EMBO reports

High-grade mesenchymal pancreatic ductal adenocarcinoma drives stromal deactivation through CSF-1

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1st Editorial Decision 2 August 2019

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you can see, the referees As you can see, referees express interest in the analysis reporting that mesenchymal PDAC tumors contain less active PSCs, mediated by CSF-1 from the cancer cells. However, they also raise significant concerns that need to be addressed before considering publication here.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

REFEREE REPORTS

Referee #1:

In this manuscript, Steins and colleagues investigate the influence of distinct PDAC subtypes on the phenotype of pancreatic stellate cells (PSC). Using human PDAC tissue samples, the authors show that collagen content negatively correlates with tumor grade. Moreover, they use PDAC cell lines with distinct subtype characteristics to link poor PSC activation within PDAC stroma with mesenchymal phenotype of PDAC. The authors identify the cytokine CSF-1 in the supernatant of mesenchymal PDAC cells and use recombinant CSF-1 or a neutralizing antibody targeting CSF-1 to show that CSF-1 can deactivate PSCs in vitro. This is an interesting study suggesting that mesenchymal PDAC tumors contain less active PSCs, mediated by CSF-1 from the cancer cells. Based on previous reports, the role of PDAC stroma in disease progression has been shown to be very complex and somewhat paradoxical. Steins et al have an opportunity to provide some insight and clarity into this paradox. However, this study does not go far enough to provide that insight, in particular because no evidence is provided for how active PSCs may affect mesenchymal PDAC cells. What are the consequences of PSC repression in this context? This, and other important concerns need to be addressed.

Major Points:

1. The authors do not show whether the changes observed in PSCs and caused by mesenchymal PDAC cells, influence cancer cell biology in any way. Does the repression of PSC activation have any functional relevance? To address this, the phenotype of PDAC cells should be analyzed, comparing mesenchymal PDAC cells in monoculture with cancer cells co-cultured with PSCs (with or without neutralizing CSF-1 antibody). The comparison between how mesenchymal and epithelial PDAC cells respond to PSC co-culture, could reveal new insights into the conflicting roles reported on PSCs within PDAC stroma. Finally, the consequences of reduced PSC activation should also be thoroughly discussed, in particular since PSCs are recognized to produce many specific factors stimulating invasion, survival or proliferation. These factors include COL1A and FN1 (used in the manuscript as markers of PSC activation) and other factors such as POSTN and MMP-2.

2. In Fig. 2, the authors analyze proliferation of PS-1 in response to conditioned medium from different PDAC lines. This analysis needs to be more rigorous. Whereas, the cells treated with mesenchymal PDAC CM cover a smaller area of the field (Fig 2A and B) they also have dramatically changed morphology, individual cells being much more slender compared to control. First, the number of cells should be counted. Second, it is not clear what the results in panel C mean and the statistics are poor. Since Ki67 stains cells that are actively cycling independent of cell cycle stage it is surprising not to observe a difference in G1 or G2 phases. The analysis needs to be more rigorous. For example, by increasing sample sizes and/or adding BrdU incorporation analysis.

3. In Fig. 1E, the difference in alpha-SMA expression between PSC co-culture with PDAC cell lines of different subtypes is not very clear. The epithelial-like cell lines Capan-2 shows intense alpha-SMA staining. However the same cannot be said about ASPC1. In addition, significant alpha-SMA staining is observed in the PANC-1 co-culture. To this reviewer the results are not conclusive.

4. It should be noted that increased expansion of mesenchymal cancer cells may contribute indirectly to reduced stroma/cancer cells ratio. This is clearly observed in Fig. 1E where the co-cultures show a major expansion of mesenchymal cell lines. The relevance of this may particularly apply to analysis of tumor samples, such as in Fig. 1A and B, where modest, yet significant, differences are observed. This should be discussed.

5. The authors analyze CSF-1 and COL1A or ACTA2 expression in PDAC patient samples (GSE62165) and show a negative correlation. What about other genes induced in mesenchymal-like cells such as CCL5 or CXCL16? Do they also negatively correlate with ACTA2 and COLA1?

6. What are the CSF-1 expression levels in the PDAC samples that were used in Fig. 1A and B?

7. The investigators perform a proteomic screen using forward-phase protein array. However they do not show which proteins are included in the 68 cyto/chemokine array. A table should be shown. In addition, it would be interesting to know how the levels of proteins that are recognized to induce PSCs activation (PDGFs and TGFbeta) change in this context. Are the PSC activators lower expressed in mesenchymal-like vs epithelial-like PDAC?

8. With the results in Fig. 4, the authors claim that the deactivation of PSCs is not impacted by filtering the conditioned medium (CM) through a 100 µm filter. However, the deactivation of PCSs is already reduced with CM, from Mia PaCa-2 cells, that has been filtered through the 100 µm pore size membrane compared to unfiltered CM. This suggests a more complex regulation of PSC repression and should be discussed.

9. In the discussion on p. 21 the authors suggest that mesenchymal PDAC can use the pro-invasive contributions of PSCs before inactivating them. Evidence for this is missing and the rationale is unclear.

Minor Points

1. On page 18, the authors state that they want to use molecular filtering to confirm that the mediator of PSC repression was a soluble factor (Fig. 4). However, the experiments with conditioned medium (Fig. 3) should already confirm this. A different rationale should be used for the filtering experiments.

2. On page 19 they state that since CSF-1 is recognized as regulator of macrophages, they want to address whether the negative correlation between CSF-1 and COL1A1 or ACTA2 were dependent on macrophage content. However, similar to minor point 1 the experiments with conditioned medium show that the effect is direct (independent of macrophages). A different rationale should be used for correlation analysis in the presence/absence of CD68.

3. In Fig. 5E the bar for ACTA2 expression in control cells is missing.

4. In the text on p.18 the FPPA analysis are said to be found in Fig. 4C. However, the experiments are shown in Fig. 5A.

5. The figure legends for Figs. S2 and S3 have been mixed up.

6. The biological replicates used are missing in many figures e.g. Figs. 2, 3, 4 and 5.

Referee #2:

This is an interesting study looking at the regulation of stroma activation by subtypes of PDAC tumor lines. The study identifies CSF1 as the mediator of this phenomenon. The study is well done but additional experimentation is needed to fully define the mechanism underlying the CSF1-dependent regulation of the stromal activation. First, the antibody blockade experiments should be validated by knockdown studies using two independent siRNA or shRNA targeting CSF1. Second, the expression of CSF1 and stromal marker correlation should be validated by IHC in an independent cohort of tumors. Third, since the immune system plays a major role in the regulation of stroma structure and dynamics, it is essential that the in vivo experiments are repeated in an immunocompetent animal using Panc02 or KPC-derived cell models. Finally, (and most important), the mechanism underlying the regulation of stroma de-activation by CSF1 should be investigated? What are the downstream molecules mediating this effect?

Referee #3:

The manuscript describes interesting findings related to the tumor - stroma crosstalk in pancreatic ductal adenocarcinoma (PDAC). Using PDAC cell lines and immortalized human PS-1 pancreatic

stellate cells the authors find that the epithelial and mesenchymal-like (more aggressive) PDAC cells differentially affect PS-1 cells. The mesenchymal PDAC cells inhibit the growth and expression of COL1A1 & ACTA2, used as stellate cell activation markers, in PS-1 cells. Authors further provide evidence suggesting that CSF-1 is a mediator of this mesenchymal PDAC-mediated stromal cell "deactivation". These results with the used cell lines appear solid, and are considered to support the contribution of PDAC-associated stromal cells and extracellular matrix deposition, often associated to PDAC aggressiveness, to tumor inhibition or restriction.

