FULL LENGTH ARTICLE

Pancreatic stromal Gremlin 1 expression during pancreatic tumorigenesis

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Abstract Chronic pancreatitis (CP) is a major risk factor of pancreatic ductal adenocarcinoma (PDAC). How CP promotes pancreatic oncogenesis remains unclear. A characteristic feature of PDAC is its prominent desmoplasia in the tumor microenvironment, composed of activated fibroblasts and macrophages. Macrophages can be characterized as M1 or M2, with tumor-inhibiting or -promoting functions, respectively. We reported that Gremlin 1 (GREM1), a key pro-fibrogenic factor, is upregulated in the stroma of CP. The current study aimed to investigate the expression of GREM1 and correlation between GREM1 and macrophages within

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the pancreas during chronic inflammation and the development of PDAC. By mRNA *in situ* hybridization, we detected GREM1 mRNA expression within α-smooth muscle actin (SMA)-positive fibroblasts of the pancreatic stroma. These designated Fibroblasts[Grem1+] marginally increased from CP to pancreatic intraepithelial neoplasia (PanIN) and PDAC. Within PDAC, Fibroblasts[Grem1+] increased with higher pathological tumor stages and in a majority of PDAC subtypes screened. Additionally, Fibroblasts[Grem1+] positively correlated with total macrophages (Mac[CD68+]) and M2 macrophages (M2[CD163+]) in PDAC. To begin exploring potential molecular links between Fibroblasts[Grem1+] and macrophages in PDAC, we examined the expression of macrophage migration inhibitory factor (MIF), an endogenous counteracting molecule of GREM1 and an M1 macrophage promoting factor. By IHC staining of MIF, we found MIF to be expressed by tumor cells, positively correlated with GREM1; by IHC co-staining, we found MIF to be negatively correlated with M2[CD163+] expression. Our findings suggest that GREM1 expression by activated fibroblasts may promote PDAC development, and GREM1/MIF may play an important role in macrophage phenotype.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies worldwide. With a five-year survival rate of less than 8%, it is predicted to be the second leading cause of cancer death in the US by 2030.[1,2] Complete surgical resection is the only curative treatment for PDAC. However, only 15—20% of patients are surgical candidates at the time of diagnosis due to the aggressively metastatic nature of the disease.[3] Aside from late stage diagnosis, chemo-resistance is a major driver of PDAC's high mortality rates. A desmoplastic tumor microenvironment surrounds the cancerous cells preventing effective drug delivery and promoting tumor progression.[4] Composed of non-malignant cells, particularly fibroblasts and infiltrating immune cells, the microenvironment communicates with the tumor to further chemo-resistance and tumor progression.[5] Numerous studies have worked to identify the role of macrophages within the pancreatic tumor microenvironment. A dichotomy of macrophage activation exists via two main pathways: classical (M1) or alternative (M2), with tumor inhibiting or promoting functions, respectively.[6,7] M2 macrophages can promote pancreatic cancer progression through a variety of mechanisms and are correlated with a poor prognosis.[8—10] For this reason, the tumor microenvironment is an active area of PDAC research, with the aim of identifying potential therapeutic targets to enhance chemotherapy and prolong patient survival.

As a widely accepted adjunct to cancer development, inflammation poses an intriguing role in predisposing patients to pancreatic cancer. Patients with chronic pancreatitis (CP), a condition characterized by progressive pancreatic fibrosis due to activated fibroblasts, have a significantly elevated risk of developing PDAC.[11] However, how activated fibroblasts promote pancreatic tumorigenesis during the inflammatory transformation to PDAC is unknown. Previously, we reported that Gremlin 1 (GREM1), a bone morphogenetic protein antagonist, is a key pro-fibrogenic factor in the stroma of CP.[12] The purpose of this study was to analyze the expression of and correlation between GREM1 and macrophages within the pancreas during chronic inflammation and the development of PDAC.

