^{R172H}-Pdx1-**C**re (KPC) mice were already elevated at the stage of pre-cancerous pancreatitis and PanIN lesions, and further increased during progression to full-blown PDAC (**Fig.1A**). Concomitantly, we observed induction of proMMP-2 (**Supplementary Fig.S1**). Within pancreatic lesions of KPC mice, MMP-9 transcripts were present in tumour cells as well as in cells of the desmoplastic stroma (**Fig.1B**). Thus, MMP-9 was present in murine PDAC and its expression correlated with disease progression.

Loss of MMP-9 promotes invasive growth of PDAC *in vivo*

To explore the role of MMP-9 in the progression of PDAC, we cross-bred KPC mice with a non-leaky MMP-9 knock-out line (Dubois et al 1999) to obtain C57BL/6-LSL-Kras^{G12D}-LSL-Trp53^{R172H}-Pdx1-Cre-MMP-9$^{-/-}$ (KPCxMMP-9$^{-/-}$) mice. First, we compared tumour progression with and without MMP-9 at the age of 8 weeks. Macroscopically, pancreata from KPCxMMP-9$^{-/-}$ mice were dramatically enlarged containing palpable tumour masses while KPC pancreata appeared macroscopically normal (**Fig.2A**). Gross histological analysis by HE staining showed that pancreata of 8 weeks old KPC mice contained PanIN lesions surrounded by normal pancreatic tissue whereas KPCxMMP-9$^{-/-}$ mice exhibited large areas of dedifferentiated, invasive PDAC and a strong desmoplastic reaction (**Fig.2B**). Invasiveness of the tumours was determined histologically by scoring the circularity of the tumour borders as previously shown for neuroendocrine pancreatic tumours (15). Tumours from 8 weeks old KPCxMMP-9$^{-/-}$ mice were profoundly more invasive than the PanIN lesions in MMP-9-competent KPC mice as reflected by a decreased circularity (**Fig. 2C**). To determine whether this increased invasiveness of KPCxMMP-9$^{-/-}$ tumours is a result of the observed promoted disease

progression or attributable to MMP-9-deficiency, we compared MMP-9-deficient carcinomas of 8 weeks old KPCxMMP-9$^{-/-}$ mice with advanced MMP-9-competent carcinomas from 14-16 weeks old KPC mice. Interestingly, the MMP-9-deficient pancreatic carcinomas were still profoundly more invasive (**Fig. 2C**). In accordance with the gross histological analysis, immunohistochemical analysis of specific progression markers showed that enhanced tumour growth in KPCxMMP-9$^{-/-}$ mice was accompanied by increased numbers of CK19$^+$ tumour cells and an increased PCNA index (**Fig.2D**). The increased areas of desmoplastic stroma in MMP-9-deficient tumours were reflected by an increased content of αSMA$^+$ myofibroblasts (**Fig.2E**). Taken together, ablation of MMP-9 promoted progression and invasive growth of pancreatic tumours.

Ablation of MMP-9 does not directly promote invasive growth of PDAC cells *in vitro*

Invasive growth is defined as a complex biological program which instructs cells to migrate, invade, proliferate and survive (27). First, we tested whether ablation of MMP-9 could directly unleash invasive growth of PDAC cells *in vitro*. MMP-9 was knocked down by lentiviral shRNAi in the murine KPC-derived pancreatic tumour cell line 9801 (19) (**Fig. 3A**). Knock-down of MMP-9 did not induce invasive growth but instead led to reduced proliferation (**Fig.3B**), viability (**Fig.3C**), migration (**Fig.3D**) and invasion (**Fig.3E**) of 9801 PDAC cells *in vitro*. Likewise, knock-down of MMP-9 in a second cell line, Panc02 murine pancreatic carcinoma cells (20), reduced invasive growth of the cells (**Supplementary Fig. S2**).