At this stage, the data provided using PDAC clinical samples and datasets as well as with cell line xenografts, "organotypic co-cultures" and patient derived cells remains less clear/convincing and thus premature to strongly support the drawn provocative conclusions.

Specific comments:

1. Figure 1A-B: What were the percentages of tumor cells in the images of the total 15 different grade 1-2 and grade 3 tumors in the staining quantification? Figure 1A seem to show mainly stromal cells in OK26 and cancer cells in OK37. Is the presented Picrosirius positivity simply reflecting the relative stroma and cancer cell numbers in the analyzed tumor tissues or areas? This is a small cohort for which the staining results could be more comprehensively presented. Markers for the cancer and/or stromal cells would also be informative.

2. In materials and methods immunohistochemical staining is described as follows: "For PSR staining of primary and PDX tumors, the entire tumor was quantified." How do the images in Figure 1A relate to the "entire tumor"? How many sections per tumor were used?

3. Figure 1C shows more collagen and aSMA in epithelial-like PDAC cell xenografts than in mesenchymal-like PDAC xenografts. It is further postulated that "the collagens are produced by activated CAFs". Therefore, expression of the stromal activation marker aSMA was assessed, which revealed that activated PSCs were present in epithelial-like tumors while these were absent in mesenchymal-like PDAC tumors. Firstly, the inconsistent description of CAFs versus PSCs here and throughout the manuscript is distracting. Secondly; are the mesenchymal PDAC cells producing collagens? Are the contrast/intensity adjustments in PSR images of MIA PaCa-2 and PANC-1 similar to the epithelial-like cell line tumors? Are these PDAC cells themselves producing and assembling more collagens than the more epithelial cells?

4. It is difficult to understand the organotypic co-culture results and drawn conclusions from the data as presented in Figures 1 and S1.

What seems clear based on Figure S1 is that PS-1 cells dramatically increase the growth of the mesenchymal-like PDAC cells. This growth induction is very prominent with MIA PaCa-2 cells, and would be important to quantify in all the samples. I cannot see that this result could be described only as an impact on initial stage of invasion. In contrast, if PS-1 cells activate both invasion and growth of the mesenchymal PDAC cells, the concept of their deactivated or tumor-restricting status should be reconsidered.

Regarding the presence or absence of aSMA positive PS-1 cells, there would be another possible explanation than "these PS-1 cells were absent in the ECM gel when co-cultured with PANC-1 and MIA PaCa-2 cells", as it could be that they will not be detected due to the mesenchymal PDAC-mediated aSMA down-regulation. Another marker that stain the PS-1 cells, as well as cultures with PS-1 cells alone would be relevant to address this issue. It also seems that only part of PANC-1 cells are positive for CK19. Means to quantify total PS-1 and PDAC cells will improve this data.

5. Legends to Figure S2 and S3 are mixed and Figure S2 requires scale bars. Are the poorly differentiated 53M and well-differentiated 67 tumors shown with same magnification? Do these tumors have equal CDH1 expression also in vivo? Can the poorly differentiated cells be considered mesenchymal, if CHD1 is equal to the well-differentiated cells? Some additional markers, such as CK19-aSMA double staining would be informative to characterize the patient derived cultures.

Materials and methods says: "For α-SMA staining of PDX tumors, a representative region of interest was and quantified." However, only the cell line xenograft tumors seem to have been quantified.

How did collagen and ACTA2/aSMA look like in the 53M and 67 tumors?

6. Figure S3: Are the CSF-1 high tumors CD68 low?

7. The terms PSC, CAF and stroma, as well as collagen could be used more carefully, systematically considering exact definitions throughout the manuscript. What is for example meant by "increased stroma deposition" or "PSC deactivation" in Abstract? Altogether, the results seem to reveal interesting new crosstalk between PDAC and stromal cells, but the results would need to be considered and interpreted more carefully.

1st Revision - authors' response 21 December 2019

POINT-BY-POINT REPLY

REVIEWER 1

In this manuscript, Steins and colleagues investigate the influence of distinct PDAC subtypes on the phenotype of pancreatic stellate cells (PSC). Using human PDAC tissue samples, the authors show that collagen content negatively correlates with tumor grade. Moreover, they use PDAC cell lines with distinct subtype characteristics to link poor PSC activation within PDAC stroma with mesenchymal phenotype of PDAC. The authors identify the cytokine CSF-1 in the supernatant of mesenchymal PDAC cells and use recombinant CSF-1 or a neutralizing antibody targeting CSF-1 to show that CSF-1 can deactivate PSCs in vitro. This is an interesting study suggesting that mesenchymal PDAC tumors contain less active PSCs, mediated by CSF-1 from the cancer cells. Based on previous reports, the role of PDAC stroma in disease progression has been shown to be very complex and somewhat paradoxical. Steins et al have an opportunity to provide some insight and clarity into this paradox. However, this study does not go far enough to provide that insight, in particular because no evidence is provided for how active PSCs may affect mesenchymal PDAC cells. What are the consequences of PSC repression in this context? This, and other important concerns need to be addressed.

Major Points:

1. *The authors do not show whether the changes observed in PSCs and caused by mesenchymal PDAC cells, influence cancer cell biology in any way. Does the repression of PSC activation have any functional relevance? To address this, the phenotype of PDAC cells should be analyzed, comparing mesenchymal PDAC cells in monoculture with cancer cells co-cultured with PSCs (with or without neutralizing CSF-1 antibody). The comparison between how mesenchymal and epithelial PDAC cells respond to PSC co-culture, could reveal new insights into the conflicting roles reported on PSCs within PDAC stroma.*

We appreciate the reviewer's positive assessment of the data presented. We agree with the reviewer that it is interesting to study the functional relevance of PSCs on PDAC cell phenotype and performed the suggested experiments. The results of these co-culture experiments are included in Fig 2D and page 20 paragraph 1 (without CSF-1 inhibition), and Fig 4F and page 24 paragraph 1 (with CSF-1 inhibition). Interestingly, we find that following co-culture with PS-1 cells, mesenchymal PDAC cells substantially *reduce* the expression of mesenchymal markers, possibly explaining the benefit of PSC repression by mesenchymal-like PDAC. This phenotypical change in mesenchymal PDAC cells remained during inhibition of CSF-1 suggesting that the functional relevance of CSF-1 signalling in high-grade PDAC is mainly directed against the tumor stroma and changing the composition of it.

Finally, the consequences of reduced PSC activation should also be thoroughly discussed, in particular since PSCs are recognized to produce many specific factors stimulating invasion, survival or proliferation. These factors include COL1A and FN1 (used in the manuscript as markers of PSC activation) and other factors such as POSTN and MMP-2.

We have now more elaborately discussed the consequences of PSC deactivation in PDAC tumors in the revised Discussion section, page 17, first paragraph. Importantly, we find that PSCs reduce

markers for EMT in PDAC cells. Also, our findings suggest that activated stroma has structural/mechanical contributions which are not beneficial to mesenchymal-like PDAC cells. This further supports the notion that deactivated PSCs and low collagen content results in a pro-tumorigenic microenvironment.

