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Inactivation of Rb in stromal fibroblasts promotes epithelial cell invasion

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 January 2012

Thank you for the submission of your manuscript to The EMBO Journal. Please, accept my apologies for the delay in responding due to the holiday season. Your manuscript was sent to three referees and we have now received comments from two of them, which are copied below. Although the third reviewer has not yet returned his report, the other two are in fair agreement, so I am making a decision on your manuscript now in order to save you from any unnecessary loss of time.

As you will see from the enclosed reports, the referees do not support publication of your manuscript. In brief, although referee #2 is more positive both, and particularly referee #1, consider that the main claim - that RB directly controls KGF expression to induce invasion - is not properly substantiated by the experimental evidence.

Given these negative opinions and the fact that the EMBO Journal can only afford to accept papers that receive enthusiastic support from a majority of referees, we see little choice other than returning the manuscript to you with the message that we cannot offer to publish it.

I am sorry that I have to disappoint you this time. I hope, however, that the referee comments will be helpful in your continued work in this area and I thank you once more for the opportunity to consider your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The authors show that stromal fibroblasts adjacent to epithelial tumors that have acquired invasive ability display reactivity with two phospho-specific pRB antibodies. In keeping with the concept that this may reflect pRB inactivation, knockdown of pRB in fibroblasts used to produce 3 dimensional cultures promotes invasion of the epithelial layer to a much greater extent than does the use of normal fibroblasts. This activity is traced to the expression of KGF by the mutant (in vitro) or proliferative (in vivo) fibroblasts, which then activates AKT and ETS2- dependent activation of MMP1 in the tumor epithelium. The role of these latter proteins and pathways in invasion not particularly novel, but the concept that pRB inactivation in the stromal fibroblasts might be the initiating event that leads to KGF production in intact tumors is an important concept. This work clearly shows that there is something different in regards to pRB modification in the stroma, and the manipulation of fibroblasts in vitro appears to support the authors' conclusions. However, the idea that pRB itself controls the expression of KGF or other factors leading to invasion in a direct manner is still very speculative based on the data provided, since the pRB modifications reported might simply be just another indicator of proliferation state, analagous to the use of EdU or KI67. Further, specificity of the antibodies used and localization of this reactivity predominantly to the stromal fibroblasts needs to be better supported. With these issues addressed as suggested below, this work would be much more novel and of higher impact.

1)The fundamental concern with this manuscript is the attempt to connect a specific RB function with the expression of KGF by stromal fibroblasts with all the sequelae reported leading to invasion. While the downstream steps are well supported by the work presented here and other studies in the literature, this reviewer is far from convinced that the phenomenon shown is linked to any specific effect beyond an increase in proliferation in the stroma, and this may not be limited to fibroblasts. To address this it is crucial to (a) simply show the proliferation state of the stroma in tumor sections stained with phosphoRB antibodies, and determine if ppRB reactivity correlates to proliferative status, or not. (b) The authors should test the relative roles of pRB inactivation and proliferation by inhibiting proliferation in fibroblasts lacking pRB in the 3D assay. If pRB null (knockdown) cells cannot proliferate, is the expression of KGF still dysregulated and is invasion promoted? This and other tests of pRB's role in these processes beyond that simply as a marker of proliferative state would greatly improve the implications and impact of the work.

2) This reviewer is not aware of previous work rigorously demonstrating that the phospho pRB antibodies specifically recognize only phospho pRB in IF/IHC. References to this should be specifically noted in the text, or better yet, controls using null or knockdown fibroblasts performed to show the absence of reactivity when pRB is absent.

3) The authors assume that reactivity with one of the two phosphopRB antibodies indicates that pRB is inactive in the stromal cells. But it is widely accepted that hypophosphorylated pRB, that is pRB phosphorylated on just a small number of possible sites, is active in binding E2F and repressing proliferation. What evidence is provided that pRB is inactive in cells that react with one ppRB antibody? Do both ppRB antibodies react with ppRB in the same cell, indicating multiple phosphorylation events? Is this only seen in cells that are KI67 positive?