Materials and methods

Human pancreatic tissue samples

Three human pancreatic tissue microarrays (TMAs), with recorded pathology diagnoses and grades, were purchased from US Biomax (Cat# BIC14011a, PA1001b, and PA961e. Rockville, MD). These commercially acquired human tissue samples are exempt from UTHSC IRB committee review. The results presented in this study were based on up to 119 cases, including 12 CP, 9 PanIN, and 98 PDAC. Based on the information provided by the manufacturer, all PanIN cases were not from PDAC, but 4 PanIN cases were from CP patients as duplicate cores are provided from each patient, one core with the disease type of PanIN and the other core with the disease type of CP.

There were 8 normal pancreatic tissue cores as baseline controls. Excluded cases were duplicates between TMAs, cancers other than PDAC, and defective cores (not present on the slide secondary to technical issue, CP and PanIN cores with almost no stroma, and PDAC cores with fibrotic and adipose stroma without cancer cells). For duplicate cores, the cores with the higher histology scores were selected for analysis.

GREM1 mRNA *in situ* hybridization assay and quantification

GREM1 mRNA *in situ* hybridization (RISH) was performed using RNAscope© 2.5 HD Detection Reagent — RED (Advanced Cell Diagnostics, Newark, CA) following manufacturer's instruction. In brief, TMA slides were baked (60 min at 60 °C), deparaffinized by xylene (2 × 5 min), followed by 100% ethanol (2 × 1 min). The slides were then treated with RNAScope© hydrogen peroxide reagent (10 min at room

temperature) to block endogenous peroxidases. Following two washes with distilled water, antigen retrieval was performed (15 min at 100 °C in a steamed pot). The slides were dipped in distilled water (15 s at room temperature) then 100% ethanol (1 × 3 min), and a barrier was drawn around each section with a hydrophobic barrier pen. Next, the slides were incubated with protease (15 min at 40 °C), washed with distilled water, and then hybridized with the target probe hGREM1 (3 h at 40 °C. Advanced Cell Diagnostics, Newark, CA). RNAscope© amplification was performed according to the manufacturer's protocol, with the exception of a prolonged Amp 5 incubation of 60 min. Following Fast-Red detection, slides were counterstained with hematoxylin, then dehydrated and mounted.

GREM1 mRNA *in situ* hybridization scoring was performed by a pathologist blinded to case identification. Scores were given according to the manufacturer's scoring guidelines: 0 (no staining or less than one dot to every 10 cells), 1 (one to three dots per cell (at 20–40× magnification)), 2 (four to ten dots per cell with very few dot clusters (at 20–40× magnification)), 3 (greater than 10 dots per cell with less than 10% positive cells having dot clusters (at 20× magnification)), and 4 (greater than 10 dots per cell with more than 10% positive cells having dot clusters (at 20× magnification)). The Grem1 RISH scoring system used in current study, adapted from the manufacturer's instruction, has been validated by published works on Grem1 RISH quantification in colorectal cancer and basal cell carcinoma.[13,14]

Immunohistochemistry and quantification

Immunohistochemistry (IHC) was performed on a Dako automated immunostainer (Agilent Technologies, Santa Clara, CA) using the antibodies and dilutions as specified in Table 1.

For quantification of Mac[CD68+] and M2[CD163+], based on literature,[9,10] one image was taken at high power (40×) for each core at the most intensely stained area. Positively stained cells, defined by cytoplasmic staining with a coinciding nucleus, were counted by two researchers blinded to case identification using NIS-Elements AR 3.2 software and were averaged per case.

For MIF scoring, based on the Remmele Scoring System,[15,16] one image was taken at high power (20×) for each core at the most intensely stained area. Two researchers

Table 1 Antibodies used for IHC.