MMP-9-deficient hosts exhibit increased levels of IL-6 in bone marrow and plasma

Ablation of MMP-9 in tumour cells did not directly promote invasive growth. We thus hypothesized that systemic alterations in the MMP-9-deficient hosts promoted the invasive growth of PDAC. Previously, we have shown that MMP-9 knock-out can result in specific induction of IL-6 in the bone marrow (BM) (28). Noteworthy, elevated systemic and tumoural IL-6 levels derived from the BM

were recently shown to be central to PDAC initiation and progression (29) by acting on various tumour-promoting processes including tumour cell proliferation, survival, migration, invasion (i.e. invasive growth) as well as angiogenesis, activation of stromal fibroblasts, and immune invasion (30). Indeed, IL-6 levels in plasma and BM of tumour-free MMP-9$^{-/-}$ animals were increased (**Fig.4A**). Noteworthy, plasma IL-6 levels in MMP-9-deficient tumour-bearing KPC mice were increased between 11- to 20-fold as compared to MMP-9-competent KPC mice (**Fig.4B**). We next tested whether the IL-6 plasma level in MMP-9$^{-/-}$ mice (~20 pg/ml) was sufficient to directly promote invasive growth of PDAC cells. KPC-derived 9801 cells *in vitro* showed increased migration, invasion, and proliferation in the presence of 20 ng/ml IL-6 (**Fig.4C**). Knock-down of IL-6R in 9801 cells (**Fig.4D**) abolished the IL-6-induced invasive growth *in vitro* (**Fig.4E, F**). IL-6-induced invasive growth is mediated *via* activation of the STAT3 pathway (30). STAT3 was not active in the PDAC cells *in vitro* but could be induced by stimulation with IL-6 (**Fig. 5A**) followed by transcription of the STAT3 target genes c*Met*, *Vegfa*, *Car9*, *Hif1a*, *Vimentin*, and *Icam-1* (**Fig. 5B**) in an IL-6R-dependent manner. KPCxMMP-9$^{-/-}$ tumours exhibited strong STAT3 activation in tumour cells and infiltrating immune cells as compared to MMP-9-competent KPC mice (**Fig. 5C**). Taken together, these results indicate that increased IL-6 levels in MMP-9-deficient hosts are able to promote invasive growth *via* IL-6R-dependent STAT3 activation in PDAC cells.

Loss of MMP-9-dependent SCF-signalling *via* STAT3 in the bone marrow is compensated by induction of IL-6

Next, we sought to determine why loss of MMP-9 resulted in induction of IL-6 in the BM. BM homeostasis is largely dependent on MMP-9 as the sheddase of stem cell factor (SCF) (31) which is necessary for proliferation and differentiation of hematopoietic stem cells *via* binding to stem cell factor receptor (SCF-R). Indeed, loss of MMP-9 led to a significant reduction of soluble SCF within BM extracellular fluid while the membrane-bound fraction of SCF was increased (**Fig. 6A**). Potential

consequences of this MMP-9-deficiency-induced ablation of SCF shedding include abrogation of downstream signalling cascades maintaining proliferation and survival of BM cells, such as STAT3, PI3K and ERK (32). We found that phosphorylation status of the PI3K regulator p85 and ERK was reduced in BM MMP-9$^{-/-}$ mice, whereas STAT3 activation was enhanced (**Fig. 6B**). STAT3 expression remained unaltered (**Fig. 6C**). Thus, MMP-9$^{-/-}$ mice maintained STAT3 activation. In addition, MMP-9$^{-/-}$ mice show no defects of the hematopoietic system in the steady-state (31). This led us to hypothesize that reduction of soluble SCF in MMP-9-deficient bone marrow is compensated by induction of another molecule, which can signal *via* STAT3, namely IL-6. We thus tested whether SCF deprivation could in principle induce IL-6 in BM cells. Indeed, BM cells cultured in absence of SCF expressed significantly higher levels of IL-6 while addition of SCF reduced IL-6 expression (**Fig. 6D**). To further investigate whether induction of IL-6 in the BM upon ablation of MMP-9 was a result of genetic manipulation, we treated mice with synthetic MMP inhibitors of varying selectivity for MMP-9. Inhibitors with a higher selectivity for MMP-9 led to a slight (Ro206-0222) or significant (SB-3CT) increase of IL-6 expression (**Fig. 6E**), while treatment with the less MMP-9- specific inhibitors (BB94 or Ro28-2653) had negligible effects. We conclude that induction of IL-6 in the BM occurs upon ablation of MMP-9.