4) A control IF using panRb antibody, to illustrate expression and localization of pRB in all fibroblasts, would help with the interpretation of the ppRB signal. Does panRB show cytoplasmic localization in a subset of cells only in the presence of tumor epithelium?

5) The authors reference work from Mittnacht indicating that phospho (inactive) pRB loses its affinity for the nucleus, which is indeed established by that group, but use this a evidence supporting the cytoplasmic localization they observe. However, the Mittnacht work showed that nuclear, phosphorylated "inactive" pRB lost affinity for that compartment without relocalizing to the cytoplasm. This should be more clearly stated. Indeed, the authors should consider using conditions

established by Mittnacht to show that pRB can be extracted from the nucleus of tumor stromal cells but not normal stroma as a robust indicator of the inactive state.

6) Figure 1A supports to some extent the authors' conclusion that ppRB signal coincides with FSP1 positive fibroblasts, but this is far less clear in Figure S1B. This needs to be more clearly established, and would be enhanced by staining for other markers of other types of stromal cells, such a macrophages, which may be proliferating and thus ppRB positive.

Referee #2:

This is a clearly written and interesting paper that highlights a potential role RB in the regulation of growth factor (GF) production by tumor-associated fibroblasts. GF (in this case, KGF) appears capable of promoting invasion by cancer cells in a paracrine fashion.

Major comments:

1. Fig 3F is a critical experiment. Need to see that the hKGF shRNA is not affecting the knockdown of Rb. Also want to see how the hKGF knockdown compares to the differences shown in Fig 3C.

2. The finding of increased MMP1 and pETS-1 in tumors could have little to do with the biology described in Fig 1-4. Both MMP1 and ETS-1, for example, are induced by hypoxia (at least in some models). Are there other markers of KGF activity that could be explored?

Minor comments:

1. The authors' data shows that fibroblasts in which Rb is inactivated can promote invasion by overproducing GF such as KGF. I don't think that is the same as showing that "stromal fibroblasts (normally) act to suppress invasion".

2. The observations described in the first paragraph indicate that you 1) need a placenta to make a viable mouse, 2) Rb plays a critical, presumably cell-autonomous, role in making a placenta, 3) it is possible to make a functional placenta if at least some of the cells are positive for Rb. Seems like a soft argument for Rb regulating neighboring cells (although I suspect this is true).

3. It is possible that some of the cancer-associated stromal cells are actually tumor cells that have undergone and EMT (as has been seen now in some other systems)?

4. "they alone are able to promote the proliferation" might suggest that fibroblasts are unique in this regard. I think the authors meant that the fibroblasts, acting alone, are sufficient to promote proliferation.

5. I can barely see the differences in Fig 3a and 3b. Can these spots be quantified? Corroborated by ELISA?

6. If Rb is "inactivated" by phosphorylation in the tumor-associated fibroblasts then it begs the question why. For example, do the authors think the cancer cells are, in turn, producing factors that promote Rb phosphorylation in fibroblasts? This should probably be discussed.

Rebuttal 13 January 2012

We feel that the reviewer's comments do not support the decision to reject the paper outright. Whilst the reviewer's comments are fair and indeed would help strengthen the current manuscript, we do not see how the decision to reject the paper was reached, as the reviewers were generally positive about concept and research carried out. They did, however, want some further information, which in some cases we already have but did not present due to space constraints. All of the other information requested could be easily attained in a short timescale.