Antibodies	Catalog (Clone)	Company	Dilution
α-SMA	IR61161-2 (1A4)	DAKO	Ready-to-Use
p40	ab166857	abcam	1:50
PDX1	AC-0131RUO (EP139)	Cell Marque Millipore Sigma	1:400
CTLA4	ab19792 (BN3)	abcam	1:100
MIST1	MA1-517 (6E8/A12/C11P1)	Thermofisher	1:100
CD68	GA60961-2 (KP1)	DAKO	Ready-to-Use
CD163	CD163-L-CE (10D6)	Leica Biosystems	1:600
MIF	ab227073	abcam	1:2000

blinded to case identification scored MIF expression semi-quantitatively. Staining intensity (SI) was scored according to the following categories: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The percentage of positive cells (PP) was estimated according to the following categories: 0 (none), 1 (<10%), 2 (10%–50%), 3 (51%–80%), and 4 (>80%). The comprehensive MIF score was then determined by multiplying the SI and PP scores for a score range of 0–12. MIF scores from each researcher were then averaged per case.

For MIF/CD163+ co-staining, CD163 IHC with magenta chromogen was performed onto the MIF IHC with DAB chromogen (Agilent Technologies, Santa Clara, CA). Two images were acquired at high power for each core at the most intensely stained area for MIF and CD163+, respectively, and quantified according to the respective protocols above.

Statistics

Data are expressed as medians with interquartile range (IQR). Non-parametric Mann–Whitney U test was used for two groups, and Kruskal–Wallis H test was used for more than two groups with Bonferroni's P adjusted values for multiple comparisons. Correlation analysis was determined using Spearman correlation. All statistics were performed using GraphPad Prism version 8 for Windows (GraphPad Software, La Jolla, CA). P values less than 0.05 are considered significant.

Results

GREM1 expression localizes in activated fibroblasts of the pancreatic stroma

Previous studies have shown GREM1 expression by stromal cells in multiple carcinomas,[13,14,17] including PDAC.[18] To date, GREM1 mRNA *in situ* hybridization (RISH) is the most specific assay for measuring GREM1 expression.[13,14,17–19] In order to confirm the location of GREM1 expression within the pancreas, we performed GREM1 RISH, along with immunohistochemistry (IHC) using an α-smooth muscle actin (α-SMA) antibody, a marker of activated fibroblasts, on human pancreatic tissue microarrays (TMAs). GREM1 mRNA localized exclusively to α-SMA positively-stained cells, supporting that activated fibroblasts within the pancreatic stroma express GREM1. These GREM1+ fibroblasts are designated as Fibroblasts[Grem1+] (Fig. 1). In addition, not all α-SMA positive cells were Grem1 positive. However, almost all Grem1 positive cells are also α-SMA positive.

Fibroblasts[Grem1+] increase in chronic pancreatitis, PanIN, and PDAC

Based on our previous works demonstrating increased GREM1 expression in chronic pancreatitis,[12] we began by investigating GREM1 expression during PDAC oncogenesis from chronic pancreatitis. GREM1 RISH was performed on 3 human pancreatic tissue microarrays (BIC14011a, PA1001b, and PA961e) containing 119 total cases, including CP (12 cases), pancreatic intraepithelial neoplasia or PanIN (9 cases), and PDAC (98 cases), followed by scoring.

Figure 1 Activated fibroblasts express GREM1. Representative images of GREM1 RISH and α-SMA IHC from normal pancreas and PDAC. Arrowhead points to GREM1 positive staining. Arrow points to α-SMA positive staining. 200× magnification; scale bar = 50 μm.

Figure 2 Fibroblasts[Grem1+] increase in CP, PanIN, and PDAC and with increasing pT stages in PDAC. **(A)** Representative images of GREM1 RISH in chronic pancreatitis (CP), pancreatic intraepithelial neoplasia (PanIN), and PDAC cases. Arrowhead points to GREM1 positive staining. 400× magnification; scale bar = 100 μm. **(B)** GREM1 RISH scores in CP, PanIN, and PDAC. **(C)** GREM1 RISH scores in the pathological tumor (pT) stages of PDAC. *$P < 0.05$. Total of 118 cases: 11 CP, 9 PanIN, and 98 PDAC.

Fibroblasts[Grem1+] marginally increased from CP to PanIN and PDAC ($P = 0.06$, Fig. 2A, B).