2. *In Fig. 2, the authors analyze proliferation of PS-1 in response to conditioned medium from different PDAC lines. This analysis needs to be more rigorous. Whereas, the cells treated with mesenchymal PDAC CM cover a smaller area of the field (Fig 2A and B) they also have dramatically changed morphology, individual cells being much more slender compared to control. First, the number of cells should be counted. Second, it is not clear what the results in panel C mean and the statistics are poor. Since Ki67 stains cells that are actively cycling independent of cell cycle stage it is surprising not to observe a difference in G1 or G2 phases. The analysis needs to be more rigorous. For example, by increasing sample sizes and/or adding BrdU incorporation analysis.*
We apologize that the analyses on cell count and cell cycle were not sufficiently in-depth and agree that different techniques and increased sample sizes improve the interpretation of these results. We have analyzed the number of cells now using bead calibrated FACS counting, and analyzed cell cycle using EdU incorporation. These results are shown in Fig 2B and C and explained on page 9, first paragraph. These experiments showed a clear reduction in the absolute number of PS-1 cells when subjected to mesenchymal PDAC CM, and explain that this is caused by a reduction of cells in S-phase and an increase of cells in G2/M phase. Since these methods are more accurate and comprehensive, we have removed the previously shown Nicoletti assay data from the manuscript, and moved the cell surface area quantification and *MKI67* mRNA expression data to Fig EV2A and B.

3. *In Fig. 1E, the difference in alpha-SMA expression between PSC co-culture with PDAC cell lines of different subtypes is not very clear. The epithelial-like cell lines Capan-2 shows intense alpha-SMA staining. However the same cannot be said about ASPC1. In addition, significant alpha-SMA staining is observed in the PANC-1 co-culture. To this reviewer the results are not conclusive.*
We agree with the reviewer that the organotypic α-SMA images are not conclusive. This is mainly caused by the AsPC-1+PS-1 organotypic co-culture, which did not show a stratified ECM and epithelial layer as did the other gels and we expected this to be due to technical error. We therefore repeated the organotypic AsPC-1 mono-, and AsPC-1 + PS-1 co-cultures which showed invasion of PS-1 cells into the gel as depicted in Fig 1F by intense α-SMA staining. Unfortunately, the AsPC-1 tumor cells stained positive for α-SMA as well. Additional CK19 (Fig EV1D) staining was performed to show that the epithelial layer was formed on top of the ECM gel containing PS-1 cells, comparable to the other epithelial PDAC cell line Capan-2. We hope that these results are now more conclusive.

4. *It should be noted that increased expansion of mesenchymal cancer cells may contribute indirectly to reduced stroma/cancer cells ratio. This is clearly observed in Fig. 1E where the co-cultures show a major expansion of mesenchymal cell lines. The relevance of this may particularly apply to analysis of tumor samples, such as in Fig. 1A and B, where modest, yet significant, differences are observed. This should be discussed.*
We agree with the reviewer that, based on the images in Fig 1F, mesenchymal-like cells seem more abundant then epithelial-like PDAC cells. To address whether high-grade PDAC represented tumors with an increased expansion of cancer cells, two independent pathologists scored the H&E stainings of the entire tumor area (see Fig EV1A) of the patient cohort from Fig 1A and B for the percentage of tumor cells. This revealed no differences in tumor cellularity. We have added this data as Fig 1C and made this more explicit on page 7 paragraph 1.

5. *The authors analyze CSF-1 and COL1A or ACTA2 expression in PDAC patient samples (GSE62165) and show a negative correlation. What about other genes induced in mesenchymal-like cells such as CCL5 or CXCL16? Do they also negatively correlate with ACTA2 and COLA1?*
We have now assessed the correlation of *CCL5* and *CXCL16* with *ACTA2* and *COL1A1* in the same dataset, which showed no correlation of *CCL5* with *COL1A1* or *ACTA2*. *CXCL16* was negatively correlated to *COL1A1*, but not *ACTA2*, and is smaller than 30 kDa and therefore not likely to be the factor inducing the effects in PSCs. The data are shown in Fig EV3A-C and discussed on page 11 and 12.

6. What are the CSF-1 expression levels in the PDAC samples that were used in Fig. 1A and B?

Of the PDAC patient cohort we used in Fig 1A and B, no tissue was available for mRNA expression levels of *CSF1*. Therefore, we have now analysed the entire cohort for CSF-1 by IHC and quantified the expression of CSF-1 in the tumor cells. This confirmed that high-grade PDAC samples had significantly more CSF-1 expressing tumor cells. These results are included in Fig 6A and B and on made explicit on page 14 paragraph 2.

7. *The investigators perform a proteomic screen using forward-phase protein array. However they do not show which proteins are included in the 68 cyto/chemokine array. A table should be shown. In addition, it would be interesting to know how the levels of proteins that are recognized to induce PSCs activation (PDGFs and TGFbeta) change in this context. Are the PSC activators lower expressed in mesenchymal-like vs epithelial-like PDAC?*
We apologize we did not present an overview of the cyto/chemokines measured with the FPPA and have now included this as Table EV1. Since PDGFs and TGF-β were not included in the list of cyto/chemokines, we assessed the expression levels of these factors in these cell lines using a publicly available dataset. This revealed no differences in expression between epithelial- and mesenchymal-like PDAC cells. A heatmap representing these results is included in Fig EV3D and explained on page 12 paragraph 1.

8. *With the results in Fig. 4, the authors claim that the deactivation of PSCs is not impacted by filtering the conditioned medium (CM) through a 100 µm filter. However, the deactivation of PCSs is already reduced with CM, from Mia PaCa-2 cells, that has been filtered through the 100 µm pore size membrane compared to unfiltered CM. This suggests a more complex regulation of PSC repression and should be discussed.*
We agree with the reviewer that 100 kDa filtration of MIA PaCa-2 CM already reduced the deactivation of PS-1 cells in terms of *ACTA2* expression (Fig 4B). As multiple isoforms of CSF-1 are reported, including a 44kDa glycoprotein CSF-1 and a 200+ kDa proteoglycan CSF-1, this could explain why filtration through both the 100 and 30 kDa filters affects *ACTA2* expression in PS-1 cells. This suggests that MIA PaCa-2 cells secrete more proteoglycan CSF-1 while PANC-1 cells secrete more glycoprotein CSF-1. Moreover, PSC deactivating effects were abolished when a CSF-1 inhibitor was added to MIA PaCa-2 CM suggesting that it is mainly driven by CSF1R signaling. This is explained on page 11 paragraph 1 and page 12 paragraph 2.

9. *In the discussion on p. 21 the authors suggest that mesenchymal PDAC can use the pro-invasive contributions of PSCs before inactivating them. Evidence for this is missing and the rationale is unclear.*
We agree with the reviewer that this discussion to explain timing effects is not well substantiated and have deleted it from the manuscript.

Minor Points
1. *On page 18, the authors state that they want to use molecular filtering to confirm that the mediator of PSC repression was a soluble factor (Fig. 4). However, the experiments with conditioned medium (Fig. 3) should already confirm this. A different rationale should be used for the filtering experiments.*
We thank the reviewer for this thoughtful comment and have changed the rationale for the filtration experiments to; 'We first set out to narrow down the list of potential candidates by defining their molecular size. To this end, we performed size exclusion experiments.'

2. *On page 19 they state that since CSF-1 is recognized as regulator of macrophages, they want to address whether the negative correlation between CSF-1 and COL1A1 or ACTA2 were dependent on macrophage content. However, similar to minor point 1 the experiments with conditioned medium show that the effect is direct (independent of macrophages). A different rationale should be used for correlation analysis in the presence/absence of CD68.*
We agree with the reviewer that the effects of tumor cell secreted CSF-1 is direct on PSCs following CM transfer and have therefore excluded the CD68 dichotomization in correlating *CSF1, CCL5* and *CXCL16* to *COL1A1* and *ACTA2*. The validation that *CSF1* expression is negatively correlated to *COL1A1* and *ACTA2* can be found in Fig EV3A.