Reviewer 1 had two major issues, i) whether the effects on invasion we are seeing is due just to the proliferative status of dermal fibroblasts and ii) Rb regulation of KGF expression and whether this is in fact related to enhanced proliferation. To address the first point, Reviewer 1 has provided suggestions to "simply" assess the proliferation in our Head and Neck cancer specimens, this could be easily done in our Head and Neck samples. However, the Reviewer also wishes to dissect Rb's role in regulating proliferation from regulation of KGF. We have addressed this by expressing CDK6 in HFFs which phosphorylates Rb and induces epithelial invasion (Fig. 2G & H), does not increase fibroblast proliferation (data we did not show), but does importantly induce KGF expression (data we did not show). This data could be included in Supplemental data. To address the second point we have already conducted experiments which demonstrate that Rb depleted cells (by mitomycin C treatment) still express elevated levels of KGF, resulting in invasion. Further to this point, the fibroblasts in our organotypic rafts are embedded in collagen I, which results in arrest of Rb depleted cells (Kono, T., et al, Arch.Dermatol. Res. 282, 258-262 (1990)). We do not see BrdU incorporation in Rb-depleted fibroblasts when they are in collagen, but we still observe elevated KGF production in these cultures. These data were not included for issues of space and could be included in a revised version. Therefore, we do not believe that increased KGF expression in Rb depleted fibroblasts is due to proliferation effects. Data addressing the issue of antibody specificity can also be included.

Reviewer 2 also had two major issues, i) whether shRNA to KGF affected levels of Rb and wanted inclusion of data showing the levels of KGF in shKGF knockdown fibroblasts. This information was omitted for space but we know that Rb levels are not affected by depletion of KGF and we can include the data showing the levels of KGF in depleted fibroblasts. ii) Are there other markers of KGF activity that could be explored? This is an important question raised by the reviewer. We considered investigating the activation status of FGFR2 however antibodies that are currently available are limited in that they detect other FGF receptors, this was one of the reasons why we investigated Ets2 phosphorylation and MMP1 expression, which are downstream targets of FGFR2. We do not believe in our organotypic raft model that hypoxia is involved in regulating Ets2 and MMP1, but we can test the tissues for evidence of hypoxic markers and so determine if this reviewer's comments are correct. Expression of additional targets could be assessed in the Head and Neck cancer specimens.
In conclusion, we hope that you would reconsider in the light of the comments above and the fact that we have most of the data the reviewers have requested and can easily collect the rest in a short period of time.

I look forward to your response.

2nd Editorial Decision

13 January 2012

Thank you for your e-mail requesting the re-consideration of our decision on your manuscript, which I have discussed again within our editorial team taking into consideration the comments from the reviewers.

First of all, I would like to apologize for not being able to express the reasons for the rejection of your manuscript clearly enough. As I mentioned in my decision letter, both referees expressed serious doubts on the conclusiveness of your experimental data and, under this circumstances, we believe that rejection was the sensible decision to make. As you might understand, we could not possibly anticipate that additional data that might come in support of your main claims was excluded from the manuscript and, most importantly, what the outcome of the review process would have been had those extra experiments been incorporated in the original manuscript. Taking these arguments into consideration, I am afraid that I do not see myself in the position of reverting our original decision.

That being said, if you can provide experimental evidence addressing the concerns of the referees - as your rebuttal letter suggests - I would be happy to reconsider a new manuscript in the near future. To be completely clear, however, I would like to stress that a new manuscript will be treated as a new submission rather than a revision and, although reviewed by the same referees, it will be re-evaluated in terms of novelty with respect to the published literature at the time of submission.

I hope that this letter has clarified the rationale behind our decision and re-emphasized the strong demands that we have to apply according to the aim and scope of our highly competitive journal.

Yours sincerely,

Editor
The EMBO Journal

Resubmission 17 March 2012

Firstly, thank you for allowing us to re-submit the paper: 80029. Reviewer 1 had a number of comments mostly revolving around whether we were looking at a proliferation effect in stromal fibroblasts rather than an effect of Rb phosphorylation. We do not believe we are looking at a proliferative effect due to the following:

1. There is little proliferation in the cancer-associated fibroblasts although we see extensive Rb phosphorylation and this is addressed fully in the accompanying letter.
2. In our organotypic raft cultures even when we inhibit proliferation of stromal fibroblasts by mitomycin C treatment, we still observe invasion of cancer cells. Therefore, on both accounts we feel we are looking at an affect of phosphorylated Rb

We have addressed this and other points of the reviewers in the following letter.