Fibroblasts[Grem1+] increase in PDAC with increasing pT stage and in a majority of subtypes

To further evaluate Fibroblasts[Grem1+] in PDAC, all 98 PDAC cases were grouped by pT stages 1—2 (48 cases) and 3—4 (50 cases). A higher number of Fibroblasts[Grem1+] was observed in pT stages 3—4 than that in pT stages 1—2 ($P < 0.05$, Fig. 2C).

The classification and stratification of PDAC is an active area of research,[20,21] promoting identification of molecular signatures for pancreatic cancer subtypes in order to develop specific targeted therapies. Four subtypes of PDAC have been grouped based on their gene expression profiles: squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine.[20] Based on this classification system, we selected four corresponding protein markers, p40 (or p40-DeltaNp63), PDX1, CTLA4, and MIST1, to determine if our previously observed increase in Fibroblasts[Grem1+] was associated with specific PDAC subtype(s). IHC was performed using antibodies to these four protein markers on a human tissue microarray containing 75 PDAC cases (PA961e). PDAC cases that stained positive for PDX1 (33), CTLA4 (21), and MIST1 (12) all demonstrated an increase in Fibroblast[Grem1+] compared to negative cases for PDX1 (42), CTLA4 (54), MIST1 (63) ($P < 0.05$), while PDAC cases that stained positive for p40 (15) or negative for p40 (60) did not show a difference (Fig. 3). These results demonstrate increased Fibroblasts[Grem1+] in the majority of PDAC subtypes screened, supporting the idea that increased Fibroblasts[Grem1+] in PDAC may be a common pathological event during pancreatic tumorigenesis.

Fibroblasts[Grem1+] correlate with increased macrophages in PDAC

As previously mentioned, macrophages play an important role in the tumor microenvironment of PDAC.[7,8] To begin profiling macrophage levels and distributions during PDAC oncogenesis, IHC was performed using CD68 as a general macrophage marker and positively stained cells (designated as Mac[CD68+]) were quantified (Fig. 4A). No significant
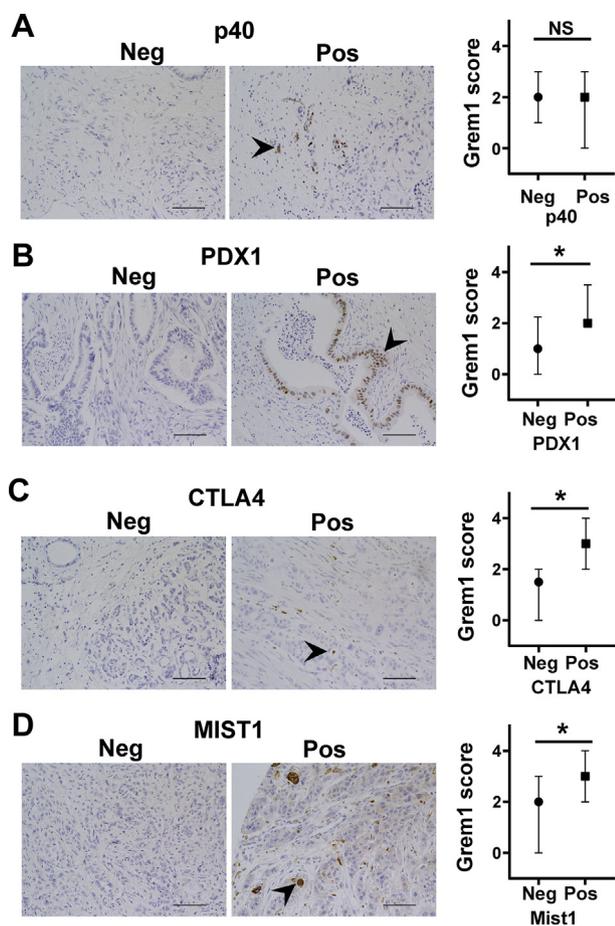
Figure 3 Fibroblasts[Grem1+] increase in a majority of subtypes of PDAC. Representative images of IHC and the quantification. **(A)** p40. **(B)** PDX1. **(C)** CTLA4. **(D)** MIST1. Arrowhead points to positive staining. 200× magnification; scale bar = 100 μm *$P < 0.05$. Total of 75 PDAC cases.

difference in Mac[CD68+] was observed between CP, PanIN, and PDAC (Fig. 4B). Mac[CD68+] increased between pT stages of PDAC ($P < 0.05$, Fig. 4C). Fibroblasts[Grem1+] positively correlated with Mac[CD68+] in PDAC ($P < 0.05$, $r = 0.39$, Fig. 4D).