3. *In Fig. 5E the bar for ACTA2 expression in control cells is missing.*

The *ACTA2* mRNA expression in PS-1 cells after exposure to MIA PaCa-2 CM was above 35 cycles on qPCR and therefore the bar was not detectable compared to the expression level of the other bar. We have added this cycle information to Fig 5F.

4. *In the text on p.18 the FPPA analysis are said to be found in Fig. 4C. However, the experiments are shown in Fig. 5A.*
We apologize for this mistake and have now referred to the correct figures.

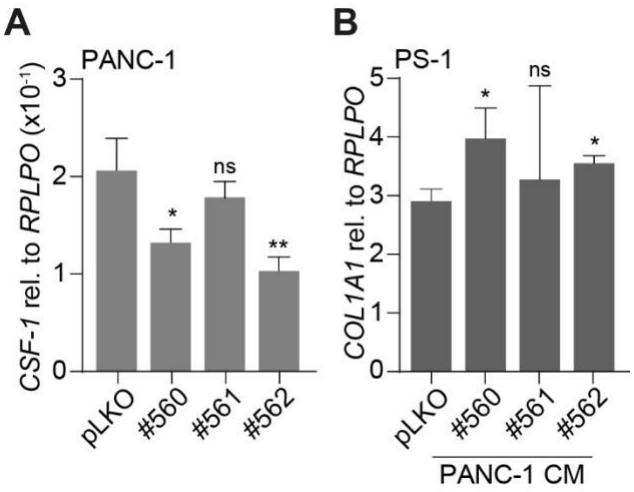
5. *The Figure legends for Figures. S2 and S3 have been mixed up*.
We apologize for this mistake and have placed the correct legends with the correct figures.

6. *The biological replicates used are missing in many Figures e.g. Figures. 2, 3, 4 and 5.*
We apologize for the missing information and have added the replicates to the figure legends or as individual data points in the graphs.

REVIEWER 2

This is an interesting study looking at the regulation of stroma activation by subtypes of PDAC tumor lines. The study identifies CSF1 as the mediator of this phenomenon. The study is well done but additional experimentation is needed to fully define the mechanism underlying the CSF1-dependent regulation of the stromal activation.
We thank the reviewer for his/her positive comments on the submitted work.

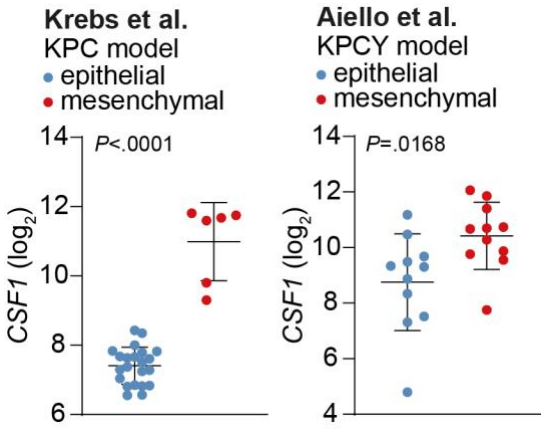
First, the antibody blockade experiments should be validated by knockdown studies using two independent siRNA or shRNA targeting CSF1.
As suggested, we have performed *CSF1* knockdown experiments in PANC-1 cells. We used three independent shRNA sequences targeting *CSF1* of which two *shCSF1* (#560 and 562) were able to significantly downregulate *CSF1* expression in PANC-1 cells compared to *pLKOctr*l (figure below, panel A). Unfortunately, we did not manage to establish a full knockdown of *CSF1* in PANC-1 cells suggesting that CSF-1 might be an obligate feature of mesenchymal-like PDAC cells. Nevertheless, exposure of PS-1 cells to CM of PANC-1 with *shCSF1* #560 and 562 resulted in a significant upregulation of *COL1A1* compared to control (figure below, panel B) thereby validating that reduced tumor cell secreted CSF-1 is proportionally correlated with increased *COL1A1* in PS-1 cells. In the manuscript we have presented *CSF1* and *COL1A1* expression in an XY correlation graph, shown in Fig 5C and D and made this more explicit on page 14 paragraph 1.



To further ascertain specificity that tumor cell secreted CSF-1 is responsible for PSC deactivation, Capan-2 cells were transduced with a lentiviral vector to overexpress CSF-1. Exposure of PS-1 cells to CM of these CSF-1 producing Capan-2 cells resulted in decreased *COL1A1* expression. These results are included in Fig 5E-G and discussed on page 14 paragraph 1.

Second, the expression of CSF1 and stromal marker correlation should be validated by IHC in an independent cohort of tumors.
We agree with the reviewer that the correlation of CSF-1 and stromal markers should be validated in a patient cohort. As the first discovery cohort was composed of PDAC patients included between 2014-2016, we composed a second independent cohort of 21 PDAC patients that were included between 2001-2014, and (immuno)histochemically stained these sections for CSF-1, PSR and α-SMA. Quantification of these stainings revealed that there was a strong correlation between CSF-1 expressed in tumor cells and collagens and a modest correlation between CSF-1 and α-SMA positive fibroblasts. These results are shown in Fig 6C-E and discussed on page 14 paragraph 2.

Third, since the immune system plays a major role in the regulation of stroma structure and dynamics, it is essential that the in vivo experiments are repeated in an immunocompetent animal using Panc02 or KPC-derived cell models.
Indeed, the immune system exerts a large impact on the stroma. There are, however, a couple of reasons why we did not perform studies in immunocompetent animals. First, we base our findings of deactivated stroma in high-grade PDAC on patient data, and validate that increased tumor cell secreted CSF-1 correlates with deactivated stroma in a second independent PDAC cohort. This indicates that our conclusions are not impacted by the absence of immune cells. Second, syngenic mouse models of PDAC carry some drawbacks. These tumors often present with little stroma and some cell lines are KRAS WT[1,2] . Moreover, to draw conclusions from these experiments a panel of epithelial-like and mesenchymal-like mouse PDAC cell lines would be needed, which is not readily available. If we want to test our hypothesis using a transgenic mouse model, such as the KPC model, we would need to create strains that have a more mesenchymal or epithelial phenotype which is beyond the scope of this paper. We therefore performed *in silico* analyses on studies that have been performed in genetically engineered mouse models of PDAC in which a more epithelial- and mesenchymal-like phenotype was observed.
Krebs and colleagues [3] use a KPC model for PDAC in which the EMT-activator *Zeb1* was conditionally ablated. From these models, tumor cell lines were derived in replicates with an epithelial or mesenchymal phenotype. Analysis of these data shows that mesenchymal cell lines significantly increase the expression of *Csf1* (figure below). Another study performed by Aiello and colleagues[4] used a KPCY model for PDAC in which tumor cells derived from these models were isolated by sorting for membranous E-cadherin high (M-ECAD+, epithelial cancer cells) and low (M-ECAD-, mesenchymal cancer cells). Analysis of these data as well revealed that mesenchymal pancreatic tumor cells have increased expression of *Csf1* (figure below) and we can validate that the upregulation of CSF-1 by mesenchymal-like tumor cells also occurs in immunocompetent animal models.