Detailed response to the reviewer's comments:

Referee #1:

The authors show that stromal fibroblasts adjacent to epithelial tumors that have acquired invasive ability display reactivity with two phospho-specific pRB antibodies. In keeping with the concept that this may reflect pRB inactivation, knockdown of pRB in fibroblasts used to produce 3 dimensional cultures promotes invasion of the epithelial layer to a much greater extent than does the use of normal fibroblasts. This activity is traced to the expression of KGF by the mutant (in vitro) or proliferative (in vivo) fibroblasts, which then activates AKT and ETS2- dependent activation of MMP1 in the tumor epithelium. The role of these latter proteins and pathways in invasion not particularly novel, but the concept that pRB inactivation in the stromal fibroblasts might be the initiating event that leads to KGF production in intact tumors is an important concept. This work clearly shows that there is something different in regards to pRB modification in the stroma, and the manipulation of fibroblasts in vitro appears to support the authors' conclusions. However, the idea that pRB itself controls the expression of KGF or other factors leading to invasion in a direct manner is still very speculative based on the data provided, since the pRB modifications reported might simply be just another indicator of proliferation state, analagous to the use of BrdU or KI67. Further, specificity of the antibodies used and localization of this reactivity predominantly to the stromal fibroblasts needs to be better supported. With these issues addressed as suggested below, this work would be much more novel and of higher impact.

1) The fundamental concern with this manuscript is the attempt to connect a specific RB function with the expression of KGF by stromal fibroblasts with all the sequelae reported leading to invasion. While the downstream steps are well supported by the work presented here and other studies in the literature, this reviewer is far from convinced that the phenomenon shown is linked to any specific effect beyond an increase in proliferation in the stroma, and this may not be limited to fibroblasts. To address this it is crucial to (a) simply show the proliferation state of the stroma in tumor sections stained with phosphoRB antibodies, and determine if ppRB reactivity correlates to proliferative status, or not. (b) The authors should test the relative roles of pRB inactivation and proliferation by inhibiting proliferation in fibroblasts lacking pRB in the 3D assay. If pRB null (knockdown) cells cannot proliferate, is the expression of KGF still dysregulated and is invasion promoted?

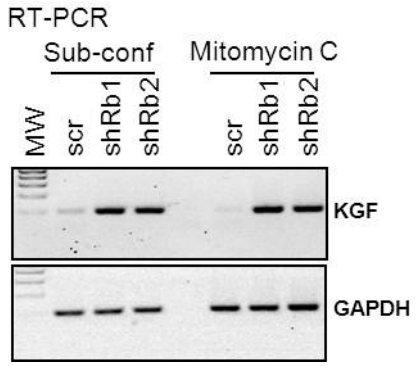
This and other tests of pRB's role in these processes beyond that simply as a marker of proliferative state would greatly improve the implications and impact of the work.

As suggested by the reviewer in their first comment, we have stained the oro-pharyngeal cancer specimens with Ki67 and phospho-Rb antibodies. This data is shown in Figure 1D and E, and also supplemental Figure 4. Phospho-Rb staining did not often co-stain with Ki67 positive cells and indeed typically they were in distinct populations of cells. For instance, across all samples there were approximately 13% of FSP1 positive cells that were Ki67 positive whereas on average there were 75% and 88% of FSP1 positive cells, which were phospho-Rb 807/811 and 821/826 positive, respectively. This is an important observation as this demonstrates that most FSP-1 positive cells are not proliferating, *per se*, but have inactivated Rb. However, this is only a snapshot of proliferation in these samples.