It has been reported that increasing M2 macrophages in PDAC correlate with a poor prognosis.[8–10] To determine if the presence of Fibroblasts[Grem1+] correlates with M2 expression during PDAC oncogenesis, IHC using CD163 as an M2 macrophage marker was performed and positively stained cells (designated as M2[CD163+]) were quantified. No significant difference in M2[CD163+] was observed between CP, PanIN, and PDAC or between pT stages of PDAC (Fig. 5A–C). A positive correlation was observed between Fibroblasts[Grem1+] and M2[CD163+] in PDAC ($P < 0.05$, $r = 0.23$, Fig. 5D).

MIF expression positively correlates with GREM1 and inversely correlates with M2[CD163+] in PDAC

As an initial step in the investigation of how Fibroblasts[Grem1+] influence macrophage activation in PDAC, the expression of macrophage migration inhibitory factor (MIF) was examined. MIF is a known pro-inflammatory mediator that promotes a classical immune response, including activation of M1 macrophages.[22,23] As an endogenous inhibitor of MIF, GREM1 has been shown to bind to and block MIF's effects on M1 macrophage activation in vascular inflammation and atherogenesis.[23,24] To begin exploring the potential role of MIF in the observed correlation between Fibroblasts[Grem1+] and M2[CD163+] in PDAC, the expression pattern of MIF within the pancreas was profiled using IHC. In contrast to GREM1 expression in activated fibroblasts, MIF was primarily expressed by tumor cells within the pancreas. A positive correlation was observed between MIF and GREM1 within PDAC ($P < 0.05$, $r = 0.32$, Fig. 6A–C). Furthermore, co-staining of MIF/CD163 was performed, which revealed an inverse correlation and distribution of MIF and M2[CD163+] in PDAC ($P < 0.05$, $r = -0.29$, Fig. 6D–F). These results suggest that MIF may be involved in M2[CD163+] distribution in PDAC.

Discussion

PDAC is one of the most lethal cancers, primarily due to late stage diagnosis and chemoresistance attributed to a fibrotic tumor microenvironment. Chronic pancreatitis patients are at an increased risk of developing PDAC, although how chronic inflammation promotes pancreatic tumorigenesis is unclear. Identifying specific molecules that advance the inflammation to neoplasia transition could lead to the development of novel therapies and biomarkers for better treatment and earlier detection in PDAC patients; thus, improving overall patient survival.

The purpose of this study was twofold, to profile and correlate Fibroblasts[Grem1+] and macrophage activation during progression of CP to PDAC, and to begin exploring potential molecular links between fibroblasts and macrophages in PDAC. Our results showed marginally increased Fibroblasts[Grem1+] from CP to PanIN and PDAC, which is likely due to the relatively small sample sizes of CP ($n = 12$) and PanIN ($n = 9$), and apparently large variations among the human samples analyzed. We demonstrated increased Fibroblasts[Grem1+] with increasing PDAC pathological tumor stages, and within the majority of PDAC subtypes screened. We also demonstrated that Fibroblasts[Grem1+] positively correlate with Mac[CD68+] and M2[CD163+] in PDAC. These results suggest that Fibroblasts[Grem1+] may influence CP progression to PDAC by promoting the activation of macrophages, specifically alternatively activating M2 macrophages.

To begin investigating the relationship between Fibroblasts[Grem1+] and M2 macrophages in PDAC, we focused on MIF. As an inflammatory mediator, MIF promotes M1 macrophages,[22,23] the effect of which can be blocked by GREM1 in vascular inflammation and atherogenesis.[23,24] However, in pancreatic disease, the interaction between GREM1 and MIF is unknown. Our results show MIF to be primarily expressed in pancreatic tumor cells, positively correlated with GREM1, and inversely correlated with M2 macrophages. The opposing correlations of GREM1 and MIF with M2 macrophages, along with a positive correlation between MIF and GREM1 expression, suggest that GREM1 inhibits MIF activity, but not MIF expression, in PDAC,
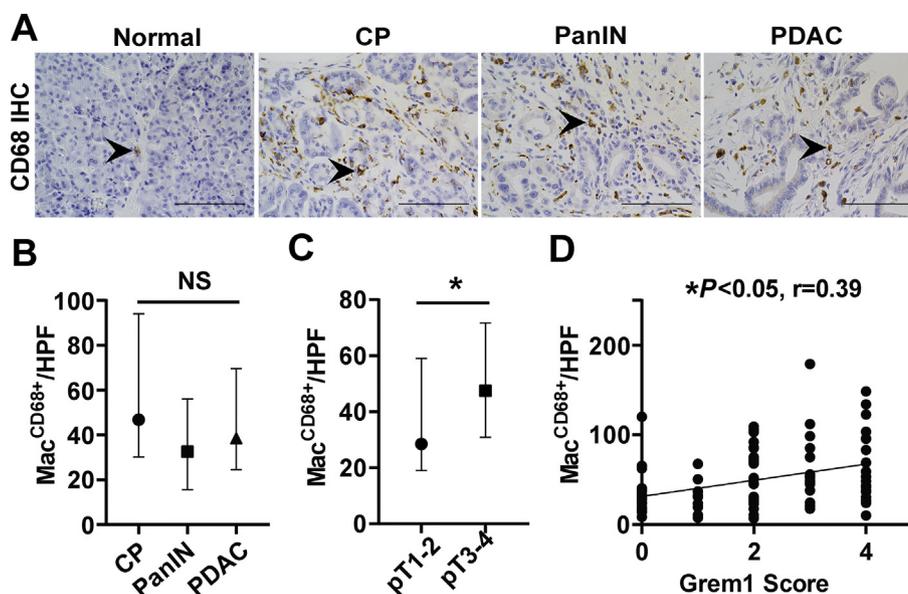
Figure 4 Fibroblasts^{Grem1+} correlate with Mac^{CD68+}. **(A)** Representative images of CD68 IHC from human normal pancreas, CP, PanIN, and PDAC. Arrowhead points to positive staining. 400× magnification; scale bar = 100 μm. **(B)** Mac^{CD68+} counts in CP, PanIN, and PDAC. **(C)** Mac^{CD68+} counts by pT stage in PDAC. **(D)** Correlation of GREM1 RISH scores with Mac^{CD68+} counts in PDAC. *$P < 0.05$, r = 0.39. Total of 114 cases: 10 CP, 7 PanIN, and 97 PDAC.
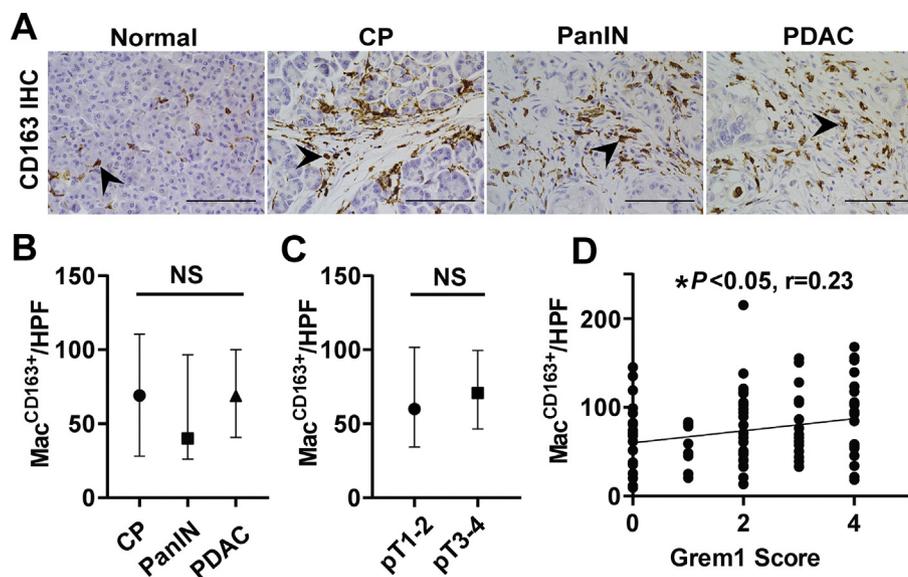
Figure 5 Fibroblasts^{Grem1+} correlate with M2^{CD163+}. **(A)** Representative images of CD163 IHC from human normal pancreas, CP, PanIN, and PDAC. Arrowhead points to positive staining. 400× magnification; scale bar = 100 μm. **(B)** M2^{CD163+} counts in CP, PanIN, and PDAC. **(C)** M2^{CD163+} counts by pT stage in PDAC. **(D)** Correlation of GREM1 RISH scores with Mac^{CD163+} counts in PDAC. *$P < 0.05$, r = 0.23. Total of 118 cases: 11 CP, 9 PanIN, and 98 PDAC.

