Author's response to reviews

Title: FAP-overexpressing fibroblasts produce an extracellular matrix that enhances invasive velocity and directionality of pancreatic cancer cells

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Author's response to reviews: see over

To the editor of BMC Cancer:

We have carefully examined the comments made by both reviewers and believe that changes made in response to their comments (marked by a thin vertical line apparent to the left of the underlined text in the new version) have significantly strengthened our study. Listed below, **in bold font**, are all comments made by the referees followed by our responses in blue:

Reviewer #1 report

Are limitations of the work clearly stated? This is the only weakness of the manuscript. In fact, the authors should take into account that the cellular components of pancreatic tumor stroma are represented not only by fibroblasts but also by pancreatic stellate cells (PSC). Hence at least the expression of FAP in PSC should be checked and discussed.

We thank the reviewer for this question and are happy to state that we have now included images of matrices derived from both stellate pancreatic cells, harvested from a surgical tissue sample far away from a human pancreatic tumor (adenocarcinoma), and matrices derived from activated tumor-associated fibroblasts harvested from the matching human pancreatic adenocarcinoma tumor sample. Results reflecting the fact that pancreatic stellate cell (e.g., normal) matrices are similar to FAP minus matrices and that matrices derived from pancreatic tumor-associated fibroblasts resemble FAP+ phenotypes are now part of the manuscript as new Figure 2. We believe that the addition of this information greatly increases the relevance of our study and thus it merits the addition of a co-author, Dr. Franco-Barraza.

Reviewer: #2

Major Compusory Revisions

The authors propose that the remodeling of fibronectin fibrils by fibroblasts affects cancer cell migration. #1-integrin or alpha #5#1 inhibition reduces cancer cell migration, however the authors did not determine if the changes in migration induced by integrin inhibition are associated with modifications in matrix remodeling.

We thank the reviewer for this question and would like to state that, although we did not assess whether the matrices underwent any changes when cancer cells were cultured within them in the presence or absence of the integrin inhibitors, we also did not see any obvious differences imparted to the matrices (by simple light microscopy examinations) which would have merited more detailed measurements/analyses under these conditions. It is important to state that our intention, using the integrin inhibitory reagents, was to disturb cancer cell-stromal (matrix) interactions subsequent to the formation of fibroblast-derived matrices and after the original fibroblasts were extracted. To this end, testing effects on the matrix architecture under the inhibition of the above-mentioned integrins during fibroblastic matrix formation would have been impossible to assess since matrix fibrillogenesis directly depends on the activity of these integrins

(Pankov et al JCB 200) and, thus, matrix production would have been inhibited under these conditions.

Furthermore, fibronectin is not a FAP substrate, so why does the inhibition of FAP activity affect fibronectin remodeling?
This is an interesting question which, unfortunately, was not the focus of the present study but which, nevertheless, merits further investigation (in the future). In this study, we observed a clear phenotypic modification of matrix architecture as a consequence of the FAP induction similar to the *in vivo* observations made by Santos et al., 2009, J. Clin. Invest., 119: 3613 (brought to our attention by this reviewer). Although it is well known that ECM plays fundamental roles in regulating diverse cell behaviors, it is also well accepted that changes in the ECM due to cellular activities (in this case FAP expression in fibroblastic cells) also impart great influence upon adjacent cells (in this case cancer cells) and modify their behaviors (in this case matrix-induced cancer cell invasion). Our only claim in this study is to state that (perhaps by indirect means) fibroblastic FAP activity plays a role in the specific production of tumor-ECMs which, in turn, facilitate cancer tumorigenic responses such as invasion. Our observations do not mean to imply that the mechanism behind the phenotypic effect resides on the fact that fibronectin is a direct substrate of FAP activity *in vivo*. We have modified the text dealing with this point in the discussion section.