As the abovementioned studies only had datasets available of the isolated tumor cells, we were not able to assess the activation status of the stroma in response to the CSF1 produced by the epithelial- or mesenchymal-like tumor cells in transgenic mouse models for PDAC.
To determine the stroma-deactivation contributions of the immune cell best known to remodel the stroma, we exposed PS-1 cells to CM from M2 macrophages. This revealed no changes in the activation status of PS-1 cells and suggests that M2 macrophages are not responsible for (additional) changes to stromal activation status, providing further support for the notion that the effects

described in the paper are largely independent from the immune system. These results are shown in Fig EV3G and H and made explicit on page 12 paragraph 2.

Finally, (and most important), the mechanism underlying the regulation of stroma de-activation by CSF1 should be investigated? What are the downstream molecules mediating this effect?
We agree with the reviewer that the mechanism underlying the PSC deactivation should be investigated. We have stimulated PS-1 cells with rh-CSF-1 and assessed activation of pathways known to be involved in CSF1R signaling. This revealed that ERK1/2 and AKT were both activated, while Src and STAT3 were deactivated in PS-1 cells upon CSF-1 stimulation. This suggests that the reduced proliferation of PS-1 cells following CSF-1 activation is mediated by inactivation of STAT3 -which is required for cell proliferation-. This data is shown in Fig 4G and is discussed on page 13, first paragraph.

REVIEWER 3

The manuscript describes interesting findings related to the tumor - stroma crosstalk in pancreatic ductal adenocarcinoma (PDAC). Using PDAC cell lines and immortalized human PS-1 pancreatic stellate cells the authors find that the epithelial and mesenchymal-like (more aggressive) PDAC cells differentially affect PS-1 cells. The mesenchymal PDAC cells inhibit the growth and expression of COL1A1 & ACTA2, used as stellate cell activation markers, in PS-1 cells. Authors further provide evidence suggesting that CSF-1 is a mediator of this mesenchymal PDAC-mediated stromal cell "deactivation". These results with the used cell lines appear solid, and are considered to support the contribution of PDAC-associated stromal cells and extracellular matrix deposition, often associated to PDAC aggressiveness, to tumor inhibition or restriction. At this stage, the data provided using PDAC clinical samples and datasets as well as with cell line xenografts, "organotypic co-cultures" and patient derived cells remains less clear/convincing and thus premature to strongly support the drawn provocative conclusions.

We appreciate the reviewer's overall positive assessment of the data presented in this manuscript and we have addressed the concerns regarding the data as outlined below.

Specific comments:

1. *Fig 1A-B: What were the percentages of tumor cells in the images of the total 15 different grade 1-2 and grade 3 tumors in the staining quantification? Fig 1A seem to show mainly stromal cells in OK26 and cancer cells in OK37. Is the presented Picrosirius positivity simply reflecting the relative stroma and cancer cell numbers in the analyzed tumor tissues or areas? This is a small cohort for which the staining results could be more comprehensively presented. Markers for the cancer and/or stromal cells would also be informative.*
To address the tumor cell percentage of each specimen in the PDAC cohort used in Fig 1A and B, two pathologists have independently scored the H&E stainings of the entire tumor area (see Fig EV1A) which revealed no differences. We have added this data to Fig 1C and made this more explicit on page 7 paragraph 1. We agree that this is a small cohort and have therefore added an independent validation cohort of 21 PDAC patients in which we confirm that tumor cell secreted CSF-1 is associated with reduced collagen I and III deposition, as well as reduced α-SMA positive fibroblasts. These results are shown in Fig 6C-E and made explicit on page 14 paragraph 2. The images included in Fig 6C show clearly that patients low in PSR and α-SMA staining do not have a higher tumor cell percentage. This suggests that there is not necessarily *less* stroma in high-grade PDAC, but that it is composed differently. We explain this in the Discussion section on page 17 paragraph 1.

2. *In materials and methods immunohistochemical staining is described as follows: "For PSR staining of primary and PDX tumors, the entire tumor was quantified." How do the images in Fig 1A relate to the "entire tumor"? How many sections per tumor were used?*
We apologize that this was not made sufficiently clear in the manuscript. For the PDAC cohort represented in Fig 1A-C, axial tissue slices of the *entire* resection specimen containing the pancreatic head and duodenum were embedded and stained, we have added images of this method in Fig EV1A. Subsequently, these large tissue sections were digitalized and the tumor area was marked

by a pathologist and quantified. We have made this more explicit in the Materials and Methods section on page 23 paragraph 1.

3. *Fig 1C shows more collagen and aSMA in epithelial-like PDAC cell xenografts than in mesenchymal-like PDAC xenografts. It is further postulated that "the collagens are produced by activated CAFs". Therefore, expression of the stromal activation marker aSMA was assessed, which revealed that activated PSCs were present in epithelial-like tumors while these were absent in mesenchymal-like PDAC tumors. Firstly, the inconsistent description of CAFs versus PSCs here and throughout the manuscript is distracting.*
We agree with the reviewer that the phrasing of *PSCs* or *CAFs* should be more consistent throughout the manuscript and have attempted to correct this: We now refer to (de)activated PSCs in our own work, and either CAFs or PSCs when referring to the work of others (based on what the authors named their stromal cell subsets).

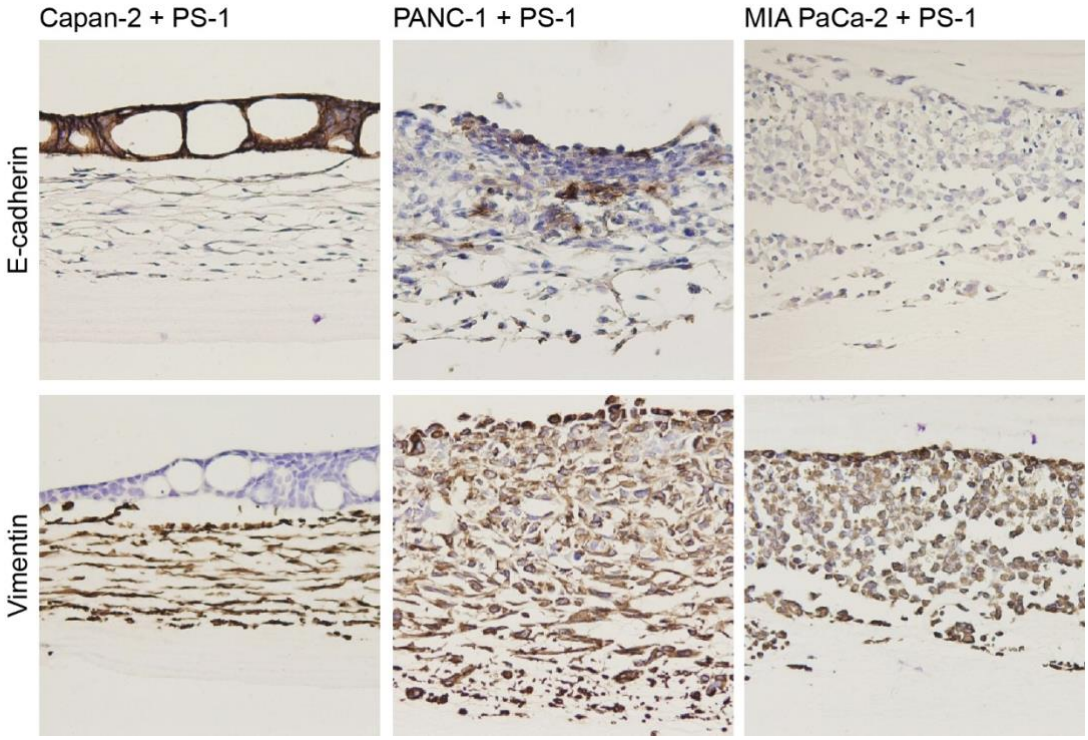
Secondly; are the mesenchymal PDAC cells producing collagens? Are the contrast/intensity adjustments in PSR images of MIA PaCa-2 and PANC-1 similar to the epithelial-like cell line tumors? Are these PDAC cells themselves producing and assembling more collagens than the more epithelial cells?
To address the collagen production of the PDAC cell lines, expression levels of *COL1A1*, *COL3A1*, *COL4A1*, *COL5A1* and *COL11A1* were assessed in publicly available gene expression datasets. This revealed no significant differences in expression levels between epithelial- and mesenchymal-like PDAC cell lines. These results are shown in Fig EV1B and discussed on page 7 paragraph 1.
Regarding the PSR images, the same contrast/intensity adjustments have been made in these images. It should be taken into account that PSR staining can differ in intensity of cytoplasmic yellow staining between sections, possibly explaining why images might look different.