To address the second point we have included a number of findings:

i) We have arrested control and knockdown fibroblasts with mitomycin C and incorporated them into our organotypic culture and proliferation then assessed by measuring Brdu-incorporation (Supplemental Figure 8A-C). The results show that, firstly, there is very little proliferation in untreated fibroblasts (~1.5%) and when this is reduced to almost undetectable levels in mitomycin C treatment, there was no reduction in the invasive potential of the epithelial cells. This suggests that proliferation capacity of the fibroblasts does not influence invasion.

ii) We have also shown that mitomycin C fibroblasts continue to express KGF at elevated levels. See figure below:



2) This reviewer is not aware of previous work rigorously demonstrating that the phospho pRB antibodies specifically recognize only phospho pRB in IF/IHC. References to this should be specifically noted in the text, or better yet, controls using null or knockdown fibroblasts performed to show the absence of reactivity when pRB is absent.

To address this issue we have stained normal fibroblasts with the phospho-Rb antibodies and total Rb antibody. These experiments show that while all cells stain with total antibody only a small portion stain with the phosphor-antibody. Furthermore, we have stained Rb null SAOS2 cells with the antibody to determine whether the antibodies detect non-specific proteins, no staining was observed with either phospho-specific antibody. (Supplemental Figure 1A and B).

3) The authors assume that reactivity with one of the two phosphopRB antibodies indicates that pRB is inactive in the stromal cells. But it is widely accepted that hypophosphorylated pRB, that is pRB phosphorylated on just a small number of possible sites, is active in binding E2F and repressing proliferation. What evidence is provided that pRB is inactive in cells that react with one ppRB antibody? Do both ppRB antibodies react with ppRB in the same cell, indicating multiple phosphorylation events? Is this only seen in cells that are KI67 positive?

We have found that the major modification determining whether cells can enter S-phase, as measured by Brdu incorporation is phosphorylation at Serines 807/11 (Supplemental Figure 1C) as BrDU and phosphorylation at 807/811 are coincide. In isolated fibroblasts the modification of Thr 821/826 is observed in a high percentage of S-phase cells, however, this modification is also observed in some cells, which are not in S-phase (Supplemental Figure 1C). This is in

keeping with similar finding where Thr 821/826 are mutated without affecting E2F binding (Knudsen & Wang, 1997).

4) A control IF using panRb antibody, to illustrate expression and localization of pRB in all fibroblasts, would help with the interpretation of the ppRB signal. Does panRB show cytoplasmic localization in a subset of cells only in the presence of tumor epithelium?

We have co-stained the oro-pharygeal cancer specimens with the phospho-Rb specific antibodies and a panRb antibody and there is clear co-localisation of the phospho-Rb and total Rb in cells where Rb is mislocalised to the cytoplasm. Some residual Rb staining can be observed in the nucleus of some of these cells (Supplemental Figure 3B).

5) The authors reference work from Mittnacht indicating that phospho (inactive) pRB loses its affinity for the nucleus, which is indeed established by that group, but use this a evidence supporting the cytoplasmic localization they observe. However, the Mittnacht work showed that nuclear, phosphorylated "inactive" pRB lost affinity for that compartment without relocalizing to the cytoplasm. This should be more clearly stated. Indeed, the authors should consider using conditions established by Mittnacht to show that pRB can be extracted from the nucleus of tumor stromal cells but not normal stroma as a robust indicator of the inactive state.

Additional references relating to the mis-localisation of Rb have been included (Jiao et al 2006).

6) Figure 1A supports to some extent the authors' conclusion that ppRB signal coincides with FSP1 positive fibroblasts, but this is far less clear in Figure S1B. This needs to be more clearly established, and would be enhanced by staining for other markers of other types of stromal cells, such a macrophages, which may be proliferating and thus ppRB positive.

The format of the supplemental figure has been altered to allow easier identification of co-localised staining (New supplemental Figure 3A). In addition, we have also included staining of the oro-pharyngeal specimens with phospho-Rb and smooth muscle actin and the macrophage marker CD68. Phosphorylated Rb did not frequently correlate with CD68 or SMA staining (Supplemental Figure 2).