Do FAP-positive fibroblasts also affect the remodeling of collagen I fibers?
We thank the reviewer for this question and are happy to state that we were able to conduct an additional (small) experiment to address this point. Our results suggest that although the collagen I expression/fibrillogenesis levels during matrix production (8 days) were not as substantial as to allow the analysis of the fiber orientation, simple microscopy observations suggest that FAP-positive fibroblasts indeed affect collagen I fiber organization. The new results are presented in supplemental figure 2. Moreover, when measuring the levels of collagen I expression (as opposed to its localization), we observed that the previously presented differences in collagen I expression were indeed significant (modified Figure 3) and concluded that, while FAP activity is important to both fibronectin and collagen architectural fiber organization, its activity is mostly important for regulating collagen I as opposed to fibronectin levels of expression. Changes were made to the text to reflect both of these observations.

Why were the effects of matrices produced by FAP-, FAP+, or FAP+inhibitor fibroblasts or the inhibition of #1 integrin or alpha #5#1 only studied with only one pancreatic cancer cell line. To determine the significance of the results other cell lines should also be tested.
We agree with this comment and would like to state that, when we first conducted the motility assay, we tested 4 different pancreatic cancer cell lines including HPAF-II, Capan-1, AsPC-1, and Panc-1 (Figure 4). Cells were tested on both FAp + and FAP – matrices and no differences were observed in FAP – matrices. Changes were made to the text highlighting these results. In addition, Panc-1 was selected to continue the study since none of the additional cell lines showed rates of movement that merited further follow-up. Nevertheless, we have now tested additional cells (breast) which

have been well characterized with regards to their tumorigenic and metastatic characteristics (new supplemental Figure 3). These are the immortalized normal epithelial human breast MCF-10A, the human breast tumorigenic MCF-7, and the invasive human breast cancer cell MDA-MB-23. Similar to the observations presented for pancreatic cells when compared to the other two cell lines (as expected), MDA-MB-231 cells moved faster and with increased persistent directionality on FAP+ matrices (new supplemental figure 4). This result prompted us to continue to characterize the FAP+-matrix effects in the presence or absence of the general beta1-integrin inhibitor mAb13 (new supplemental figure 5). Our results were comparable to the ones observed with Panc-1, and thus we concluded that fibroblastic FAP-dependent matrix alterations and the importance of beta1 integrins in the regulation of cancer cell motility are effects that are not necessarily restricted to pancreatic cancers and that additional cancers such as breast, where stromal FAP expression levels have been shown to be increased (Mersmann et al, *IJC* 92(2):240–248, 2001), show similar behaviors. Changes to the text and new figures were added to the new version of the manuscript.

The upregulation of FAP in fibroblasts increases their expression of collagen I, fibronectin and #SMA and treatment of FAP+ matrices with a FAP inhibitor further increases the expression of fibronectin and #SMA. It is unclear if the increase in fibronectin and #SMA expression is significantly higher between matrices treated with or without the FAP inhibitor.

Alpha-SMA and fibronectin expression levels increased in FAP+inhibitor matrices compared to FAP+ matrices were found to be 'not significant' although they were significant when compared to FAP - matrices. Also, collagen I expression was found to be significantly down-regulated in FAP+inhibitor matrices ($p=0.007$) compared to FAP+ suggesting that FAP enzymatic activity regulates collagen I, but not alpha-SMA and fibronectin, protein levels. Changes reflecting these observations were made to both modified Figure 3 and the relevant text.

Also, the treatment of experimental tumors with a FAP inhibitor increases the intratumoral collagen content and decreases the number of myofibroblasts (#SMA-positive cells) (Santos et al., 2009, J. Clin. Invest., 119: 3613). In contrast, in the present study the inhibition of FAP activity does not affect the expression of collagen I and increases the #SMA-positive expression. These differences in FAP activity between in vitro and in vivo results should be acknowledged and explained.