4. It is difficult to understand the organotypic co-culture results and drawn conclusions from the data as presented in Figures 1 and S1.
What seems clear based on Fig S1 is that PS-1 cells dramatically increase the growth of the mesenchymal-like PDAC cells. This growth induction is very prominent with MIA PaCa-2 cells, and would be important to quantify in all the samples. I cannot see that this result could be described only as an impact on initial stage of invasion. In contrast, if PS-1 cells activate both invasion and growth of the mesenchymal PDAC cells, the concept of their deactivated or tumor-restricting status should be reconsidered.
We thank the reviewer for these thoughtful comments. We looked closer into the MIA PaCa-2 monocultured organotypic cultures and found that the image we had included represented a specimen in which the epithelial layer had detached from the gel during the IHC workup. The culture, embedding and cutting of the organotypics is a very delicate procedure, and unfortunately these technical issues do occur. We apologize for including an erroneous image and have now included the H&E and CK19 stained images of MIA PaCa-2 monocultures in Fig EV1C and D in which the epithelial layer is intact.
Regarding the question whether PS-1 cells activate growth of mesenchymal PDAC cells; based on these organotypic images comparing monocultures to co-cultures we conclude that the thickness of the epithelial layer is comparable, though the cells are differentially dispersed throughout the gel. However, it is hard to make statements about an absolute cell quantity based on organotypic cultures because the gels are lens shaped and the thickness of the epithelial layer is not uniform throughout the gel.

Regarding the issue if PS-1 cells activate invasion of mesenchymal PDAC cells; we have performed co-cultures of mCherry positive PS-1 cells and PDAC cells, which revealed that mesenchymal-like tumor cells in fact *decrease* the expression of mesenchymal markers CDH2, VIM and ZEB1. This implies that the invasive capacity is also decreased in these cell lines in the presence of PS-1 cells thereby contributing to a tumor-restricting environment (Fig 2D and page 9 paragraph 1). The migration of mesenchymal-like PDAC cells when co-cultured with PS-1 cells in organotypics is not based on increased invasion or migration of these cells. Instead, we suspect the PS-1 cells affect the composition of the ECM architecture to make migration easier for mesenchymal-like PDAC cells. We have excluded the statements that PS-1 cells activate or are involved in invasion of PDAC from the manuscript and discussed these issues on page 8 paragraph 1 and page 9 paragraph 1.

Regarding the presence or absence of aSMA positive PS-1 cells, there would be another possible

explanation than "these PS-1 cells were absent in the ECM gel when co-cultured with PANC-1 and MIA PaCa-2 cells", as it could be that they will not be detected due to the mesenchymal PDAC-mediated aSMA down-regulation. Another marker that stain the PS-1 cells, as well as cultures with PS-1 cells alone would be relevant to address this issue. It also seems that only part of PANC-1 cells are positive for CK19. Means to quantify total PS-1 and PDAC cells will improve this data.
We agree with the reviewer that it would be relevant to have an additional marker that could stain PSCs in these co-cultures. Unfortunately, as is also shown in Fig 3A, *all* stromal markers go down in these deactivated PSCs. Likewise, epithelial markers such as E-cadherin are highly expressed in Capan-2 cells while PS-1 cells as well as PANC-1 and MIA PaCa-2 cells are negative (figure below, upper panel). On the contrary, mesenchymal markers such as Vimentin are negative in Capan-2 cells and positive in PS-1 cells as well as PANC-1 and MIA PaCa-2 cells (figure below, lower panel). These IHC stainings thus cannot give conclusive results and we did not manage to find an additional IHC marker to address the presence of deactivated PSCs.

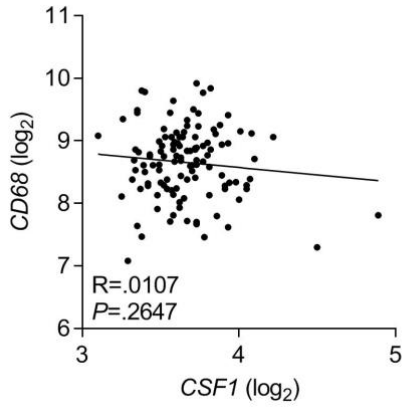


Therefore, we addressed the presence of epithelial cells in these cultures. For MIA PaCa-2 cells we can be quite certain that no PS-1 cells are present since all cells are CK19 positive in mono and co-culture, while monoculture PS-1 cells are CK19 negative. Since PANC-1 cells were partly negative for both CK19 and E-cadherin, we stained these cultures for EpCAM which revealed that all cells were positive in both mono and co-cultures while PS-1 monoculture cells were EpCAM negative. Based on these findings, combined with the patient cohorts, PDXs and *in vitro* findings we conclude that PSCs become deactivated, reduce their proliferation and collagen deposition when exposed to (CM of) high-grade PDAC cells. We can, however, not say with certainty that PSCs are absent and have therefore changed these statements into '*activated* PSCs are absent'. The PANC-1 and PS-1 organotypic stainings are shown in Fig EV1E and F and made explicit on page 8 paragraph 1.

5. *Legends to Fig S2 and S3 are mixed and Fig S2 requires scale bars. Are the poorly differentiated 53M and well-differentiated 67 tumors shown with same magnification? Do these tumors have equal CDH1 expression also in vivo? Can the poorly differentiated cells be considered mesenchymal, if CHD1 is equal to the well-differentiated cells? Some additional markers, such as CK19-aSMA double staining would be informative to characterize the patient derived cultures.*
Materials and methods says: "For α-SMA staining of PDX tumors, a representative region of interest was and quantified." However, only the cell line xenograft tumors seem to have been quantified. How did collagen and ACTA2/aSMA look like in the 53M and 67 tumors?
We apologize for these omissions and have changed the figure legends, the scale bars and the magnification of 53M and 67 H&E images. We have stained the 53M and 67 PDXs for E-cadherin,

showing that 67 cells have a stronger membranous E-cadherin expression then 53M cells. To characterize these cultures we added the suggested CK19, α-SMA and PSR stainings of these PDXs which show convincingly that 67 tumors have α-SMA and PSR positive stroma while this is not the case in 53M tumors. These results are shown in Fig EV2D-F and explained on page 10 paragraph 1.