Promotion of invasive growth in MMP-9-deficient hosts is dependent on IL-6R

In the final set of experiments, we determined whether increased IL-6 levels in MMP-9-deficient hosts were directly able to promote invasive growth of tumour cells. We employed the 9801 and Panc02 PDAC cells in experimental metastasis assays in MMP-9 knock-out mice. Tumour cells were *lacZ*-tagged to enable highly sensitive detection at the single cell level (33). In MMP-9-deficient mice, we observed an induction of *in vivo* invasion of 9801 cells (**Fig.7A**) and Pan02 cells (**Fig.7B**) into livers and lungs. In addition, we observed promoted outgrowth of 9801 (**Fig.7C**) and Panc02 metastases (**Fig.7D**) in MMP-9$^{-/-}$ mice. Interestingly, invasion of MMP-9-deficient tumour cells was also promoted in MMP-9-deficient hosts (**Fig.7E**). We next determined the role of IL-6/IL-6R signalling in the induction of invasive growth in MMP-9-deficient hosts. Knock-down of IL-6R in 9801 cells abolished

the invasive growth-promoting effects of MMP-9-deficient hosts *in vivo* (**Fig.7F**). Taken together, ablation of MMP-9 in the host, but not in the tumour cell itself, promoted invasive growth of pancreatic tumour cells *via* induction of IL-6.

Discussion

In the present study we addressed the consequences of specific long-term loss-of-function of MMP-9 in the context of PDAC. PDAC is among the most aggressive malignancies and currently no effective treatment options exist for this fatal disease (1) so that new therapeutic targets are urgently needed (2,3). Inhibition of MMP-9 is currently tested as therapeutic approach in clinical trials (11), despite the seminal failure of MMP inhibitors in the past (5), because increased selectivity of inhibitors for target MMPs is thought to be key to the expected anti-tumourigenic and anti-metastatic therapeutic potential of MMP inhibition (8,9). However, functions of specific MMPs can be context-dependent (28). Thus, critical assessment of potential target MMPs in relevant disease contexts is imperative to their evaluation as therapeutic targets (12). Here, we report that even highly specific, complete ablation of MMP-9 has detrimental effects in PDAC and dramatically promoted disease progression in a clinically relevant genetic PDAC mouse model. We identified the underlying mechanism and report that ablation of MMP-9 in tumour cells did not directly promote invasive growth. Instead, systemic ablation of MMP-9 induced a tumour-promoting host microenvironment by inducing detrimental locoregional alterations in the BM, where loss of MMP-9 activity led to impaired SCF shedding. The resulting distortion of SCF-release induced IL-6 expression in the BM leading to increased systemic IL-6 to a level that was able to activate STAT3 signalling and induce invasive growth of pancreatic tumour cells. Thus, ablation of systemic MMP-9 initiated a fatal communication between the physiological function of MMP-9 in BM homeostasis and invasive growth of tumour cells *via* the IL-6/IL6R/STAT3 axis.

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MMP-9 ablation-induced invasive growth of pancreatic cancer

We used a non-leaky knock-out mouse line that was 13 times back-crossed into C57BL/6J background and corresponding WT mice to determine the consequences of specific, long-term inhibition of MMP-9 on PDAC progression in order to model a `perfect therapy', mimicking full and selective MMP-9 inhibition while avoiding the complexities of pharmacological agents, such as unselective off-target binding, incomplete inhibition and toxicity. Subsequent dissection of the roles of tumour cell MMP-9 *vs*. host MMP-9 revealed that these play controversial roles in pancreatic cancer progression: ablation of MMP-9 in PDAC cells reduced their invasive growth, which would be in accordance with the common notion of a pro-invasive and growth-promoting role of MMP-9 in cancer (9–11). However, systemic ablation of MMP-9 created a tumour-promoting microenvironment that facilitated invasive growth and metastasis of PDAC cells. In KPCxMMP-9$^{-/-}$ mice both host and tumour cells are MMP-9-deficient, and invasive growth was dramatically promoted. This suggests that pro-tumourigenic alterations in the MMP-9-deficient host dominate over the tumour-inhibiting effects of MMP-9-deficiency in tumour cells ultimately resulting in promotion of disease upon systemic loss of MMP-9 function. Additional studies have already hinted that systemic loss of MMP-9 activity can increase the aggressiveness of malignant disease. In a genetic mouse model of neuroendocrine pancreatic tumours, knock-out of MMP-9 impaired tumour angiogenesis but promoted tumour invasion in association with a shift in inflammatory cell content to cathepsin-expressing CD11b/Gr1-positive cells (15). Neuroendocrine tumours are relatively rare (1-5% of pancreatic cancers) while the KPC model used in the current study is a widely used ´gold standard´ model for PDAC (14,34), the most common form of pancreatic cancer accounting for >90% of the diagnosed cases. Due to differences in the cellular origin, the biology of exocrine and endocrine pancreatic tumours is likely to be different so that it seems that ablation of MMP-9 promotes aggressiveness of various pancreatic tumour types. In addition, several studies have suggested that ablation of MMP-9 has detrimental effects in various other tumour entities: In a model of skin carcinogenesis, MMP-9 deficiency decreased the total number of tumours but the emerging tumours were significantly more aggressive (35) and in a genetic model of prostate cancer, MMP-9-deficiency increased the number of invasive foci (36). Moreover, MMP-9 clearly plays a