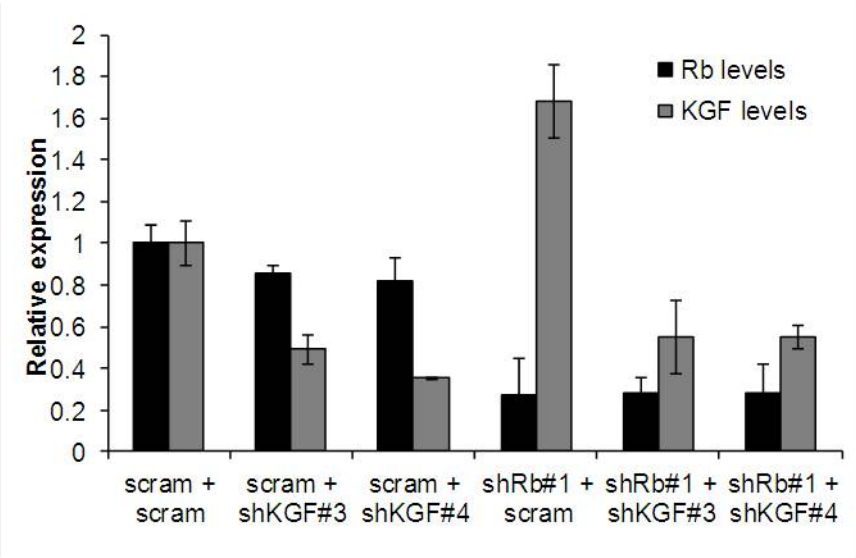
Referee #2:

This is a clearly written and interesting paper that highlights a potential role RB in the regulation of growth factor (GF) production by tumor-associated fibroblasts. GF (in this case, KGF) appears capable of promoting invasion by cancer cells in a paracrine fashion.

Major comments:

1. Fig 3F is a critical experiment. Need to see that the hKGF shRNA is not affecting the knockdown of Rb. Also want to see how the hKGF knockdown compares to the differences shown in Fig 3C.

KGF shRNA does not affect the levels of Rb as shown in the Figure below, while shRb affects KGF levels:



2. The finding of increased MMP1 and pETS-1 in tumors could have little to do with the biology described in Fig 1-4. Both MMP1 and ETS-1, for example, are induced by hypoxia (at least in some models). Are there other markers of KGF activity that could be explored?

We cannot discount the possibility that the elevated staining of MMP1 and p-Ets2 may be a result of stresses such as hypoxia. However, the results correlate with our finding in the organotypic model. We have also stained oro-pharyngeal cancer specimens with interleukin 1 alpha (IL1A), which was found to be induced by KGF treatment in our model, but which is also known to be induced in non-transformed epithelia in response to KGF treatment. IL1A staining was evident in the epithelium component of head and neck cancers in the majority of cases. This data has not been included as this may form part of a future study.

Minor comments:

1. The authors' data shows that fibroblasts in which Rb is inactivated can promote invasion by overproducing GF such as KGF. I don't think that is the same as showing that "stromal fibroblasts (normally) act to suppress invasion".

We have re-worded the abstract where this was stated.

2. The observations described in the first paragraph indicate that you 1) need a placenta to make a viable mouse, 2) Rb plays a critical, presumably cell-autonomous, role in making a placenta, 3) it is possible to make a functional placenta if at least some of the cells are positive for Rb. Seems like a soft argument for Rb regulating neighboring cells (although I suspect this is true).

In the chimeric mice the defects in Rb-null embryos of elevated apoptosis and loss of tissue differentiation are also rescued. This suggests that in these tissues Rb expression in one cell can regulate the growth/differentiation/survival of neighbouring Rb-null cells.

3. It is possible that some of the cancer-associated stromal cells are actually tumor cells that have undergone and EMT (as has been seen now in some other systems)?

This is a very good point and at this stage we cannot discount this possibility. However, EMT is not a complete process and it is likely that some epithelial markers would persist in cancer cells that have gone through this process. We did stain the stroma with antibodies against keratins 5 or 14, which are basal epithelial non-differentiated keratins and did not find any FSP-1 fibroblasts that were positive for keratins 5 or 14. We believe this would be a very difficult scenario to comprehensively prove one way or the other.