resulting in a predominance of M2 macrophages. Pertinent functional experiments are being planned in our ongoing studies to elucidate the interactions of GREM1 and MIF and strengthen our current findings that GREM1 inhibits MIF activity.

GREM1 expression by stromal cells has been reported in a variety of cancers, including basal cell carcinoma, esophageal, colon, lung, breast, and pancreas,[13,14,17,18] which our results support. Our findings of the increased Fibroblast^{Grem1+} from CP to PanIN and PDAC, although marginal, shed light on a potential role of Fibroblasts^{Grem1+} in the transition from chronic inflammation to neoplasia. Although currently molecular subtypes are not used clinically to diagnose patients or influence treatment, the identification and classification of PDAC subtypes is an ongoing research aim to improve prognostication and treatment regimen decision.[25] Based on genomic expression profiling, four subtypes of PDAC have been identified:
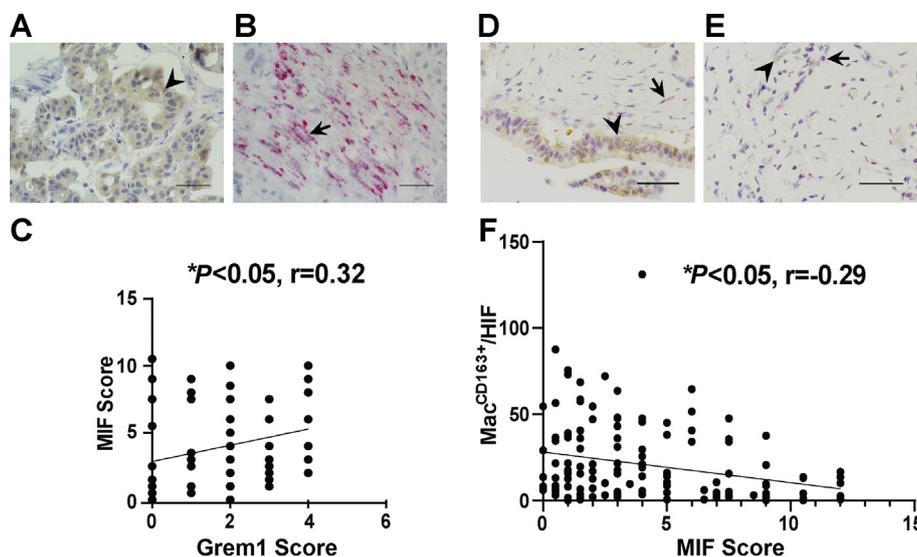
Figure 6 MIF expression positively correlates with GREM1 and inversely correlates with M2[CD163+] in PDAC. Representative images from the same PDAC case with: **(A)** MIF IHC (pointed by arrowhead), and **(B)** GREM1 RISH (pointed by arrow). 400× magnification; scale bar = 50 μm. **(C)** Correlation of MIF and GREM1 in PDAC. *P < 0.05, r = 0.32. Total of 70 PDAC cases. Representative IHC images from the same PDAC case by co-staining with MIF (pointed by arrowhead) and CD163 (pointed by arrow). **(D)** The most densely stained area of MIF, and **(E)** The most densely stained area of CD163. 400× magnification; scale bar = 50 μm. **(F)** Correlation of MIF scores with M2[CD163+] counts in PDAC. *P < 0.05, r = −0.29. Total of 68 PDAC cases.