We thank the reviewers for bringing this important study to our attention. Indeed in Santos et al, the authors suggest a stromagenic inhibition under FAP blockage. Interestingly, when looking at figure 8 (in Santos el at) where the authors show the *in vivo* collagen effects observed under FAP blockage one can clearly observe an increase in collagen disorganization. These types of observations (in the absence of FAP inhibitory effects) have been previously explained by Provenzano et al 2006 where the relationship between collagen density/organization and tumorigenesis was well established in breast cancers. The stroma surrounding normal epithelium and mammary tumors in intact tissues was visualized by multi-photon microscopy using second harmonic generation of light. Provenzano et al carefully studied the local

alterations in collagen density and found tumor-associated collagen signatures that facilitate tumor invasion. The globally increased collagen concentration and wavy fiber structure surrounding small tumors (Fig. 4A, Provenzano et al., 2006) greatly resembled the net impact of inhibiting or depleting FAP *in vivo* in Santos et al 2009. In both studies, collagen morphology was dramatically modified in regions of tumor masses undergoing growth and invasion. The majority of the tumor-associated fibers are straightened (taut) and aligned in the direction of cell invasion, which coincidentally also resembled the architectural alterations of fibronectin and collagen fibers presented in our study that were induced by FAP activity. Thus, although we cannot explain the *in vivo* vs. *in vitro* as well as lung vs. pancreas and breast discrepancies with regards to collagen expression levels, our results showing FAP dependent matrix organization in parallel fiber patterns (fibronectin as well as collagen) are in tune with the *in vivo* observation of breast invasive stromal architectures in general (as in Provenzano et al 2006) and regarding FAP blockage effects upon stromagenic inhibition (as in Santos et al 2009) in particular. Changes reflecting these claims were added to the discussion section of the manuscript.

For the migration studies the cancer cells were plated on the matrix formed by the fibroblasts. However, it is not specified if cancer cell migration was measured and reported for cells on top or inside the gel.
As we previously suggested in Cukierman et al., *Science* 2001, confocal images of 90^0 rotated projections showed a clear cell intercalation within these types of cell-derived matrices following overnight incubation of cells under these conditions. In the present study, we believe we have followed cells migrating both on top and within the matrices since control matrices used were exactly the same as in Cukierman et al 2001. In addition, BSA blocking of extracted matrices prior to cancer cell re-plating is believed to have prevented cells from reaching the bottom of the culturing plates (underneath the matrix). This point was clarified by additional details provided in the Materials and Methods section of the manuscript.

How many experiments were performed to determine the average values of migration velocity and directionality?
All motility experiments in the absence of inhibitors were performed at least 3 times using different batches of matrices (Figure 5). Motility experiments using inhibitory conditions and their controls were performed twice also using two batches of matrices (Figure 6). In any case, for each experiment a minimum of 10 cells were recorded. All conditions tested passed the normality test before conducting statistical analyses using the statistical software. Changes to the text (Materials and methods) were made to reflect these facts.

Discretionary Revisions
What is the composition of the matrix produced by the fibroblasts, is there a significant accumulation of collagen type I fibers in the matrix?
Although tested matrix components included fibronectin, tenascin-C, collagen I, collagen IV, collagen VI, versican, and decorin, we conducted statistical analyses and included results measuring only tenascin-c, fibronectin and collagen I In this study. No

changes, other than the ones stated above with regards to collagen I were made to the text to reflect this point.

The immunostaining results suggest that FAP is expressed by most fibroblasts in pancreatic cancer tissue sections. In a more detailed study it was found in pancreatic cancer sections that the expression of FAP by fibroblasts decreases has a function of the distance from tumor cells (Cohen et al., 2008, Pancreas 37: 154). The expression of FAP in pancreatic cancer tissue presented in figure 1 is not really necessary and should be removed.

We agree with the comments and have decided to move previous Figure 1 to appear as new supplemental Figure 1. A decision was made not to completely take the figure away since it contains tumor xerografts of the four pancreatic cell lines used in the study where the host stroma (but not the xenografted tumor cells) shows clear up regulation of FAP expression. We believe this figure underscores the physiologic/pathologic relevance of the study.

We thank you (the editor) for your consideration and the reviewers for their thoughtful comments. In addition, we would be happy to provide any additional needed information.

Best regards,

Jonathan Cheng and Edna Cukierman