4. "they alone are able to promote the proliferation" might suggest that fibroblasts are unique in this regard. I think the authors meant that the fibroblasts, acting alone, are sufficient to promote proliferation.

This has been removed.

5. I can barely see the differences in Fig 3a and 3b. Can these spots be quantified? Corroborated by ELISA?

This has been quantified and is now shown in Figure 3A.

6. If Rb is "inactivated" by phosphorylation in the tumor-associated fibroblasts then it begs the question why. For example, do the authors think the cancer cells are, in turn, producing factors that promote Rb phosphorylation in fibroblasts? This should probably be discussed.

We do believe that the communication between the stroma and the cancer cells is a two way process and are investigating the possible pathways involved. This is at an early stage, however, we have discussed this possibility in the discussion.

References:

Knudsen ES, Wang JY (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. *Mol Cell Biol* 17: 5771-5783

Jiao W, Lin HM, Datta J, Braunschweig T, Chung JY, Hewitt SM, Rane SG (2008) Aberrant nucleocytoplasmic localization of the retinoblastoma tumor suppressor protein in human cancer correlates with moderate/poor tumor differentiation. *Oncogene* 27: 3156-3164

3rd Editorial Decision 17 April 2012

Thank you for the re-submission of your manuscript to The EMBO Journal. It has been sent to one of the original reviewers, who now considers that, pending very minor changes, your manuscript is basically ready for publication.

Following the recommendation of the referee as you will see below, I would like to ask you to add a few lines in the discussion section concerning the interplay between CAFs and tumor cells. Also, browsing through the manuscript myself I have noticed that the micrographs in your figures lack scale bars, which we require for clarity. In addition, the statistical analysis of the results is not properly described. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed. If the number of independent experiments is less than three, use of error bars is not appropriate and one representative experiment should be provided, clearly indicating this fact. Along these lines, albeit not absolutely necessary, we recommend the use of statistical significance analysis tools to further strengthen the interpretation of the results. The statistical significance analysis tool chosen must be also clearly stated.

As a novel initiative in The EMBO Journal, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any

questions regarding this initiative do not hesitate to contact me.

Thank you very much for your patience and congratulations in advance on a successful publication. Once these minor changes suggested are incorporated into the manuscript, you will receive an official acceptance letter with further instructions on how to proceed with the publication process.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Referee #1:

The authors have significantly improved this manuscript, and the data that purport to show Rb inactivation by phosphorylation are now robust. The mechanistic characterization of the effects of RB-deplete fibroblasts on epithelial tumors is also strong, and the manuscript now makes a well-supported, highly significant contribution.

Although the mechanism of KGF's effects on the epithelial cells in terms of invasion are well investigated here, the events that lead to increased RB phosphorylation in CAFs is far less clear. The in vitro system used is a reasonable model of this, but cannot provided information about how this RB inactivation is achieved in vivo. It is certainly beyond the scope of the current work to ask the authors to figure this out, and they have included a reasonable speculation in the discussion that posits that now well-known factors secreted by tumor cells may alter CAFs, which then reciprocate by altering tumor cells. This is attractive, but somewhat unsatisfying in one sense, since one might then expect that tumor cells would "convert" normal fibroblasts to CAFS at least in terms of RB inactivation by phosphorylation, and that removal of CAFs from the tumor environment would cause them to revert. I don't believe that either of these events is observed, and the authors should add 2-3 more sentences to the appropriate section of the discussion addressing this apparent, non-transient alteration in RB function in CAFs. Other than this, the overall manuscript is quite compelling and will open up new territory for both basic and therapeutic studies focused on tumor-stroma interactions.

1st Revision - Authors' Response 24 April 2012

Please find attached a modified manuscript (EMBOJ-2012-81425) which includes:

i) an additional brief discussion of the cross talk between the tumour and stroma, highlighted in the text file, as requested by Referee 1
ii) Scale bars and statistical analysis that have now been added to the figure files and are highlighted in the figure legend text, as requested by yourself