squamous (31% incidence, 13.3 months survival rate), pancreatic progenitor (19% incidence, 23.7 months survival rate), immunogenic (29% incidence, 25.6 months survival rate), and aberrantly differentiated endocrine exocrine (21% incidence, 30.0 months survival rate).[20,26] Our study found an increase in Fibroblast[Grem1+] expression in all subtypes screened, with the exception of squamous, suggesting a potential significant role of Fibroblast[Grem1+] in PDAC development. Previous investigators have reported that stromal fibroblasts influence M2 polarization in PDAC.[27] Our study is the first to correlate an increase in macrophage presence, specifically M2 macrophages, with an increase in Fibroblast[Grem1+] in PDAC.

There are several limitations in this study. First, we used GREM1 RISH for detection of GREM1 mRNA expression. Although this method is specific, and the results possibly correlate with GREM1 protein level, it is understood that this is not exactly equal to the GREM1 protein level. We took effort and performed IHC using several commercially available Grem1 antibodies, but unfortunately, did not produce quality IHC. Therefore, specific custom antibodies against human Grem1 for quality IHC are warranted for future studies. However, even at the mRNA level, the presence of GREM1 during the chronic inflammation to cancer transition, and the interplay between GREM1 and macrophages, appear evident based on our findings. Furthermore, although all correlations found in this study were significant, we recognize that only poor–fair correlations were observed. However, our focus in current study is to establish an evident and clinical relevance by analyzing human pancreas tissue samples from various disease stages. The outcome from the current study will lay a solid foundation for further investigation of the cellular and molecular mechanisms during

pancreatic tumorigenesis. Pertinent functional experiments are warranted in further mechanistic studies to determine causation.[28,29] Finally, access to clinical samples of human chronic pancreatitis and PanIN has been a significant challenge in the field of pancreas research. Due to the lack of availability of human pancreatic tissue samples, relatively fewer cases of normal, CP, and PanIN were acquired and analyzed, which may account for the finding that no significant difference in Mac[CD68+] and M2[CD163+] between CP, PanIN, and PDAC was observed, as others have reported the contrary.[30] With the analysis of 98 cases of PDAC, we revealed significant differences in Fibroblast[Grem1+] expression between CP and PanIN. However, the lack of data on patients' and disease characterization, tumor stage, patients' survival and genetic information from these commercially acquired pancreatic TMAs hampers further detailed analysis. Nonetheless, additional human chronic pancreatitis and PanIN samples should be acquired through clinical collaborations in future studies.

Overall, our findings suggest that Fibroblasts[Grem1+] may influence the chronic inflammation to cancer transition within the pancreas, and may promote PDAC progression through activation of M2 macrophages, potentially via MIF inhibition. Further *in vitro* studies are warranted to delineate these important cellular and molecular interactions, with hope of identifying molecular biomarker and treatment targets for PDAC patients.

Author contributions

Joy M. Davis: Conceptualization, methodology, software, data curation, analysis, manuscript - original draft preparation and revision.

Binglu Cheng, Madeline M. Drake, Qiang Yu, Baibing Yang, Jing Li, Chunhui Liu: Data curation, analysis, manuscript reviewing and editing.

Mamoun Younes: Conceptualization, data curation, analysis, manuscript reviewing and editing.

Xiurong Zhao, Jennifer M. Bailey, Qiang Shen: Conceptualization, manuscript reviewing and editing.

Tien C. Ko, Yanna Cao: Conceptualization, manuscript reviewing and revision, and supervision.

Conflict of interests

The authors have no conflict of interests to declare.

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