protective role in colitis-associated cancer (37). Taken together, the suitability of MMP-9 *per se* as a therapeutic target in cancer and especially in pancreatic cancer, is highly questionable. Thus, we suggest that MMP-9 inhibition can only be a valid strategy against pancreatic cancer when combined with effective and highly specific targeting of tumour cells.

MMP-9-deficient mice exhibited increased IL-6 levels in the BM. Addressing the underlying mechanism we found that loss of MMP-9-dependent SCF-signalling in the BM of MMP-9-deficient mice led to reduced activation of PI3K/Akt and ERK pathways, but increased STAT3-activation. In addition, despite distortion of this central MMP-9-dependent pathway in BM homeostasis, MMP-9-deficient mice do not show any defects in the immune system under steady-state conditions (31). We thus propose that abrogation of MMP-9-mediated SCF-release is compensated by induction of IL-6 to maintain STAT3 signalling and thereby BM homeostasis and function. In accordance, absence of SCF was sufficient to induce IL-6 expression BM cells. IL-6 levels as seen in MMP-9-deficient mice were sufficient to induce invasive growth and STAT3 activation in pancreatic cancer cell lines *in vitro*. IL-6 was in fact discovered as a hybridoma and plasmacytoma growth factor (38). IL-6 owns a broad spectrum of pathobiological activities and can affect various tumour-promoting processes including tumour cell proliferation, survival, migration, invasion (i.e. invasive growth) as well as angiogenesis, activation of stromal fibroblasts, and immune invasion (30). Invasive growth is defined as a complex biological program concomitantly instructing cells to migrate, invade, proliferate and survive (27). IL-6 was shown to induce invasive growth of tumour cells in skin cancer (39) and breast carcinomas (40) and was identified as a central driver of PDAC initiation and progression (29,30). IL-6 mediates its potent effects in PDAC progression *via* STAT3 activation and both, ablation of IL-6 and of STAT3 are essential drivers of pancreatic tumour formation in Kras[G12D]-driven pancreatic carcinogenesis (29). We observed no basal STAT3 activation in pancreatic cancer cells *in vitro*. In accordance, it was suggested previously that STAT3 activation in pancreatic cancer cells is non-cell-autonomous but depends on IL-

6 derived from the microenvironment (29). Importantly, not only genetic but also pharmacologic inhibition of MMP-9 induced IL-6 expression in the bone marrow. In conclusion, ablation of systemic MMP-9 dramatically promoted invasive growth and metastasis of PDAC tumours *via* distant locoregional alterations in the BM. We thus believe that such detrimental net effects in the protease web upon MMP-9 ablation are an important caveat for the use of MMP-9 as therapeutic target in pancreatic cancer.

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Figure Legends.

Figure 1: MMP-9 was present in tumours of KPC mice

(A) MMP-2 and MMP-9 protein levels in pancreas lysates from KPC (+) mice or healthy (-) littermates at an age of 5-6 weeks, 8-10 weeks and 12-14 weeks (n = 3 per group). Quantitative gelatine zymography. *Columns*, mean; *Bars*, SEM. Statistical testing by One-way ANOVA: *, $p<0.05$; **, $p<0.01$.