6. *Fig S3: Are the CSF-1 high tumors CD68 low?*
We have correlated *CSF1* and *CD68* expression in this dataset, which showed no correlation between these markers as shown in the figure below. Moreover, as Reviewer 1 suggested that our conditioned medium experiments already showed that the effects of CSF-1 on PSCs are direct and not dependent on macrophage content, we excluded the CD68 dichotomization in the correlation of *CSF1* and *COL1A1* and *ACTA2* in Fig EV3A. At the editor's discretion we can include the data with the CD68 dichotomization that were taken out in response to reviewer 1's concern.



7. *The terms PSC, CAF and stroma, as well as collagen could be used more carefully, systematically considering exact definitions throughout the manuscript. What is for example meant by "increased stroma deposition" or "PSC deactivation" in Abstract? Altogether, the results seem to reveal interesting new crosstalk between PDAC and stromal cells, but the results would need to be considered and interpreted more carefully.*
We agree with the reviewer that these terms should be used more carefully and consistently, since we do not see an increase in stroma but rather a change in the composition of the stroma. We have replaced 'increased stroma deposition' with the observation 'increased collagen deposition'. And have characterized this PSC deactivation as the downregulation of *COL1A1* and *ACTA2* and reduced proliferation. Throughout the manuscript we have changed this in which we want to emphasize that the tumor stroma composition (i.e. the total of extracellular matrix components) is changed in high-grade PDAC tumors, have made this more explicit in the Discussion section on page 17 paragraph 1.

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3. Krebs AM, Mitschke J, Lasierra Losada M, Schmalhofer O, Boerries M, Busch H, Boettcher M, Mougiakakos D, Reichardt W, Bronsert P, et al. (2017) The EMT-activator Zeb1 is a key factor for cell plasticity and promotes metastasis in pancreatic cancer. *Nat Cell Biol* **19**: 518–529.
4. Aiello NM, Maddipati R, Norgard RJ, Balli D, Li J, Yuan S, Yamazoe T, Black T, Sahmoud A, Furth EE, et al. (2018) EMT Subtype Influences Epithelial Plasticity and Mode of Cell Migration. *Dev Cell* **45**: 681-695.e4.

2nd Editorial Decision 31 January 2020

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees. My apologies for this unusual delay in getting back to you. It took longer than anticipated to receive the referee reports due to the recent holiday season.

As you can see, referees find that the study is significantly improved during revision and recommend publication here. Before I can accept the manuscript, I need you to address some minor points below:

• Please address the remaining concerns of the referees #2 and 3 by textual additions/changes.

REFEREE REPORTS

Referee #1:

The authors have made substantial revisions to the manuscript and added important new data. Overall, I think the study has improved significantly. Most of my concerns have been addressed. However, there are a few things that need to be considered.

1. It is hard to draw significant conclusions based on the results in Fig 2D and Fig 4F. In light of all the other new relevant data that has been added in the revised manuscript to strengthen the mechanistic insight into PSC repression by mesenchymal PDAC, one could argue that Fig 2D and 4F are superfluous for this submission. The results do not provide much insight.

2. The analysis of PSC proliferation in response to PDAC (Fig 2A-C) has improved significantly. The counted cell number, EdU incorporation (S-phase) and Ki67 results, all line up in support of mesenchymal PDAC conditioned medium repressing PSC growth. Thus, the general conclusion is that these cells simply proliferate less. However, the relevance of G0/G1 and G2/M results are not clear to me. Are the cells treated with CM from mesenchymal PDAC stuck in G2/M phase? Are the results robust enough to conclude on the specifics of cell cycle distribution?

Referee #2:

The authors have been responsive to the reviewers critiques. I do not have any further comments.

Referee #3:

The revised manuscript by Steins et al. has improved substantially in presenting the highly interesting finding of CSF-1 mediated PDAC-PSC crosstalk. New important data has been added to address the comments and all major concerns. Only very minor revisions are recommended for more precisely interpreted results and carefully drawn conclusions as follows:

1. New figure 1F: Should the bottom layer marked as "medium" in AsPC1+PS-1 image represent ECM gel?

2. In the same figure, rather than being absent, activated PSCs seem to be reduced "when co-cultured with PANC-1 and MIA PaCa-2 cells (Fig 1F and Fig EV1F)"

3. Term "monolayer" (p.8) is typically used to describe a single cell layer, not multiple layers of one type of cells.

4. Why conclude from Fig 2D that "in our models, PSCs do not contribute to a mesenchymal phenotype of PDAC cells" , when the results show "a substantial reduction in the mesenchymal markers N-cadherin (CDH2), Vimentin (VIM) and Zinc finger E-box-binding homeobox 1 (ZEB1) in mesenchymal-like PDAC cells" ?

5. Could the scales be the same in Fig. 3A and B?

6. P.11 Suggestion for Fig 4A-B conclusion; soluble factors > 30 kDa were "able to deactivate PSCs"?

7. P. 12 The suggestions of differential expression of proteoglycan and glycoprotein CSF-1 forms are speculative and better suited to Discussion.

8. P. 13 As above the speculation of STAT3 in proliferation or Src in actin remodeling could go to Discussion.

9. Fig 5A and B show anti-CSF-1 experiments, not "selective inhibition of CSF1R".

10. The new results presented in Fig 6 are excellent additions to the study, but areas with quite different types of tumor cell colonies are shown for OK7 in CSF-1/aSMA and PSR images.

11. Discussion: Mechanical properties were not addressed, and therefore the presented findings could suggest rather than indicate "that the activated stroma acts as a mechanical barrier with no beneficial signaling, prompting the rapid deactivation of surrounding stroma".

12. Abstract: Term differentiation grade (correlating negatively with collagen deposition) can be confusing, as high-grade tumors are poorly differentiated. The suggestion would be to consider comparing to either (low) differentiation or (high) grade.

2nd Revision - authors' response 11 February 2020

Referee comments

Referee #1:

The authors have made substantial revisions to the manuscript and added important new data. Overall, I think the study has improved significantly. Most of my concerns have been addressed. However, there are a few things that need to be considered.

1. *It is hard to draw significant conclusions based on the results in Fig 2D and Fig 4F. In light of all the other new relevant data that has been added in the revised manuscript to strengthen the mechanistic insight into PSC repression by mesenchymal PDAC, one could argue that Fig 2D and 4F are superfluous for this submission. The results do not provide much insight.*
We thank the reviewer for the positive assessment of the data. We agree with the reviewer that the co-culture information in figure 4F perhaps do not add to the main message of the paper. However, we do think that Fig 2D adds valuable information given that we find that PSCs decrease markers for invasion in PDAC cells when co-cultured. This contradicts the prevailing dogma that PSCs are strictly tumor-promoting and potentially explains why PSCs are repressed by high-grade PDAC. We have added an extra EV Fig, and moved the data in Fig 2F to Fig EV2C and from Fig 4F to Fig EV4I. Nevertheless, if the Editor feels we should remove these figures, we are willing to do so.

2. *The analysis of PSC proliferation in response to PDAC (Fig 2A-C) has improved significantly. The counted cell number, EdU incorporation (S-phase) and Ki67 results, all line up in support of mesenchymal PDAC conditioned medium repressing PSC growth. Thus, the general conclusion is that these cells simply proliferate less. However, the relevance of G0/G1 and G2/M results are not clear to me. Are the cells treated with CM from mesenchymal PDAC stuck in G2/M phase? Are the results robust enough to conclude on the specifics of cell cycle distribution?*
Indeed, we find that there is reduced proliferation in PSCs when exposed to CM of mesenchymal-like PDAC cells and that this is the result of G2/M phase arrest. We think these data are sufficiently robust to conclude this and have made this more explicit on page 9 paragraph 1.