(B) Visualisation of MMP-9 mRNA in tissue sections from KPC mice by *in situ* hybridization.

Figure 2: Invasive growth of MMP-9-deficient pancreatic tumours in mice

(A) Whole mounts of pancreata from 8 weeks old KPC and KPCxMMP-9$^{-/-}$ mice (n = 6). Representative images.

(B) HE stained sections from pancreata from 8 weeks old KPC and KPCxMMP-9$^{-/-}$ mice (n = 6). Representative images. *S*, desmoplastic stroma; *N*, normal pancreatic tissue; ***, PanIN lesion; *#*. Invasive PDAC. *Bars*, 100 µm.

(C) Tumour circularity of lesions from 8 weeks old KPCxMMP-9$^{-/-}$ mice (n = 6) compared to 8 weeks old (n = 5) and 14-16 weeks old (n = 5) KPC mice.

(D) CK19^{+} tumour cells and PCNA^{+} proliferating cells in sections from pancreata from 8 weeks old KPC and KPCxMMP-9$^{-/-}$ mice (n = 5). *Top*, quantification; *bottom*, representative images. *Bars*, 50 µm.

(E) αSMA^{+} stromal cells in sections from pancreata from 8 weeks old KPC and KPCxMMP-9$^{-/-}$ mice (n = 5). *Left*, quantification; *right*, representative images. *Bars*, 50 µm.

(C-E) *Columns*, mean; *Bars*, SEM. Statistical testing by One-way ANOVA: *, $p<0.05$; **, $p<0.01$.

Figure 3: Knock-down of MMP-9 did not promote invasive growth of

pancreatic cancer cells *in vitro*

(A-E) Analysis of invasive growth of 9801 pancreatic carcinoma cells after lentiviral knock-down of

MMP-9 (n = 3).

(A) Lentiviral knock-down of MMP-9 in 9801 cells. MMP-9 mRNA determined by qRT-PCR.

(B) Proliferation determined by AlamarBlue assays.

(C) Viability determined by AlamarBlue assays.

(D) TransWell Migration.

(E) TransWell Invasion.

(A,C-E) *Columns*, mean; *Bars*, SEM.

(A-E) Statistical testing by One-way ANOVA: *, p<0.05; **, p<0.01.

Figure 4: IL-6 levels in MMP-9-deficient hosts promoted invasion and growth of PDAC cells

(A) IL6 levels of MMP-9$^{-/-}$ mice and MMP-9-kompetent littermates determined in plasma by ELISA

(*left*) and in bone marrow by qRT-PCR (*right*) (n = 5).

(B) IL-6 plasma levels in KPC and KPCxMMP-9$^{-/-}$ mice determined by ELISA (n = 3 per group).

(C) Invasive growth of 9801 pancreatic carcinoma cells in presence of 20 ng/ml IL-6 (n = 3).

Proliferation determined by AlamarBlue assays (*left*), TransWell Migration (*middle*), TransWell

Invasion (*right*).

(D) Lentiviral knock-down of IL-6R in 9801 cells. IL6R mRNA determined by qRT-PCR (n = 3).

(E,F) Invasive growth of 9801 pancreatic carcinoma cells in presence of 20 ng/ml IL-6 (n = 3).

(E) Proliferation determined by AlamarBlue assays,

(F) TransWell Migration (*left*), TransWell Invasion (*right*)

(A-D, F*) Columns*, mean; *Bars*, SEM.

(A-F) Statistical testing by One-way ANOVA: *, p<0.05; **, p<0.01.

Figure 5: Activation of STAT3 in pancreatic cancer cells *via* IL-6/IL-6R

(A) P-STAT3 levels in 9801 pancreatic carcinoma cells in presence or absence of 20 ng/ml IL-6 determined by immunoblotting (n = 3). Blots were cropped to the indicated bands.

(B) Expression of STAT3 downstream targets c*Met*, *Vegfa*, *Car9*, *Hif1a*, *Vimentin*, and *Icam-1* in 9801 pancreatic carcinoma cells in presence or absence of 20 ng/ml IL-6 determined by qRT-PCR (n = 3). *Columns*, mean; *Bars*, SEM. Statistical testing by One-way ANOVA: *, $p<0.05$; **, $p<0.01$.

(C) P-STAT3 staining by IHC on sections from pancreata from 8 weeks old KPC and KPCxMMP-9$^{-/-}$ mice (n = 3). *Bars*, 100 µm.

Figure 6: Induction of IL-6 and STAT3 in the bone marrow upon ablation of MMP-9

(A) Extracellular and membrane-bound SCF in bone marrow fractions from MMP-9$^{-/-}$ mice and MMP-9-kompetent littermates determined by immunoblotting (n = 5). *Left*, representative (of three) blots; *right*, densitometrical analysis. Blots were cropped to the indicated bands.

(B) P-STAT3, P-p85 and P-ERK levels in bone marrow lysates from MMP-9$^{-/-}$ mice and MMP-9-kompetent littermates determined by immunoblotting. Representative (of three) blots. Blots were cropped to the indicated bands.

(C) STAT3 and P-STAT3 levels in bone marrow lysates from MMP-9$^{-/-}$ mice and MMP-9-kompetent littermates determined by immunoblotting (n = 5). *Left*, representative (of three) blots; *right*, densitometrical analysis. Blots were cropped to the indicated bands.

(D) IL-6 mRNA in bone marrow cells in the presence or absence of 2.5ng/ml SCF determined by qRT-PCR (n = 3).

(E) IL-6 levels in bone marrow lysates from MMP-9-kompetent mice after treatment with MMP inhibitors and un-treated MMP-9$^{-/-}$ mice (n = 5) determined by ELISA.

(A,C-E) *Columns*, mean; *Bars*, SEM. Statistical testing by One-way ANOVA: *, $p<0.05$; **, $p<0.01$.

Figure 7: Increased metastasis formation of pancreatic cancer cells in MMP-9-deficient hosts depended on IL-6R

(A-F) Invasive growth of pancreatic carcinoma cells in MMP-9$^{-/-}$ mice and MMP-9-kompetent littermates. Experimental metastasis assays (n = 6). *Columns*, mean; *Bars*, SEM. Statistical testing by One-way ANOVA: *, $p<0.05$; **, $p<0.01$.

(A) *In vivo* invasion of 9801 cells into livers (*left*) and lungs (*right*).

(B) *In vivo* invasion of Panc02 cells into livers (*left*) and lungs (*right*).

(C) Out-growth of metastatic colonies of 9801 cells in livers (*left*) and lungs (*right*).

(D) Out-growth of metastatic colonies of Panc02 cells in livers (*left*) and lungs (*right*).

(E) *In vivo* invasion of 9801 cells with and without knock-down of MMP-9 into livers.

(F) *In vivo* invasion of 9801 cells with and without knock-down of IL6R into livers.

Visual Overview: Loss of host MMP-9 promotes invasive growth and metastasis in pancreatic cancer by inducing IL-6 in the bone marrow.

Proposed mechanism: loss of host MMP-9 abrogates SCF shedding in the bone marrow which leads to induction of IL-6. Elevated IL-6 levels activate STAT3 in tumour cells in the pancreas and in the circulation thereby promoting pancreatic tumour progression as well as metastasis.

Supplementary Figure Legends.

Supplementary Figure S1: MMP2 in KPC mice

MMP-2 quantification from gelatine zymography analysis shown in Figure 1A.

Supplementary Figure S2: Knock-down of MMP-9 did not promote invasive growth of

Panc02 pancreatic cancer cells *in vitro*

(A-E) Analysis of invasive growth of Panc02 pancreatic carcinoma cells after lentiviral knock-down

of MMP-9.

(A) Lentiviral knock-down of MMP-9 in Panc02 cells. MMP-9 mRNA determined by qRT-PCR.

(B) Proliferation determined by AlamarBlue assays.

(C) Viability determined by AlamarBlue assays.

(D) TransWell Migration.

(E) TransWell Invasion.

(A,C-E) *Columns*, mean; *Bars*, SEM.

(A-E) Statistical testing by One-way ANOVA: *, $p < 0.05$; **, $p < 0.01$.