Referee #2:

The authors have been responsive to the reviewers critiques. I do not have any further comments.
We thank the referee for this positive review.

Referee #3:

The revised manuscript by Steins et al. has improved substantially in presenting the highly interesting finding of CSF-1 mediated PDAC-PSC crosstalk. New important data has been added to address the comments and all major concerns. Only very minor revisions are recommended for more precisely interpreted results and carefully drawn conclusions as follows:

1. *New figure 1F: Should the bottom layer marked as "medium" in AsPC1+PS-1 image represent ECM gel?*
We thank the reviewer for this thoughtful comment and have adjusted Fig 1F.

2. *In the same figure, rather than being absent, activated PSCs seem to be reduced "when co-cultured with PANC-1 and MIA PaCa-2 cells (Fig 1F and Fig EV1F)"*
We have changed the PSCs being 'absent' to 'reduced' on page 7 and 8 of the results section.

3. *Term "monolayer" (p.8) is typically used to describe a single cell layer, not multiple layers of one type of cells.*
The reviewer is right, this was a misnomer. We have changed the term 'monolayer' into 'stratified epithelium' on page 8 paragraph 1.

4. *Why conclude from Fig 2D that "in our models, PSCs do not contribute to a mesenchymal phenotype of PDAC cells", when the results show "a substantial reduction in the mesenchymal markers N-cadherin (CDH2), Vimentin (VIM) and Zinc finger E-box-binding homeobox 1 (ZEB1) in mesenchymal-like PDAC cells" ?*
As we see a substantial reduction of markers for invasion in PDAC cells when cocultured with PSCs, this implies that PSCs do not contribute to the mesenchymal phenotype of PDAC cells. This contradicts the prevailing dogma and is therefore a valuable finding potentially explaining in part why mesenchymal-like PDAC cells would want to repress PSCs. To make this more explicit, we have adjusted our conclusion on page 9 paragraph 1. Also note that in response to Reviewer 1's comment, we have moved these data out of the main Figure.

5. *Could the scales be the same in Fig. 3A and B?*
In Fig 3A and Fig 3B all data is Log2 transformed but the type of data representation is different (graph vs heatmap) so unfortunately scales cannot be the same. For the Fig 3B heatmap representation the data is normalized to Suit-2, which is set to 1, to compare relative changes in *COL1A1* and *ACTA2* expression. We have explained this more in depth in the legend of Fig 3.

6. *P.11 Suggestion for Fig 4A-B conclusion; soluble factors > 30 kDa were "able to deactivate PSCs"?*
We have changed the conclusion to; 'This suggests that mesenchymal-like PDAC cells secrete soluble factors larger than 30 kDa that are able to deactivate PSCs.'

7. *P. 12 The suggestions of differential expression of proteoglycan and glycoprotein CSF-1 forms are speculative and better suited to Discussion.*
We agree with the reviewer that this is more suitable for the Discussion section. This information can now be found on page 18 paragraph 2.

8. *P. 13 As above the speculation of STAT3 in proliferation or Src in actin remodeling could go to Discussion.*
Again, we agree that this would be more suitable for the Discussion section and this information is now on page 17 paragraph 2.

9. *Fig 5A and B show anti-CSF-1 experiments, not "selective inhibition of CSF1R".*
We apologize for this mistake, and have changed a-CSF-1 to a-CSF1R in Figs 5A and B and the text.

10. *The new results presented in Fig 6 are excellent additions to the study, but areas with quite different types of tumor cell colonies are shown for OK7 in CSF-1/aSMA and PSR images.*
Unfortunately these stainings could not be performed on consecutive slides. Therefore, fully similar tumor cell regions could not be represented in these images.

11. *Discussion: Mechanical properties were not addressed, and therefore the presented findings could suggest rather than indicate "that the activated stroma acts as a mechanical barrier with no beneficial signaling, prompting the rapid deactivation of surrounding stroma".*
We have changed this to ‘suggest’.

12. *Abstract: Term differentiation grade (correlating negatively with collagen deposition) can be confusing, as high-grade tumors are poorly differentiated. The suggestion would be to consider comparing to either (low) differentiation or (high) grade.*
We apologize for this potentially confusing nomenclature and have changed this to: ‘We found in primary tissue that high-grade PDAC had reduced collagen deposition compared to low-grade PDAC.’

Accepted 18 February 2020

Thank you for submitting your revised manuscript. I have now taken a look at everything and all looks fine. Therefore I am very pleased to accept your manuscript for publication at EMBO Reports.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Maarten F. Bijlsma
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-48780V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For clinical studies, to ensure adequate power sample sizes were calculated with the online available sample size calculator tool (https://clincalc.com/stats/samplesize.aspx)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, we stained and quantified one tumor per cell line for stroma deposition to validate whether this was in agreement with in vitro observations
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animal or patient samples were excluded from our observations/analyses for this study
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	no randomization was used because the animals used in this study were not subjected to treatment
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	no blinding was done because the animals used in this study were not subjected to treatment
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes, we tested whether data was normally distributed using SPSS
Is there an estimate of variation within each group of data?	yes

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.consort-statement.org/checklists/view/32-consort/66-title

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies were used in this study; α-SMA (ab5694, Abcam), CK19 (MU246-UC, BioGenex), E-cadherin (EP700Y, Abcam), EpCAM (ab32392, Abcam), CSF-1 (EP1179Y, Ab52864, Abcam), anti-CDH2 (Ab8978, Abcam), anti-VIM (HPA027524, Sigma), anti-ZEB1 (TA503933/2G7, OriGene), phospho-p44/42 MAPK (Erk1/2) (9101, Cell Signaling), phospho-AKT (4060/D9E, Cell Signaling), phospho-p70 S6 Kinase (Thr389) (2211S; Cell Signaling), phospho-Src Family (6943; Cell Signaling), phospho-STAT3 (9131; Cell Signaling), α-Tubulin (sc-23948; Santa Cruz), Goat anti rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling, #7074)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Capan-2, AsPC-1, PANC-1, MIA PaCa-2, Suit-2, PL-45 and BxPC-3 cells were all purchased from ATCC. PS-1 cells were kindly provided by Hemant Kocher. Primary tumor cells were established in our own lab. Cell lines were recently profiled and tested monthly for mycoplasm contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	For animal experiments, male NSG mice were used (JAX 005557) that were around 6-12 weeks of age. Animals were housed in in cages with 2-5 mates.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Grafting of PDAC cell lines, breeding and maintaining of mice at the local animal facility was performed according to the local legislation and under ethical approval of the animal ethical committee (LEX102348; LEX268AD).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All patients included in this study signed informed consent according to the procedures approved by the Amsterdam UMC, location AMC, ethical committee.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All patients included in this study signed informed consent according to the procedures approved by the Amsterdam UMC, location AMC, ethical committee (METC2013_254 (NCT01989000), Netherlands Trial Registry NTR3709, METC_A1 15.0122, METC 2018_181, METC 01/288#08.17.1042, METC2016_325 and METC2014_181) and conform the principles set out in the WMA Declaration of Helsinki and the Department of Health Services Belmont Report
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	MIPA study; METC2013_254, NCT01989000, registered November 2013, https://clinicaltrials.gov/ct2/show/NCT01989000, and PREOPANC study; NTR3709, registered 8 November 2012, https://www.trialregister.nl/trial/3525
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	We confirm we have followed these guidelines

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA